ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 58(1), March 2021

PROCEEDINGS
OF THE PAKISTAN ACADEMY OF SCIENCES:
B. Life and Environmental Sciences

PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

PAKISTAN ACADEMY OF SCIENCES

Founded 1953

President: Khalid Mahmood Khan
Secretary General: Tasawar Hayat
Treasurer: Amin Badshah

Proceedings of the Pakistan Academy of Sciences B. Life and Environmental Sciences is the official flagship, the peer-reviewed quarterly journal of the Pakistan Academy of Sciences. This journal publishes original research articles and reviews in the field of Agricultural and Biological Sciences (all), Biochemistry, Genetics and Molecular Biology (all), Environmental Science (all), Health Sciences (all). Authors are not required to be Fellows or Members of the Pakistan Academy of Sciences or citizens of Pakistan.

Editor:

Irum Iqrar, Pakistan Academy of Sciences, Islamabad, Pakistan; editor@paspk.org

Discipline Editors:

Agricultural Sciences: Kadambot Siddique, The UWA Institute of Agriculture, The University of Western Australia, Perth, Australia
Animal Sciences: Abdul Rauf Shakoori, School of Biological Sciences, University of the Punjab, Lahore, Pakistan
Biological Sciences: Azra Khanum, University Institute of Biochemistry and Biotechnology, PMAS Arid Agriculture University Rawalpindi, Pakistan
Environmental Sciences: Bin Chen, State Key Joint Laboratory of Environmental Simulation and Pollution Control School of Environment, Beijing Normal University, China
Environmental Sciences: Zahir Ahmad Zahir, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan
Food Sciences: G. Sarwar Gilani, Nutrition, and Health Advisor (Independent), Ottawa, Canada
Health Sciences: Khalid Iqbal, Department of Neurochemistry, New York State Institute for Basic Research, New York, USA
Health Sciences: Anwar-ul-Hassan Gilani, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan
Pharmaceutical Sciences: Ilkay Erdogan Orhan, Faculty of Pharmacy, Gazi University, Ankara, Turkey
Plant Sciences: Munir Ozturk, Faculty of Science, Ege University, Izmir, Turkey
Plant Sciences: Zabta K. Shinwari, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Editorial Advisory Board:

Mohammad Perwaiz Iqbal, School of Science, University of Management and Technology, Lahore, Pakistan
Nancy Connell, Johns Hopkins Center for Health Security, Baltimore Maryland, USA
Kamal Chowdhury, School of Natural Sciences & Mathematics, Claflin University, USA
Mohammad Wasay, Department of Medicine, The Aga Khan University, Karachi, Pakistan
Shahid Mansoor, Department of Biological Sciences, NIBGE, Faisalabad, Pakistan
Muhammad Ansar, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
Muhammad Shahzad Aslam, School of Traditional Chinese Medicine, Xiamen University, Malaysia
Muhammad Farooq, Department of Agronomy, University of Agriculture, Faisalabad, Pakistan
Syed Ghulam Musharraf, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan
Muhammad Zaffar Hashmi, Department of Chemistry, COMSATS University, Islamabad, Pakistan
Syed Sikandar Azam, National Center for Bioinformatics, Quaid-i-Azam University, Islamabad, Pakistan

Annual Subscription: **Pakistan:** Institutions, Rupees 4000/- ; Individuals, Rupees 2000/- (Delivery Charges: Rupees 150/-)
Other Countries: US$ 200.00 (includes air-lifted overseas delivery)

© *Pakistan Academy of Sciences.* Reproduction of paper abstracts is permitted provided the source is acknowledged. Permission to reproduce any other material may be obtained in writing from the Editor.

The data and opinions published in the *Proceedings* are of the author(s) only. The *Pakistan Academy of Sciences* and the *Editors* accept no responsibility whatsoever in this regard.

HEC Recognized, Category Y

Published by **Pakistan Academy of Sciences**, 3 Constitution Avenue, G-5/2, Islamabad, Pakistan
Tel: 92-51-9207140; **Fax:** 92-51-920 6770; **Websites:** www.paspk.org/proceedings/; www.ppaspk.org

Printed at **Graphics Point.,** Office 3-A, Wasal Plaza, Fazal-e-Haq Road Blue Area, Islamabad, Pakistan
Ph: 051-2806257; E-mail: graphicspoint16@gmail.com

PROCEEDINGS
OF THE PAKISTAN ACADEMY OF SCIENCES:
B. Life and Environmental Sciences

CONTENTS

Volume 58, No. 1, March 2021 **Page**

Research Articles

Obituaries

Submission of Manuscripts: Manuscripts may be submitted as an e-mail attachment at editor@paspk.org or submit online at http://ppaspk.org/index.php/PPASB/about/submissions. Authors must consult the ***Instructions for Authors*** at the end of this issue or at the Website: www.paspk.org/proceedings/ or www.ppaspk.org.

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 1-42 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)349

Pakistan Academy of Sciences

Research Article

Subtractive Proteomics Supported with Rational Drug Design Approach Revealed ZINC23121280 as a Potent Lead Inhibitory Molecule for Multi-drug Resistant *Francisella tularensis*

Naima Javed[+], Sajjad Ahmad[+], Saad Raza, and Syed Sikander Azam[*]

Computational Biology Lab, National Center for Bioinformatics,
Quaid-i-Azam University, Islamabad-45320, Pakistan

Abstract: *Francisella tularensis* is a Gram-negative bacterium and is the etiological agent of taluremia. The prolonged use of antibiotics is the reason for pathogen resistance to antibiotics such as beta-lactams and macrolides. This leads to the search to explore novel drug targets for *F. tularensis* to inhibit its growth. Subtractive proteomics revealed Glucose-1-phosphate thymidylyltransferase (G1PTT) as the most promising protein as a drug target. A pharmacophore model was generated for virtual screening of a druglike library comprised of 1,000,000 drug molecules. Based on a pharmacophore-based search, a set of 152 compounds was predicted as the most potent inhibitors against this enzyme. The screened hits were docked with the target enzyme; which unveiled ZINC23121280 as the best-docked inhibitor having Autdock Vina binding energy of -7.2 kcal/mol and the GOLD score of 64.06. Moreover, the time-dependent dynamic behavior of the complex was analyzed using Molecular Dynamics (MD) simulation studies that revealed a stable system with a Root Mean Square Deviation (RMSD) average value of 2.25 Å and Root Mean Square Fluctuations (RMSF) of 1.16 Å. Radial Distribution Function (RDF) predicted strong hydrogen interactions between the ligand and Trp221 from the enzyme active pocket. The higher affinity of the antagonist for the enzyme was further supported by Molecular Mechanics Energies combined with the Poisson–Boltzmann and Surface Area (MMPBSA) and or Generalized Born and Surface Area (MMGBSA) with the estimated binding free energy of −1.07 kcal/mol and −29.59 kcal/mol, respectively. Findings from this present computational framework may provide the foundation for future drug discovery against *F. tularensis.*

Keywords: *Francisella tularensis*; Subtractive proteomics; Glucose-1-phosphate thymidylyltransferase; Pharmacophore; Molecular docking; Molecular Dynamic simulation; MMPB/GBSA.

1. INTRODUCTION

Francisella tularensis is a pleomorphic Gram-negative coccobacilli and is the etiologic agent of a zoonotic disease of the northern hemisphere, tularemia [1, 2]. *F. tularensis* is highly virulent to a wide range of animals and humans [3]. The pathogen may cause epizootics or epidemics [4]. No human-to-human transmission is observed whereas transmission to humans occurs by four main routes: (i) through direct contact with the infected animals, infectious animal fluids or tissues, (ii) through arthropod bites, (iii) by inhaling infective aerosols, and (iv) by ingesting contaminated food or water [5, 6]. Tularemia is highly prevalent in Sweden, Finland, and Turkey [7]. According to the pre-antibiotic era, the reported mortality rate of tularemia was 30%–60% [8]. The mortality rate associated with respiratory tularemia is as high as 5 – 30% [9]. According to Center for Disease Control and Prevention (CDC) statistics, *F. tularensis* is common in south-central America, and Massachusetts, and cases of Tularemia have been reported from all states of the USA except Hawaii. The number of cases from 1950 declined from 927 to 229 in 2018 (https://www.cdc.gov/tularemia/statistics/index.html). This bacterium is considered an aerosol biological weapon by several countries in the past [10]. Various subspecies include tularensis, holarctica, mediasiatica, and

Received: March 2020; Accepted: March 2021
*Corresponding Author: Syed Sikander Azam <ssazam@qau.edu.pk; syedazam2008@gmail.com>
[+]Both authors contributed equally to this work

novicida, where tularensis type A and holarctica type B is the most significant clinical subspecies of *F. tularensis* [11, 12]. Strains of the *F. tularensis* subspecies are common in North America where they cause rapidly progressive diseases [13] leading to prominent lymph node enlargement and flu-like symptoms [14]. The pathogen is also capable of infecting many types of eukaryotic cells and tissue macrophages [15]. With the increasing trend of antibiotic resistance to beta-lactams and macrolides in *F. tularensis* and the absence of a licensed vaccine for boosting the immune response to infections, identification of novel drug targets for designing novel antibiotics is an imperative need of time [4, 16].

The first phase of the drug designing process is the identification of potential drug targets against bacterial pathogens [17]. In traditional drug discovery, this process is time and resource-consuming and often results in failures [18]. On the other hand, using computational power and available genomic and proteomic data of bacterial pathogens is now common to discover new antibiotics, optimization, and development [19, 20]. In this context, subtractive proteomics is a widely used approach that in a step-wise process, filters proteins of high pathogen specificity [21]. The use of such in silico methodologies, not only saves extensive labor cost and time but also expedites the process of characterization of bacterial host's non-homologous and essential proteins to eradicate the disease with fewer side effects [22]. In the present study, a subtractive proteomics approach was employed in combination with the applications of computer-aided drug designing (CAAD) for the identification of potential drug candidates for the potential druggable protein against *F. tularensis* reference strain SCHU4 [23, 24]. The pathogen is investigated for host non-homologous proteins followed by essential proteins mapping using the Database of Essential Genes (DEG) [25]. The Glucose-1-phosphate thymidylyltransferase (G1PTT) is a target of choice for novel antibacterial discovery. The G1PTT is the first enzyme in the dTDP-L-rhamnose biosynthesis pathway that acts as an L-rhamnose precursor. L-rhamnose is an important component of bacterial surface antigens such as the O-lipopolysaccharide [26]. In addition, it aids in mediating pathogen adhesion to host tissues and virulence [26]. The best-docked complex was simulated to unveil the enzyme dynamics in the presence of ligand [27]. To further explore the ligand affinity towards the enzyme active site, binding free energies were estimated using Molecular Mechanics Energies combined with the Poisson–Boltzmann or Generalized Born and Surface Area Continuum Solvation (MMPB/GBSA) [28].

2. MATERIALS AND METHODS

The workflow for characterizing potential drug targets in *F. tularensis* proteome and subsequent steps of pharmacophore generation, molecular docking, MD simulations, and binding free energies is illustrated in Fig. 1.

2.1 Subtractive Proteomics

The Uniprot database [29] was used to retrieve the complete proteome of the reference strain of *F. tularensis* labeled as SCHU S4 [30]. The proteome was subjected to the subtractive proteomics where proteins relevant to antibiotics design were extracted in a step-wise manner [31]. In the first step, the CD-HIT suite [32] was applied to eliminate the redundant proteins sharing the identity of 80%. Redundant proteins are paralogous proteins that arise because of duplication during evolution and are not conserved across bacterial species. Non-redundant proteins, in contrast, are orthologous and are well conserved across bacterial species and strains; thus can be targeted for the design of broad-spectrum inhibitors [23, 33]. The BLASTp search of the National Center for Biotechnological Information (NCBI) was performed against reference human proteome (*Homo sapiens*: Tax id. 9606) to obtain proteins specific to the pathogen with a percentage identity threshold $\geq$ 30% and the Expectation value (E-value) of 10-5. The host non-homologous proteins were then used in BLASTp of DEG [25] with the threshold E-value of 10-10, sequence identity of $\geq$ 30%, and bit score of 100 to predict pathogen essential proteins. The identified essential proteins were then allowed to enter the metabolic pathway mapping stage, where the proteins were mapped to the metabolic pathways of the pathogen [34]. In order to predict protein sequences involved in different metabolic pathways of the organism, the KEGG Automatic Annotation sever (KAAS) [35] was used. Further in the framework, virulence

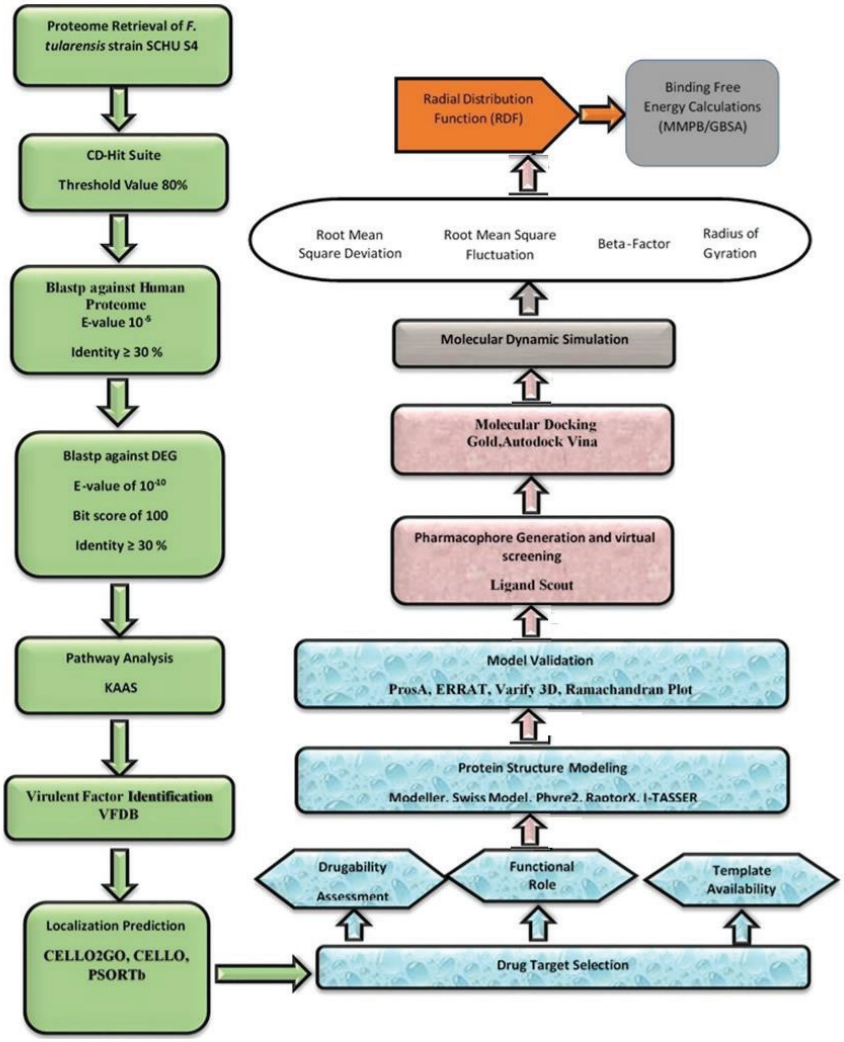


Fig. 1. Workflow of the methodology used in the present study

proteins of the bacteria were screened as they aid in bacteria adherence, colonization, invasion, evasion of host defense, and disease etiology as such are attractive targets to deactivate the pathogen [36]. For virulent protein identification, the Virulent Factor Database (VFDB) was accessed [37]. The unique proteins from *F. tularensis* metabolic pathways were subjected to BLASTp of VFDB to screen proteins having a threshold bit score ≥100 and identity ≥ of 50%. In the concluding step of the subtractive proteomics, cytoplasmic proteins were identified using a comparative subcellular localization prediction approach. In this approach, the cellular localization of selected proteins was determined using three online servers: PSORTb [38] CELLO [39], and CELLO2GO [40]. The cytoplasmic proteins are presumed to be attractive targets because of the higher availability of drugs [41].

2.2 Physiochemical Characterization of Cytoplasmic Proteins

The physicochemical properties of the cytoplasmic proteins were unraveled to evaluate several vital parameters of the targets important from an experimental validations point of view [42]. The characterization was done based on molecular weight, theoretical pI, atomic composition, instability index, and grand average of hydropathicity (GRAVY). For this, an online server of ProtParam [43] was used.

2.3 Drug Target Selection

The selection of a drug target was done based on their subcellular localization, virulence, and physiochemical properties of the protein [16, 17]. Another parameter for the target selection was

the non-availability of experimentally determined structure. The Protein Data Bank (PDB) was explored for the availability of protein experimental structures. Enzymes in the shortlisted targets were especially targeted because of the following reasons: (i) Enzymes are essential to life, (ii) dysregulated enzymes lead to disease states, (iii) Enzymes are highly amenable to inhibition by small druglike molecules, (iv) Majority of the pharmaceutical companies (50% - 75%) around the globe focused on enzyme as primary target [44].

2.4 Comparative Structure Modeling and Validation

The availability of 3D structures of the targeted protein was checked using an online BLASTp tool of NCBI against PDB. The 3D structure is a prerequisite of the subsequent molecular docking study, MD simulation, and binding free energy calculations. In the absence of 3D structure, the sequence of proteins was used in a comparative structure modeling approach. First, in this approach, the amino acid sequence of the selected proteins was BLASTp against PDB for the identification of the appropriate template structure. Once template structure was identified, an automated protein modeling program, Modeller 9.14, was run to predict the most optimal 3D structure of proteins. Parallel to Modeller, several online severs: ReptorX [45], Phyre2 [46], SWISS-MODEL [47] and I-TASSER [48] were used. To check the thermodynamic stability of the generated models, online structure quality assessment tools: ERRAT, Verify3D [49], ProSA [50], and Ramachandran Plot [51] were utilized. The protein was then energetically minimized using UCSF Chimera [52] to improve the quality of the structure by removing steric clashes. Minimization was performed for 1500 steps which can be split into 750 conjugate gradient steps and 750 steepest descent steps under Tripos Force Field [53].

2.5 Pharmacophore Model Generation and Virtual Screening

For ligand-based pharmacophore model generation, 15 compounds against the target protein were retrieved from the extensive literature reviews and binding database as illustrated in Table S1 [38]. The pharmacophore model was generated using LigandScout 4.5. Pharmacophoric sites: aromatic ring, hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), positive and negative ionizable groups, and hydrophobic sites were characterized carefully. To incorporate associated features of the utilized compounds, merge feature model generation and atom overlapping scoring function of the LigandScout was employed. The pharmacophore model with the best score was selected and used in virtual screening of Zinc database druglike libraries containing 1 million compounds. Once the screening was done, Lipinski's rule of five filters was additionally applied to the shortlisted inhibitors for filtering drug molecules with properties: HBA < 10, HBD < 5, molecular weight < 500 Da, and the value of log P < 5 [54].

2.6 Molecular Docking

The binding site in the selected enzyme for the screened set of inhibitors was predicted using a combined approach of online binding site prediction tools and Multiple Sequence Alignment (MSA) [55]. Meta pocket [56] was used first to predict the enzyme active cavity, followed by aligning the orthologues of G1PTT enzyme through ClustalW [57] to look for the most conserved active site pocket residue. Genetic Optimization for Ligand Docking (GOLD) [58] and AutoDock Vina (AD-Vina) [59] were used for docking of the compounds to enzyme active pockets. The coordinates of the oxygen atom from Gly09 were set as the point of inhibitors binding. The GOLD docking was accomplished with a genetic run for each compound was set to 10. In AD-Vina, the same active site coordinates used in GOLD were used with the grid box size set to 15 Å along with the X, Y, and Z-axis. The docking results were visualized using LIGPLOT [60], UCSF Chimera [60], Visual Molecular Dynamics (VMD) [61], and Discovery Studio (DS).

2.7 MD Simulation

To determine the dynamic behavior of the enzyme in complex with the ligand, MD simulation for 100-ns was carried out [62]. Assisted model building with the energy refinement 14 (AMBER 14) [63] was used to design and perform simulation protocol. Initial libraries of the complex were generated using the Antechamber program. The docked complex was integrated into a TIP3P water

box with a padding distance size of 12 Å between the protein and water box boundary conditions. This was accomplished using ff14SB force field using Leap program [64]. The addition of 12 Na+ ions was involved in neutralizing the hydrated complex. During minimization, hydrogen atoms, water box, carbon alpha atoms of the complex were minimized for 1000 cycles whereas non-heavy atoms were relaxed for 300 steps [65]. After that, the system was subjected to a heating step, where a temperature of 300 K for 20-ps with the restraint of 5 kcal/mol. on alpha-carbon atoms are used. SHAKE algorithm [66] was used to heat the system while Langevin dynamics was used for maintaining system temperature. The system equilibrium was achieved for 100-ps with a time scale of 2-fs [67]. The NPT ensemble was employed for 50-ps to maintain system pressure. The system was then equilibrated for 1-ns. In production phase, the Berendsen algorithm combined with NVT ensemble was used with cut-off value of non-bonded interactions set to 0.8 Å. The production run was carried out for 100-ns. For simulation trajectories analysis, CPPTRAJ program of AMBER was used [68].

2.8 Binding Free Energy Calculations

The MM-PBSA and MM-GBSA of AMBER14 were used to estimate the binding free energies of the system [62]. A total of 500 frames were extracted from the simulation trajectories. The prmtop files of receptor, ligand, and complex were generated using the anti-MMPBSA.py module of AMBER whereas for estimating binding free energy MMPBSA.py module was used.

3. RESULTS AND DISCUSSION

3.1 Subtractive Proteomics

The emergence of resistance in bacteria is rapid and occurring worldwide thus endangering the efficacy of life-saving antibiotics [69]. This resistance to antibiotics has been attributed to misuse and overuse of these medications, in addition, to the lack of new antibiotic development by pharma industries due to challenging regulatory requirements and lesser economic incentives [70]. Because of many limitations of conventional drug target identification and drug discovery, computational identification of potentially druggable proteins and subsequent drug designing played a major role in providing therapeutically important molecules against several medical complications [71]. The drug target identification is the first step in drug discovery and can be applied to a range of biological entities that may include protein, DNA, and RNA [72] associated with the disease. Subtractive proteomics is now a widely used approach for the identification and validation of bacterial proteins involved in regulating essential biological processes [73]. This is a step-wise approach that gradually reduces the number of proteins involved in the host's non-homologous, essential and unique pathways of the bacterial pathogen [31]. Using this approach, the complete proteome of *F. tularensis* strain SCHU S4 that contains 1556 proteins were thoroughly screened first for non-redundant proteome. The non-redundant proteins are attractive targets for antibiotics because of their broad-spectrum conservation across bacterial species and strains [31]. On the other hand, redundant proteins are paralogous that are less conserved and not preferred as drug targets [31]. The CD-HIT analysis revealed 28 duplicated proteins and thus excluded them from *F. tularensis* proteome. The 1528 orthologous proteins were forwarded to the homology check. At this check, homologous proteins between the host (*Homo sapiens*) and bacteria were compared using an online BLASTp tool of NCBI. The homologous proteins sharing 30% of identity were discarded whereas those having identity of <30% were passed to the next step of DEG analysis. A homology check revealed 1218 proteins as host non-homologous proteins as a potential target for drug discovery. Screening host non-homologs is vital as targeting host homologous proteins could lead to autoimmune reactions and adverse side effects [73]. The essential proteins are the foundation to bacterial life without such proteins the bacterium is unable to survive, and as such are attractive targets for designing novel antibiotics [73]. The essentiality analysis unraveled 732 proteins in number while the remaining 486 proteins are non-essential hence excluded from further analysis [73]. Mapping essential proteins to organism metabolic pathways are vital as it provides an array of opportunities to block pathogen survival. The KAAS mapped a total of 262 proteins to metabolic pathways including 11 unique and 251 common proteins. The output of KASS was investigated first enzymatic and non-enzymatic proteins. The enzymatic proteins

were recognized through its Enzyme Classification (EC) number and were 71% of the total proteins compared to the non-enzymatic proteins that were 29%. The metabolic proteins were categorized into the following: 1) cellular process, 2) environment information processing pathways, 3) metabolism 4) human diseases and drug development and 5) organismal systems. Cellular processes can be divided into two main systems including peroxisome cell-cycle –caulobacter. Genetic information processing includes proteins that take part in folding, translation, transcription, sorting and degradation, repair, and replication. Environmental information processing include transport systems including phosphotransferase system (PTS), ABC transporters, two-component system and bacterial secretion system. The virulent proteins were identified through VFDB. Six proteins were found virulent including wcaJ, oppF, qseC, phnA, wbtL and tolQ (Table 1). The WcaJ is considered as initiating enzyme in the synthesis of colonic acid (CA) [74]. OppF plays part in transporting oligonucleotides. QseC is a member of two-component regulatory system (QseB/QseC) and functions by activating the flagella regulon of FlhDC [75]. The PhnA is involved in hydrolysis of phosphonoacetate [76]. The WbtL drives formation of dTDP-glucose from glucose 1-phosphate and dTTP [26]. The TolQ is a part of Tol-Pal system which plays a role in outer membrane invagination during cell division and is important for maintaining outer membrane integrity [77]. Knowledge of subcellular localization is significant in identification of therapeutic targets. Cytoplasmic proteins are preferred as drug targets compared to membrane proteins because of the following reasons: (i) membrane proteins have low permeation rate thus can block the access of drugs to the biological target, (ii) the presence of energy driven efflux systems may use the drug as effluxing substrate for broad specificity. The comparative subcellular localization for the virulent proteins is illustrated in Fig. 2. According to PSORTb, majority of the proteins were found in the cytoplasmic membrane (73%), followed by cytoplasmic

Table 1. Virulent proteins screened in the study.

Gene	Protein Name	Bit score	Identity
wcaj	Hydrogen peroxide-inducible genes activator	226	65
oppF	Oligopeptide transport ATP-	157	56
qseC	Sensor histidine	107	31
phnA	Protein PhnA	32	3
wbtL	Glucose-1-phosphate thymidylyltransferase	381	64
tolQ	Biopolymer transport protein TolQ	86	43

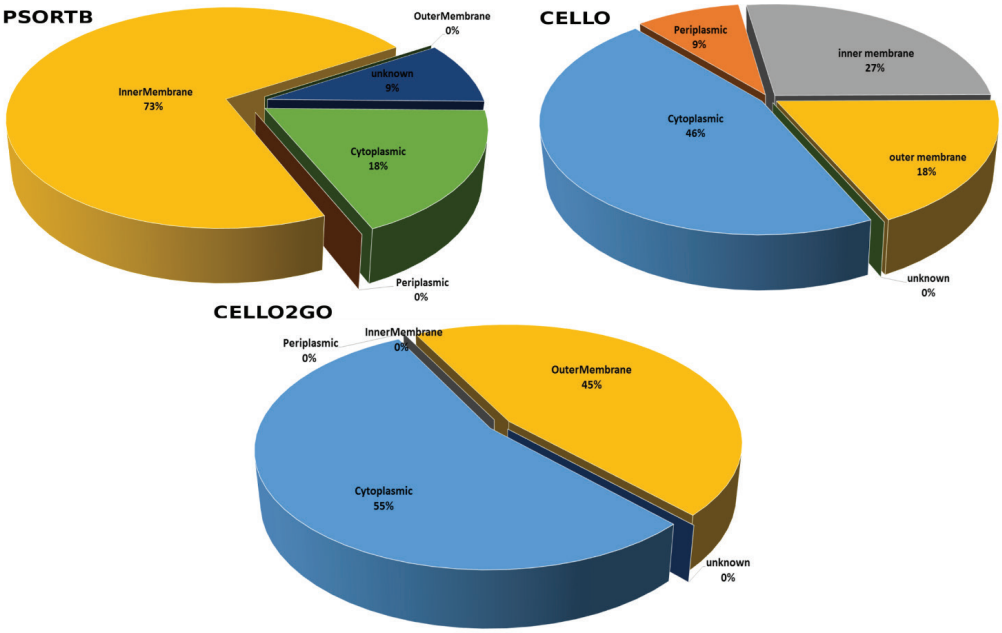


Fig. 2. Sub-Cellular localization of *F. tularensis* virulent proteins.

proteins (18%), 9% of unknown proteins, 0% of outer membrane, inner membrane and periplasmic proteins. Cello demonstrated majority of the proteins as cytoplasmic (46%), inner membrane (27%), outer membrane (18%), periplasmic (9%), cytoplasmic membrane and unknown (0%). Lastly, Cello2Go revealed 55% cytoplasmic, 45% outer membrane, 0% of cytoplasmic membrane, periplasmic, and unknown proteins. It was found that around 5 proteins were cytoplasmic, 1 was periplasmic, 3 in inner membrane and 2 were in outer membrane. By comparative analysis, only 3 proteins: wcaj, qseC, and wbtL were selected as cytoplasmic proteins and forwarded along the framework.

3.2 Physicochemical Characterization

The physicochemical characterization of shortlisted proteins was an important consideration for shedding light on the suitability of selected proteins for a wet lab analysis [42]. The major parameter during this analysis was to compute the molecular weight of proteins. The proteins having <110 kDa molecular weight are preferred as drug and vaccine targets because of their easy purification [42, 53]. It was estimated that all four proteins have a molecular weight less than the threshold and can be used in the development of the novel drug. The stability of the protein is the next most important characteristic of protein. The proteins having an estimated <40 stability index are considered stable while those proteins with >40 are regarded as unstable. For all three proteins, stability were calculated <40 while no protein stability value was > 40. The GRAVY negative score of all the stable proteins indicates the hydrophilic nature of the proteins. The theoretical pI value of 2 proteins (wcaJ, qseC) were greater than 7 represented the basic nature of the protein and the 1 protein (wbtL) with less than 7 pI value indicating the acidic nature of drug-protein. The aliphatic index value for the proteins ranged from 117.9 - 95 show the high thermal stability of proteins. The physicochemical parameters for the shortlisted 3 proteins are tabulated in Table 2.

3.3 Selection of Drug Target

The G1PTT enzyme was selected as the potential drug target against the pathogen based on its cytoplasmic location, involvement in virulent pathways, suitable molecular weight, theoretical PI, instability index, and GRAVY index. Another parameter for the target selection was the non-availability of experimental structure and availability of a suitable template. The G1PTT is involved in the biosynthesis of different antibiotics, polyketide sugar unit biosynthesis, and acarbose and validamycin biosynthesis pathways. As the target is an enzyme, involved pathogen-specific and selective pathways, structure modeling, molecular docking, and dynamics simulation can provide an excellent platform for designing antibiotics against this target enzyme.

3.4 Comparative Structure Modelling

The 3D structure of the selected protein was not

Table 2. Physiochemical characterization of selected cytoplasmic proteins.

Uniprot ID	Gene	Protein Name	Residues length	Molecular weight (KDa)	Theoretical pI	Instability Index	Aliphatic index	GRAVY
Q5NEZ2	wcaJ	Hydrogen peroxide-inducible genes activator	205	23.3	9.4	33.1	95.5	-0.073
Q5NIH6	qseC	Sensor histidine kinase QseC	4745	54.7	7.1	31.8	105.0	-0.22
Q5NF04	wbtL	Glucose-1-phosphate thymidylyltransferase (G1PTT)	294	32.4	5.5	39.1	104.1	-0.052

present in the PDB, therefore, a comparative structure prediction approach was used. The X-ray crystallographic structure of a template "PDB id: 1H5T" was selected for the model building process with 98% query coverage and 64% sequence identity. For the selection of the best model, a detailed comparison of stereochemical properties was performed as tabulated in Table 3. Based on quality assessment measurements, Phyre2 predicted structure was selected having 97.9% residues in the favored region of the Ramachandran plot. The number of residues in the allowed and outlier region was 1.7% and 0.3%, respectively. ERRAT, Verify3D, and Z-score were 93.116, 93.10%, and -8.41, respectively thus further confirmed the reliability of the optimal model selected for antibiotics screening. Moreover, the superimposed structure of the template and the target unraveled Root Mean Square Deviation (RMSD) of 0.001 Å is in a highly acceptable range and shown in Fig. 3. The tertiary structure of G1PTT can be found in Fig. 4.

3.5 Pharmacophore Model Generation and Virtual Screening

A pharmacophore model was generated to shortlist druglike compounds from Zinc database druglike libraries that share molecular features necessary for recognizing a ligand by druggable biological macromolecules. Pharmacophore model-based virtual screening of 1,000,000 drug molecules was then performed to shortlist the best possible drug molecules. The screening filtered 152 compounds. Structures of these compounds are tabulated in Table S2. These compounds were energetically minimized using the MMFF94 force field and further utilized in molecular docking studies.

3.6 Molecular Docking

The sequence 8GGSGTR13 was found conserved in all orthologues of the G1PTT enzyme. The coordinates oxygen atom from Gly9 was selected for molecular docking studies. Comparative docking performed using two different tools: GOLD and AD-Vina. In molecular docking, structure-based virtual screening of 152 drug-like compounds extracted based on pharmacophore-based virtual screening. All the inhibitors were docked into the enzyme active site using GOLD, and AD-Vina. The top ten best inhibitors based on descending order of GOLD fitness score together with AD-Vina binding energy and drug-likeness are shown in Table 4. The correlation coefficients between GOLD fitness score and AD-Vina binding energy of the compounds can be found in Fig. 5. Compound ZINC23121280 ((3R)-N-(6-amino-1-benzyl-2,4-dioxo-pyrimidin-5-yl)-1-cyclopentyl-5-oxo-N-propyl-pyrrolidine-3-carbox) was selected as the best-docked inhibitor with GOLD fitness score and AD-Vina binding energy of 64.02 and -7.2 kcal/mol, respectively. The complex was selected based on several parameters including strong interactions between ligand and target protein, drug-likeness of the compound, and its pharmacokinetics. In both tools, the inhibitor was investigated to dock in the same position and interacts with almost the same residues of the active site (Fig. 6). Visual inspection of complexes from both GOLD and AD-Vina revealed inhibitor binding with active residues: Leu6, Ala7, Gly8, Gly9, Ser10, Gly11, Arg13, Lys23, Gln24, Pro83, Gly85, Leu86, Leu106, Gly107, Asp108, Glu194, and Gly225.

Table 3. Stereo-chemical properties of comparative homology modeled structure.

Structure Resources	Favored region	Allowed region	outlier region	Errat	Z-score	Verify-3D
Modeller 1	279 (96.5%)	9 (3.1%)	1 (0.3%)	83.039	-8.41	88.32%
Modeller 2	279 (96.5%)	6 (2.1%)	4 (1.4%)	84.099	-8.41	90.38%
Modeller 3	280 (96.9%)	8 (2.8%)	1 (0.3%)	83.746	-8.73	96.22%
Modeller 4	279 (96.5%)	8 (2.8%)	2 (0.7%)	80.565	-8.32	94.50%
Modeller 5	279 (96.5%)	9 (3.1%)	1 (0.3%)	84.452	-8.47	92.44%
Phyre 2	282 (97.9%)	5 (1.7%)	1 (0.3%)	93.116	-8.41	93.10%
Swiss-Model 1	1073 (93.1%)	55 (4.8%)	24 (2.1%)	96.791	-8.39	93.10%
Swiss-Model 2	220 (93.6%)	12 (5.1%)	3 (1.3%)	83.772	-8.04	99.16%
I-TASSER	271 (92.8%)	14 (4.8%)	7 (2.4%)	97.895	-8.75	98.64%
RaptorX	280 (95.9%)	10 (3.4%)	2 (0.7%)	80.42	-8.4	98.80%

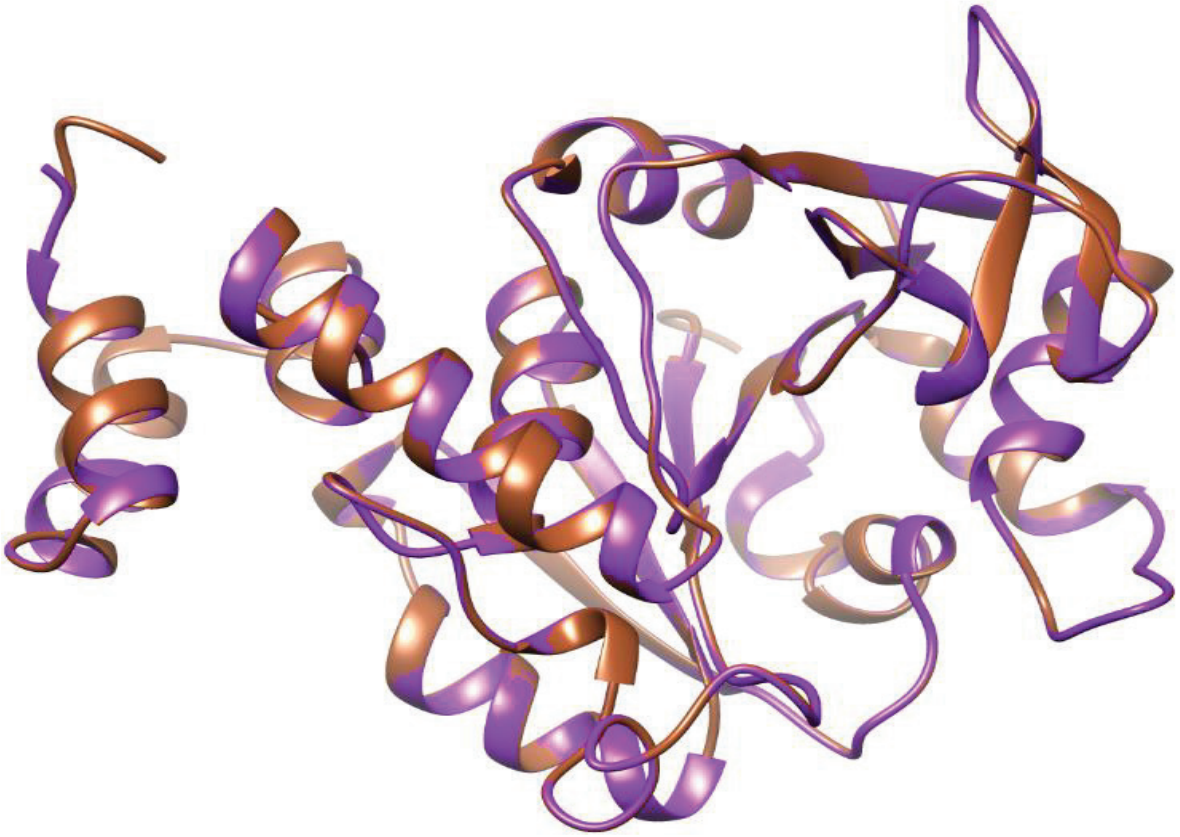


Fig. 3. Superimposition of selected optimum model (purple) over the template (sienna).

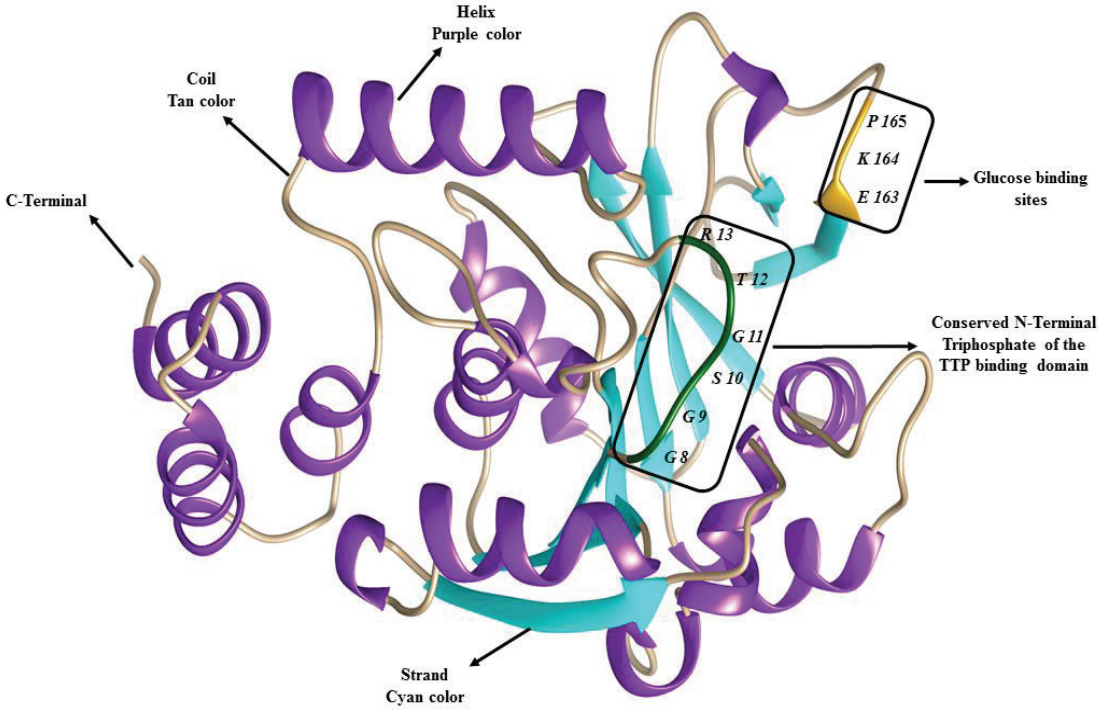


Fig. 4.Tertiary structure of G1PTT enzyme.

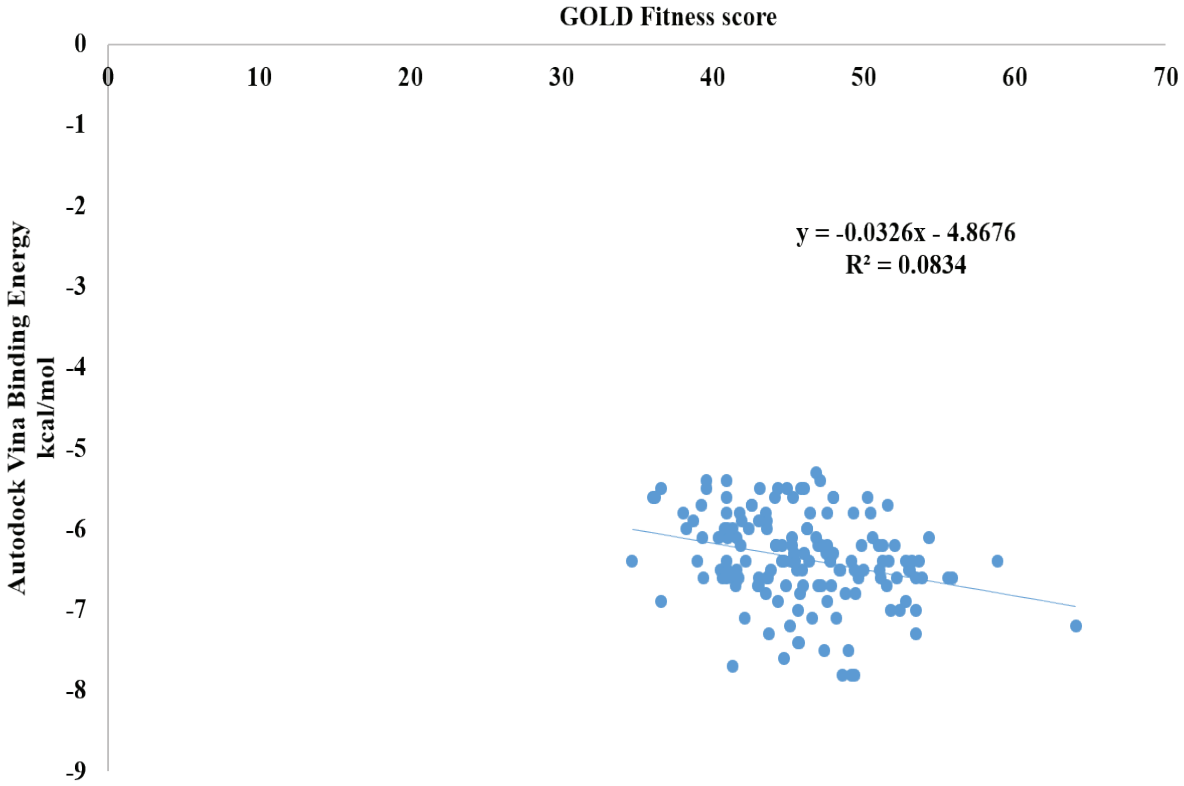


Fig. 5. Correlation coefficient between GOLD scores and binding free energies of 152 inhibitors.

The binding mode of the compound in the G1PTT pocket in GOLD was positioned as such to allow deep binding of 6-amino-1-(cyclohexylmethyl)-5-(ethyl(methyl)amino)-hexahydropyrimidne-2,4-diol ring and covered the major portion of the active site (Fig. 7). The oxygen atom of the ring was observed in hydrogen bond interaction with Gly85 and Pro83. The ring, (4R)-4-(hydroxymethyl) pyrrolidin-2-ol, bind in the cavity of the active site and the nitrogen atom of the ring was observed to interact hydrophilically with Gly09. It also forms interactions with Ser10, Leu106, Asp108, and Lys23.

At the drug design stage, unveiling drug-likeness and pharmacokinetic behavior of drugs are important as it reduces the number of unsuccessful hits in clinical trials [78]. In addition, it also enables chemists to select the most appropriate compounds for lead optimization and novel drug development. The compound completely follows Lipinski's rule of five: molecular weight (453.53 g/mol), number of H-bonds acceptors (4), number of H-bonds donors (2), topological polar surface area (TPSA) value (130.78 Å²), and LogP value (1.53). The number of heavy atoms in the compound is 33, while aromatic heavy atoms and rotatable bonds are 12 and 8, respectively. The molar refractivity of the compound is 127.95. An important consideration of drugs is their lipophilicity, which describes the compound's ability to be dissolved in lipophilic solutions (non-aqueous). Lipophilicity determines the compound's ability to permeate across different biological membranes [79]. Higher LogP, higher the capacity of drugs to cross biological membranes and hence can access targets for inhibition. The efficient delivery of a drug to the target site depends on the tendency of drugs to retain in blood for an extended period [78]. This was disclosed using the plasma protein binding (PPB) feature of the compound (described as LogK). The different ADMET properties of the best 10 inhibitors screened in the study can be found in Table 5.

3.7 Molecular Dynamics Simulation

The MD Simulations for 100-ns of the enzyme complex were carried out to investigate system stability. There are different examples in which time-dependent MD simulations have been applied

Table 4. Docking scores of top ten docked inhibitors.

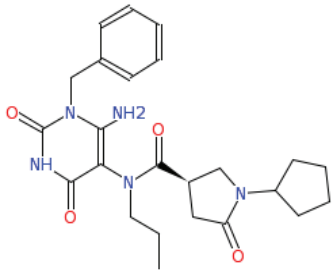
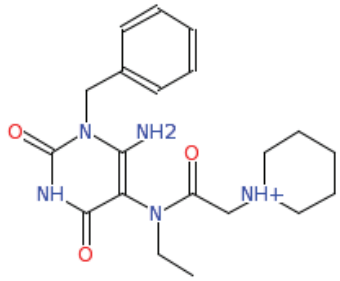
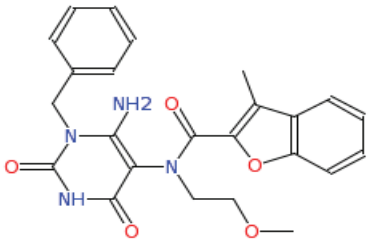
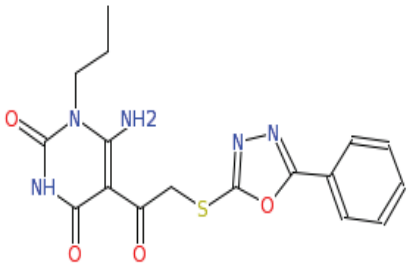
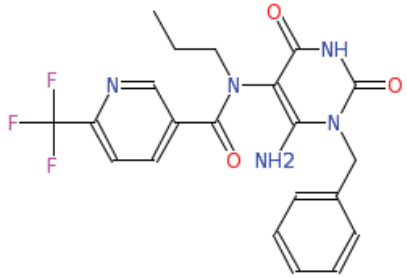
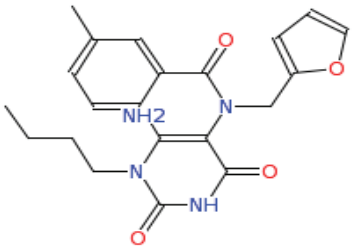
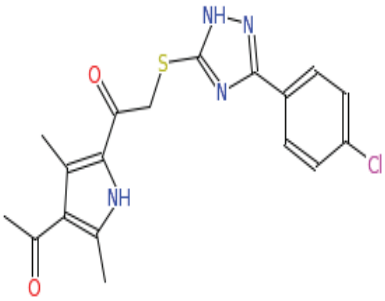
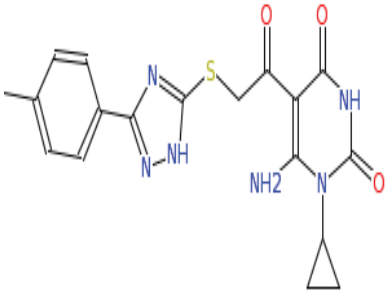
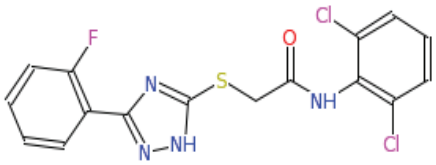
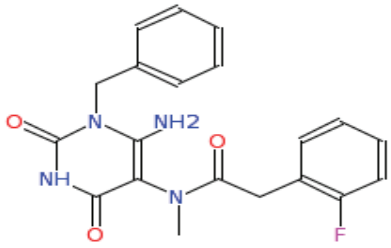
Compounds	GOLD Fitness Score	AD-Vina binding energy (kcal/mol)	Druglikenss rule violations
 ZINC23121280	64.06	-7.2	No violations
 ZINC02629047	58.94	-6.4	No violations
 ZINC14511277	55.86	-6.6	Egan rule (1 violations: TPSA>131.6)
 ZINC03348170	55.62	-6.6	Veber rule (1 violation: TPSA>140), Egan rule (1 violation: TPSA>131.6)
 ZINC12763362	54.31	-6.1	No violations

Table 4. Docking scores of top ten docked inhibitors.

Compounds	GOLD Fitness Score	AD-Vina binding energy (kcal/mol)	Druglikenss rule violations
 ZINC06221653	53.85	-6.6	No violations
 ZINC28807288	53.69	-6.4	No violations
 ZINC08343860	53.46	-6.6	Veber rule (1 violations: TPSA>140), Egan rule (1 violations: TPSA>131.6), Muegge filter (1 violation: TPSA> 150)
 ZINC05678255	53.44	-7.3	No violations
 ZINC13135410	53.44	-7	No violations

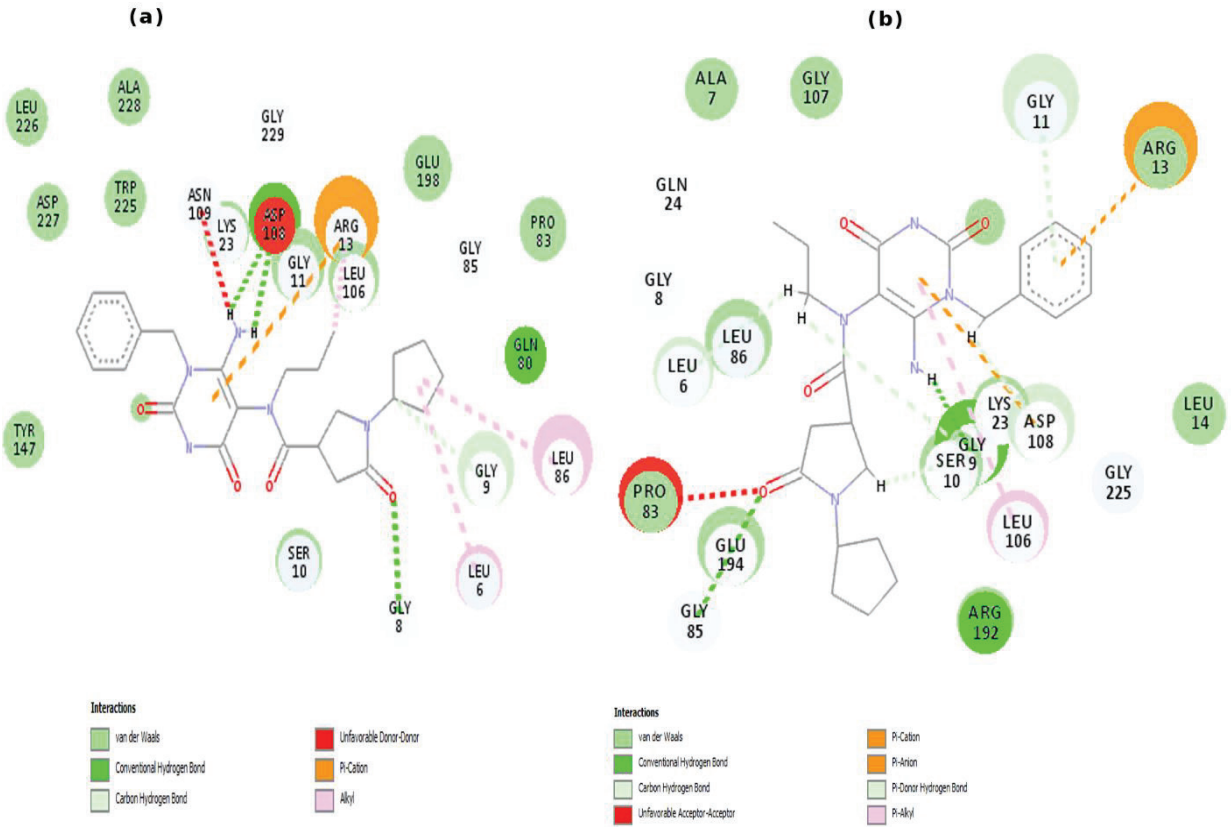


Fig. 6. Interacting residues of the enzyme with the ligand in AD-Vina (a) and GOLD (b).

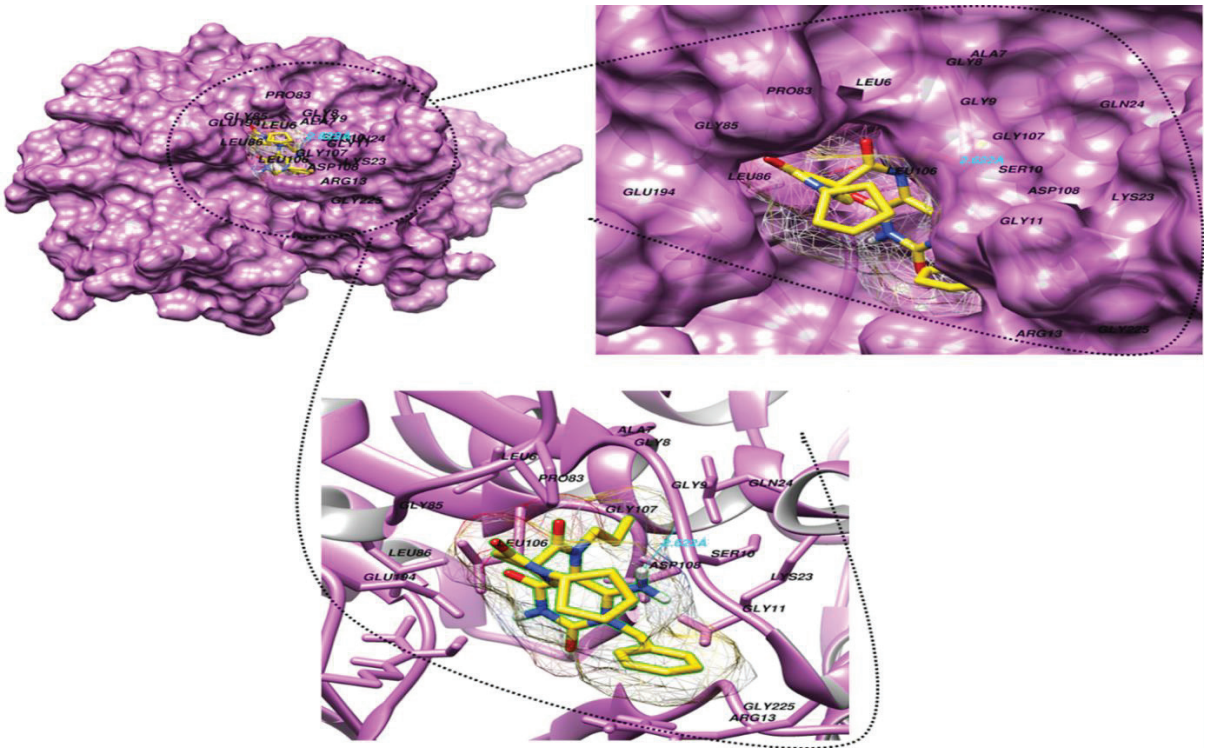


Fig. 7. Binding mode and interactions of ZINC23121280 in the binding pocket of the G1PTT enzyme.

Table 5. AdmetSAR properties of selected Compounds

Selected compounds	Blood-brain barrier (BBB) (probability)	Human intestinal absorption (HIA) (probability)	Caco2 permeability (probability)	CYP450 2D6 inhibitor (probability)	Carcinogens (probability)	Acute oral toxicity (probability)	Aqueous solubility (LogS)	Rat acute toxicity (LD50, mol/kg)	Fish toxicity (pLC50, mg/L)	Ames toxicity (probability)	Honey bee toxicity (HBT) (probability)
ZINC 23121 280	BBB- 0.7	HIA+ 0.9	Caco2- 0.7	Non-substrate 0.8301	Non-carcinogens 0.8	0.6	-2.6	2.5	1.9	Non-AMES toxic 0.7	Low HBT 0.8
ZINC 02629 047	BBB- 0.7	HIA+ 0.9	Caco2- 0.6	Non-substrate 0.8090	Non-carcinogens 0.8	0.6	-2.4	2.5	2.2	Non-AMES toxic 0.6	Low HBT 0.8
ZINC 14511 277	BBB- 0.5	HIA+ 0.8	Caco2- 0.7	Non-substrate 0.8273	Non-carcinogens 0.9	0.5	-2.5	2.6	1.6	Non-AMES toxic 0.6	Low HBT 0.6
ZINC 03348 170	BBB + 0.7	HIA+ 0.9	Caco2- 0.6	Non-substrate 0.8322	Non-carcinogens 0.8	0.6	-3.1	2.6	1.3	Non-AMES toxic 0.5	Low HBT 0.8
ZINC 06221 653	BBB + 0.5	HIA+ 0.9	Caco2- 0.6	Non-substrate 0.8	Non-carcinogens 0.9	0.6	-3.0	2.6	1.3	Non-AMES toxic 0.6	Low HBT 0.8
ZINC 28807 288	BBB + 0.9	HIA+ 1.0	Caco2- 0.5	Non-substrate 0.8	Non-carcinogens 0.6	0.6	-3.7	2.4	1.2	Non-AMES toxic 0.5	Low HBT 0.7
ZINC 08343 860	BBB + 0.9	HIA+ 1.0	Caco2- 0.5	Non-substrate 0.8	Non-carcinogens 0.7	0.6	-3.1	2.4	1.4	Non-AMES toxic 0.5	Low HBT 0.8
ZINC 05678 255	BBB + 0.9	HIA+ 1.0	Caco2- 0.5	Non-substrate 0.8	Non-carcinogens 0.6	0.5	-3.6	2.7	1.2	Non-AMES toxic 0.6	Low HBT 0.8

Table 5. AdmetSAR properties of selected Compounds

Selected compounds	Blood-brain barrier (BBB) (probability)	Human intestinal absorption (HIA) (probability)	Caco2 permeability (probability)	CYP450 2D6 inhibitor (probability)	Carcinogens (probability)	Acute oral toxicity (probability)	Aqueous solubility (LogS)	Rat acute toxicity (LD50, mol/kg)	Fish toxicity (pLC50, mg/L)	Ames toxicity (probability)	Honey bee toxicity (HBT) (probability)
ZINC 13135 410	BBB + 0.8	HIA+ 0.9	Caco2- 0.5	Non-substrate 0.8	Non-carcinogens 0.9	0.6	-3.0	2.4	1.6	Non-AMES toxic 0.6	Low HBT 0.9

on docked complexes to explore the protein-ligand interactions, conformational fluctuations, structural, architectural changes, and dynamical shifts of the proteins [80]. The MD simulations aid in understanding the dynamic behavior of the complex and also highlight the important residues playing a vital role in identifying and binding the ligand [81]. To shed light on biomolecular movements within a solvated environment, RMSD, RMSF, B-factor, and radius of gyration were plotted as a function of time (Fig. 8). Investigation of the enzyme in ligand-bounded form led to the assessment of structural minor structural variations and atomic level transitions [27]. The deviation of the backbone Cα atoms was observed first for the complete production run. The average RMSD value calculated for the complex is 2.25 Å, with a maximum of 3.10 Å at 70th ns. No substantial structural movements were reported that elucidates complex stability. The average RMSF value for the complex was 1.16 Å. The regions illustrating higher fluctuation were loops: that involve the regular conversion of sheets into helix and helix into the sheet. The graph indicates that most of the residues of the active site have remained stable. The highest peak of the graph indicates the region in the loop region. The β-factor is a thermal disorderness calibrating function which stipulates the structural stability at the atomic position in term of RMSF. Therefore, its value depends on the level of atomic fluctuations which collectively contribute to the global vibrational movements of the protein and its thermal stability. The pattern of β-factor for protein is consistent with the RMSF trend. The β-factor average value calculated for the complex was 47 Å. To evaluate the structural compactness, radius of gyration was calculated as a time function. The average value of 25.3 Å for the docked protein denotes the stability of the protein structure. Snapshots at different ns of the docked enzyme complex over simulation period of 100-ns is presented in Fig. 9.

3.8 RDF Analysis

The RDF is a key tool to describe the probability of the distance 'r' between two particles in a system [82]. The distribution of atoms, molecules, and species around a specific residue of targeted protein can be described by RDF. For this, the first vital residues of the enzyme involved in hydrogen bonding with the inhibitor toward the end of the simulation were identified using an in-house script in VMD. It was found that TRP221 is the main residue from the enzyme active pocket that contributes significantly to ligand binding. The RDF graphs were generated for all the three hydrogen interactions of TRP221 atoms: HE1 and HH2 as illustrated in Fig. 10. The highest distribution was observed between HE1 atom of TRP221 and ligand N atom, at a distance of 4.50 Å with a g(r) value of 0.16. The highest distribution of TRP221: HE1 and N1 atom of ligand was observed at 3.89 Å having a g(r) value of 0.20. The third plot describes the highest distribution at 3.39 Å with a g(r) value of 0.26 between the HH2 atom of TRP221 and the N4 atom of the ligand.

3.9 Binding Free Energy Calculations

MM_PBSA/GBSA methods of the AMBER14 were used to describe the binding free energy of the

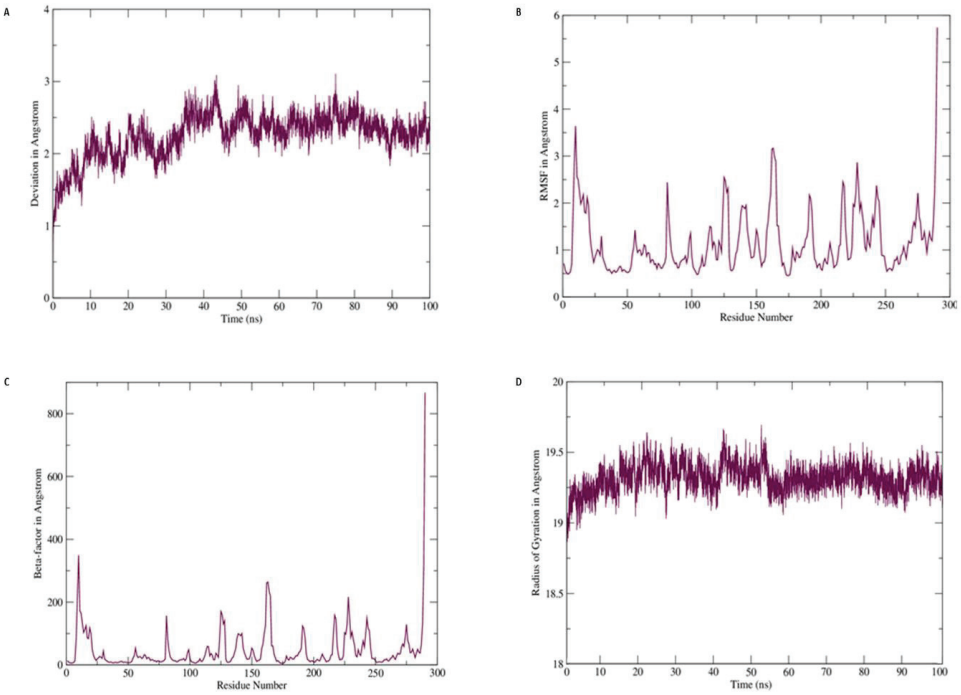


Fig. 8. Trajectories analysis.

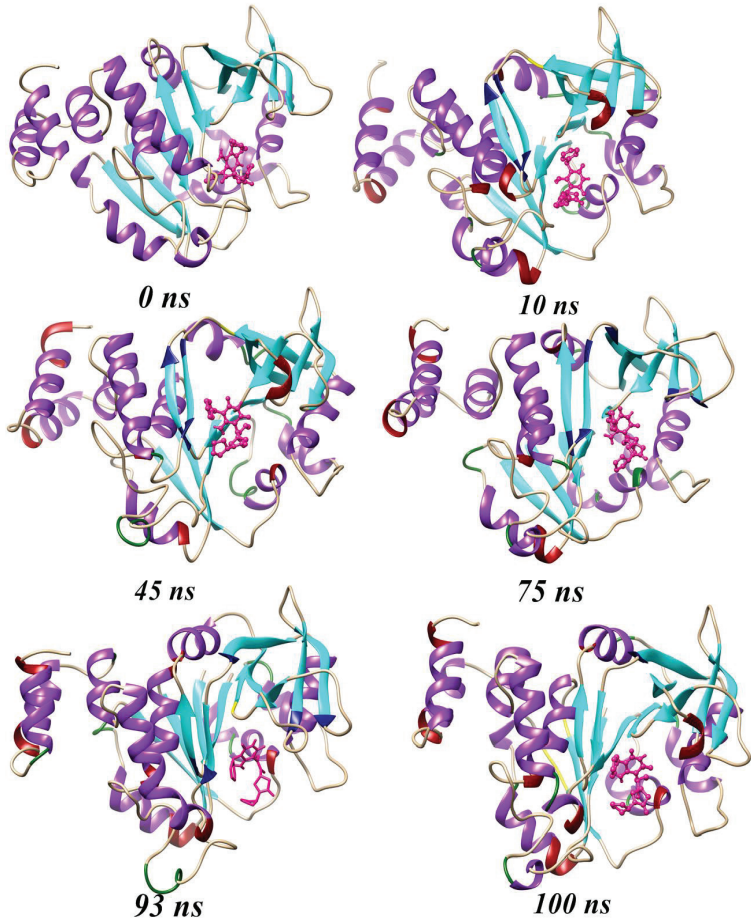


Fig. 9. Snapshots of structural variations of the docked complex at different ns. Dark red color indicates loop converted into the helix, the navy blue color indicates loop converted into the sheet, dark green indicates helix converted into a loop, and yellow color indicates the sheets convert into loops

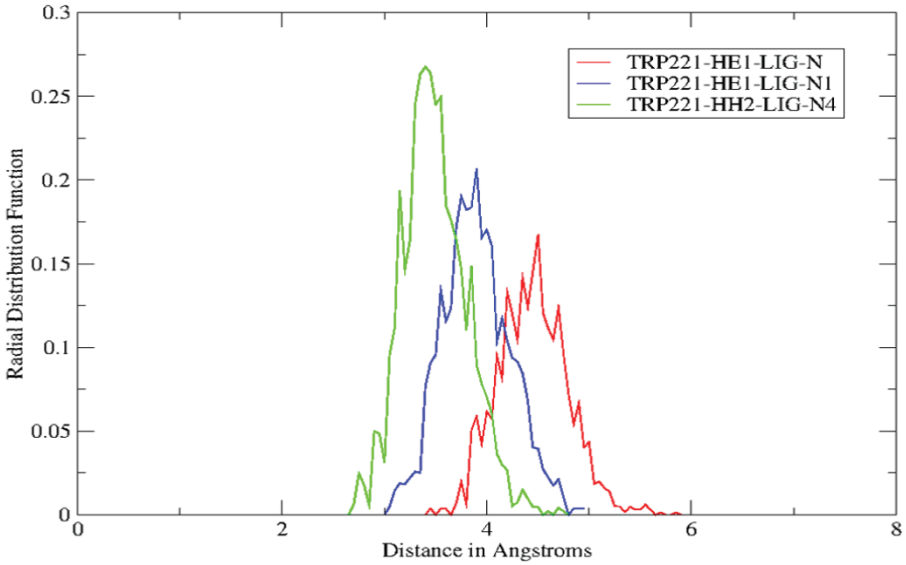


Fig. 10. RDF plots for G1PTT enzyme TRP221 atoms: HE1 and HH2.

system as well as molecular interactions between the protein and ligand. The MM_PBSA/GBSA technique combines the molecular mechanical energies with the continuum solvent approaches. The values of binding free energy were explained in Table 6. The entropy term is eliminated because of convergence problems in some cases, and it cannot be calculated.

The formation of the complex leads to highly favorable columbic interactions (−30.15 kcal/mol) as opposed to non-favorable contributions from the polar part of solvation free energy (44.79 kcal/mol in case of PB and 40.93 kcal/mol in GB). The total electrostatic contribution is 10.41 kcal/mol in GB and 15.71 in PB calculations, respectively. The binding energy value for van der waal interactions is -35.93 kcal/mol, which depicts system stability. The total binding free energies in PB and GB were determined as -1.07 and -29.59 kcal/mol, respectively. The difference in values due to the solvation energy which was 66.11 kcal/mol in MMPBSA compared to 36.85 kcal/mol from MMGBSA. The binding energy of active site residues are as follow: Leu6 (-0.2 kcal/mol), Ala7 (-0.0 kcal/mol), Gly8 (-0.03 kcal/mol), Gly9 (-0.50 kcal/mol), Ser10 (-0.55 kcal/mol), Gly11 (-0.09 kcal/mol), Arg13 (-0.36 kcal/mol), Lys23 (-0.13 kcal/mol), Gln24 (-0.01 kcal/mol), Pro83 (-0.04 kcal/mol), Gly85 (-0.0 kcal/mol), Leu86 (-0.58 kcal/mol), Leu106 (-1.22 kcal/mol), Gly107 (-0.02 kcal/mol), Asp108 (-0.56 kcal/mol), Glu194 (0.24 kcal/mol) and Gly225 (-0.17 kcal/mol). These values indicate that the overall system was stable as described in RMSD and RMSF earlier. The fluctuations pattern observed in RMSD and MM(PB/GB)SA analyses were almost identical and indicates the system stability.

Table 6. Binding energies values.

Contribution	Energy Values (kcal.mol^{-1})
Van der Waals energy	− 35.93
Columbic energy	− 30.15
Gas phase energy	− 66.44
Polar solvation free energy (PB)	44.79
Solvation free energy (PB)	66.11
Total binding free energy (PB)	− 1.07
Polar solvation free energy (GB)	40.93
Solvation free energy (GB)	36.85
Electrostatic energy (PB)	15.71
Electrostatic energy (GB)	10.41
Total binding free energy (GB)	− 29.59

4. CONCLUSION

The current study was based upon a combinatorial approach highlighting the G1PTT enzyme of *F. tularensis* as a potential drug target. The findings revealed ZINC23121280 ((3R)-N-(6-amino-1-benzyl-2,4-dioxo-pyrimidin-5-yl)-1-cyclopentyl-

5-oxo-N-propyl-pyrrolidine-3-carbox) as potential inhibitor of the enzyme. Although the inhibitor seems to show good binding efficacy for the enzyme the still these predictions required experimental in *vivo* and *in vitro* validation. These findings can be used to design and develop more specific, efficient, and potent drugs against *F. tularensis*.

5. ACKNOWLEDGEMENTS

All the authors are indebted to Pak-US Science and Technology Cooperation Program under the grant No. Pak-US2017/360 for providing financial assistance to make this possible.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. REFERENCES

1. M.E. Evans., D.W. Gregory.,W. Schaffner, and Z.A. McGee. Tularemia: a 30-year experience with 88 cases. *Medicine* 64:251–269 (1985).
2. L.D. Thomas, and W. Schaffner. Tularemia pneumonia. *Infection Disease Clinics* 24:43–55 (2010).
3. I. Golovliov., V. Baranov., Z. Krocova., H. Kovarova, and A. Sjostedt. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. *Infection Immunity* 71:5940–5950 (2003).
4. World Health Organization, WHO guidelines on Tularaemia: epidemic and pandemic alert and response (2007).
5. A. Johansson., A. Larkeryd., M. Widerstrom., S. Mortberg., K. Myrtannas., C. Ohrman., D. Birdsell., P. Keim., D.M. Wagner., M. Forsman, and P. Larsson. An Outbreak of Respiratory Tularemia Caused by Diverse Clones of *Francisella tularensis*. *Clinics Infection Diseases* 59:1546–1553 (2014).
6. A.M. Hauri., I. Hofstetter., E. Seibold., P. Kaysser., J. Eckert., H. Neubauer, and W.D. Splettstoesser. investigating an airborne tularemia outbreak, Germany. *Emerging Infectious Diseases* 16:238–243 (2010).
7. A. Desvars., M. Furberg., M. Hjertqvist., L. Vidman., A. Sjostedt., P.Ryden, and A.Johansson. epidemiology and ecology of tularemia in Sweden, 1984–2012. *Emerging Infectious Diseases* 21:32–39 (2015).
8. A. Sjostedt. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. *Annals of the New York Academy of Sciences* 1105:1–29 (2007).
9. J. Ellis., P.C.F. Oyston., M. Green, and R.W.Titball. Tularemia. *Clinical Microbiology Review* 15:631–646 (2002).
10. J. Su., J. Yang., D. Zhao., T.H. Kawula., J.A. Banas, and J.R. Zhang. Genome-Wide Identification of *Francisella tularensis* virulence determinants. *Infection Immunity* 75:3089–3101 (2007).
11. J.E. Staples., K.A. Kubota., L.G. Chalcraft., P.S. Mead, and J.M. Petersen. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. *Emerging Infectious Diseases* 12:1113–1118 (2006).
12. K.J. Kugeler., P.S. Mead., A.M. Janusz., J.E. Staples., K.A. Kubota., L.G. Chalcraft, and J.M. Petersen. Molecular epidemiology of *Francisella tularensis* in the United States. *Clinical Infectious Diseases* 48:863–870 (2009).
13. J. Celli, and T.C. Zahrt. Mechanisms of *Francisella tularensis* intracellular pathogenesis. *Cold Spring Harbor Perspective Medicine* 3:a010314 (2013).
14. W.J. Dai., W. Bartens., G. Kohler., M. Hufnagel., M. Kopf, and F. Brombacher. Impaired macrophage listericidal and cytokine activities are responsible for the rapid death of Listeria monocytogenes-infected IFN-gamma receptor-deficient mice. *Journal of Immunology* 158:5297–5304 (1997).
15. A. Tarnvik. Nature of protective immunity to *Francisella tularensis*. *Reviews of Infectious Diseases* 11:440–451 (1989).
16. S. Ahmad., S. Raza., S.W. Abbasi, and S.S. Azam. Identification of natural inhibitors against Acinetobacter baumannii D-alanine-D-alanine ligase enzyme: A multi-spectrum in silico approach. *Journal of Molecular Liquids* 262:460–475 (2018).
17. S. Ahmad., S. Raza., R. Uddin, and S.S. Azam. Comparative subtractive proteomics based ranking for antibiotic targets against the dirtiest superbug: Acinetobacter baumannii. *Journal of Molecular Graphics and Modelling* 82:74–92 (2018).
18. G.R. Marshall. Computer-Aided Drug Design. *Annual Reviews in Pharmacology Toxicology* 27:193–213 (1987).
19. S. Ahmad., S. Raza., A. Abro., K.R. Liedl, and S.S. Azam. Toward novel inhibitors against KdsB: a highly specific and selective broad-spectrum bacterial enzyme. *Journal of Biomolecular Structure and Dynamics* 0:1–20 (2018).
20. R. Gupta., D. Pradhan., A.K.. Jain, and C.S. Rai.

TiD: Standalone software for mining putative drug targets from bacterial proteome. *Genomics* 109:51–57 (2017).

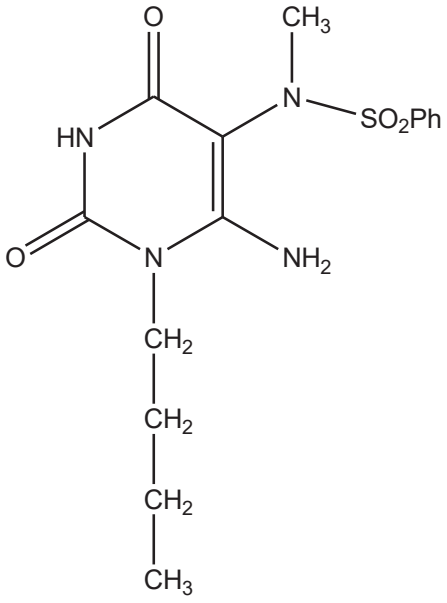
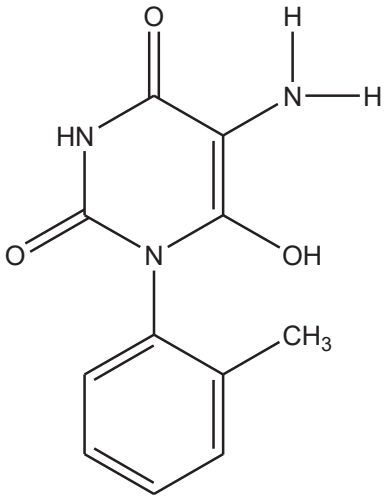
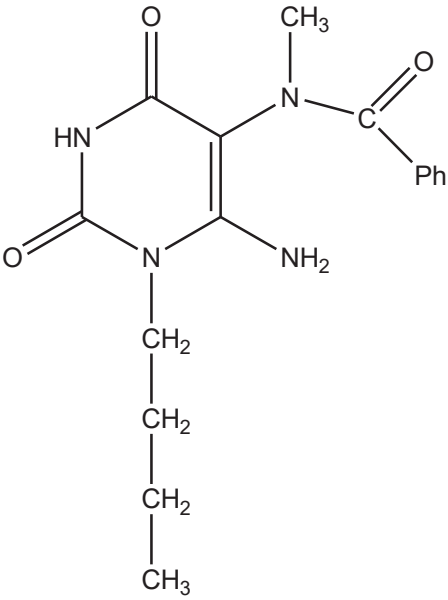
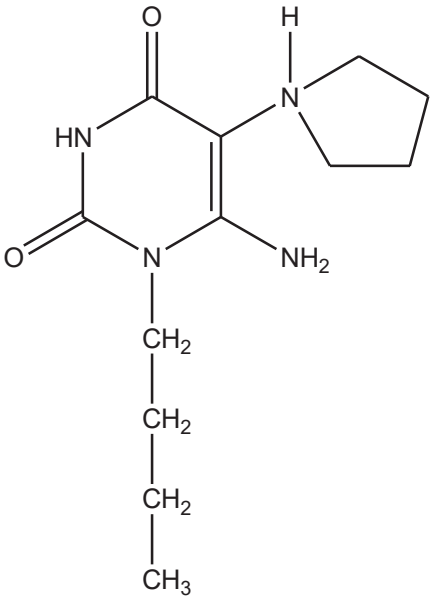
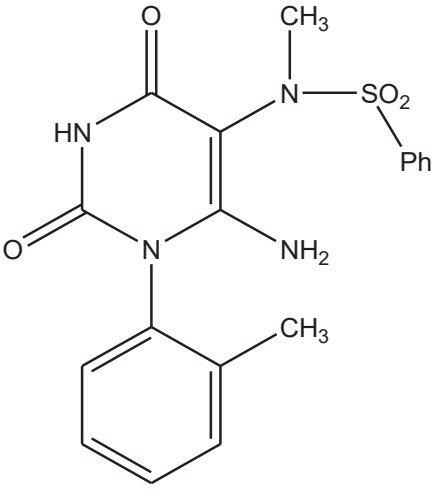
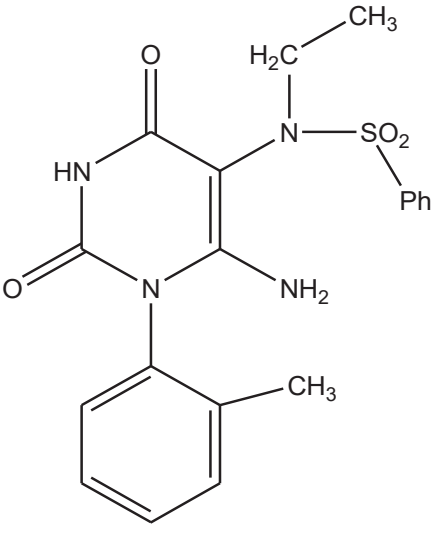
21. Z .Khalid., S. Ahmad., S. Raza, and S.S. Azam. Subtractive proteomics revealed plausible drug candidates in the proteome of multi-drug resistant *Corynebacterium diphtheriae. Meta Gene* 17: 34-42 (2018).
22. A.L. Harvey. Natural products in drug discovery. *Drug Discovery Today* 13:894–901(2008).
23. G. Sanober., S. Ahmad, and S.S. Azam. Identification of plausible drug targets by investigating the druggable genome of MDR Staphylococcus epidermidis. *Gene Reports* 7:147–153 (2017).
24. S. Ahmad., S. Raza., R. Uddin, and S.S. Azam. Comparative subtractive proteomics based ranking for antibiotic targets against the dirtiest superbug: *Acinetobacter baumannii. Journal of Molecular Graphics and Modelling* 82:74-92 (2018).
25. R. Zhang, and Y. Lin. DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucleic Acids Research* 37:D455–D458 (2009).
26. S. Zuccotti., D. Zanardi., C. Rosano., L. Sturla., M. Tonetti, and M. Bolognesi. Kinetic and crystallographic analyses support a sequential-ordered bi-bi catalytic mechanism for *Escherichia coli* glucose-1-phosphate thymidylyltransferase. *Journal of Molecular Biology* 313:831–843 (2001).
27. S. Ahmad., S. Raza., R. Uddin, and S.S. Azam. Binding mode analysis, dynamic simulation and binding free energy calculations of the MurF ligase from *Acinetobacter baumannii. Journal of Molecular Graphics and Modelling* 77:72–85 (2017).
28. C. Wang., P.H. Nguyen., K. Pham., D. Huynh., T.B.N. Le., H. Wang, P. Ren, and R. Luo. Calculating protein–ligand binding affinities with MMPBSA: method and error analysis. *Journal of Computational Chemistry* 37:2436–2446 (2016).
29. UniProt. UniProt: the universal protein knowledgebase. *Nucleic Acids Research* 46:2699 (2018).
30. S. Twine., M. Bystrom., W. Chen., M. Forsman., I. Golovliov., A. Johansson., J. Kelly., H. Lindgren., K. Svensson., C. Zingmark., W. Conlan, and A. Sjostedt. a mutant of *Francisella tularensis* strain Schu S4 lacking the ability to express a 58-kilodalton protein is attenuated for virulence and is an effective live vaccine. I*nfection Immunity* 73:8345–8352 (2005).
31. G. Sanober., S. Ahmad, and S.S. Azam. Identification of plausible drug targets by investigating the druggable genome of MDR *Staphylococcus epidermidis. Gene Reports* 7:147–153 (2017).
32. Y. Huang., B. Niu., Y. Gao., L. Fu, and W. Li. CD-HIT Suite: a web server for clustering and comparing biological sequences. *Bioinformatics* 26:680–682 (2010).
33. AR. Ortiz., P. Gomez-Puertas., A. Leo-Macias., P. Lopez-Romero, and E. Lopez-Vinas. Computational Approaches to Model Ligand Selectivity in Drug Design. *Current Topics in Medicine Chemistry* 6:41–55 (2006).
34. R. Cloete., E. Oppon., E. Murungi., W.D. Schubert, and A. Christoffels. Resistance related metabolic pathways for drug target identification in *Mycobacterium tuberculosis. BMC Bioinformatics* 17: 75 (2016).
35. Y. Moriya., M. Itoh., S. Okuda., A.C. Yoshizawa, and M. Kanehisa. KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Research* 35:182-185 (2007).
36. A. Naz., F.M. Awan., A. Obaid., S.A. Muhammad., R.Z. Paracha., J. Ahmad, and A. Ali. Identification of putative vaccine candidates against *Helicobacter pylori* exploiting exoproteome and secretome: A reverse vaccinology based approach. *Infection Genetics Evolution* 32:280–291 (2015).
37. L. Chen., J. Yang., J. Yu., Z. Yao., L. Sun., Y. Shen, and Q. Jin. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Research* 33:325-328 (2005).
38. N.Y. Yu., J.R. Wagner., M.R. Laird., G. Melli., S. Rey., R. Lo., P. Dao., S.C. Sahinalp., M. Ester., L.J. Foster, and F.S.L. Brinkman. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26:1608–1615 (2010).
39. C.S. Yu., Y.C. Chen., C.H. Lu, and J.K. Hwang. Prediction of protein subcellular localization. *Proteins Structure Function Bioinformatics* 64:643–651 (2006).
40. C.S.Yu., C.W. Cheng., W.C. Su., K.C. Chang., S.W. Huang., J.K. Hwang, and C.H. Lu. CELLO2GO: a web server for protein subcellular localization prediction with functional gene ontology annotation. PLOS ONE 9:e99368 (2014).
41. S.S. Azam, and A.Shamim. An insight into the exploration of druggable genome of Streptococcus gordonii for the identification of novel therapeutic candidates. *Genomics* 104:203–214 (2014).

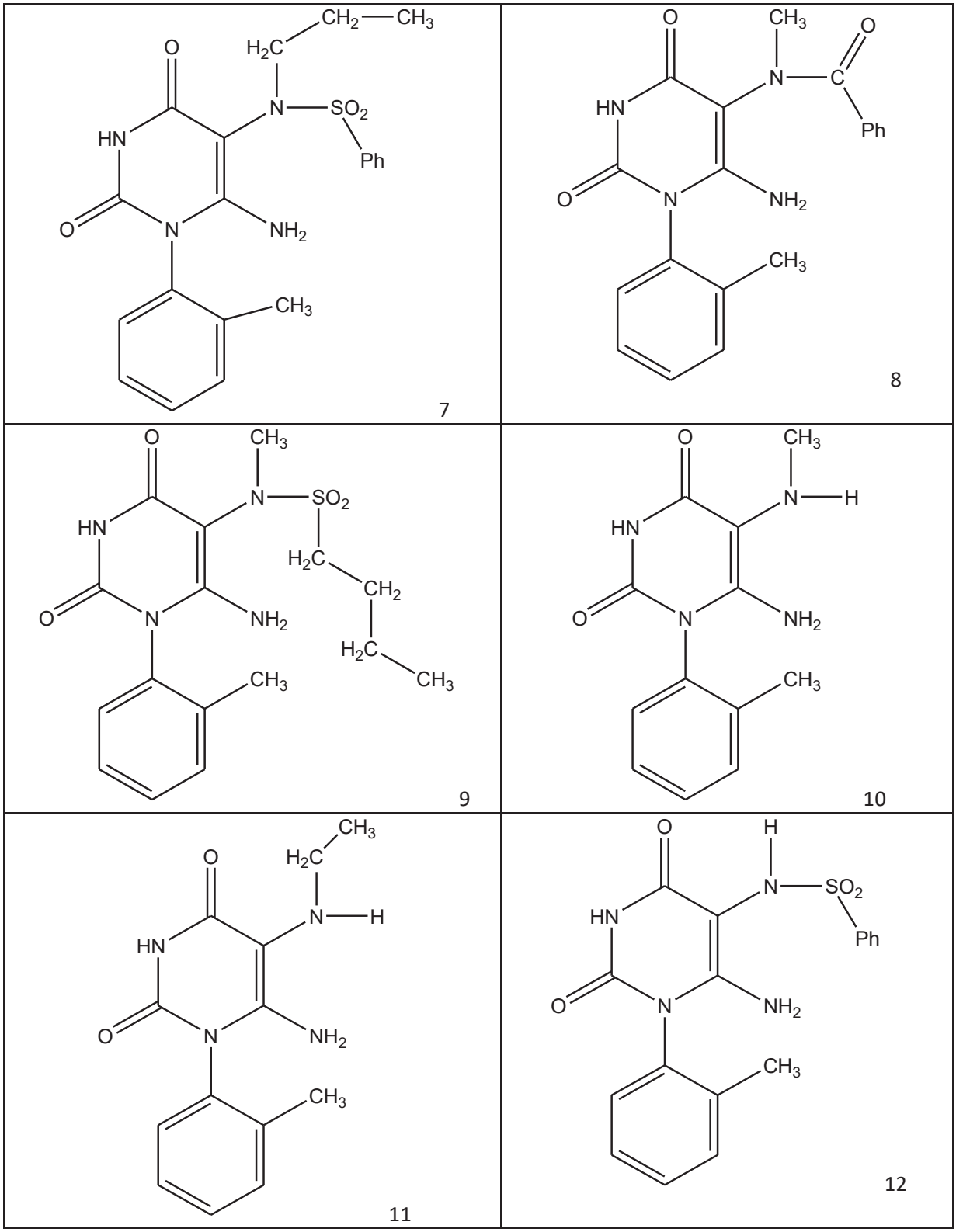
42. Y.Asad., S. Ahmad., T. Rungrotmongkol., K.E. Ranaghan, and S.S. Azam. Immuno-informatics driven proteome-wide investigation revealed novel peptide-based vaccine targets against emerging multiple drug resistant *Providencia stuartii. Journal of Molecular Graphics and Modelling* 80:238–250 (2018).
43. E.Gasteiger., C. Hoogland., A. Gattiker., S. Duvaud., M.R. Wilkins., R.D. Appel, ands Bairoch, A. Protein identification and analysis tools on the expasy server. In: the proteomics protocols handbook. Humana Press, pp 571–607 (2005).
44. B. Hesp, and A.K. Willard. Enzymes as targets for drug design. In: Enzyme Chemistry. Springer, Dordrecht, pp 119–161 (1984).
45. M. Kallberg., H. Wang., S. Wang., J. Peng., Z. Wang., H. Lu, and J. Xu. Template-based protein structure modeling using the RaptorX web server. *Nature Protocol* 7:1511–1522 (2012).
46. Kelley, L.A. and Sternberg, M.J.E. Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocol* 4:363–371 (2009).
47. K.Arnold., L. Bordoli., J. Kopp, and T. Schwede. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22:195–201 (2006).
48. A. Roy., A. Kucukural, and Y. Zhang. I-TASSER: a unified platform for automated protein structure and function prediction. *Nature Protocol* 5:725–738 (2010).
49. D. Eisenberg., R. Luthy, and J.U. Bowie. VERIFY3D: Assessment of protein models with three-dimensional profiles. In: methods in enzymology. Academic Press, pp 396–404 (1997).
50. M.J. Wiederstein, and M. Sippl. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research* 35:407-10 (2007).
51. R.A. Laskowski., J.A. Rullmannn., M.W. MacArthur., R. Kaptein, and J.M. Thornton. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *Journal of Biomolecular NMR* 8:477–486 (1996).
52. E.F. Pettersen., T.D. Goddard., C.C. Huang., G.S. Couch., D.M. Greenblatt., E.C. Meng, and T.E. Ferrin. UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25:1605–1612 (2004).
53. S. Baseer., S. Ahmad., K.E. Ranaghan, and S.S. Azam. Towards a peptide-based vaccine against Shigella sonnei: A subtractive reverse vaccinology based approach. *Biologicals* 50:87–99 (2017).
54. C.A. Lipinski., F. Lombardo., B.W. Dominy, and P.J. Feeney. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46:3–26.
55. J. Lange., L.S. Wyrwicz, and G. Vriend. (2016) KMAD: knowledge-based multiple sequence alignment for intrinsically disordered proteins. *Bioinformatics* 32:932–936 (2001).
56. B.Huang. MetaPocket: A meta approach to improve protein ligand binding site prediction. OMICS: *A Journal of Integrative Biology* 13:325–330 (2009).
57. J.D. Thompson., J. Gibson, and D.G. Higgins. Multiple sequence alignment using clustalw and clustalx. Current Protocols in Bioinformatics 1:2-3 (2002).
58. M.L. Verdonk., J.C. Cole., M.J. Hartshorn., C.W. Murray, and R.D. Taylor. Improved protein–ligand docking using GOLD. *Proteins Structure Function Bioinformatics* 52:609–623 (2003).
59. O. Trott, and A.J. Olson. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry* 31:455–461 (2010).
60. E.F. Pettersen., T.D. Goddard., C.C. Huang., G.S. Couch., D.M. Greenblatt., E.C. Meng, and T.E.L. Ferrin. UCSF Chimera—A visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25:1605–1612 (2004).
61. W. Humphrey., A. Dalke, and K.Schulten. VMD: visual molecular dynamics. *Journal of Molecular Graphics* 14:33–38 (1996).
62. A.Abro, and S.S. Azam. Binding free energy based analysis of arsenic (+3 oxidation state) methyltransferase with S-adenosylmethionine. *Journal of Molecular Liquids* 220:375–382 (2016).
63. D.A. Case., V. Babin., J. Berryman., R.M. Betz., Q. Cai., D.S. Cerutti., I.T.E. Cheatham., T.A. Darden., R.E. Duke., H. Gohlke, and A.W. Goetz. Amber 14.
64. P.K. Weiner, and P.A. Kollman. AMBER: Assisted model building with energy refinement. A general program for modeling molecules and their interactions. *Journal of Computational Chemistry* 2:287–303 (1981).
65. S. Andleeb., I.U. Din., M.K. Rauf., S.S. Azam., A. Badshah., H. Sadaf., A. Raheel., M.N. Tahir, and S. Raza. A one-pot multicomponent facile synthesis of dihydropyrimidin-2(1H)-thione derivatives using triphenylgermane as a catalyst and its binding

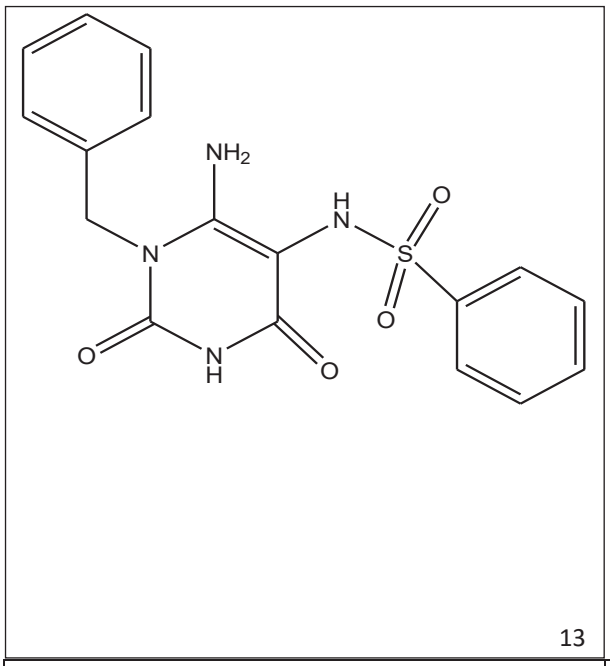
pattern validation. *RSC Advances* 6:79651–79661 (2016).

66. V. Krautler., G.W.F. Van, and P.H. Hunenberger. A fast SHAKE algorithm to solve distance constraint equations for small molecules in molecular dynamics simulations. *Journal of Computational Chemistry* 22:501–508 (2001).
67. H.C. Andersen. Molecular dynamics simulations at constant pressure and/or temperature. *Journal of Chemical Physics* 72:2384–2393 (1980).
68. D.R. Roe, and T.E. Cheatham. PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. *Journal of Chemical Theory and Computation* 9:3084–3095 (2013).
69. World Health Organization. WHO publishes list of bacteria for which new antibiotics are urgently needed. WHO Geneva Switzerland (2017).
70. C.L. Ventola. The Antibiotic Resistance Crisis. *Pharmacy and Therapeutics* 40:277–283 (2015)
71. B.M. Hassan., K. Ahmad., S. Roy., A.J. Mohammad., M. Adil., S.S. Haris., S. Khan., K.M. Amjad., I. Provazník, and I. Choi. Computer aided drug design: success and limitations. *Current pharmaceutical design* 22: 572-581 (2016).
72. J.Drews. Drug Discovery: A Historical Perspective. *Science* 287:1960–1964 (2000).
73. T.Hossain., M. Kamruzzaman., T.Z. Choudhury., H.N. Mahmood., A.H.M.N Nabi, and M.I. Hosen. Application of the subtractive genomics and molecular docking analysis for the identification of novel putative drug targets against Salmonella enterica subsp. enterica serovar Poona. *BioMed Research International* 2017: (2017)
74. K.B. Patel., E. Toh., X.B. Fernandez., A. Hanuszkiewicz., G.G. Hardy., Y.V. Brun., M.A. Bernards, and M.A. Valvano. Functional Characterization of UDP-Glucose:Undecaprenyl-Phosphate Glucose-1-Phosphate Transferases of Escherichia coli and Caulobacter crescentus. *Journal of Bacteriology* 194:2646–2657 (2012).
75. V. Sperandio., A.G. Torres, and J.B. Kaper. Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli. *Molecular Microbiology* 43:809–821 (2002).
76. G. McMullan, and J.P. Quinn. In vitro characterization of a phosphate starvation-independent carbon-phosphorus bond cleavage activity in Pseudomonas fluorescens 23F. *Journal of Bacteriology* 176:320–324 (1994).
77. R.E. Webster. The tol gene products and the import of macronmolecules into Escherichia coli. *Molecular Microbiology* 5:1005–1011 (1991).
78. A. Wadood., M. Ghufran., S.F. Hassan., H. Khan., S.S. Azam, and U. Rashid. *In silico* identification of promiscuous scaffolds as potential inhibitors of 1-deoxy-d-xylulose 5-phosphate reductoisomerase for treatment of Falciparum malaria. *Pharmaceutical Biology* 55:19–32 (2017).
79. E. Rutkowska., K. Pajak, and K. Jozwiak. Lipophilicity-- methods of determination and its role in medicinal chemistry. *Acta Poloniae Pharmaceutica -Drug Research* 70:3–18 (2013).
80. S.A. Adcock, and J.A. McCammon. Molecular dynamics: survey of methods for simulating the activity of proteins. *Chemical Reviews* 106:1589–1615 (2006).
81. S. Abbasi., S. Raza., S.S. Azam., K.R. Liedl, and J.E. Fuchs. Interaction mechanisms of a melatonergic inhibitor in the melatonin synthesis pathway. *Journal of Molecular Liquids* 221:507–517 (2016).
82. J. Donohue. Radial distribution functions of some structures of the polypeptide chain. *Proceedings of the National Academy of Sciences of the United States of America* 40:377–381 (1954).

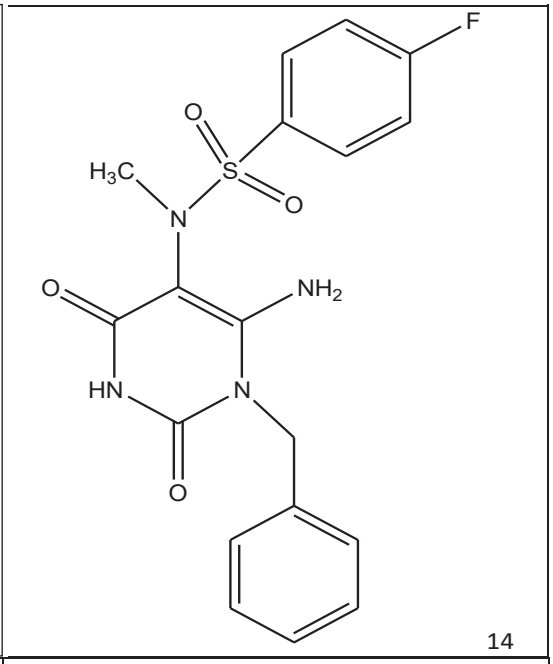
Table 1S. Supplementary Table 1

 1	 2
 3	 4
 5	 6

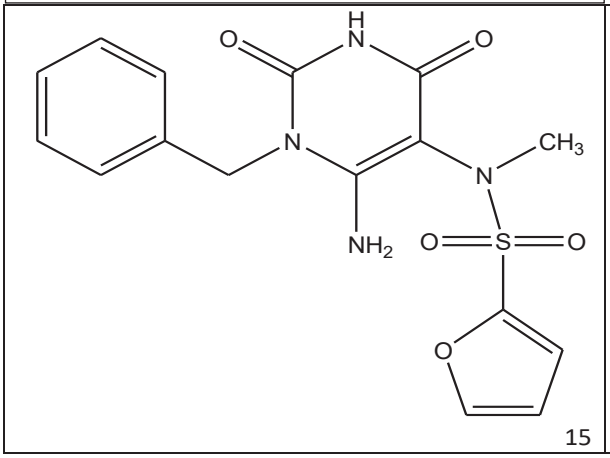

7
8
9
10
11
12



13



14

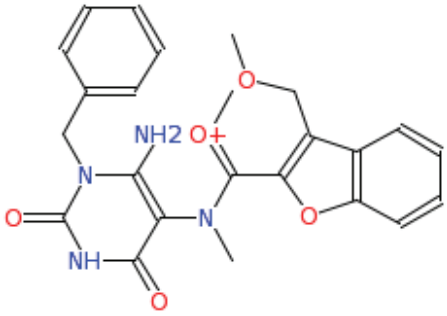
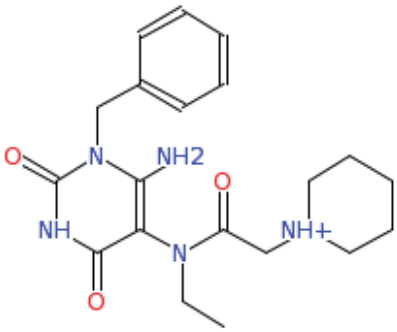
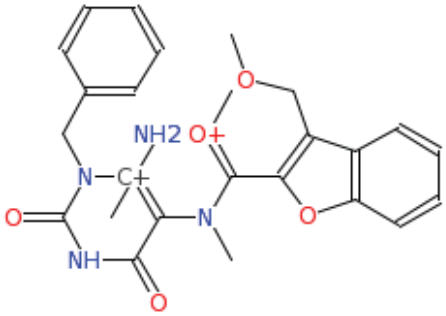
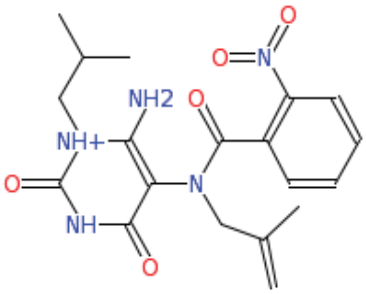
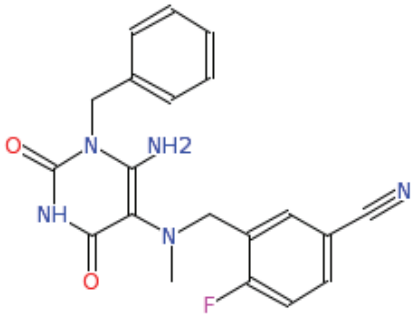
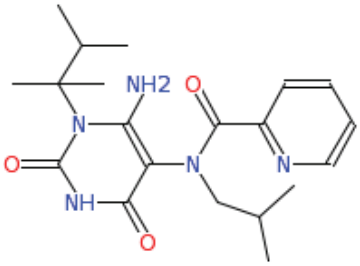
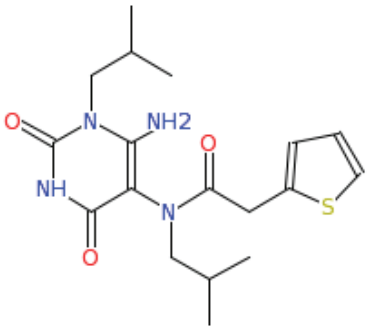
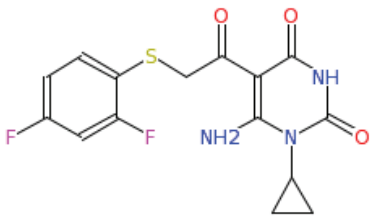


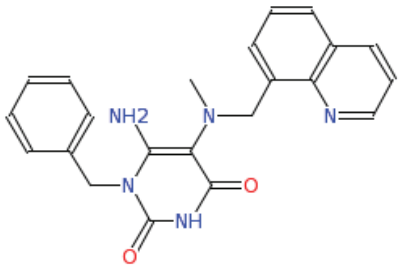
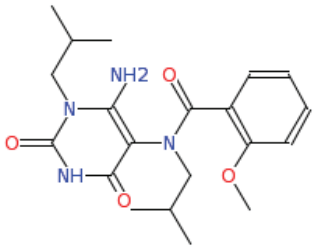
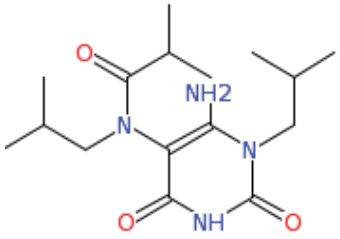
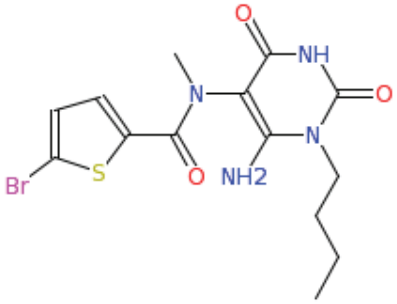
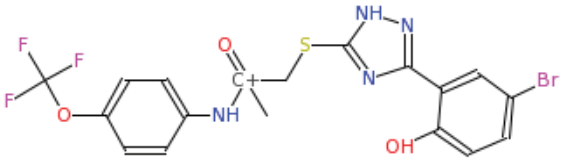
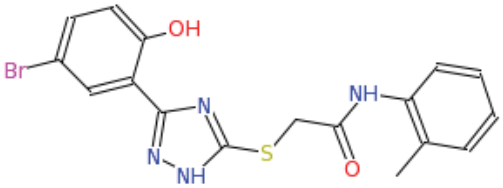
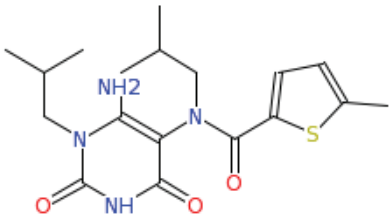
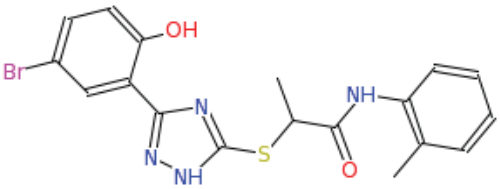
15

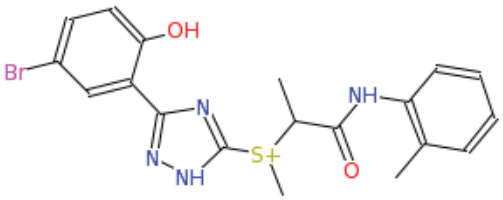
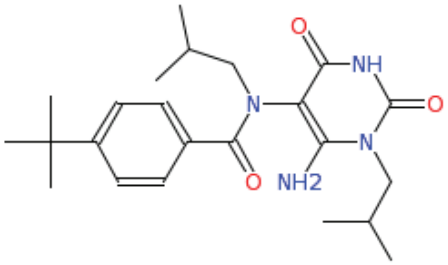
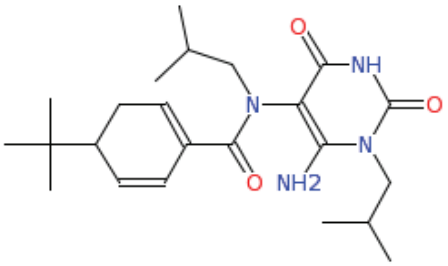
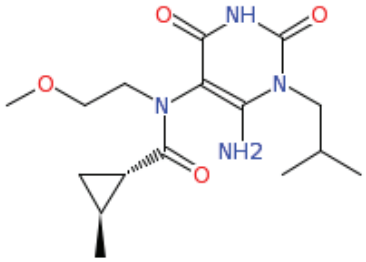
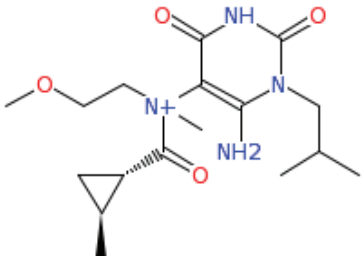
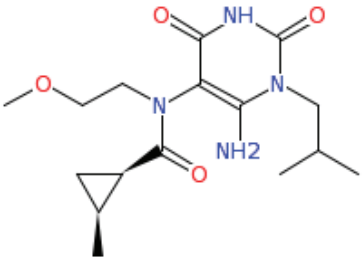
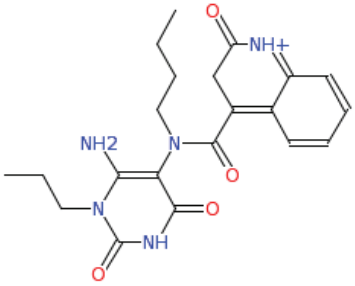
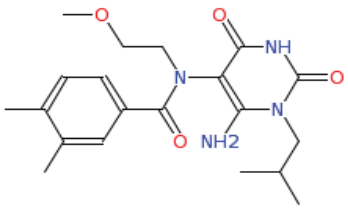
Name of Compounds

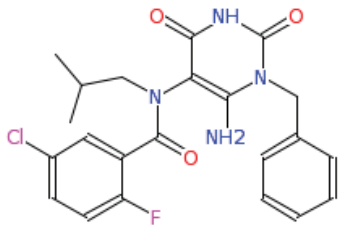
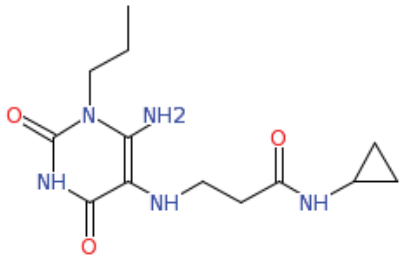
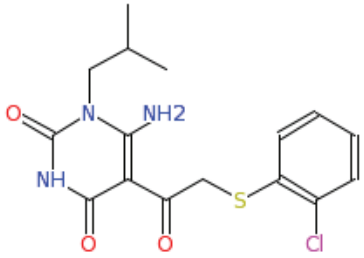
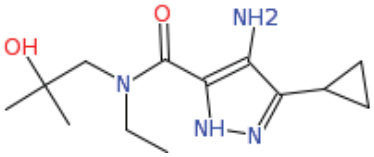
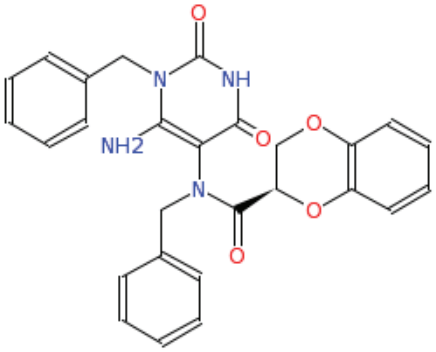
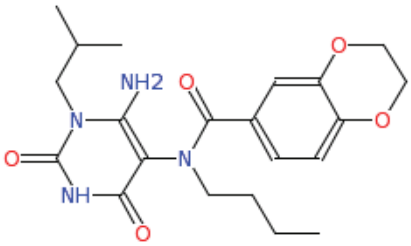
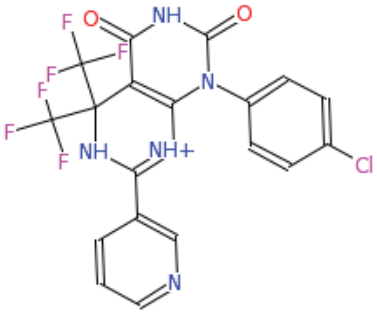
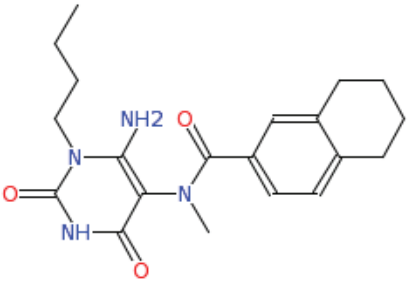
1. N-(6-amino-1-butyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylbenzenesulfonamide
2. 5-amino-6-hydroxy-1-o-tolylpyrimidine-2,4(1H,3H)-dione
3. N-(6-amino-1-butyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylbenzamide
4. N/A
5. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylbenzenesulfonamide
6. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)-N-ethylbenzenesulfonamide
7. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)-N-propylbenzenesulfonamide
8. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylbenzamide
9. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylbutane-1-sulfonamide
10. 6-amino-5-(methylamino)-1-o-tolylpyrimidine-2,4(1H,3H)-dione
11. 6-amino-5-(ethylamino)-1-o-tolylpyrimidine-2,4(1H,3H)-dione
12. N-(6-amino-2,4-dioxo-1-o-tolyl-1,2,3,4-tetrahydropyrimidin-5-yl)benzenesulfonamide
13. N-(6-amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)benzenesulfonamide
14. N-(6-amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-4-fluoro-N-methylbenzenesulfonamide
15. N-(6-amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-N-methylfuran-2-sulfonamide

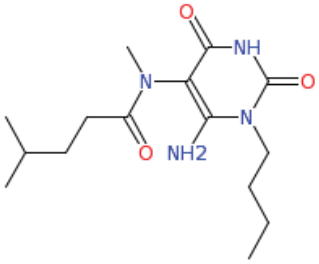
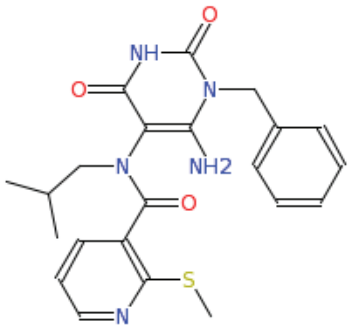
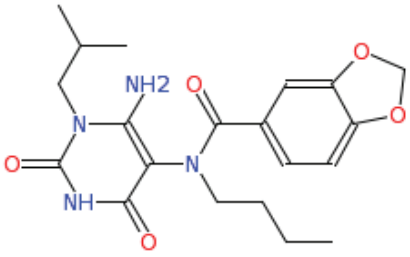
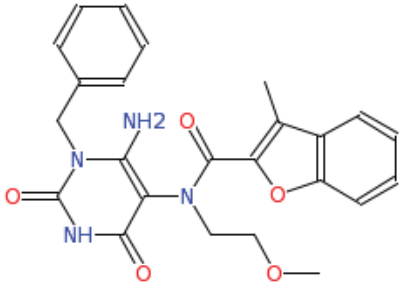
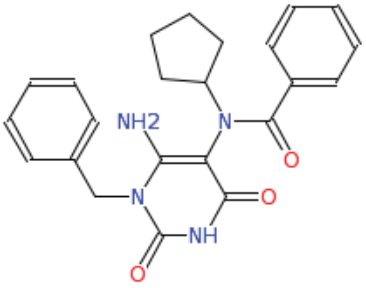
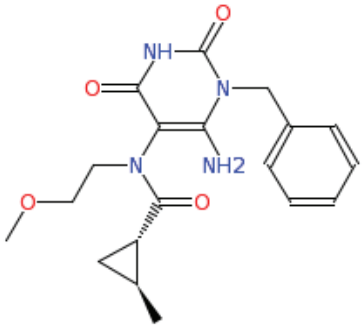
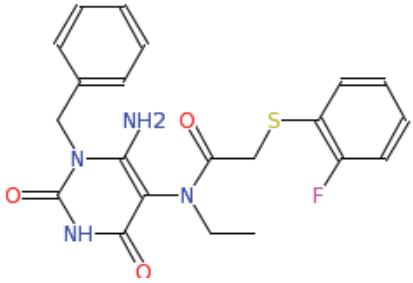
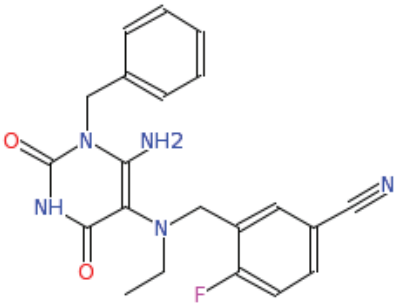
Table 2S. Supplementary Table 2

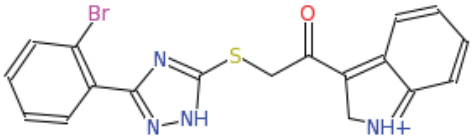
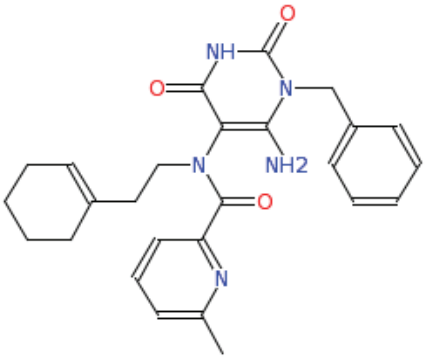
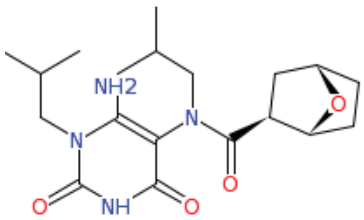
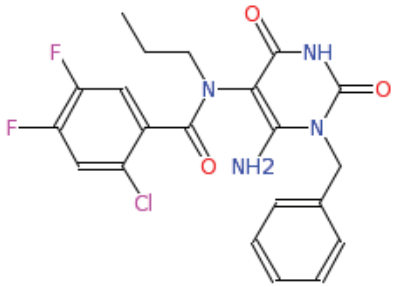
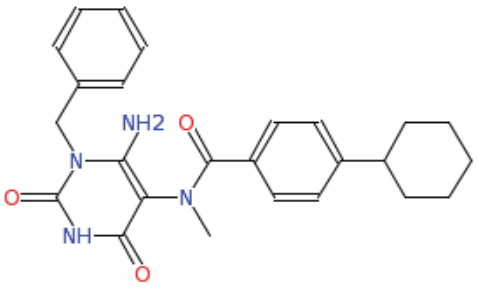
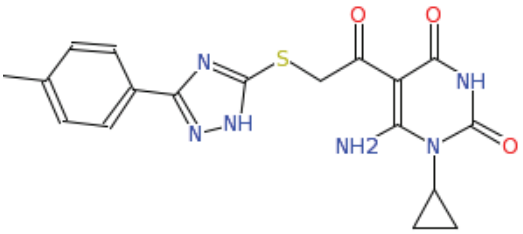
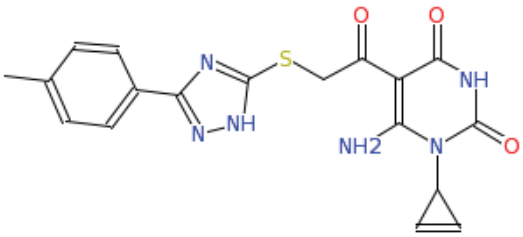
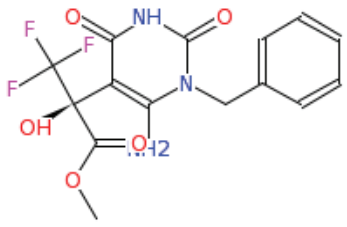
ZINC09255350 	ZINC02629047 
ZINC21083072 	ZINC20968846 
ZINC12688361 	ZINC20968850 
	ZINC09503746 

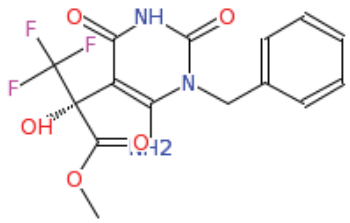
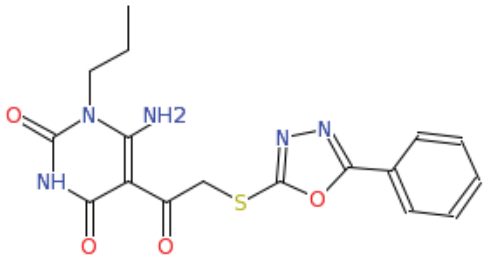
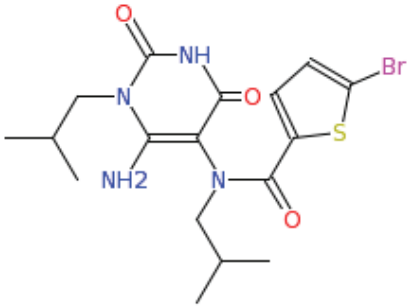
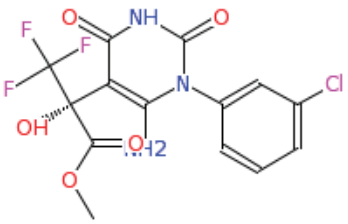
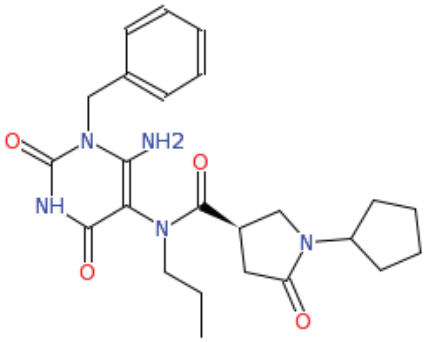
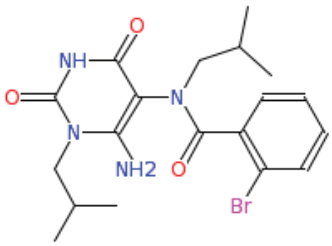
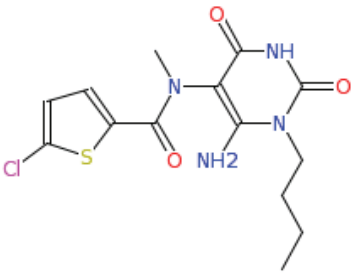
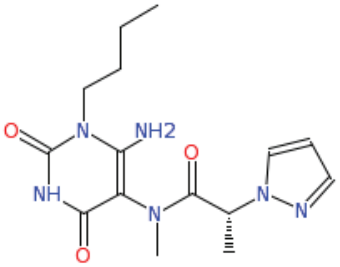
ZINC12688372 	ZINC21056754 
ZINC20968860 	ZINC14656215 
ZINC20968864 	ZINC06973908 
ZINC20968868 	ZINC14670047 

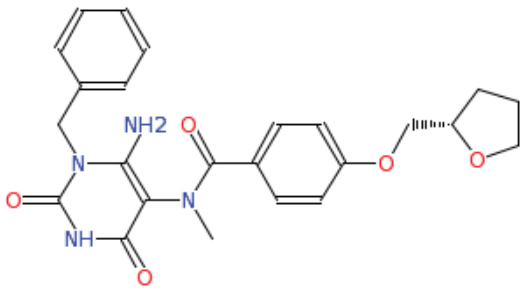
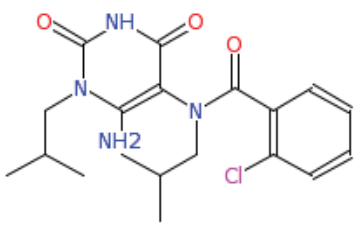
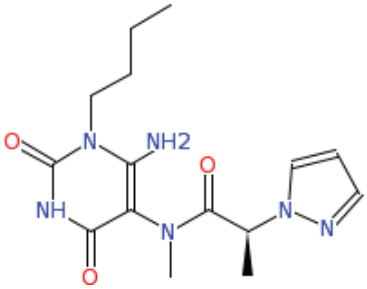
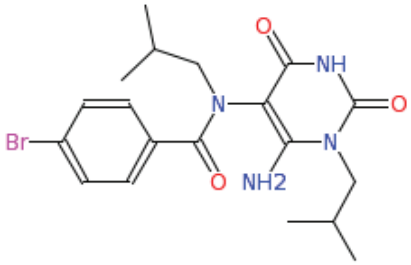
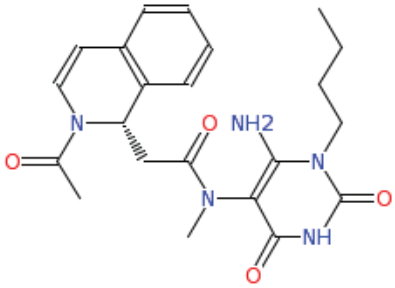
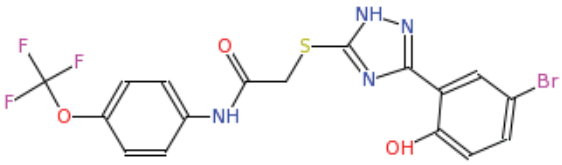
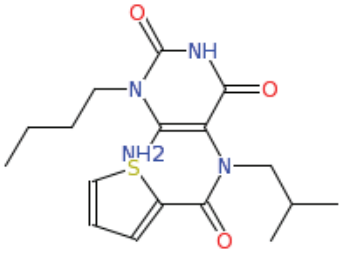
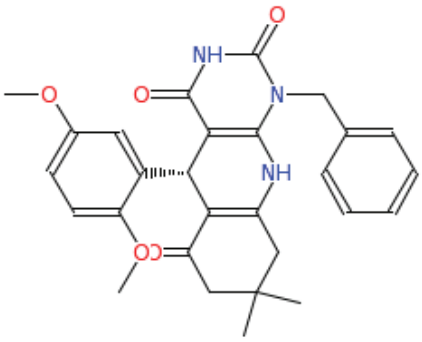
ZINC20968872 	ZINC21056767 
ZINC14656232 	ZINC57329128 
ZINC57329133 	ZINC57329136 
ZINC39944073 	ZINC57329148 

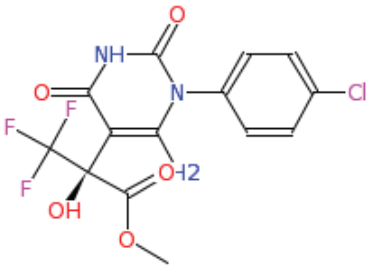
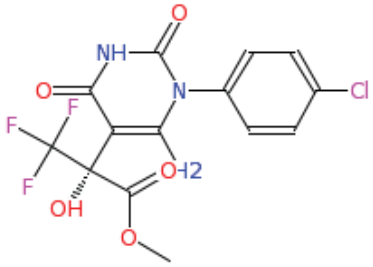
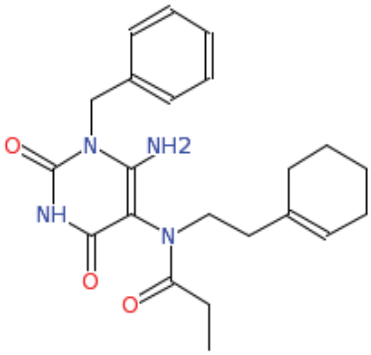
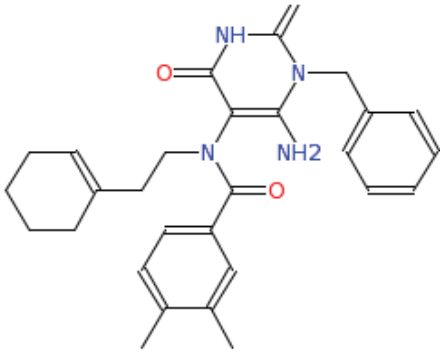
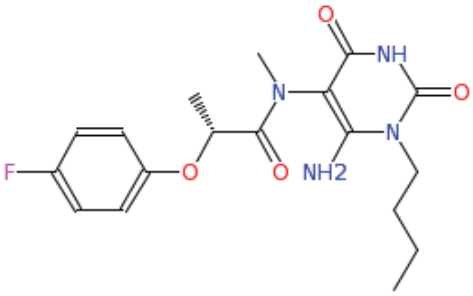
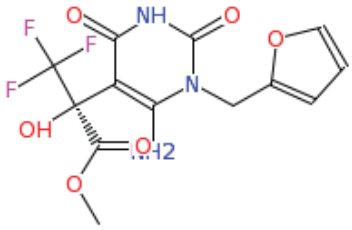
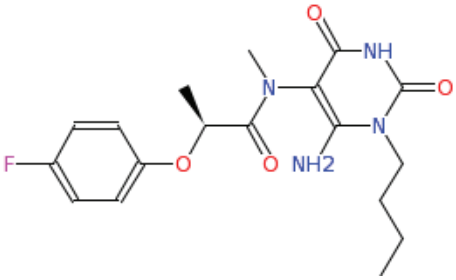
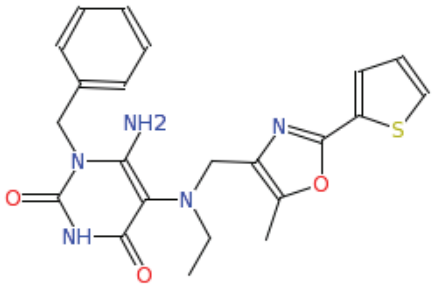
ZINC13006236 	ZINC92996529 
ZINC22066775 	ZINC92958387 
ZINC12568770 	ZINC14720788 
ZINC19848781 	ZINC15480656 

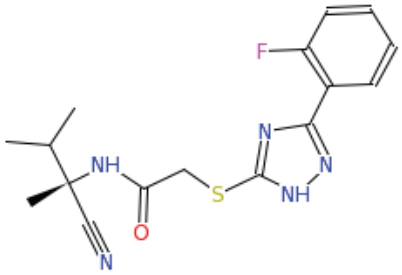
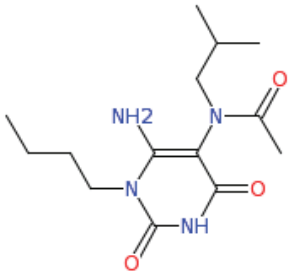
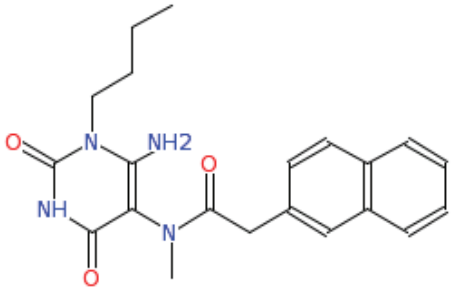
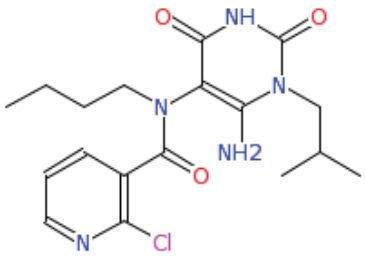
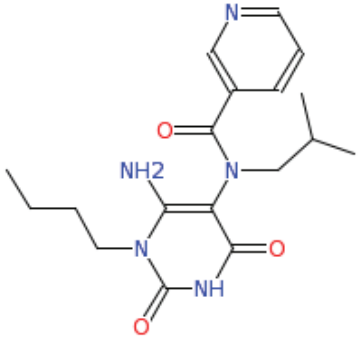
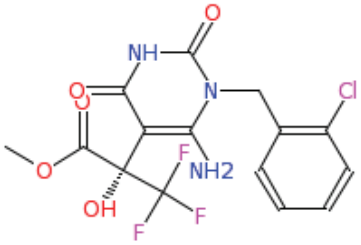
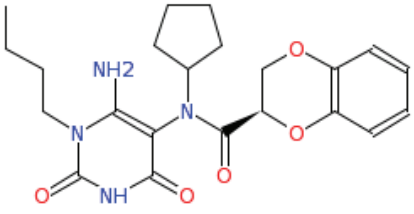
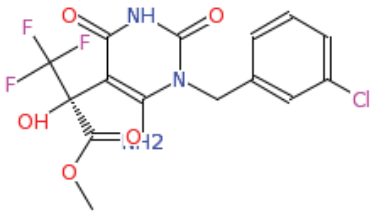
ZINC14655663 	ZINC12935584 
ZINC14720612 	ZINC14511277 
ZINC13098937 	ZINC14512098 
ZINC14584341 	ZINC10431910 

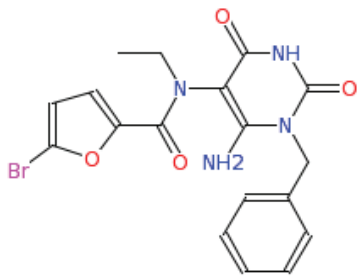
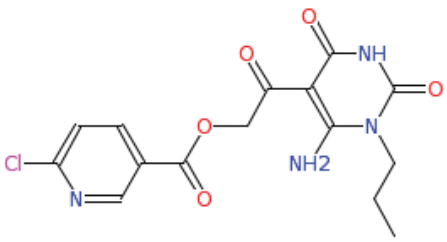
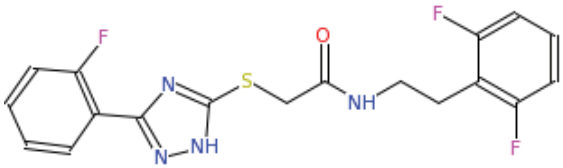
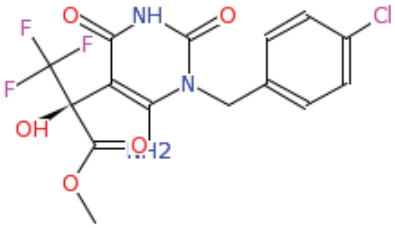
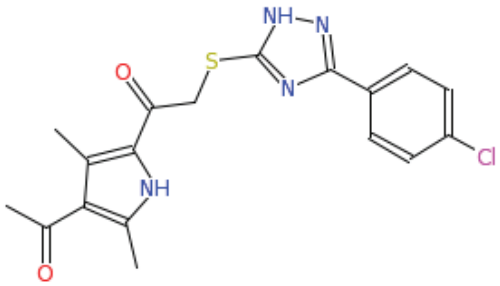
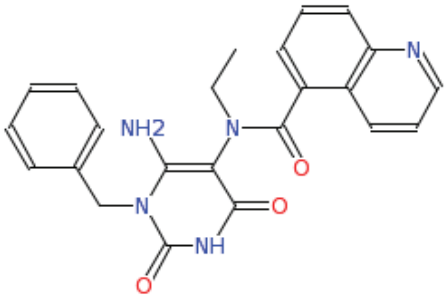
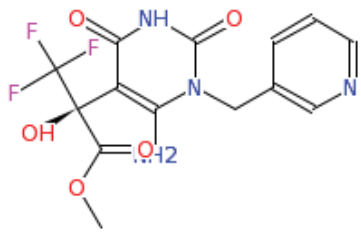
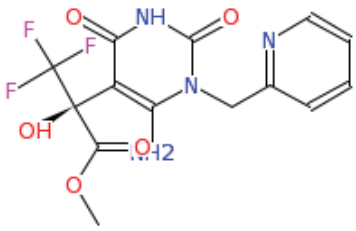
ZINC40061682 	ZINC24616811 
ZINC72645280 	ZINC12821917 
ZINC09255029 	ZINC08343860 
ZINC20989147 	ZINC12417381 

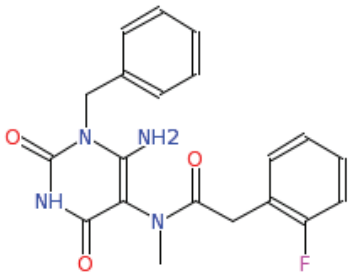
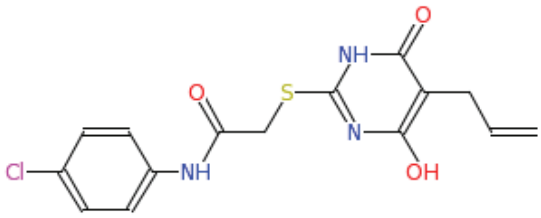
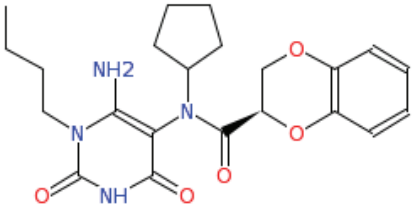
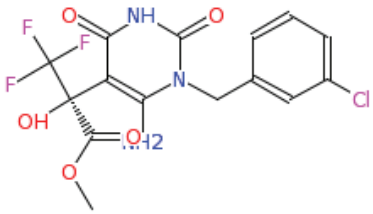
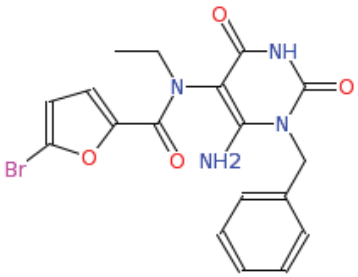
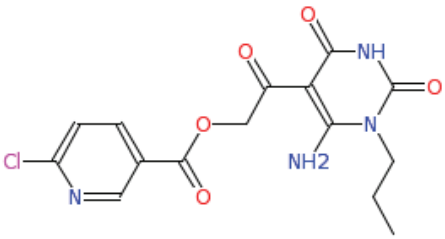
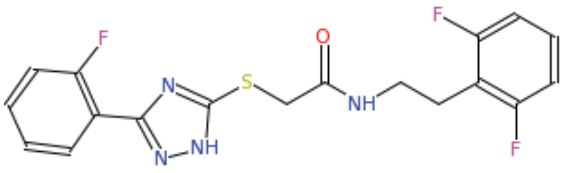
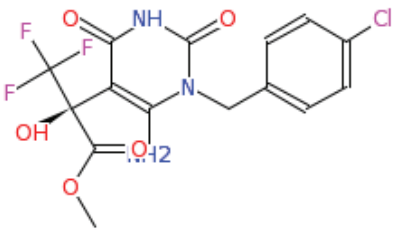
ZINC12417384 	ZINC03348170 
ZINC20989150 	ZINC78396862 
ZINC23121280 	ZINC21056728 
ZINC14656171 	ZINC65433956 

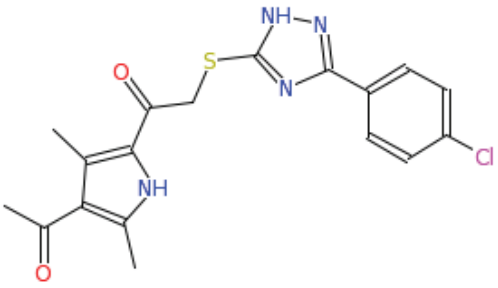
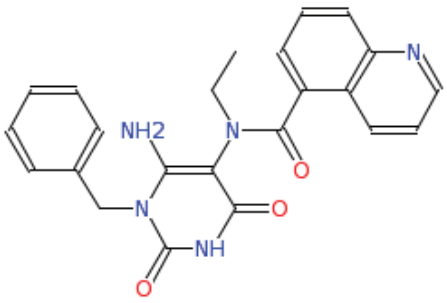
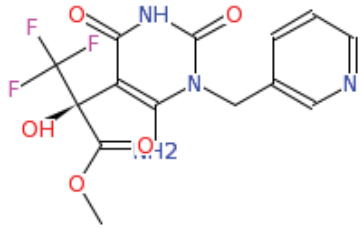
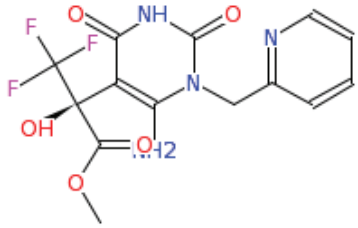
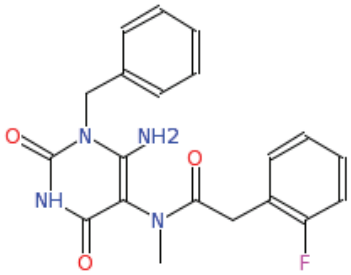
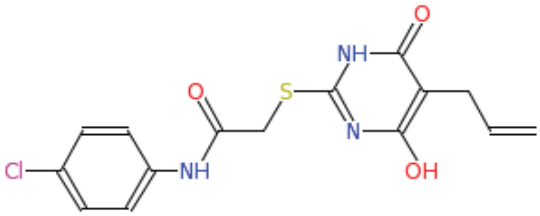
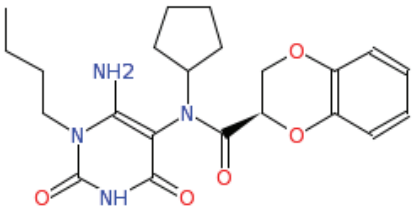
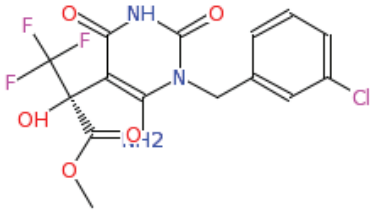
ZINC09255338 	ZINC21056731 
ZINC65433958 	ZINC21056736 
ZINC15605260 	ZINC09235457 
ZINC14670114 	ZINC44974019 

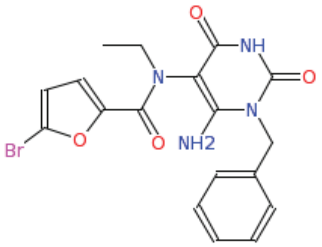
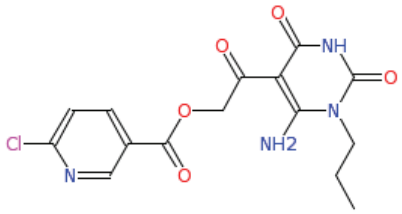
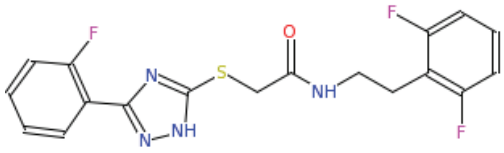
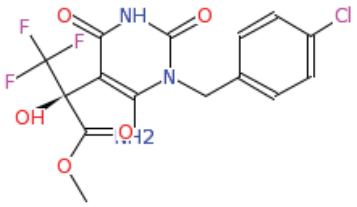
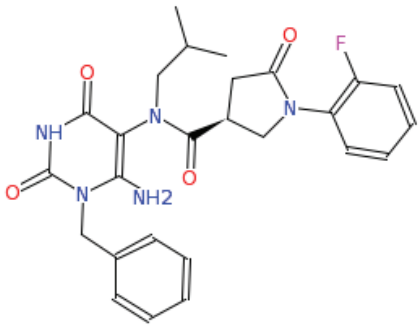
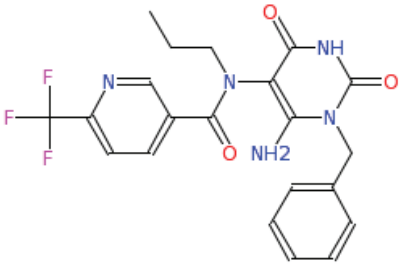
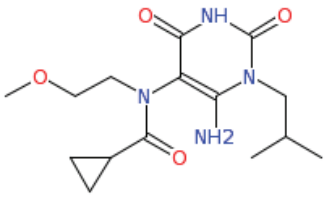
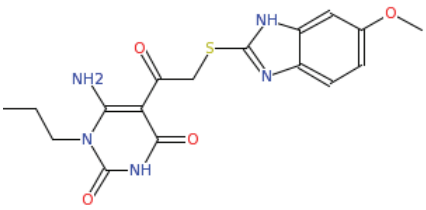
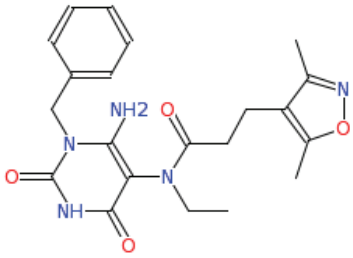
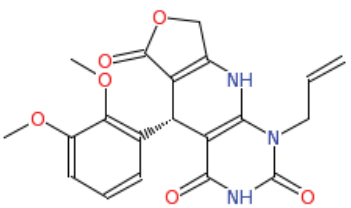
ZINC78396864 	ZINC78396865 
ZINC60392593 	ZINC14697665 
ZINC14655861 	ZINC78396872 
ZINC14655864 	ZINC11094122 

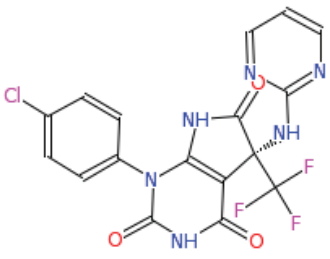
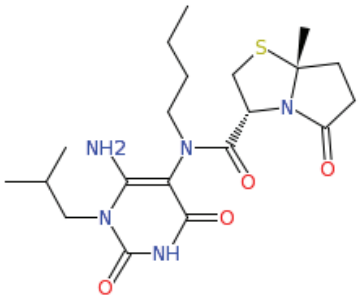
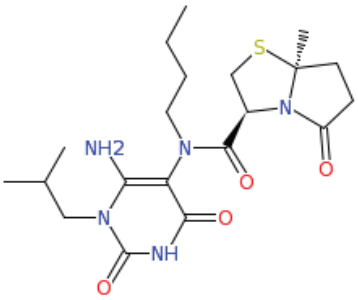
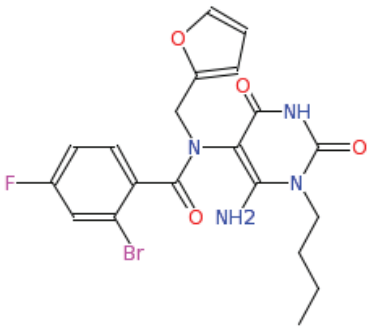
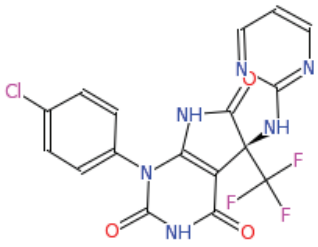
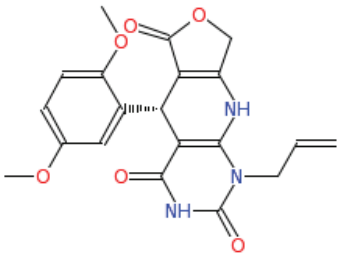
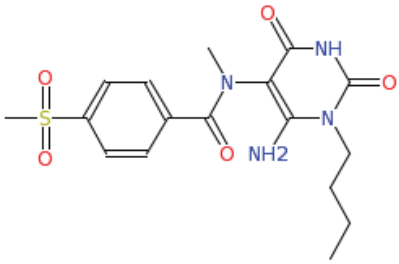
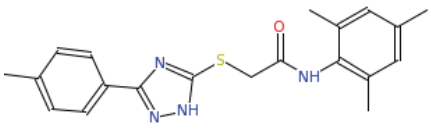
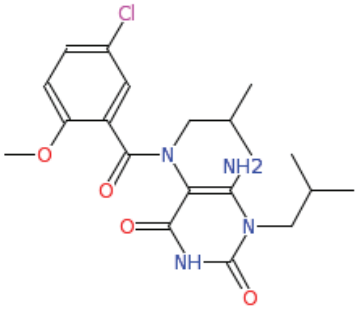
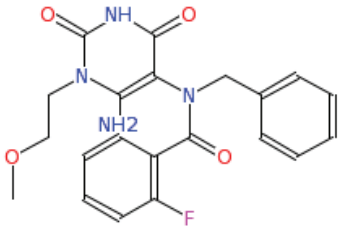
ZINC05677220 	ZINC72498202 
ZINC14655870 	ZINC14720573 
ZINC72498204 	ZINC78396879 
ZINC12804529 	ZINC78396883 

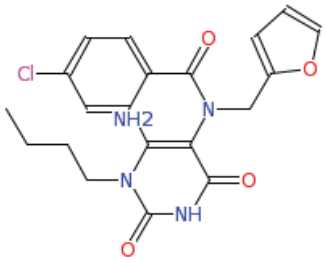
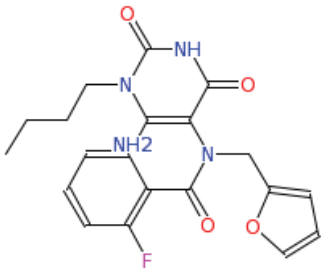
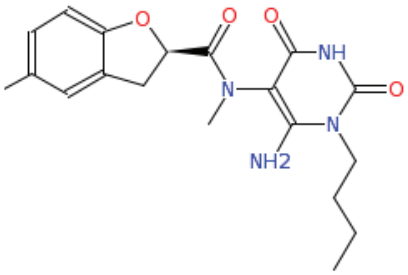
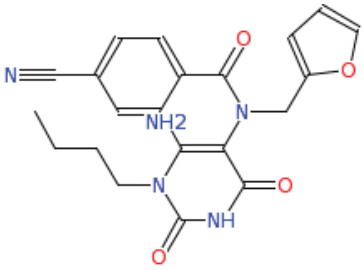
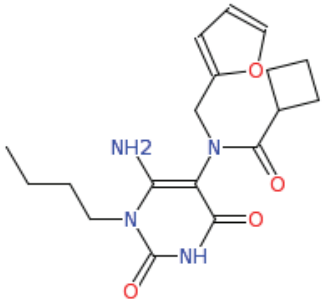
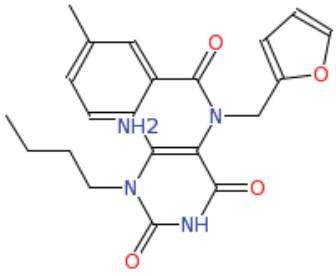
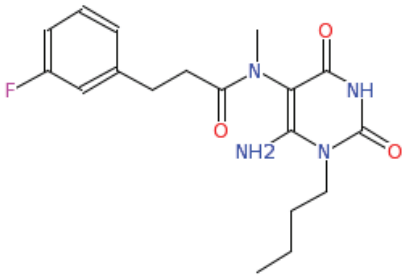
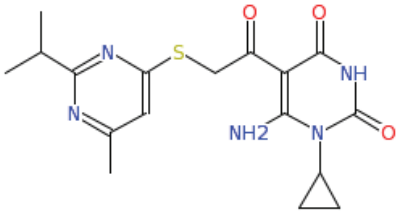
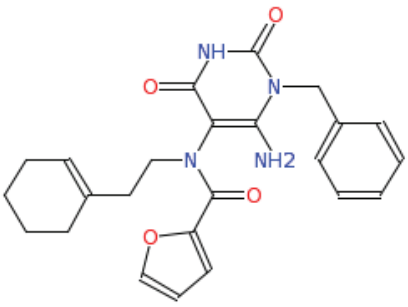
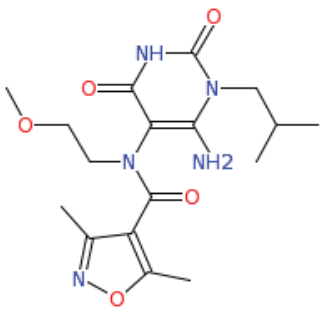
ZINC09467411 	ZINC09452073 
ZINC53264670 	ZINC78396886 
ZINC28807288 	ZINC12270700 
ZINC78396891 	ZINC78396894 

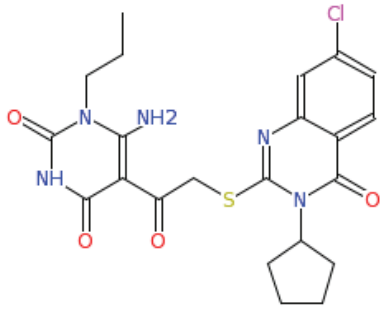
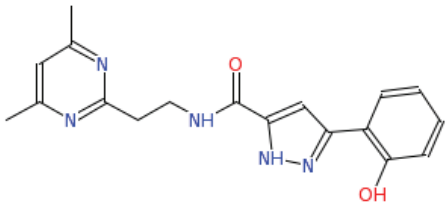
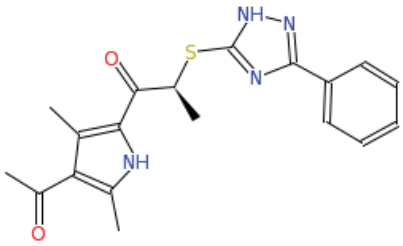
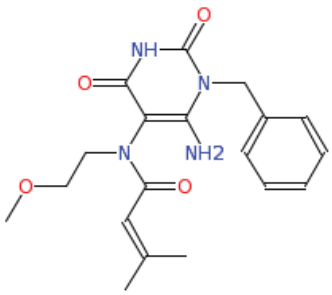
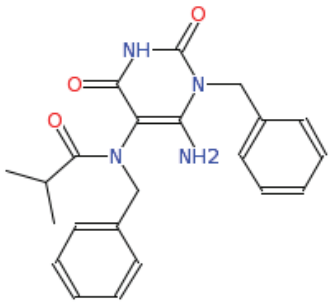
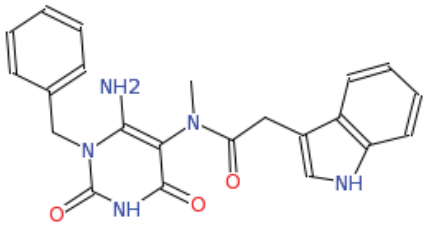
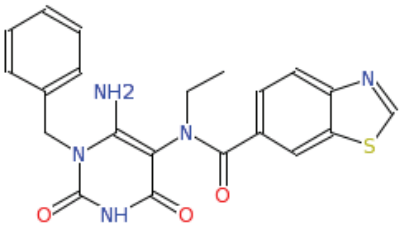
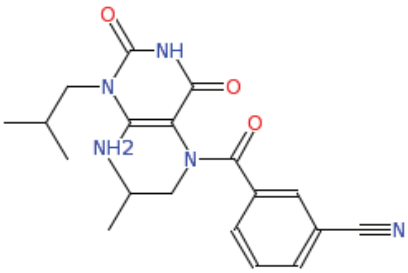
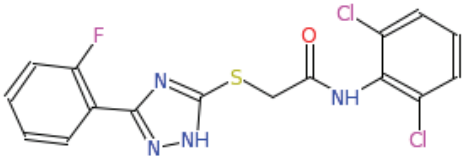
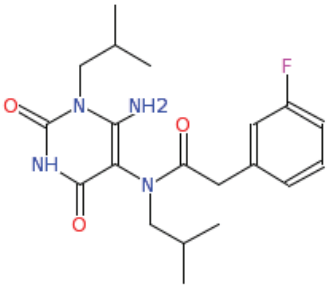
ZINC13135410 	ZINC13536709 
ZINC12804529 	ZINC78396883 
ZINC09467411 	ZINC09452073 
ZINC53264670 	ZINC78396886 

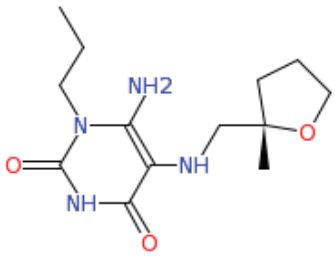
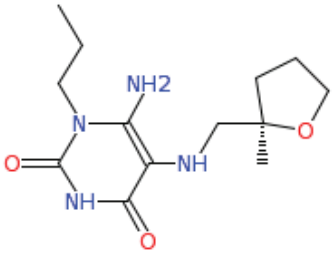
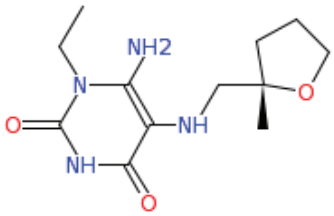
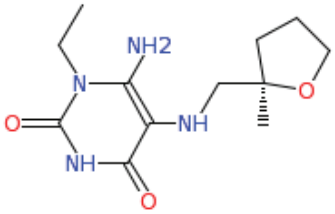
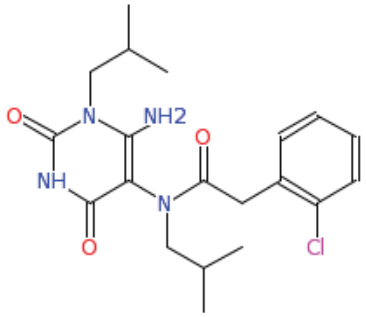
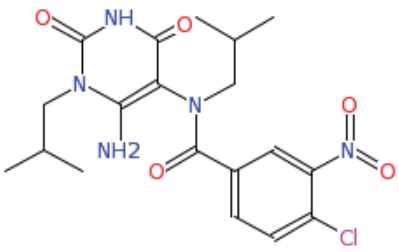
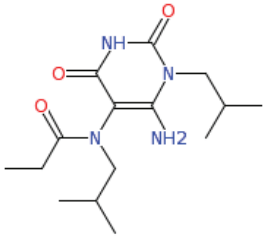
ZINC28807288 	ZINC12270700 
ZINC78396891 	ZINC78396894 
ZINC13135410 	ZINC13536709 
ZINC12804529 	ZINC78396883 

ZINC09467411 	ZINC09452073 
ZINC53264670 	ZINC78396886 
ZINC24833725 	ZINC12763362 
ZINC68713803 	ZINC04273915 
ZINC23121423 	ZINC44974155 

ZINC12360655 	ZINC24004947 
ZINC24004945 	ZINC12922825 
ZINC12360656 	ZINC44974164 
ZINC14656013 	ZINC06205277 
ZINC21082940 	ZINC08486806 

ZINC06221643 	ZINC06221644 
ZINC14119574 	ZINC06221650 
ZINC06221651 	ZINC06221653 
ZINC10442496 	ZINC08548182 
ZINC09244451 	ZINC14721420 

ZINC09509328 	ZINC95366855 
ZINC07763936 	ZINC30858638 
ZINC12873547 	ZINC07983874 
ZINC14584621 	ZINC03528638 
ZINC05678255 	ZINC21083027 

ZINC94804951 	ZINC94804952 
ZINC94804953 	ZINC94804954 
ZINC21083040 	ZINC21056718 
ZINC57329501 	

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 43-51 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)353

Pakistan Academy of Sciences

Research Article

Prevalence of Methicillin-Resistant *Staphylococcus aureus* using Molecular Biological Methods and its Antibiotic Resistance Patterns in Al-Ahsa Region of Saudi Arabia

Zafar Iqbal[1*], Muhammad Absar[1, 2], Khowlah Al-Sayel[1], Munira Al-Mulhim[1], Shouq Al-Qahtani[1], Sarah Al-Dawasari[1], Nourah Al-Mulhim[1], Nouf Fallatah[1], Maha Alomari[1], Kanza Adeel[1], Aysha Bhalli[1], Mughisuddin Ahmed[1,2], and Nawaf Alanazi[1,3]

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS)/ KAIMRC/SSBMT, National Guards Health Affairs, Al-Ahsa, Kingdom of Saudi Arabia
[2]Microbiology Section, Department of Pathology, King Abdulaziz Hospital, Al-Ahsa, Saudi Arabia
[3]Department of Pediatrics, King Abdulaziz Hospital, Al-Ahsa, Saudi Arabia

Abstract: Methicillin-Resistant *Staphylococcus aureus* (MRSA) causes many clinical manifestations. In modern healthcare systems, the frequency of MRSA infections is used as the benchmark for the quality of healthcare. MRSA-associated clinical manifestations and antibiotic resistance differ in different regions of the world. No studies had previously been carried out on MRSA in Al-Ahsa Saudi Arabia. Therefore, we studied the prevalence and antibiotic resistance patterns of MRSA in our region. Overall, 2661 patients were tested for MRSA by employing GeneXpert-based PCR assay during Jan- Dec 2018, and data was analyzed using SPSS version 24. 146 patients were MRSA positive (5.48%), with a mean age of 45.17 years and a male to female ratio of 1:1.03. The highest frequency of MRSA was in the age group 60-79 years (25.43%) The prevalence of MRSA infection was highest between August to September (p-value < 0.001). Anemia, hypoalbuminemia, and leukocytosis were associated with MRSA infections ($p < 0.001$). 87.67% of patients had community-acquired infections (CA-MRSA) ($p < 0.001$). Prevalence of CA-MRSA was the highest among the age group 60-70 years while the patient age group ≥ 80 years had the highest frequency of hospital-acquired infections ($p = < 0.0003$). Vancomycin and Linezolid showed 100% susceptibility, Penicillin, Cefoxitin, and Cefazoline 100% resistance while Oxacillin showed 98.9% resistance. The highest frequency of MRSA was found during scorching summer while the majority of the patients had CA-MRSA which necessitates precautions to be taken during top summer months. MRSA infections were significantly associated with anemia, hypoalbuminemia, and leukocytosis. Vancomycin and Linezolid could be drugs of choice for MRSA infections.

Keywords: MRSA, Epidemiology, Co-morbidities, Antibiotic resistance, Electrolyte imbalance.

1. INTRODUCTION

Staphylococcus aureus is considered as one of the most clinically significant infectious agents due to its innate pathogenicity, leading to a lot of life-threatening infections [1-2]. It can adapt to a variety of environmental conditions that lead to MRSA infections in different parts of the world in every clinical setting [2-3]. Due to this, it is the major cause of inpatient clinical infections globally [4]. Nevertheless, as many patients are treated for infections outside hospitals (outpatient) as well, MRSA is rapidly increasing as community-acquired infection [5]. Although many antibiotics are available to treat *S. aureus*, including methicillin, tetracyclines, fluoroquinolones, linezolid, and daptomycin, most of these drugs quickly lose their clinical effectiveness as *Staphylococcus aureus* can develop drug resistance [1, 6].

Received: December: 2020; Accepted: March 2021
*Corresponding Author: Zafar Iqbal <drzafarmedgen@yahoo.com>

Methicillin-resistant *S. aureus* was reported in October 1960 when some *Staphylococcus aureus* strains were found to be resistant to new antibiotic methicillin discovered just a year before [7]. MRSA had been regarded as the serious nosocomial infectious agent causing infections throughout the world that has severely affected health care and resulted in tremendous increases in health care costs [7]. Therefore, the quality of healthcare is benchmarked against the frequency of MRSA infections in modern healthcare systems. Accordingly, reliable testing of MRSA infections and their antibiotic resistance patterns is an integral component of an infection control program at any good hospital [3-6].

S. aureus isolates develop methicillin resistance when staphylococcal cassette chromosome (SCCmec) with mecA gene is inserted in the bacteria. mecA gene transcribes a modified penicillin-binding protein (PBP-2a) that cannot be inhibited by methicillin and other b-lactam antibiotics, thus causing methicillin resistance [8–11]. Many methods are available to detect and diagnose MRSA, that include oxacillin screening test, oxacillin minimum inhibitory concentration test, and oxacillin and/or cefoxitin disk diffusion method [11-12]. Nevertheless, these standard antimicrobial tests are reported to have false positive and negative results [8-10]. On the other hand, molecular biological methods including PCR-based MRSA assays that directly detect mecA gene present only in all MRSA strains and completely absent in methicillin-sensitive *S. aureus* (MSSA) isolates are regarded as the most sensitive, specific, and reproducible tests for diagnosing MRSA [13-20]. Furthermore, PCR-based assays are also recommended as confirmatory tests for the detection of MRSA in clinical samples identified by standard MRSA detection methods [7, 14]. Therefore, we employed a PCR-based molecular biological assay for the detection of MRSA in our subjects. Clinical manifestations and comorbidities associated with MRSA were also studied along with antibiotic resistance patterns.

2. MATERIALS AND METHODS

Overall, all 2661 patients suspected of MRSA infections visiting King Abdulaziz Hospital in Al-Ahsa during January 2018 – December 2018 in Saudi Arabia were included in the study. To detect MRSA in clinical samples, a fully automated PCR-based molecular biological assay (GeneXpert® MRSA assay, Cepheid, Sunnyvale, CA, USA) was employed that carried out all steps of pre-PCR (DNA extraction), PCR amplification, and post-PCR (fluorescent detection of PCR-amplified fragments) per manufacturer's instructions [14]. All the specimens were processed by using standard microbiological methods and susceptibility testing for determining resistance to different antibiotics was carried out by broth microdilution using Vitek-II (fully-automated identification and susceptibility testing instrument) per Clinical and Laboratory Standards Institute (CLSI) guidelines 2012 [15].

Data obtained from the electronic medical records (EMR) was incorporated and stored in Microsoft excel. Statistical analysis was carried out using SPSS version 24 (IBM Corp., Armonk, N.Y., USA) and conducted in form of descriptive and inferential statistics. A p-value of equal to or less than 0.05 (corresponding to 95% confidence level) was taken as significant for all statistical tests. The study was approved from the Scientific Committee (SC) and Institutional Review Board (IRB) of our institute.

3. RESULTS

Out of a total of 2661 patients, 146 (5.45%) patients were detected positive for MRSA with 74 females (50.7%) and 72 males (49.3%). The ratio of male to female patients was 1:1.03 ($p = 0.47$). Patients' mean age in the study was 45.17 years. MRSA infections were most common in the age group 60-79 years (25.43%) Figure 1.

Out of 146 patients, 87.67% of patients had a community-acquired infection and 12.23% acquired during hospitalization (Figure 2: Table1) which was based on whether the infection was detected from the sample taken within 48 hours of hospital admission or later, respectively. Our result clearly illustrated a significantly higher incidence rate of community-acquired MRSA infections than hospital-acquired ($p < 0.001$).

Findings indicate that the patient group ≥ 80 years had the highest frequency of hospital-

Table 1. Frequency of hospital-acquired, and community-acquired MRSA infections

Acquisition of infections	Number	Percentage %	P-value
Hospital-Acquired MRSA	18	12.33%	<0.001
Community-Acquired MRSA	128	87.67%	

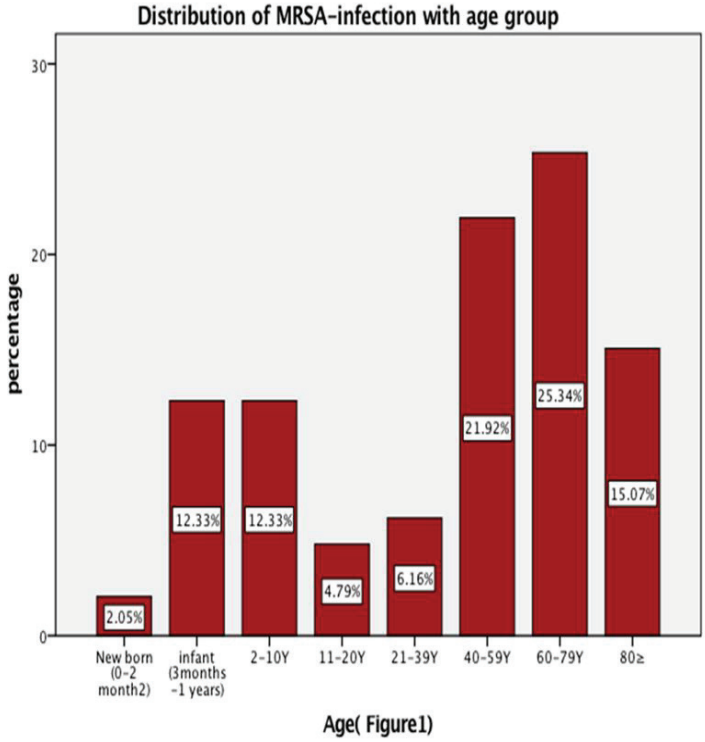


Fig. 1. Frequency of MRSA infections in different age groups

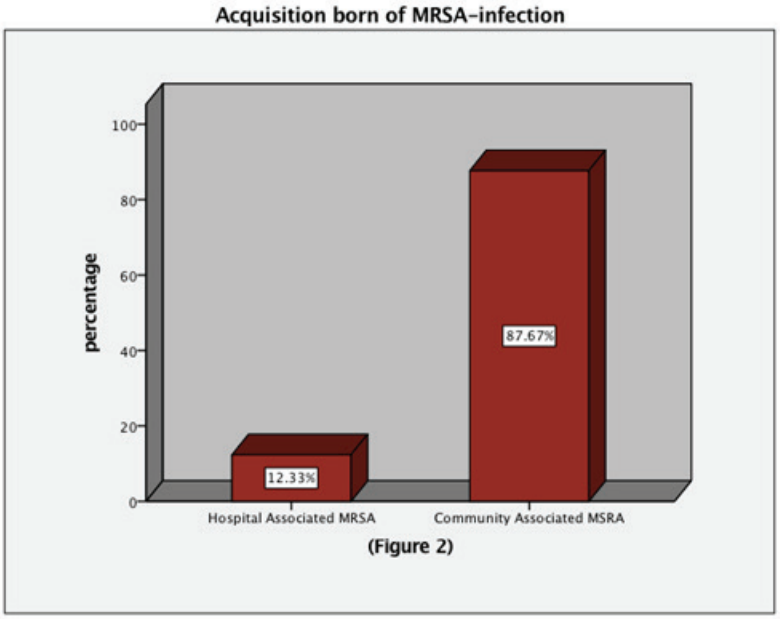


Fig. 2. Prevalence of hospital-acquired, and community community-acquired infections

acquired infections. However, in community-acquired MRSA age groups, 60-70 years had the highest frequency followed by 40-59 years age group with a (p-value < 0.0003) Figure 3.

A high prevalence of MRSA infection was observed during the scorching summer of 2018. Out of 146 cases, 20 (13.70%) were diagnosed in August, and 17 (11.64%) cases were diagnosed in September. Thus, it indicates the highest rate of infection with MRSA was in the period between August to September with a p-value of < 0.001 (Figure 4).

The Association of MRSA infections with different hematological parameters was studied. MRSA patients had leukocytosis in 26 cases (16.4%), neutrophilia in 32 cases (20.1%), eosinopenia in 21 (13.2%) cases, low RBC count in 40 cases (25.2%), decreased hemoglobin in 62 cases (39%), and decreased hematocrit in 55 cases (34.6%) (p < 0.001) Table 2.

The effect of MRSA infection on different electrolytes was also studied in our patients. There was a decrease in carbon dioxide in 60 MRSA patients (37.7%), hypoalbuminemia in 39 patients (24.5%), hypocalcemia in 33 patients (20.8%), and hyponatremia in 58 patients (36.5%). On the other hand, there was a noted elevation in the anion gap in 59 patients (37.1%), and creatinine in 32 patients (20.1%) (p < 0.001) (Table 3).

There was a significant association between various age groups and albumin levels in MRSA patients. Among patients ≥60 years of age 25 patients (17.1%) presented hypoalbuminemia. (P=0.002) (Table 4).

Antimicrobial susceptibility minimum inhibitory concentration (MIC) testing for MRSA isolates indicated that all tested samples (100%) were resistant to Penicillin, Cefoxitin, Cefazoline, 98.9% resistant to Oxacillin, 22.3% resistant to Clindamycin, 29.4% resistant to Erythromycin, 27% resistant to Sulfamethoxazole, 12.9% resistant to Moxifloxacin, 8.2% resistant to Nitrofurantoin, 10.5% resistant to Gentamicin, and 1.1% resistant to Tigecycline. Our results were based on using *S.aureus* (ATCC29213, MRSA negative control), *S.aureus* (ATCC43300, MRSA positive control), and *S. aureus* (ATCCBAA-1026, Cefoxitin screen MRSA positive control) in MIC studies. Moreover, 77.6% of the isolates were susceptible to Clindamycin, 72.9% for Sulfamethoxazole 70.5% for Erythromycin 87% for Moxifloxacin, 91.7% for Nitrofurantoin, 89.4% for Gentamicin, 98.8% for Tigecycline, 98.9% for vancomycin, and 100% susceptibility to Linezolid. Therefore, our results indicate that Linezolid, vancomycin, and Tigecycline are the best treatment options for MRSA infections in our region. Because in the cases of vancomycin only one case 1.1% presented a resistance response however linezolid showed no resistance. No significant correlation was found between the acquisition of infection and the antimicrobial susceptibility (MIC) (Table 5).

4. DISCUSSION

Our findings show that the prevalence of Methicillin-Resistant *S. aureus* is higher in elderly patients in Al- Ahsa region. Our results are following a report from Kuwait documenting MRSA frequency of 81.6% infections in elderly patients [21]. The reason for the higher rate of infection among elderly patients might be due to a weakened immune system at older ages [21]. Our study also showed that community-acquired MRSA infections are significantly higher than hospital-acquired infections in our region, which has been reported by others as well [2, 3, 21]. The factors contributing to higher frequencies of community-acquired MRSA infections may be sharing of contaminated personal equipment, living in overcrowded poor areas, unsealed wounds, etc. [3, 21]. This necessitates the need to increase public awareness about MRSA as part of infection control programs at the community level.

We also report an increase in the incidence of MRSA infections in the warmer seasons especially between the months from August to September. Our results are endorsing other similar reports in this regard. For example, a study conducted in Iowa, in the USA showed 47.3% of MRSA patients infected during summer [22]. The heat rise and subsequently more sweating along with compromised personal hygiene increase chances of the infection transmission in summer [22]. The Centers for Disease Control and Prevention (CDC) and the National Collegiate Athletic Association

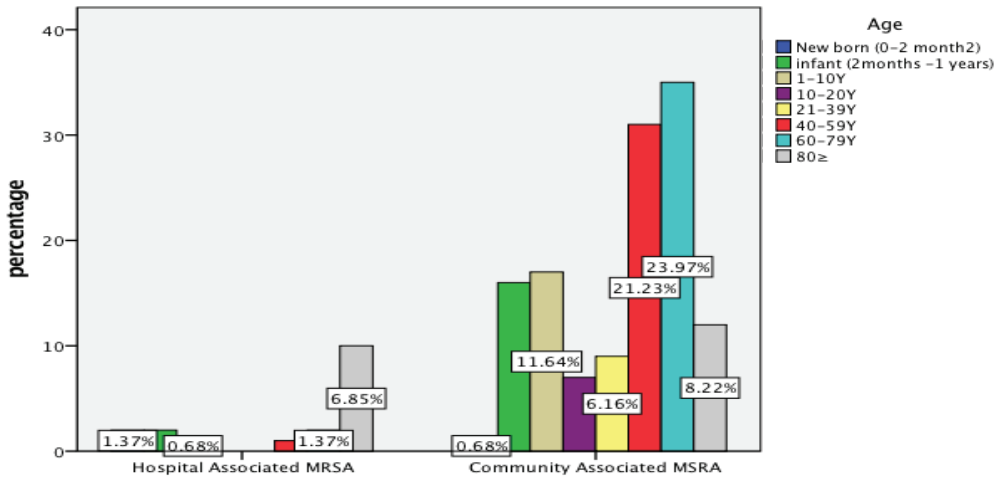


Fig. 3. Association of age groups with the hospital & Acquired MRSA infections

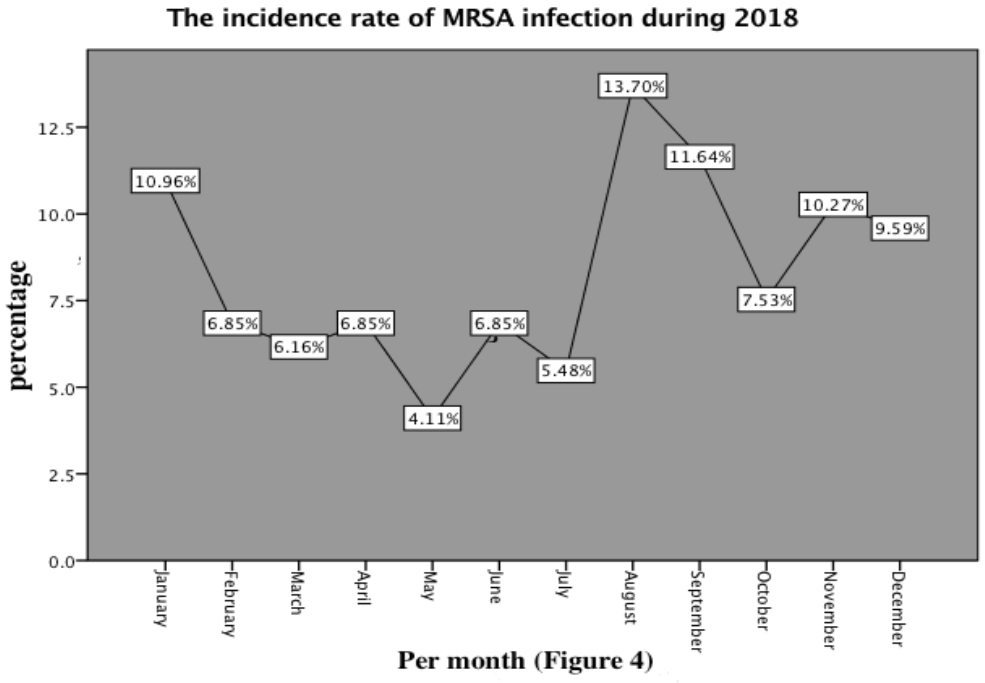


Fig. 4. Frequency of MRSA infections with different periods in the year

Table 2. Association of MRSA infections with different hematological parameters

CBC Result	Low	High	Critical	P-value
White blood cell	6 (3.8%)	26 (16.4%)	1 (0.6%)	< 0.001
Neutrophil	3 (1.9%)	32 (20.1%)	0%	< 0.001
Eosinophil	21 (13.2%)	2 (1.3%)	0%	< 0.001
Red blood cell	40 (25.2%)	14 (8.8%)	0%	< 0.001
Hemoglobin	62 (39%)	3 (1.9%)	6 (3.8%)	< 0.001
Hematocrit	55 (34.6%)	3 (1.9%)	0%	< 0.001

Table 3. Effect of MRSA infection on different electrolyte

Chemistry results	Low	High	Critical	P-value
Carbon dioxide	60 (37.7%)	5 (3.1%)	2 (1.3%)	(p < 0.001).
Anion gap	1 (0.6%)	59 (37.1%)	0 (0 %)	(p < 0.001).
Albumin	39 (24.5%)	0 (0 %)	0 (0 %)	(p < 0.001).
Calcium	33 (20.8%)	5 (3.1%)	1 (0.6%)	(p < 0.001).
Sodium	58 (36.5%)	7 (4.4%)	1 (0.6%)	(p < 0.001).
Creatinine	6 (3.8%)	32 (20.1%)	5 (3.1%)	(p < 0.001).

Table 4. Association between MRSA infection and hypoalbuminemia

Age	Low	Normal	High	Not available
60-79Y	11 (7.5%)	21 (14.4%)	0	5 (3.4%)
≥80Y	14 (9.6%)	6 (4.1%)	0	2 (1.4%)

Table 5. Antibiotic resistance and susceptibility patterns of MRSA isolates

Antibiotic susceptibility	Cefazoline	Cefoxitin	Penicillin	Linezolid	Oxacillin	Tigecycline	Nitrofurantoin	Gentamicin	Moxifloxacin	Vancomycin	Clindamycin	Sulfamethoxazole	Erythromycin
Resistance	**100%**	**100%**	**100 %**	**0%**	**98.8%**	**1.1%**	**8.2%**	**10.5%**	**12.9%**	**1..1%**	**22.3%**	**27%**	**29.4%**
Susceptible	**0%**	**0%**	**0%**	**100 %**	**1.1%**	**98.8%**	**91.7%**	**89.4%**	**87%**	**98.8%**	**77.6%**	**72.9%**	**70.5%**

(NCAA) have formulated relevant guidelines to suppress the spread of MRSA among athletes by educating them on personal hygiene and taking showers after working out.

We studied the association of different hematological parameters with MRSA infections. Our findings showed that 25.2% of patients had anemia and 24.5% had hypo-albuminuria. A study conducted in Kuwait showed that "most patients were anemic and presented with hypoalbuminemia" [21] which supports our results. It has been reported in a study carried out by Japan Epidemiological Association that the patients with hypoalbuminemia had an enhanced risk for MRSA infections (P<0.005) [6]. A study conducted in India showed that low hemoglobin levels were significantly associated with MRSA infections [23]. These findings may have important implications in the characterization and clinical management of patients with MRSA infections.

In this study, we found that most of our MRSA isolates manifested resistance to Penicillin, Cefoxitin, Cefazoline, and Oxacillin. The process of the resistance is arbitrated through the "mec operon", a portion of the Staphylococcal cassette chromosome (SCCmec), and the resistance is manifested due to the expression of the mecA gene [8-11]. This gene transcribes penicillin-binding

protein 2a (PBP2a), a protein with reduced binding capacity for b-lactamase inhibitors including methicillin [24]. Moreover, our analyses showed that 27% of the cases presented resistance against sulfamethoxazole. On the other hand, several studies showed that sulfamethoxazole is as potent as vancomycin [25]. Some other studies reported an emerging resistance of MRSA against sulfamethoxazole globally and it was found to be low in developed countries (20% or less) as compared to developing countries, i.e. in India 85-97%% MRSA isolates were reported resistant to sulfamethoxazole [26, 27]. A possible cause for this resistance in developing countries could be the unregulated use of antibiotics [28]. In the present study, Clindamycin showed 77.6% Susceptibility (18). Inconsistent percentages of clindamycin resistance were revealed in two other countries, i.e. the USA and India [29, 30]. MRSA causing hospital-acquired infections are frequently resistant to erythromycin and clindamycin. (CDC) [28]. Furthermore, our findings show 89.4% susceptibility to Gentamycin. A similar study conducted in Egypt reported above 90% susceptibility to gentamycin and clindamycin by Community-acquired MRSA isolates [31], Therefore these antimicrobial agents are a better treatment choice for MRSA infections. In the same study carried out in Egypt, the optimal response elucidated by MRSA isolates was found to be for Linezolid (100 % susceptibility) and for Vancomycin and Tigecycline (98.8% susceptibility) [31] which is in accordance with our findings. It is globally acknowledged that Vancomycin is the gold standard for treating MRSA [32]. Nevertheless, the use of Vancomycin has some limitations, for example, it is expensive, causes toxicity, has a short half-life, and needs refrigeration [33]. Linezolid is a good substitute for vancomycin and it has got the USA and European drug regulatory authorities' approvals for the treatment of nosocomial pneumonia already [34]. Tigecycline has recently been reported to be a safe and potent antibiotic for the treatment of MRSA infections, with 99.9% effectiveness in a clinical setting that almost equals linezolid [35]. It shows the clinical significance of our studies to show other antibiotics as a substitute for vancomycin in case of resistance to this effective drug by MRSA in our region.

In the present study, a correlation between the hospital-acquired or community-acquired MRSA infections and susceptibility of different antibiotics was not statistically significant that could be attributed to the low number of patients with MRSA infections. Globally, various studies show that community acquired-MRSA strains manifest less resistance to non-beta-lactamase inhibitors than hospital-born MRSA strains due to intrinsic genetic differences between two types of MRSA isolates [36, 37]. A recent study carried out in Shandong China reported that most community-acquired MRSA isolates showed clindamycin and erythromycin resistance (88.6% and 78.3%, respectively) while 91.7% of hospital-acquired strains MRSA isolated showed resistance to clindamycin only. Nevertheless, it reported 100% susceptibility to vancomycin, linezolid, and tigecycline by the hospital- as well as community-acquired MRSA isolates [36] that are in accordance with our findings. Moreover, it further manifests the clinical significance of our studies that provides important information about the clinical management of antibiotic-resistant MRSA isolates.

5. CONCLUSION

The highest frequency of MRSA was found during the peak summer season. The majority of the patients had community-acquired MRSA infections which necessitate more hygienic habits to be adopted during scorching summer months, specifically for elderly patients who had statistically high infection rates. Our study finds that MRSA infections were significantly associated with anemia, hypoalbuminemia, and leukocytosis along with electrolyte imbalance that may help in the better clinical management of patients with MRSA infections. Vancomycin could be the most effective antibiotic while Linezolid and Tigecycline could be good options for treating MRSA infections in case of vancomycin resistance.

6. ACKNOWLEDGEMENTS

The author acknowledge the cooperation of staff working at Clinical Microbiology Unit, Department of Pathology, and ITS Department at King Abdulaziz Hospital, Al-Ahsa, Saudi Arabia.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. REFERENCES

1. N.A. Turner, B.K. Sharma-Kuinkel, S.A. Maskarinec, E.M. Eichenberger, P.P. Shah, M. Carugati, T.L. Holland, and V.G. Fowler. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nature Reviews of Microbiology,* 17(4):203-218 (2019).
2. J.H. Song, P.R. Hsueh, D.R. Chung, K.S. Ko, C.I. Kang, K.R. Peck, et al. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *Journal of Antimicrobial Chemotherapy,* 66:1061–1069 (2011).
3. M. Gajdács. The Continuing Threat of Methicillin-Resistant *Staphylococcus aureus*. *Antibiotics (Basel)*, 8(2):52 (2019).
4. NNIS System CDC National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992-April 2001, issued August 2001. *American Journal of Infection Control,* 29:404–421 (2011).
5. M.G. Sowash, and A.C. Uhlemann. Community-associated methicillin-resistant *Staphylococcus aureus* case studies. Methods in Molecular Biology, 1085:25-69 (2014).
6. M. Washio, T. Mizoue, T. Kajioka, T. Yoshimitsu, M. Okayama, T. Hamada, et al. Risk factors for methicillin-resistant *Staphylococcus aureus* (MRSA) infection in a Japanese geriatric hospital. *Public Health,* 111(3):187-190 (1997).
7. J.M. Luteijn, G.A.A. Hubben, P. Pechlivanoglou, M.J. Bonten, and M.J. Postma. Diagnostic accuracy of culture-based and PCR-based detection tests for methicillin-resistant *Staphylococcus aureus*: a meta-analysis. *Clinical Microbiology and Infection,* 17:146–154 (2011).
8. A. Japoni, A. Alborzi, F. Orafa, M. Rasouli, and S. Farshad. Distribution patterns of methicillin resistance genes (mecA) in *Staphylococcus aureus* isolated from clinical specimens. *Iran Biomedical Journal*, 8(4):173–178 (2004).
9. P.E. Makgotlho, M.M. Kock, A. Hoosen, R. Lekalakala, S. Omar, M. Dove, et al. Molecular identification and genotyping of MRSA isolates. FEMS Immunology and Medical Microbiology, 57(2):104–115 (2009).
10. C. Cuny, and W. Witte. PCR for the identification of methicillin-resistant *Staphylococcus aureus* (MRSA) strains using a single primer pair specific for SCCmec elements and the neighbouring chromosome-borne orfX. *Clinical Microbiology and Infection,* 11(10):834–837 (2005).
11. K.M. Mohanasoundaram, and M.K. Lalitha. Comparison of phenotypic versus genotypic methods in the detection of methicillin resistance in *Staphylococcus aureus*. *Indian Journal of Medical Research,* 127(1):78–84 (2008).
12. A. Tübbicke, C. Hübner, A. Kramer, N.O. Hübner, and Fleßa S. Transmission rates, screening methods and costs of MRSA-a systematic literature review related to the prevalence in Germany. *European Journal of Clinical Microbiology and Infectious Diseases,* 31:2497–2511 (2012).
13. M. Hallin, N. Maes, B. Byl, F. Jacobs, Y. De Gheldre, and M.J. Struelens. Clinical impact of a PCR assay for identification of *Staphylococcus aureus* and determination of methicillin resistance directly from blood cultures. *Journal of Clinical Microbiology,* 41(8):3942–3944 (2003).
14. A.C. Oh, J.K. Lee, H.N. Lee, Y.J. Hong, Y.H. Chang, S.I. Hong, and D.H. Kim. Clinical utility of the Xpert MRSA assay for early detection of methicillin-resistant *Staphylococcus aureus*. *Molecular Medicine Reports,* 7(1):11-5 (2012).
15. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests f or Bacteria That Grow Aerobically; Approved St andard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute (2012).
16. M. Bühlmann, K. Bögli-Stuber, S. Droz, and K. Mühlemann. Rapid screening for carriage of methicillin-resistant *Staphylococcus aureus* by PCR and associated costs. *Journal of Clinical Microbiology,* 46(7):2151–2154 (2008).
17. A.O. Shittu, K. Okon, S. Adesida, O. Oyedara, W. Witte, and B. Strommenger, et al. Antibiotic resistance and molecular epidemiology of *Staphylococcus aureus* in Nigeria. *BMC Microbiology,* 11:92 (2011).
18. N.R. Davoodi, J.V. Yousefi, N. Harzandi, A. Hajrafi, B. Rajaei, S. Gerayesh-Nejad, et al. Molecular detection of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase-negative *Staphylococcus (CoNS)* in Iran. *African Journal of Microbiology Research,* 6(16):3716–3721 (2016).
19. N. Torimiro, and S.E.A. Torimiro. Antibiotic resistance profile in community-associated *Staphylococcus aureus* strains isolated from a Nigerian peri-urban community. *African Journal of Biotechnology,* 11(94):16071–16076 (2012).

20. D. L Valle, P. A. Paclibare, E. C. Cabrera, and W. L. Rivera. Molecular and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* isolates from a tertiary hospital in the Philippines. *Tropical Medicine and Health*, 44(1) (2016).
21. S.A. Abdallah, K.K. Al-Asfoor, M.F. Salama, and B.M. Al-Awadi. Prospective Analysis Methicillin-resistant *Staphylococcus aureus* and its Risk Factors. *Journal of Global Infectious Diseases*, 5(1):19-25 (2013).
22. P. Van De Griend, L.A. Herwaldt, B. Alvis, M. DeMartino, K. Heilmann, et al. Community-associated methicillin-resistant *Staphylococcus aureus*, Iowa, USA. *Emerging Infectious Diseases,* 15(10):1582-9 (2009).
23. J. V. Sathish, P. Malleshappa, M. K. Yashaswini, and S. Shariff. Influence of methicillin-resistant *Staphylococcus aureus* (MRSA) nasal carriage on MRSA bloodstream infections among patients on chronic hemodialysis. *Diálisis y Trasplante*, 35(4), 143-147 (2014).
24. F. S. Mah, R. Davidson, E. J. Holland, J. Hovanesian, T. John, J. Kanellopoulos, et al. Current knowledge about and recommendations for ocular methicillin-resistant *Staphylococcus aureus*. J*ournal of Cataract & Refractive Surgery,* 40(11), 1894-1908 (2014).
25. M. Paul, J. Bishara, D. Yahav, E. Goldberg, A. Neuberger, N. Ghanem-Zoubi, et al. Trimethoprim-sulfamethoxazole versus vancomycin for severe infections caused by meticillin resistant *Staphylococcus aureus*: randomised controlled trial. *British Medical Journal,* 350:h2219 (2015).
26. G. Pappas, A.P. Athanasoulia, D.K. Matthaiou, and M.E. Falagas. Trimethoprim-sulfamethoxazole for methicillin-resistant *Staphylococcus aureus*: a forgotten alternative? J*ournal of Chemotherapy,* 21(2):115-26 (2009).
27. Y. Mehta, A. Hegde, R. Pande, K.G. Zirpe, V. Gupta, J. Ahdal, et al. Methicillin-resistant *Staphylococcus aureus* in Intensive Care Unit Setting of India: A Review of Clinical Burden, Patterns of Prevalence, Preventive Measures, and Future Strategies. Indian *Journal of Critical Care Medicine,* 24(1):55-62 (2020)
28. J.A. Ayukekbong, M. Ntemgwa, and A.N. Atabe. The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrobial Resistance and Infection Control*, 6:47 (2017).
29. M. Patel, K. B. Waites, S. A. Moser, G. A. Cloud, and C.J. Hoesley. Prevalence of inducible clindamycin resistance among community- and hospital-associated *Staphylococcus aureus* isolates. Journal of Clinical Microbiology, 44(7):2481–2484 (2006).
30. P. R. Vysakh, and M. Jeya. A comparative analysis of community acquired and hospital acquired methicillin resistant *Staphylococcus aureus*. *Journal of Clinical and Diagnostic Research,* 2013;7(7):1339–1342 (2013).
31. M. Abdel-Maksoud, M. El-Shokry, G. Ismail, S. Hafez, A. El-Kholy, E. Attia, and M. Talaat. Methicillin-Resistant *Staphylococcus aureus* Recovered from Healthcare- and Community-Associated Infections in Egypt. *International Journal of Bacteriology,* 2016:5751785 (2016).
32. P. Waitayangkoon, A. Thongkam, T Benjamungkalarak, M. Rachayon, A. Thongthaisin, T. Chatsuwan, et al. Hospital epidemiology and antimicrobial susceptibility of isolated methicillin-resistant *Staphylococcus aureus*: a one-year retrospective study at a tertiary care center in Thailand. *Pathogens and Global Health* 7:1-6 (2020).
33. M. W. Pletz, O. Burkhardt, and T. Welte. Nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia: linezolid or vancomycin?-Comparison of pharmacology and clinical efficacy. *European Journal of Medical Research,* 15(12), 507 (2010).
34. N.M. Fusco, C.J. Meaney, C.A. Frederick, and W.A. Prescott. Comparative Effectiveness of Vancomycin Versus Linezolid for the Treatment of Acute Pulmonary Exacerbations of Cystic Fibrosis. *Annals of Pharmacotherapy,* 54(3):197-204 (2020).
35. A. Shariati, M. Dadashi, Z. Chegini, A. van Belkum, M. Mirzaii, S.S Khoramrooz, et al. The global prevalence of Daptomycin, Tigecycline, Quinupristin/Dalfopristin, and Linezolid-resistant *Staphylococcus aureus* and coagulase-negative staphylococci strains: a systematic review and meta-analysis. *Antimicrobial Resistance and Infection Control,* 9(1):56 (2020).
36. H. Peng, D. Liu, Y. Ma, and W. Gao. Comparison of community- and healthcare-associated methicillin-resistant *Staphylococcus aureus* isolates at a Chinese tertiary hospital, 2012-2017. *Scientific Reports*, 8(1):17916 (2018).
37. F. Liao, Z. Mo, W. Gu, W. Xu, X. Fu, and Y. Zhang. A comparative genomic analysis between methicillin-resistant *Staphylococcus aureus* strains of hospital acquired and community infections in Yunnan province of China. *BMC Infectious Diseases,* 20(1):137 (2020).

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 53-63 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)638

Pakistan Academy of Sciences

Research Article

Preliminary Phytochemical Analysis, Anthelmintic, Insecticidal and Protective Effect of *Dicliptera bupleuroides* Nees in Ethanol-induced Gastric Mucosal Damage Rats

Shehla Akbar[1], Saiqa Ishtiaq[1*], Muhammad Ajaib[2], and Uzma Hanif [3]

[1]Department of Pharmacy, Punjab University College of Pharmacy,
University of the Punjab, Lahore, Pakistan
[2]Department of Botany, Mirpur University of Science and Technology, Mirpur, Pakistan
[3]Department of Botany, Government College University, Lahore, Pakistan

Abstract: *Dicliptera bupleuroides* Nees.is traditionally used as a general tonic, as a diuretic, in skin diseases, in snake bite, in fever, and in stomach troubles but limited literature found with the scientific ground. The objective of the present research is to prove the medicinal use of *Dicliptera bupleuroides* in anthelmintic, insecticidal, and anti-ulcer activity. Reference drug was albendazole (20 mg/ml) in anthelmintic, permethrin (239.5 µg/cm^2) in insecticidal, ranitidine (50 mg/kg), omeprazole (20 mg/kg) and sucralfate (100 mg/kg) were used in antiulcer activity respectively. Time of paralysis and death was calculated in anthelmintic activity while the rate of % age mortality was calculated in insecticidal activity. Parameters such as mean ulcer indices and percentage ulcer inhibition, gastric volume, pH, mucous and protein contents were assessed in the ethanol-induced ulcer model. In the anthelmintic study ethyl acetate and n- butanol fraction showed the more significant results in comparison to reference drug (53/54 min paralysis and 92/108 min death time). No extracts showed insecticidal activity. N-hexane extract showed significantly ($p < 0.05$) reduced gastric lesion by 54.9% in rats at 50 mg/kg when compared to ranitidine at 54.2%, omeprazole at 69.5%, and sucralfate 53.6% respectively. Gastric volume, as well as total acidity, decreased when compared with positive control 3.9 ± 0.15, 98.1 ± 3.8 and chloroform fraction was 1.8 ± 0.2, 11.3 ± 4.6. Gastric volume, pH, and total acidity of chloroform extract were 1.8 ± 0.2, 6.8 ± 0.9, and 11.3 ± 4.6. Mucous and protein content of chloroform extract versus standard was 505.1 ± 16.9, 37.8 ± 4.4 439.0 ± 11.9, 454.3 ± 14.1, and 432.6 ± 14.8 respectively. The above findings concluded that *Dicliptera bupleuroides* have medicinal importance in various pharmacological aspects.

Keywords: Anthelmintic, Anti-ulcer, *Dicliptera bupleuroides*, Gastric volume, Insecticidal, paralysis, % age mortality.

1. INTRODUCTION

Dicliptera bupleuroides Nees. of the family Acanthaceae is a perennial herb. It is found in the planes of Pakistan and Afghanistan. It is a flowering herb, length up to 90 cm, branched with hairy twigs, leaves ovate or acuminate, and with linear bracts [1]. It is used in traditional medicines for applying on the wound of snakebite, in fever, in stomach troubles, and also used in bone fracture [2]. The common name in Urdu is kaali boti [3]. *Dicliptera bupleuroides* possessed antioxidant, hepatoprotective, antimicrobial, and other biological activities. It contained phenols, flavonoids, ascorbic acid, lipids, starch, glycosides, and many other compounds [4 - 6]. Infection caused by helminths is the most common and more persistent form of infection. It is a degenerative disorder infecting a large proportion of the population around all over the world. Other contributing factors are malnutrition, pneumonia, eosinophilia, and anemia which pose a great threat to human health particularly in developing countries [7]. These helminths parasites and their larvae are passing through the human intestinal tract by eating contaminated food and also subsist into their body tissues [8]. Most of the helminths diseases are chronic, devastating in nature; they probably cause greater social and

Received: December 2020; Accepted: March 2021
*Corresponding Author: Saiqa Ishtiaq <saiqa.pharmacy@pu.edu.pk>

economic deprivation among different groups of living organisms, the ratio of morbidity may become also high. To control the overspread of worms by improving our management system coupled with the chemical control of helminths [9, 10]. In the treatment of helminths infection, the development of resistance is a foremost problem against conventional anthelmintics [11]. However, it is important to choose an alternative treatment against intestinal nematodes, which helps us to acquire knowledge of medicinal plants that have anthelmintic activity.

Several infections such as malaria, dengue fever, yellow fever, and many other infections can be transmitted from insects to human beings. These infections are symptoms of diseases. From the epidemiological point of view, dengue fever is the most serious disease based on its morbidity and mortality rates. A virus acquires about 10,000 deaths each year all around the world, in approximately 60 million people who are infected by viral infection [12, 13]. More focused on the prevention strategies to control insects' larvae because of lack of vaccines against a particular disease. So now a day's most common approach is the development of synthetic insecticides by using medicinal plants which has insecticidal potential [14]. Gastrointestinal infection is the major complication worldwide widely, peptic ulcer is most common among them remain the cause of significant morbidity and major burden for health care organization [15]. Even though various famous antiulcer drugs available in the market, having various toxicities and adverse effects. Thus there is a need to focus on searching for new alternative drugs [16]. The ulcer is a denegation of the digestive tract mucous membrane which is inflamed due to external factors [17]. There are various factors which are responsible for peptic ulcer [18]. Symptoms of ulcer are a pain in the stomach with a burning sensation, episodes of distress, pain after food intake or empty stomach, other common symptoms are vomiting, intolerance to fatty diet, and loss of appetite [19].

Physiologically, reactive oxygen species are the usual cause of various illnesses i.e peptic ulcer [20]. The safest treatment is the use of natural antioxidants which are responsible to react with free oxygen species to reduce the consequences of illness. *D. bupleuroides* has an antioxidant activity which is proved by literature [6] so it is used for gastroprotective activity has not been studied yet. The aim of the present study to evaluate the anthelmintic, insecticidal and antiulcer activity of *D. bupleuroides.*

2. MATERIAL AND METHODS

2.1 Plant Material Collection and Extraction

The plant was collected from Bhimber (Shamani), Kotli, Azad Kashmir and got authenticated by Dr. Uzma Hanif, Department of Botany, Government College University (GCU) Lahore, Pakistan. A specimen of the plant was deposited in the herbarium of GCU under voucher No: GC. Herb.Bot.3402. The plant was dried under shade, powdered whole herb. This powdered herb was dipped in commercial methanol for 7 days, filtered, and evaporated by using a rotary evaporator. After extraction fractionation was done by using different solvents according to polarity. The active fraction will be separated by using small column chromatography, preparative TLC [21].

2.2 Helminths, Insects, and Animals

Earthworms collected from crops field of Sialkot, test insects (*Tribolium castaneum*, *Sitophilus oryzae*, and *Rhyzopertha dominica*), and Wistar rats (250 ± 30g) were used. Use of animals following the rules set by the Institutional Ethical Committee for Animal Care and Experimentation under No. 416, College of Pharmacy, University of the Punjab, Lahore, Pakistan provided by Zoological Society of University of the Punjab [26].

2.3 Phytochemical Analysis

The whole herb was carried out according to the standard procedures for phytochemical analysis [22].

2.4 Total Phenolic Content

Estimation of total polyphenolic contents in plant samples was done according to the method described by Liaudanskas with little modifications [23]. Gallic acid was used as a standard.

2.5 Total Flavonoid Content

Flavonoid determination was by the method

reported by Ejikeme *et al.* and Boham [24, 25]. The percentage of flavonoid was calculated.

2.6 Acute Toxicity

Preliminary experiments were carried out in mice (n=6). Methanolic extract of *D. bupleuroides* Nees. was administered in doses (500, 1000, and 2000 mg/kg/p.o) to find out toxicity which causes zero and 100 % mortality of animals.

2.7 Anthelmintic Activity

The anthelmintic activity was carried out according to the method of Ajaiyeoba [27]. The experimental procedure carried on adult Indian earthworm which is anatomically and physiologically resemble human intestinal worms. These earthworms were divided into groups of six and put into each petri dish containing three different concentrations (25, 75, 100 mg/ml) of all fractions of the extract of whole herb *D. bupleuroides* Nees. Albendazole (20 mg/ml) is used as standard drug. Then note the time of paralysis or any physiological change and mortality.

2.8 Insecticidal Activity

For insecticidal activity impregnated filter paper method was followed [28]. Filter paper placed in a petri dish, sample loaded over filter paper, left for 24 hrs for complete evaporation, put 10 healthy and active insects *(Tribolium castaneum, Sitophilus oryzae,* and *Rhyzopertha dominica)* of same size and age of each species. Incubate them at 27C for 24 hrs with 50% relative humidity in the growth chamber. Count the number of survival of each species and calculate the percentage of morality. Standard insecticide (permethrin) at a concentration of 239.5 µg/cm2 was used.

Percentage Mortality
=100 - (No of insects alive in test) / No of inscets alive in control)×100

2.9 Evaluation of Antiulcer Activity

2.9.1 Ethanol-induced Gastric Ulcer Model

Wistar albino rats were divided into 10 groups randomly (n=6), followed the method of Mizui [29]. After 24 hours of fasting, animals were allowed free access to water for 2 hr before the experimental procedure [30]. (Group I served as normal (-ve control) received 5 ml/kg of distilled water, Group II (+ve control) was treated with absolute ethanol 1ml/animal. Groups III treated with reference drugs ranitidine; 50 mg/kg, omeprazole 20 mg/kg, and sucralfate 100 mg/kg followed by ethanol 1ml. Group IV (Extract treated) received methanolic extract; 500 mg/kg, followed by 1 ml ethanol, aqueous extract, n-hexane, chloroform, ethyl acetate, n-butanol 200 mg/kg, followed by 1 ml ethanol respectively [31 - 33].

2.9.2 Measurement of ulcer index

Stomachs were examined for a hemorrhagic lesion in the mucosal lining of the stomach. To remove blood contaminants, the stomach along the greater curvature was rinsed with cold saline. For determining ulcer index, all the stomach lesions were measured with the help of a transparent millimeter scale and magnifying glass [34]. Percentage inhibition was calculated by applying the formula:

% Gastro protection/Inhibition = (UIC – UIT)/UIC X 100

Where UIC is Ulcer Index in the control and UIT is ulcer index in the test rats [35].

2.9.3 Determination of Total Acidity

Gastric acidity was determined by the method of shay [35]. For this gastric content was collected from the stomach into graduated tubes. These tubes were centrifuged for 15 min at 2000 rpm, the supernatant was used for gastric volume, pH, and total acidity by titrating with 0.01N NaOH.

2.9.4 Estimation of Gastric Mucus and Protein Content

Glandular portion of stomach weighed, dipped immediately into 0.1 % alcian blue solution and wash with 0.25 M sucrose solution after 15, 45 min interval respectively to remove the excess dye. This dye made a complex with gastric walls. Then this blue extract was shaken with diethyl ether, resulting emulsion centrifuged at 3000 rpm, and recorded its absorption at 580 nm. The alcian blue quantity extracted was determined per gram of glandular

mucous from the standard curve of alcian blue [36]. The protein content of the stomach was estimated with the standard curve of bovine albumin solution (BSA standard curve) according to the Modified Lowery method [37].

2.9.5 Histological analysis of gastric ulcer

A small portion of the stomach from each group was fixed in preservative for histopathological studies. Thin sections of 5 µ were cut by using a microtome and stained with eosin and hematoxylin. These sections were studied for degenerative features [38].

2.10 Statistical analysis

The results were represented as Mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons. The significance of the difference was accepted at $p < 0.05$. Graphical representation was made by using graph prism pad 6.

3. RESULTS

3.1 Phytochemical Analysis

Results are shown in Tables 1 and 2. Crude extract indicated the presence of all major classes of compounds carbohydrates, alkaloids, glycosides, tannins, flavonoids, triterpenoids, etc. all other fractions have different concentrations of polyphenols and flavonoids compared with Gallic acid and quercetin respectively. Standard curves are given in Figure 1 (A) and (B).

3.2 Anthelmintic Activity

Indian earthworms were divided into 22 groups, each extract divided into three concentrations 25, 75, and 100mg/ml, n=6. The time of paralysis and death in the control group is 37.4 ± 3.43, 55.2 ± 6.37 respectively at 100 mg. While ethyl acetate and n- butanol fractions showed results comparable to the control group. These results are given in Table 3, paralysis time and time of death are given.

3.3 Insecticidal Activity

For insecticidal three kinds of insects *(Tribolium castaneum, Sitophilus oryzae,* and *Rhyzopertha dominica)* at 200 mg/3 ml, permethrin is standard insecticidal (conc. 239.5 µg/cm^2). The % age mortality is calculated. Results are given in Table 4.

3.4 Ethanol-induced Ulcer Model

Wistar albino rats were used for ethanol-induced ulcer model, n-hexane fraction showed maximum % age inhibition of ulcer after it chloroform showed significant inhibition. As result shown in Table 5. The graphical representation is given in figure 4 for all parameters. Gastric volume increased in the ethanol group while the decrease in standard drugs treated groups as well as extract treated groups. PH

Table 1. Preliminary phytochemical analysis of *Dicliptera bupleuroides* Nees.

Phytochemical group	Test	Methanolic extract
Terpenoids	Salkowaski test	+ +++
	Liebermann's test	+ +++
Tannins	Ferric Chloride test	+++
	Bromine water test	+ ++
Glycosides	Keller killani test	+ +
	Legal's test	+
Flavonoids	Alkaline reagent test	+ ++
	Lead acetate test	+ ++
Alkaloids	Mayer 'test	+ +
	Wagner 'test	+ +
	Hager's test	+ +
	Dragendroff 's test	+ +
Proteins	Millon's test	++
	Ninhydrin test	+ +
Carbohydrates	Molisch 's test	+ ++
	Benedicts's test	+ + +
Saponins	Foam test	+
Fats and Fixed oil	Spot test	++

Table 2. Determination of Polyphenols and Flavonoids content.

Parameters	%Total polyphenols		%Total flavonoids	
	Mean	SD	Mean	SD
Methanol	0.947	± 0.019	0.074	± 0.006
n-hexane	0.564	± 0.021	0.055	± 0.003
Chloroform	0.906	± 0.015	0.171	± 0.003
Ethyl acetate	0.886	± 0.011	0.185	± 0.006
n-butanol	0.779	± 0.004	0.185	± 0.004
Aqueous	0.55	± 0.009	0.046	± 0.005

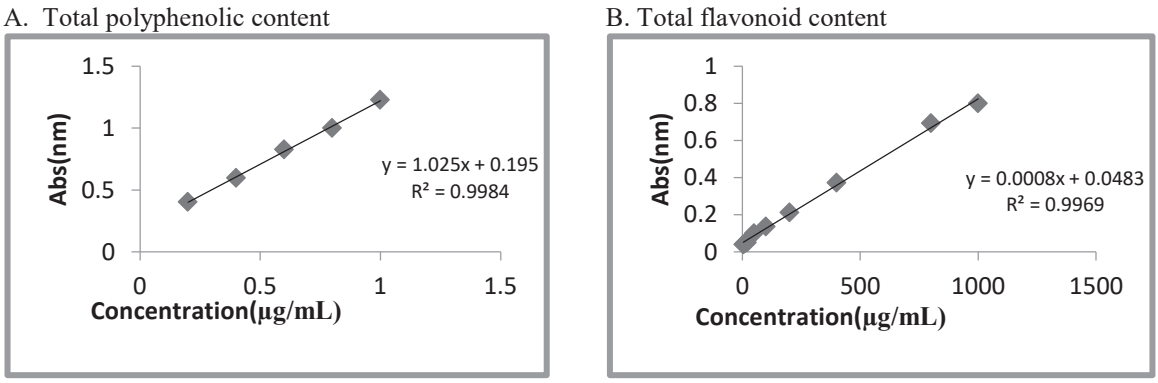


Fig. 1. (A) Graph of estimation of total phenolic content. (B) Graph of estimation of total phenolic content.

Table 3. Anthelminthic activity of *Dicliptera bupleuroides* Nees.

Treatment	Concentration (mg/ml)	Paralysis Time (min)	Death time (min)
+ve control	-	-	-
Standard (Albendazole)	25	48.2 ± 3.96	80 ± 7.90
	75	45.4 ± 3.64	70.2 ± 5.76
	100	37.4 ± 3.43	55.2 ± 6.37
Methanolic Extract	25	65.8 ± 2.58	133 ± 3.46
	75	62 ± 2.12	128.4 ± 5.94
	100	58 ± 4.30	112.6 ±7.98
Hexane fraction	25	186 ± 8.39	514.4 ± 11.26
	75	175 ± 4.12	493.6 ± 11.61
	100	167.8 ± 1.92	473 ± 8.36
Ethyl acetate fraction	25	65 ± 2.23	142.6 ± 7.98
	75	60.4 ± 2.07	122.6 ± 7.98
	100	53 ± 2.91	92.4 ± 10.01
Chloroform fraction	25	84 ± 3.08	193.2 ± 8.34
	75	75 ± 4.12	184.8 ± 4.43
	100	66.2 ± 5.71	173.6 ± 3.04
n-Butanol fraction	25	62.8 ± 3.96	137.6 ± 5.59
	75	59.8 ± 2.86	126.6 ± 5.45
	100	54.6 ± 4.21	108.8 ± 7.39
Aquous fraction	25	238.6 ± 12.03	676 ± 9.61
	75	223.6 ± 8.50	666.8 ± 7.12
	100	194.8 ± 10.13	617 ± 12.04

Results are shown as Mean± SEM. Significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, ns=not significant

Table 4. Insecticidal activity of different fractions of *Dicliptera bupleuroides* Nees.

Extract/Fraction	% Mortality (Mean ± SD)		
	Tribolium castaneum	*Sitophylus oryzae*	*Rhzopertha dominica*
-ve Control	0	0	0
+ve Control (Permethrin) (20 mg / 3 ml)	100	100	100
Methanol (200 mg / 3 ml)	0	0	0
n-Hexane (100 mg / 3 ml)	0	0	0
Ethyl acetate (100 mg / 3 ml)	0	0	0
Chloroform (100 mg / 3 ml)	0	0	0
Butanol (100 mg / 3 ml)	10	0	0
Aqueous (100 mg / 3 ml)	0	0	0

Table 5. Effect of different fractions of *Dicliptera* on the ethanol-induced gastric ulcer.

Group name	Ulcer no.	Ulcer score	Incidence of ulcer (%)	Ulcer index	Inhibition of ulcer (%)
Normal (10 ml/kg p.o)	0.00 ± 0.00***	0.00 ± 0.00***	0	0	0
Ethanol (10 ml/kg p.o)	7.83 ± 0.60	5.75 ± 0.57	100	11.36	0
Ranitidine (50 mg/kg p.o)	1.00 ± 0.52***	1.00 ± 0.47***	50	5.2	54.22
Omeprazole (20 mg/kg p.o)	0.67 ± 0.42***	0.58 ± 0.37***	33.33	3.46	69.56
Sucralfate (100 mg/kg p.o)	1.17 ± 0.54***	1.50 ± 0.67***	50	5.27	53.63
Methanol (500 mg/kg p.o)	1.33 ± 0.42***	0.83 ± 0.28***	83.33	8.55	24.73
Aqueous (200 mg/kg p.o)	1.17 ± 0.40***	0.83 ± 0.28***	83.33	8.53	24.88
Hexane (200 mg/kg p.o)	0.50 ± 0.22***	0.67 ± 0.33***	50	5.12	54.95
Chloroform (200 mg/kg p.o)	1.50 ± 0.50***	1.25 ± 0.48***	60.67	6.94	38.88
Ethyl acetate (200 mg/kg p.o)	1.83 ± 0.31***	1.83 ± 0.42***	100	10.37	8.73
Butanol (200 mg/kg p.o)	1.67 ± 0.31***	0.83 ± 0.21***	83.33	8.53	24.88

Results are shown as Mean± SEM. Significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, ns=not significant

decreased in the ethanol-treated group, increased in all other groups.

Total acidity increased in the ethanol-treated group, all groups showed a decrease in acidity, given in Table 6. Mucous content decreased and protein content increased in the ethanol-treated group and vice versa in all other treated groups, given in Table 6.

3.5 Macroscopic Examination

With the help of magnifying glass, all stomachs were examined for measurement of ulcer index, streak, spot, hemorrhage, and lesions in all treated groups, results given in Figure 2.

3.6 Histopathological Studies

As shown in Figure 3, the stomach in the group received chloroform and butanol similar to the group treated with standard drugs ranitidine, omeprazole, and sucralfate in Figure 2. The results indicate that the dose of chloroform has maximum protection of gastric mucosa of the stomach in the ethanol-induced model as compared to all other

Table 6. Gastric juice parameters in ethanol induced acute gastric ulcer.

Group name	Gastric volume (ml)	pH	Total acidity (mEq/L)
Normal (10 ml/kg p.o)	1.35 ± 0.06***	4.00 ± 0.11*	28.33 ± 2.03***
Ethanol (10 ml/kg p.o)	3.95 ± 0.15	2.45 ± 0.17	98.17 ± 3.82
Ranitidine (50 mg/kg p.o)	2.13 ± 0.23***	5.77 ± 0.39***	38.17 ± 2.95***
Omeprazole (20 mg/kg p.o)	1.80 ± 0.29***	5.96 ± 0.49***	32.33 ± 2.80***
Sucralfate (100 mg/kg p.o)	1.77 ± 0.20***	5.45 ± 0.33***	39.17 ± 3.44***
Methanol (500 mg/kg p.o)	1.72 ± 0.22***	4.90 ± 0.25***	53.67 ± 5.13***
Aqueous (200 mg/kg p.o)	2.02 ± 0.19***	4.60 ± 0.27**	44.42 ± 4.80***
Hexane (200 mg/kg p.o)	2.28 ± 0.25***	4.68 ± 0.27***	46.42 ± 6.57***
Chloroform (200 mg/kg p.o)	1.85 ± 0.21***	6.88 ± 0.91***	11.33 ± 4.62***
Ethyl acetate (200 mg/kg p.o)	2.52 ± 0.24***	5.80 ± 0.41***	40.42 ± 4.24***
Butanol (200mg/kg p.o)	2.03 ± 0.31***	6.65 ± 0.57***	08.83 ± 3.77***

Results are shown as Mean± SEM. Significant at P < 0.05, P < 0.01, P < 0.001, ns=not significant

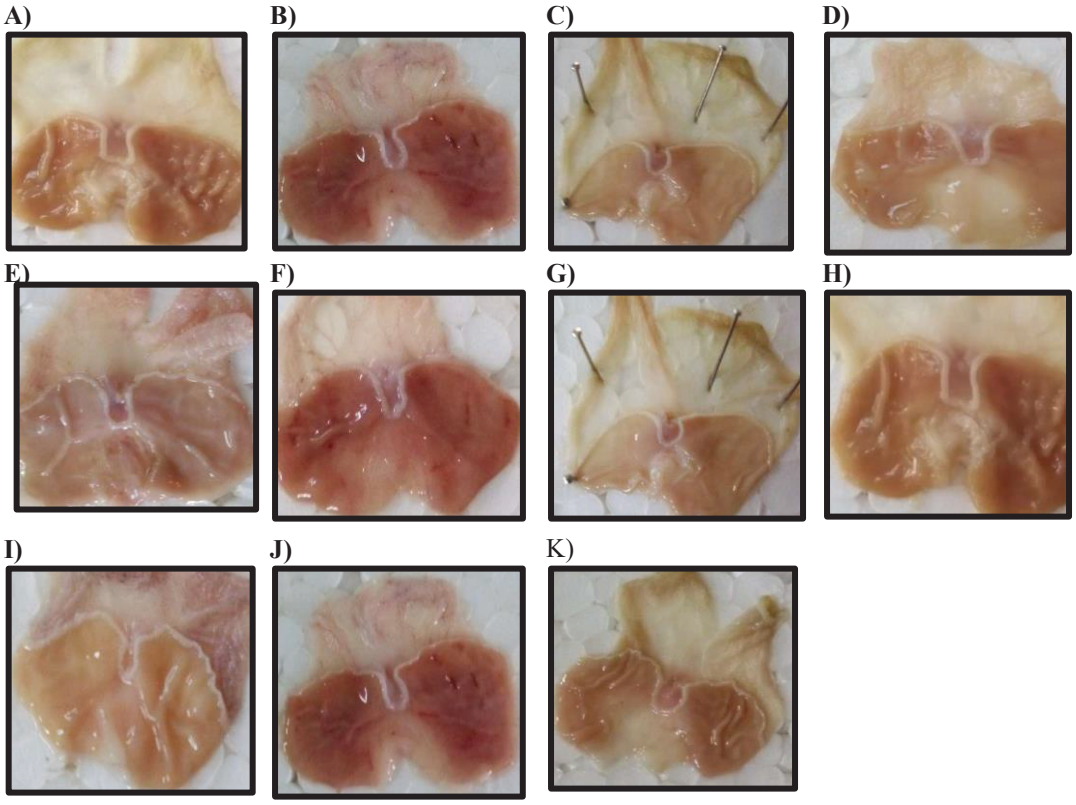


Fig. 2. Macroscopic examination of stomachs in different groups e.g., A) Normal, B) Ethanol, C) Ranitidine, D) Omeprazole, E) Sucralfate, F) Methanolic, G) Aqueous, H) n-Hexane, I) Chloroform, J) Ethyl Acetate, K) Butanol.

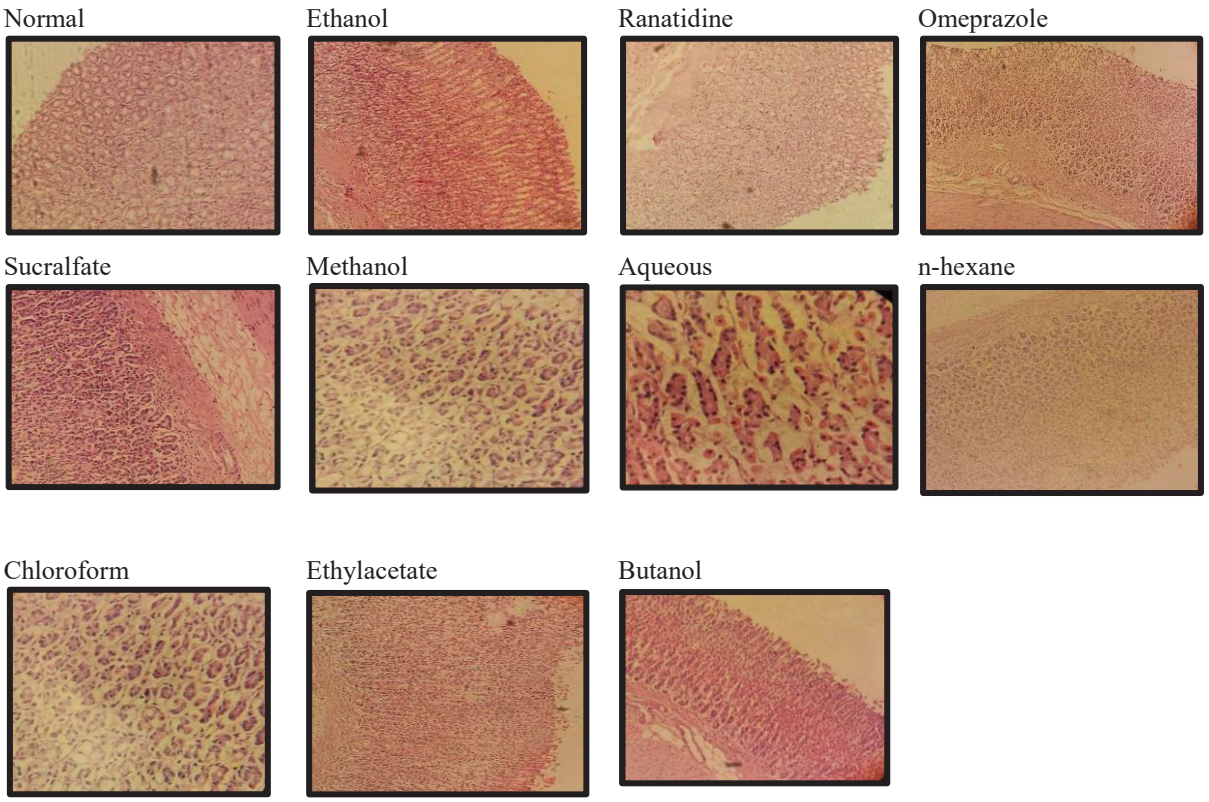


Fig.3. Sections stained with hematoxylin and eosin (H&E) displaying the regenerated glandular epithelium width in stomachs of rats treated with ranitidine, omeprazole, and sucralfate, methanolic, aqueous, n-hexane, chloroform, ethyl acetate, and butanol extract of *Dicliptera bupleuroides* in ethanol-induced ulcer model.

fractions, these results are comparable to standard treated groups

4. DISCUSSION

Nature is the ultimate source of human health, we can say that human being entirely dependent upon natural resources to fulfill their requirements [39]. For this purpose, the demand for medicinal plants and phytochemicals is increased by overcoming the increasing demand for comfort and the beneficial needs of society [40]. Our present project in this aspect to the development of alternative therapies for the treatment of various ailments. In this study, we evaluate the anthelmintic, insecticidal, and antiulcer activity of a natural herb found in the region of Pakistan [41]. The preliminary phytochemical screening of *Dicliptera bupleuroides* Nees. shown in Table 1. The extract showed the presence of polyphenolic compounds, saponins, flavonoids, and alkaloids. The content of total phenolic and flavonoids were expressed as follows:

Y= 1.025X + 0.195, R_2=0.9984 for polyphenol standard gallic acid.

Y=0.0008X + 0.0483, R_2=0.9969 for flavonoid standard is quercetin.

Results were shown in Table 2 and Figure 1(A) and (B).

In plants, polyphenolics and flavonoids are a major class of compounds that naturally possess the antioxidant potential to show therapeutic action in numerous biological ailments. Antifungal, antiviral, anti-inflammatory,anti-allergic, anticarcinogenic, antithrombic hepatoprotective, and cytotoxic effects. So that plants have flavonoids showed the greatest interest for researchers. It exerts beneficial effects on lipid peroxidation, which is a major cause of various diseases. In pharmacological profile, flavonoids have free radical scavenging activity, antioxidants, and also interact with protein phosphorylation [31].

The data revealed that the ethyl acetate and butanol fraction showed the maximum mortality/

paralysis time at 100mg/ml in comparison to other fractions. Helminths are parasitic, causing severe effects in the animal as well as in men. Human infections by helminths exist throughout the world and it may increase day by day due to travel and immigration from developing countries. However various advances have occurred in the development of new synthetic drugs but serious side effects and development of resistance are still a major hurdle in the treatment of these parasitic infections. These factors paved the focus on the development of new alternative herbal remedies for helminths [42]. Nowadays medicinal plants are screened for their major constituents which are responsible for the anthelmintic property. The use of *Dicliptera bupleuroides* Nees. crude extract and its fractions have shown significant anthelmintic activity, it would be used against intestinal nematodes. Ethyl acetate and n-butanol fraction showed the maximum anthelmintic property.

The fractions of *Dicliptera bupleuroides* Nees. were also screened for their insecticidal effects against Tribolium castaneum, Sitophilus oryzea, and Rhyzopertha dominica using permethrin as a standard drug [42]. There was no insecticidal effect on all tested samples against Tribolium castaneum, Sitophilus oryzae, and Rhyzopertha dominica (Table 3).

Antiulcer activity of *Dicliptera bupleuroides* Nees was investigated in the ethanol-induced ulcer model. The ulcer can be induced by different factors, the most commonly involved factors are the administration of non-steroidal anti-inflammatory drugs, H-pylori, environmental factors, intake of ethanol, and lifestyle factors [17]. Daniel and his colleagues induced ulcer 1st time in rats by using sucralfate as a protective drug [32]. Continuous use of alcohol also causes gastric mucosal damage by stimulating parietal cells which increases the level of cAMP and histamine. This may lead to an increase in gastric and mucosal secretion which made the grounds for stomach lesions, hemorrhage, and inflammation and blood congestion [43, 44] Standard drugs were ranitidine, omeprazole, and sucralfate, they belong to different classes and their significant results are agreed with the work of other authors [31, 32, 33].

In the present study, the *Dicliptera bupleuroides* Nees of all fractions were evaluated for anti-ulcer activity by the ethanol-induced ulcer model. When results of %age of ulcer protection compared to +ve control group (0%) showed following indices i.e methanolic extract (24.73%), aqueous (24.88%), n-hexane (54.95%), chloroform (38.88%), ethyl acetate (8.73%) and butanol fraction (24.88%) given in Table 1. The Gastric volume and total acidity of reference drugs and extract/fractions treated groups decrease, these values given in Table 2. Gastric volume and total acidity of the +ve group were 3.95 ± 0.15 and 98.17 ± 3.82. The standard group showed values of gastric volume (2.13 ± 0.23, 1.80 ± 0.29, 1.77 ± 0.20) and total acidity was ($38.17 \pm 2.95, 32.33 \pm 2.80, 39.17 \pm 3.44$) respectively. Mucous and protein content increased in standard and extract-treated groups in comparison to the ethanol group (Table 3). The mechanism of gastro protection of *Dicliptera bupleuroides* Nees. maybe due to the cytoprotective, antisecretory, and antioxidant potential of phytoconstituents present in the extract [44, 45].

5. CONCLUSION

All the extracts/fractions of *Dicliptera bupleuroides* Nees. were used for evaluating various biological activities such as anthelmintic, insecticidal, and anti-ulcer activity. Ethyl acetate and butanol fraction showed good results in comparison with the reference drug-treated group in the anthelmintic study. No significant results showed in the insecticidal study. n-hexane and chloroform extract showed a good protective effect in antiulcer activity when compared to the ethanol-treated group.

6. ACKNOWLEDGEMENTS

We are thankful to our dean and principal of Punjab University College of Pharmacy for their help and encouragement in all aspects of our research work

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. REFERENCES

1. H. Singh., T. Husain., P. Agnihotri., P.C. Pande., and S. Khatoon. An ethnobotanical study of medicinal plants used in sacred groves of Kumaon Himalaya, Uttarakhand, India. *Jornal of Ethnopharmacology.* 154(1): 98-108 (2014).

2. G. Panigrahi, and A.K. Dubey. *Dicliptera bupleuroides* var. roxburghiana, a New Name for D. roxburghiana CB Clarke, Non Nees (Acanthaceae). *Taxon.* 1: 286-288 (1983).
3. M.U. Ajaib., Z. Khan, and A.N. Zikrea. Ethnobotanical survey of some important herbaceous plants of District Kotli, Azad Jammu & Kashmir. *Biologia (Pakistan)*. 60: 11-22 (2014).
4. R.P. Bahuguna., J.S. Jangwan., T. Kaiya, and J. Sakakibara. Flavonoids and fatty acids of Dicliptera roxburghiana. International Journal of Crude Drug Research. 25:177-178 (1987).
5. Y. Luo., C. Feng., Y. Tian, and G. Zhang. Glycosides from *Dicliptera riparia. Phytochemistry.* 61(4): 449-454 (2002).
6. B. Ahmad., M.R. Khan., N.A. Shah, and R.A. Khan. In vitro antioxidant potential of *Dicliptera roxburghiana. BMC Complementary and Alternative Medicine.* 13(1): 1-10 (2013).
7. D.A.P. Bundy. The global burden of intestinal nematode disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene. 8*8(3):259-261 (1994).
8. K.P. Tripathi. Essentials of meditional pharmacology, 5th ed. Jaypee Brothers Medical Publishers (P) LTD., New Delhi, INDIA, p.759 (2003).
9. G.C. Coles. Nematode control practices and anthelmintic resistance on British sheep farms. *Veterinary Record.* 141(4): 91-93 (1997).
10. B. Asha., M. Krishnappa, and R. Kenchappa. In vitro Anthelmintic Activity of Different Extracts of Memecylon umbellatumBurm. *Science, Technology and Arts Research Journal.* 4(4):127-130 (2015).
11. S. Tagboto, and S. Townson. Antiparasitic properties of medicinal plants and other naturally occurring products. *Advances in Parasitology.* 50: 199-295 (2001).
12. A. Wilder-Smith, and P. Byass. The elusive global burden of dengue. *The Lancet Infectious Diseases.* 16(6):629-631 (2016).
13. J.D. Stanaway., D.S. Shepard., E.A. Undurraga., Y.A. Coffeng., L.E. Brady., O.J. Hay., S.I. Bedi., N. Bensenor., I.M. Castañeda-Orjuela, and T.W. Chuang. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. *The Lancet Infectious Diseases.* 16(6): 712-723 (2016).
14. A.D. DeZure., N.M. Berkowitz., B.S. Graham, and J.E. Ledgerwood. Whole-inactivated and virus-like particle vaccine strategies for chikungunya virus. *The Journal of Infectious Diseases.* 214(suppl_5): S497-S499 (2016).
15. N.F. Tanih., M. McMillan., N. Naidoo., L.M. Ndip., L.T. Weaver, and R.N. Ndip. Prevalence of Helicobacter pylori vacA, cagA and iceA genotypes in South African patients with upper gastrointestinal diseases. *Acta tropica.* 116(1): 68-73 (2010).
16. V. Prabhu, and A. Shivani. An overview of history, pathogenesis and treatment of perforated peptic ulcer disease with evaluation of prognostic scoring in adults. *Annals of Medical and Health Sciences Research*. 4(1): 22-29 (2014).
17. D. Pradhan., P. Biswasroy., G .Singh, and K.A. Suri. Anti-ulcerogenic activity of Ethanolic Extract of Cucumissativus L. against NSAID (Aspirin) induced Gastric Ulcer in wistar albino rats. *International journal of herbal medicines.* 1(3): 115-119 (2013).
18. P. Malfertheiner., F.K. Chan, and K.E. McColl. Peptic ulcer disease. *The lancet.* 374:1449-1461 (2009).
19. K. Ramakrishnan, and R.C. Salinas. Peptic ulcer disease. *American Family Physician*. 76(7):1005-1012 (2007).
20. A. Garber, and S. Jang. Novel therapeutic strategies in the management of non-variceal upper gastrointestinal bleeding. *Clinical Endoscopy.* 49(5): 421 (2016).
21. S. Li., Q. Han., C. Qiao., J. Song., C.L. Cheng, and H. Xu. Chemical markers for the quality control of herbal medicines: an overview. *Chinese Medicine.* 3(1):1-18 (2008).
22. S. Ishtiaq., T. Ali., B. Ahmad., F. Anwar., M.S.K. Afridi, and H. Shaheen. Phytochemical and biological evaluations of methanolic extract of *Amaranthus graecizans* subsp. silvestris (Vill.) Brenan. *British Journal of Pharmaceutical Research.* 15:1-11 (2017).
23. M. Liaudanskas., K. Zymonė., J. Viškelis., A. Klevinskas, and V. Janulis. 2017. Determination of the phenolic composition and antioxidant activity of pear extracts. *Journal of Chemistry.* (2017).
24. C. Ejikeme., C.S. Ezeonu, and A.N. Eboatu. Determination of Physical and Phytochemical Constituents of some Tropical Timbers Indigenous to nigerdelta area of nigeria. *European Scientific Journal.* 10(18): 247-270 (2014).
25. B.A. Boham, and R. Kocipai-Abyazan. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium vaticulatum* and *V. calycinium. Pacific Science.* 48(4): 458-463 (1974).
26. Guideline Document on Acute oral Toxicity Testing, Series on Testing and Assessment No. 423. Paris: Organization for Economic Co-Operation and

Development, OECD Environment, Health and Safety Publications; (1996).
27. E.O. Ajaiyeoba., P.A. Onocha, and O.T. Olarenwaju. In vitro anthelmintic properties of *Buchholzia coriaceae* and *Gynandropsis gynandra extracts. Pharmceutical Biology.* 39(3): 217-220 (2001).
28. R.F. Shoukat., S. Freed, and W.A. Kanwar. Assessment of binary mixtures of entomopathogenic fungi and chemical insecticides on biological parameters of culex pipiens (diptera: culicidae) under laboratory and field conditions. *Pakistan Journal of Zoology.* 50(1): 300-309 (2018).
29. T. Mizui., H. Sato., F. Hirose, and M. Doteuchi. Effect of antiperoxidative drugs on gastric damage induced by ethanol in rats. *Life Sciences.* 41(6): 755-763 (1987).
30. R.S. Alphin, and J.W. Ward. Actions of hexopyrronium bromide on gastric secretion in dogs and on gastric secretion and ulceration in rats. *Archives Internationales de Pharmacodynamie et de. Therapie.* 168(1): 82-100 (1967).
31. M. Balmik., G. Dubey., S.K. Ghauary and P.K. Jain. Phytochemical Screening and Evaluation of Antiulcer Activity of Hydroalcoholic Extract of Lilium Candidum Flowers. *Journal of Drug Delivery and Therapeutics.* 9(4-A):646-650 (2019).
32. D. Hollander., A. Tarnawski., W.J. Krause, and H. Gorgely. Protective effect of sucralfate against alcohol-induced gastric mucosal injury in the rat: macroscopic, histologic, ultrastructural, and functional time sequence analysis. *Gastroenterology*. 88(1): 366-374 (1985).
33. M.M. Zangeneh., S. Salmani., A. Zangeneh., E. Bahrami, and M. Almasi. Antiulcer activity of aqueous extract of leaves of Mentha piperita in Wistar rats. *Comparative Clinical Pathology.* 28(2): 411-418 (2019).
34. M.G. Magaji., A.H. Yaro., A. Mohammed., A.U. Zezi., Y. Tanko, and T.Y. Bala. Preliminary antidiarrhoeal activity of methanolic extracts of Securinega virosa (Euphorbiaceae). *African Journal of Biotechnology.* 6(24): 2752-2757 (2007).
35. H. Shay. A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology*. 5: 43-45 (1945).
36. O.H. Lowry., N.J. Rosebrough., A.L. Farr, and R.J. Randall. Protein measurement with the Folin phenol reagent. *Journal of biological Chemistry.* 193: 265-275 (1951).
37. A.A. Boligon., M.M. Machado, and M.L. Athayde. Technical evaluation of antioxidant activity. *Medicinal chemistry.* 4(7): 517-522 (2014).
38. L. Kokoska., P. Kloucek., O. Leuner, and P. Novy. Plant-derived products as antibacterial and antifungal agents in human health care. *Current medicinal chemistry*. 26(29): 5501-5541 (2019).
39. H.S. Elshafie, and I. Camele. An overview of the biological effects of some mediterranean essential oils on human health. *Biomed Research International.* (2017).
40. M.F. Akhtar., A. Saleem, and M. Saleem. A Comprehensive Review on Ethnomedicinal, Pharmacological and Phytochemical Basis of Anticancer Medicinal Plants of Pakistan. *Current cancer drug targets.* 19(2): 120-151 (2019).
41. S. Partap., S. Kumar., A. Kumar In-vitro anthelmintic activity of Luffa cylindrica leaves in Indian adult earthworm. *Journal of pharmacognosy and phytochemistry.* 1(2): 27-30 (2012).
42. F. Ayaz., N. Küçükboyacı., B. Bani., B. Sener, and M.I. Choudhary. Phytotoxic, Cytotoxic and Insecticidal Activities of Chrysophthalmum dichotomum Boiss. and Heldr. *Computer software.* 8: 9 (2018).
43. Z.A. Zakaria., E.A. Hisam., M.S. Rofiee., M. Norhafizah., M.N. Somchit., L.K. Teh, and M.Z. Salleh. In vivo antiulcer activity of the aqueous extract of Bauhinia purpurea leaf. *Journal of ethnopharmacologyl.* 137(2):1047-1054 (2011).
44. J.K. Moon, and T. Shibamoto. Antioxidant assays for plant and food components. *Journal of agricultural and food chemistry*. 57(5): 1655-1666 (2009).
45. D.M. de Oliveria., G. Barreto., D.V.G. De Andrade., E. Saraceno., L. Aon-Bertolino., F. Capani, and L.D. Giraldez. Cytoprotective effect of Valeriana officinalis extract on an in vitro experimental model of Parkinson disease. *Neurochemical Research*. 34(2): 215-220 (2009).

Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 58(1): 65-76 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)641

Pakistan Academy of Sciences

Research Article

Productive Use of Natural Resources for Promotion of Horticultural Crop Production through Rooftop Rainwater Harvesting in Rain-Fed Hilly Areas of Punjab

Abid Hussain*, Sidra Majeed, Muhammad Z. Khan, and Waqas Farooq

Social Sciences Research Institute, National Agricultural Research Centre, Islamabad, Pakistan

Abstract: Rainfall variability often results in low crop and fruit productivity in rain-fed hilly areas. Rooftop Rainwater Harvesting (RTWH) Technology can play a promising role in achieving agricultural production potential in these areas. Its adoption makes the supply of water sustainable for vegetables, fruit, and crop farming as well as domestic use. According to key informants, RTWH is being adopted in the study area on technical lines since the early 1990s. However, the availability of literature about economic aspects of the technology in the context of Pakistan is quite limited. This study is an effort to document the economic aspects of the technology including cost structure, potential benefits, net returns, and returns on investment. Thus, the study is based on a purposively selected sample of thirty farmers from Kotli Sattian and Murree tehsils of Rawalpindi district having operational RTWH systems installed at farms. The data have been analyzed for descriptive statistics and financial evaluation. Moreover, technical discussions with key informants and a detailed review of literature have also been made to substantiate the findings of the study. In the study area, farming families have diversified income sources with a considerably low share of agriculture in family income (19.5%). The mean command area of the RTWH systems at sample farms was 0.33 acres, which is allocated to different vegetables, and mainly to guava & citrus orchards. Benefit-cost ratios of vegetables and fruit farming through RTWH is 1.16, with returns on investment of 15 %, and a rate of return to labour of 0.95 in the first year of installation. Thus, technology is economically viable in the study area. Moreover, the financial gains of RTWH can be improved by enhancing storage capacity and increasing the command area.

Keywords: Rooftop Rainwater Harvesting, Vegetables, Fruits, Adoption, Irrigation, Benefit-cost Ratio, Hilly Areas, Punjab

1. INTRODUCTION

Rainfed agriculture plays an important role in global food production. Its importance can be gauged from the fact that it constitutes 80% of the world's cropland and produces 60% of the global cereal grains [1]. While, rainfed areas are also the hotspots of poverty, food insecurity, malnutrition, poor physical & financial infrastructure, and severe land degradation [2]. In these areas, smallholder subsistence farming systems have limited opportunities to cope with ecosystem changes [3]. Water is a vital ingredient of every living thing on the earth and a basic need for every creature. The minimum domestic water requirement per capita per day is about 50 liter, including water for drinking, cooking, and washing, etc. Considering the national average household size of 6.39 members [4], the daily water requirement of an average-sized family is about 320 liter. However, water required for food production is much more e.g. on average to produce one kg cereals crops (wheat & rice) and pulses it requires about 1000 liter of water [5].

Pakistan is one of the world's most water-stressed countries [6]. The national annual per capita availability of water is below 1000 cubic meters, which is an internationally recognized threshold of water scarcity [7]. Pakistan extracts three quarters (74.3%) of its freshwater annually

Received: May 2020; Accepted: March 2021
*Corresponding Author: Abid Hussain <abid.parc@gmail.com>

thereby exerting tremendous pressure upon renewable water resources [8]. In this scenario, maintaining water security which is defined as 'the sufficient availability and equitable access to water as an input to agricultural production and associated human well-being' is much essential [9].

The core of rainwater harvesting interventions is to reduce the effects of temporal rainfall shortages for domestic and productive uses. The water thus obtained may improve access, agricultural production, sanitation and health status of people. Ultimately all such improvements may lead to poverty reduction [10]. While rainwater harvesting (RWH) creates synergies between good ecosystem management and human well-being. It is also very useful for soil conservation which would otherwise erode due to the flash flow of rains [3]. Adoption of water harvesting improves resilience to drought and dry spells that result in both risk reduction and yield improvement [11]. RWH is an important source of domestic water in many rural areas of the developing world. It provides all or a portion of domestic, commercial, and agricultural water needs. Thus, RWH is considered the most promising source for supplying fresh water. The technology is being reflected in the water policies of many developing countries [12]. In this context, it is stated that improvements in rainwater harvesting techniques due to recent technological developments may guarantee the availability of food for the growing population [13].

RWH techniques have long been implemented around the world to cope with inter and intra annual variability in precipitation and maintain human well-being. Various studies professed RWH as an effective source of drinking water, livestock watering, and irrigation in drought-prone and rural areas viz. [12, 14, 15]. Moreover, in rural and hilly areas, high costs and low success rates make it difficult, time-consuming, and expensive to provide water supply schemes. Though RWH systems provide impressive results their adoption is much less widespread and slow than the potential to improve the livelihood of land-poor farmers [16]. The main reason is the risk involved in making agricultural investments in semi-arid environments that can be attributed to poverty and bad experiences. While researchers and development agents, more often share with farmers the positive aspects of technology and rarely highlight related risks and constraints [15].

Foregoing these facts in view, it has been endorsed that rainwater harvesting technology is the most appropriate and feasible approach for hilly areas of Pakistan [6]. According to key informants in a hilly tract of Kotli Sattian and Murree tehsils of Rawalpindi district of Punjab province, the rooftop rainwater harvesting (RTWH) technology was introduced by UNDP and IUCN in the 1990s. Many household-based productive activities viz. kitchen gardening, livestock raising, and micro enterprises are dependent on adequate supplies of domestic water [17]. While links between various activities further enhance farming income e.g. waste products from food-based micro-enterprises are used for livestock rearing.

It helps to overcome water stress periods due to changes from wet to dry season, or during within seasonal droughts through supplemental irrigation [3]. The use of RWH makes possible sowing of the crop at the desired time by providing a sufficient supply of water. Alternatively, crop selection should be made keeping in view the availability of irrigation water [18]. Moreover, RWH helps adapt the production of high-value crops through timely nursery sowing of vegetables and planting of fruit plants. High-value crops mostly make a considerable share in farmers' income and result in very high returns for them [19].

In the preview of increasing pressure on existing water resources, there is a need to reconsider actual and potential rainwater harvesting levels, as a viable alternative solution for water shortage [20]. Despite the importance of rainwater harvesting in the socio-economic development of communities, the availability of information about socio-economic aspects of technology adoption in the existing literature is quite limited. RWH technology has the potential to increase crop productivity and reduce the risk of crop failure. Moreover, it saves energy and farm maintenance costs [21]. RTWH being the most commonly used technique among RWH technologies in Makueni County and the vicinity of Nairobi city, capital of Kenya to overcome water scarcity as well for supplemental irrigation [22]. It is considered one of the potential water harvesting techniques for agricultural purposes in West Aisa

and North Africa. Similarly, it has been adopted at a building scale in Nairobi and in many cities of Australia, North America, Europe, and Asia to meet household water demand [12].

In Pakistan, Earthquake Reconstruction and Rehabilitation Authority (ERRA) executed a development project in earthquake-affected areas of Khyber Pakhtunkhwa and Azad Jammu and Kashmir to construct forty thousand houses and 400 public/ community institutions equipped with RTWH. The average annual rainfall in areas affected by the earthquake of 2005 is 1500 mm. Thus, RTWH was promoted as an alternative method to preserve natural rainwater [23]. Few researchers including declared RTWH suitable for Islamabad and Lahore, respectively [24 , 25]. It is stated that 22 percent of the yearly household demand for water in the capital city can be met by using RTWH. It is found that RTWH is economical, environmentally friendly, and easy to install the system. It is declared as the best functional technique to avert the present and future water crisis in Pakistan [25].

A generic rooftop rainwater harvesting system as shown in Figure 1 contains several components e.g. the catchment of RTWH is the roof surface that directly receives the rainfall and provides water to the system. Gutter lines are used around the boundaries of a slanted roof to gather and transport rainwater to the storage tank. Conduits are pipelines that drain rainwater from the catchment or rooftop area to the harvesting point. The most commonly used conduit materials are polyvinyl chloride (PVC) and galvanized iron (GI). Another important segment of the RTWH system is the filter that functions to confiscate pollutants from harvested rainwater over the roof before it reaches the storage chamber. According to space and requirement, cemented tanks/ pools, plastic tanks, and buckets are used for storage of the harvested water [26].

The technology has been promoted in the study area through a research and development project that was executed by Climate Change, Energy and Water Resources Institute (CEWRI), NARC in collaboration with the ICARDA-Pakistan office from 2014 - 16 by organizing farmers field days for knowledge dissemination, providing them with technical support, convincing them for the adoption, as well as logistically supporting them in the installation of RTWH systems. Thereafter, from 2017 to 2018, few of the area farmers also adopted it on small scale on their own. However, the rate of adoption is still low and there is a knowledge gap about utilization, economic gains, and up-scaling potential of the technology for the production of vegetables and fruit crops in the study area.

2. MATERIAL AND METHODS

The study is based on a purposively selected sample of thirty farmers from Kotli Sattian and Murree tehsil of Rawalpindi district of Punjab with

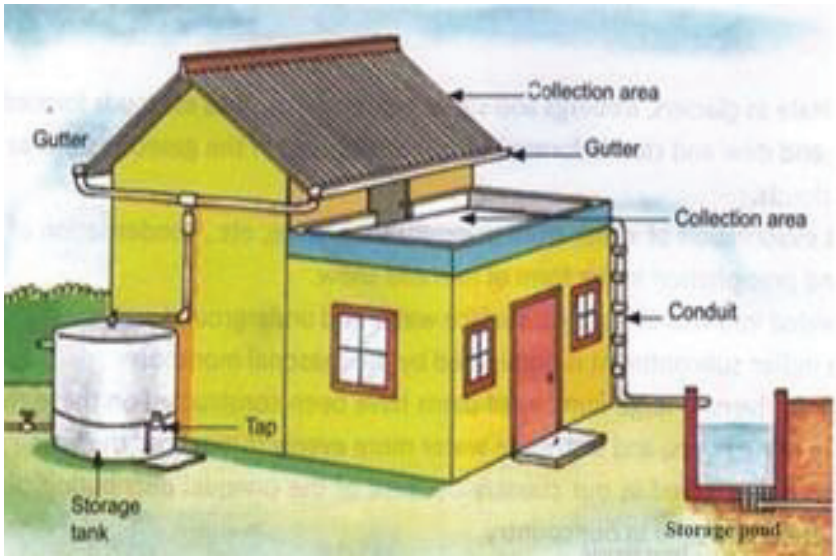


Fig. 1. Rooftop rainwater harvesting system (Adopted with the addition of few details from the website of IndiaMART: an Indian customer to customer sales services providing company)

operational RTWH systems installed at their farms. Thus, lists of such farmers were obtained from NRSP, Kotli Sattian. Moreover, a detailed review of cutting-edge published literature has also been made to substantiate the findings of the study. A field survey for the study was conducted in January-February, 2018. A comprehensive questionnaire was used to collect primary data that contains details about socioeconomic attributes, technology awareness, suitability, detailed cost of installation, and benefits. The questionnaire was pretested and then a formal survey was carried in the field area. Besides a formal survey of thirty farms with RTWH systems in operational conditions, focused formal and informal discussions were also held with key informants from National Rural Support Programme (NRSP) & Climate Change, Alternate Energy & Water Resources Institute (CAEWRI), and farmer respondents to obtain necessary complementary information. Selected areas were Bhagaand Nomal-Arokus valleys and adjoining areas of Kotli Sattian and Murree tehsils of Rawalpindi district, respectively.

Since the RTWH systems are integrated into existing buildings, the costs of land and roof were not included in the analysis. The key aspects considered are the roof gutters (collection), pipes and fittings (convey-ance), overhead /overground/ underground storage tanks, water filters, system maintenance, design life, and running cost. The variable cost of RTWH includes land preparation cost (tractor or manual), seed/ seedling cost, sowing cost, fertilizer cost, and farmyard manure (FYM), and labour cost to perform farm operations to grow the vegetables/ fruits and repair and maintenance cost of the system.

The primary data collected for the study were analyzed by using Statistical Package for Social Sciences (SPSS-22) for descriptive statistics, estimation of cost of the system installation and production of vegetables & fruits, and financial analysis; gross & net returns, benefit-cost ratios, and rate of return to labour. It is worth mentioning here that the financial benefits of RTWH as a single entity are minimal [27]. Thus, investigating the feasibility in monetary terms may provide a shortsighted perspective. Even when the economic feasibility of RWH does not lead to a favorable conclusion, a casual consideration of the non-monetary benefits can alter the conclusion. Non-monetary benefits associated with RTWH include simplicity of operation, low energy requirement, increased crop yield (> 30%), reduced emission of CO_2 increased infiltration & groundwater recharge, and reduced soil erosion [28].

The monthly volume of water that is fetched through the adoption of RTWH is also estimated, by considering the mean catchment area of roofs and using expression (1) that was also applied by [14, 29 - 32]. Water availability through the system has also been compared with the standard per capita daily water requirement of 48 liters (excluding two liters for drinking purposes) on monthly basis for the entire calendar year.

$$VR = I \times Har \times Cr \quad (1)$$

Where VR =Average volume of rainwater harvested in an hour through roof-top system
I = Rainfall intensity (mm)
Har = Water harvesting/Catchment area (m^2)
Cr = Coefficient of runoff

The coefficient of runoff for any catchment is defined as the ratio of the volume of water that run off to the volume of rain that falls on the surface [33], it depends on the dimensions of the roof as well on the material used [29], and is given by expression (2).

$$C(r) = Vr/Vw \quad (2)$$

Where Vr= Volume of runoff
Vw = Volume of rainwater that falls on the surface

The runoff coefficient for the study area has been taken as 0.8 which is taken as a standard for the designing of roof catchment systems [33]. Although, it may range from 0.8 to 0.9 for roofs made with tiles, and from 0.7 to 0.9 in the case of roofs made with corrugated metal sheets [34]. The rate of return to labour (RRL) is determined by expression (3). The benefit-cost ratio (BCR) of vegetables and fruit farming through RTWH is calculated by expression (4).

$$RRL = (GR\text{-}TEL)/TCP \quad (3)$$

Where GR = Gross Revenue
TEL = Total cost excluding labour

TCP = Total cost of production

$$BCR = REV/TC \quad (4)$$

Where REV = Total revenues

TC = Total cost

3. RESULTS AND DISCUSSION

3.1 Climate and Farming System

Rooftop rainwater harvesting (RTWH) has the potential to be adopted in rain-fed hilly, semi-hilly, plain, and desert ecologies [12]. In rain-fed areas of Pothwar, specifically in the study area with the humid environment, the technology has good potential to be adopted by farmers. Murree and KotliSattian area are situated in a subtropical highland climate zone, with mean annual precipitation of 1,440 mm in Murree and 990 mm in Kotli Sattian. Thus, the mean annual rainfall in the study area is 1215 mm. The temperature ranges from 0°C to 35°C, with an average of 26°C at Murree and from -4°C to 47°C, with an average of 22°C at Kotli Sattian [35].In the study area, crop and livestock farming, which was the main livelihood earning activity in the past has now been taken as secondary sources of income due to change in resource endowment of the people and availability of employment opportunities in non-agriculture sectors. With time area people have entered both public & private sector services, small businesses, and unskilled/ skilled labour markets. Specifically, in Muree tehsil tourism and hoteling opened up new earning avenues for the people.

Wheat is the main Rabi season crop which is generally followed by maize crop. Sample respondents told that loquat, apricot, peach, walnut, guava, plum, lemon, jamen, persimmon, orange, pear are the fruits grown in the area. Cropping mainly depends on rains as there are few seasonal springs and farmers use the water for watering livestock and raising crops. Crop productivity in the study area is low, the average yield of wheat and maize crops are 12 and 24 mounds per acre, respectively. Lower than recommended use of inputs is the reason for low crop productivity. Most people keep livestock to meet their household milk needs. Similarly, [36] stated that crop and livestock productivities are quite low in the study area. It is reported that milk productivity of dairy animals is low, with average daily milk productions of buffaloes, cows, and goats of 8.0, 4.0, and 0.75 liters, respectively. It is further stated that women of the area are actively involved in farming and allied activities. Women grow vegetables, hoe, and harvest crops. Moreover, livestock management is the complete responsibility of the womenfolk. Foregoing this in view, it is stated that RTWH technology is much compatible with existing crop and livestock farming systems in rain-fed hilly areas of Punjab.

3.2 Characteristics of Sample Households

Technological advancements, economic conditions, institutional support, and human-specific factors are the determinants of the adoption of agricultural technologies and practices in developing counties [37]. Socioeconomic characteristics of sample adopter farmers of rooftop rainwater harvesting (RTWH) are presented in (Table 1, Section-I). The adopters were in the late adulthood group, with mean age and formal education of 51.8 and 8.4 years, respectively. They were well experienced in crop farming with a mean experience of 22.6 years. The study area has hilly topography, where crop farming is practiced generally on terraces, with small landholdings. The mean operational landholding of sample farmers was 2.1 acres. Which is predominantly rain-fed. Similarly, due to the limitation of land, fodder supplies are much limited resulting in small livestock holdings. Mean livestock holding in the study area was 3.6 animals per farm, with one to two cows and two three goats. Farming households in the study area have diversified income sources with very limited dependency on crop and livestock farming. Crop and livestock income shares were 13.7 and 5.8 % in the household income, respectively (Table 1, Section-II). Non-farm income shares more than one-third of the household income (35.9%). Small enterprises and remittances share 26.7% and 17.9% in the income of farming households respectively.

3.3 Awareness of the Technology, Access to Materials and Support Services

Availability of effective information as characterized by [38] to be accurate, timely and relevant, reduces the uncertainty of the user and results in the best choice among the alternates available to him. While, adoption of water harvesting for supplementation irrigation depends on observed risk reduction of crop failure and economic benefits for farming households as was evident in Burkina

Table 1. Demographic characteristics of adopter farmers

Farmers' Characteristics	**Mean**	**Standard Deviation**
Age of the farmer (Year)	51.8	13.6
Education of the farmer (Year) Farming	8.4	2.1
experience (Year)	22.6	21.3
Family size (Number)	6.2	2.7
Total Operational holding (Acre)	2.0	1.2
Livestock holding (Number)	3.6	3.3
Household Income Sources (Rs./ annum)	**Mean**	**Percent**
Crops	41100	13.7
Livestock	17400	5.8
Small enterprises & trade	80400	26.7
Remittances	54000	17.9
Job/ Non-Farm	108000	35.9
Total	309000	100.0

Faso, Kenya. [39]. Similarly, diversification of the cropping system causes the adoption of RWH and supplementation of irrigation [19]. In agreement with this, it stated that a one-unit increase in diversity of irrigated crops, especially high-value crops, increases the chances of adoption of RWH technology by 6.98 units (about seven times) [40].

Similarly, successful experiences with the RTWH technology have been reported by [40] from China and [41] from Rwanda. In the study area, rainwater harvesting is the indigenous practice as people store rainwater in small ponds, ditches, and tanks for household use for generations. In the study area, RTWH technology was introduced by UNDP and IUCN in the 1990s, thus twenty percent of the sample farmers reported having knowhow about it for more or less 25 years. While out of the remaining (80%), forty percent each were apprised about the technology and its benefits by technical personnel of CEWRI, PARC-NARC, and by their fellow farmers, each. Similarly, market access is a key factor to improve the productivity and selection of product portfolios. Better market access results in the adoption of new beneficial production technologies and techniques. One-half of the farmers reported that materials required for installation of rooftop water harvesting systems are available in local markets on an average at two locations to them. While remaining half reported purchasing it from non-local markets in Bhara Kahu, Islamabad, and Raja Bazar, Rawalpindi at an average distance of 42 km from their farms.

Adoption of rainwater harvesting depends on educational status, number of active family labourers, contact with extension agents, participation in public sector initiatives, and optimistic attitude toward the technology. These are reported to have a statistically significant positive effect on the adoption by [40]. Other factors that facilitate the process of the adoption include farmers' assets holding and practical training [22], technical support [39], membership to a community/ farmer organization [42], and access to credit [43]. While it is also stated that RWH techniques require a considerable amount of economic and physical resources, which are often inaccessible to specific farm households having small landholdings in rain-fed areas, thus makes it an unaffordable venture for them. [39] Sixty percent 60% of the sample farmers reported that technical services for installation of RTWH systems and repair and maintenance were available in their vicinity to a sufficient extent. Eighty percent of them were of the view that educated and illiterate people in their localities have the almost same level of understanding about technology. Similarly, eighty-three percent of the farmers were of the view that technology is beneficial, and if materials required for installation are made available in their area it will accelerate the adoption. Eighty-seven percent 87% of the adopter farmers reported getting training about the technology installation, repair, and maintenance from PARC-NARC and NRSP. While thirteen percent of the farmer reported adopting the technology on their own as it is indigenous and quite easy to install.

In the study area, people use rooftop harvested water for livestock watering, household uses, crop and fruit farming, etc. The majority of the sample farmers (75%) reported having access to credit mainly provided by NRSP. However, none of them reported obtaining a loan from NRSP for investing it in rooftop rainwater harvesting infrastructure. NRSP provides individual loans of just Rs. 5000 with annual charges of Rs.1000 (20%). Which is quite insufficient to meet the installation cost of the system.

3.4 Potential of Rainwater Harvesting & Utilization

RTWH results in the availability of a sufficient quantity of water for household consumption. Rainfall water harvesting potential through RTWH in the study area is presented in Table 2. The mean annual rainfall in the study area is 1215 mm. This results in monthly water availability of 4072 to 43552 liter per household by considering the average roof catchment area of 221.3 m^2 and coefficient of runoff of 0.8. In the study area, the average monthly water availability through the system is 17926 liter. Daily water availability per capita ranges from a minimum of 22 liters in November to a maximum of 231 liters in August. Thus, RTWH provides a sufficient quantity of water to meet daily household water needs from January to September. While during October to December rainfall water harvesting through the system is less than the standard per capita daily water requirement of 48 liters by nine-liter in October to the highest gap of 26 liters in November. However, in the winter season decrease in requirements of water for both household use as well crop production occurs. Hence it can be stated that rainfall in the study area is sufficient to meet the water requirement of the people for household use if it would be attached with double storage capacity than the current level averaged at 360 ft^3 (10,194 liters). However, farmers are unable to enhance storage capacity due to resource constraints. Similar, findings of inadequate storage capacity of tanks for rainwater harvesting and its use for domestic water supply are reported by [20] from the Edo State of Nigeria. It was stated that the majority of people got empty tanks mid-way into the dry season. Thus, the water supply for production for the production of vegetables and fruits can be stabilized by increasing the water storage capacity.

3.5 Cost-benefit Analysis of RTWH for Kitchen Gardening

Economic benefits of rainwater harvesting depend on the amount of rainfall and its timings, as well as on the construction design i.e. catchment area, water storage capacity, and irrigation facilities [41]. Similarly, these factors play important role in determining the costs of installation of the RTWH

Table 2. Rainfall water harvesting potential through RTWH

Months	Rain Fall (mm per month)			Monthly water Availability per household (liter)	Daily water availability per capita	Daily excess (+)/ deficit (-) in per capita water requirement* (liter)
	Kotli Sattian	Murree	Mean			
January	62	103	83	14694	79	31
February	72	107	90	15934	86	38
March	97	130	114	20183		61
April	70	105	88	15580	84	36
May	50	76	63	11154	60	12
June	57	98	78	13809	74	26
July	201	285	243	43021		183
August	201	290	246	43552		186
September	90	125	107	18943		54
October	32	50	41	7259	39	-9
November	19	27	23	4072	22	-26
December	34	44	39	6905	37	-11
Total	985	1440	1215	215104	-	-

system. On sample farms, crop operational area was ranged from about one to four-acre, with a mean of 2.1 acres. The catchment area of the RTWH systems was ranged from 121.4 to 526.1 m^2, with a mean of 221.3 m^2. Command area of the system at sample farms, averaged at 0.33 acres (16.7% of the operational area), ranged from 0.06 to 2.50 acres. Cost and benefit analysis of RTWH on an average farm basis with a command area of 0.33 acres is presented in Table 3.

The fixed cost of the rooftop rainwater harvesting system includes the cost of gutter pipes, conduits, and filters. Which is found to be about Rs. 10,000. The annual fixed cost of installation of the system was Rs.1764 by considering the life of the system 5.75 years, thus it shares 9.22 % in the total cost of vegetable/ fruit production through the RTWH system. Twenty-five percent of the sample farmers reported having sand filters installed with the system for water cleaning and quality improvement. Half of them (50%) reported practicing manual cleaning of systems on annual basis, while the remaining (25%) responded not to clean the systems at all. Fixed cost of water storage tank (concrete or plastic) is averaged at Rs. 9933, by considering operational life of 8.67 years, it shares 5.69 percent in the total cost of production. Thus, the fixed cost of the RTWH system shares 14.91 % in the total cost of production of vegetables and fruits.

RTWH systems are used to irrigate vegetables and fruit orchards mostly in the vicinity of homes. Vegetable production is economically more viable due to higher returns and shorter growth periods and high demand in semi-arid environments [15]. Sample farmers reported a growing variety of vegetables, including garlic, onion, turnip, radish, spinach, peas, fenugreek, carrot, coriander, cucumber, gourds, and okra. Sample farmers reported using harvested rainwater for irrigation of fruit plants (guava and citrus). However, the whole of the fruit production is consumed at home or gifted to relatives and friends. Considering the cost of family labour at prevailing wage rates, the total variable cost was Rs.16087 per annum (84.99% of the total cost).

Total revenues from produce per annum (including the value of products used at home or gifted at retail market prices) were Rs.21920. Net revenue from the RTWH is Rs.2992 per annum by considering the cost of family/shared labour at prevalent market rates thus, the benefit-cost ratio (BCR) of crops and fruit farming through RTWH systems was 1.16 with a return on investment of 15%. The rate of return to labour cost was 0.95. This is quite substantial considering limited employment opportunities specifically for women folk in rain-fed hilly areas of Punjab. The results are in line with [44], as the BCR of RWH ranges from 1.0 to 1.6 in semi-arid areas of Tanzania. It is stated that RWH improves gross margin as well as returns to labour. A review of the literature revealed that BCR greater than 1.0 is considered a feasible goal for RWH [45-47].

The impact of RTWH for rural communities in India has been studied and it is reported economically viable, with a positive impact on the productivity, employment, and income of the rural poor households [48]. Farmers having RTWH systems installed at their farms in the study area obtain economic gains from the adoption despite the subsistence level of farming. It also provides partial employment opportunities for people in general and women in specific. Financial gains could be improved further by enhancing storage capacity, increasing command area under RTWH, selecting crops with water requirements process that coincides with water supply through the system, or through the adoption of high-efficiency irrigation systems like drip, drip-bucket, and sprinkler, etc. Similarly, the making of micro-catchments around fruit plants can also help to minimize the effect of water stress [49]. Likewise, the use of harvested rainwater can be made more economical if used for multiple purposes like kitchen gardens, livestock and household uses to reduce water hauling cost/ utility bills. It is reported that it resulted in increased annual income of rural households in India [50]. In the study area, RTWH results in net revenue of Rs.14385 per annum without including the opportunity cost of family labour, with a substantial benefit-cost ratio of 2.91.

Water is considered the most important factor limiting agricultural production in rain-fed hilly areas. Increasing water scarcity is also mounting pressure on other natural resources. Thus, it is required to augment the water supply in these areas through accelerated adoption of RTWH by exploring feasible sites. Moreover, farmers are

Table 3. Cost and benefits of adoption of rooftop rainwater harvesting system (Rs. per farm)

	Units	Quantity	Price	Value	Life (years)	Cost annum^{-1}
A. Fixed Costs						
i. Water harvesting						
Conveyance component Gutter lines	feet	35.0	65.5	2643	5.75	460
Conduit	feet	15.0	413.3	6200	6.67	930
Water quality improvement component Coarse mesh/ valve/ filters	No.	0.33	600.0	200	1.0	200
Installation cost	M/day	1.0	1000.0	1000	5.75	174
Sub total				10043	-	1746 (9.22%)
ii. Storage component						
Tank (Concrete/plastic)	No.	1.0	9933.3	9933.3	8.67	1077
Sub total				9933.3	-	1077 (5.69%)
Total (i+ii)	**Rs.**	**-**	**-**	**19975.8**	**-**	**2841**
B. Variable costs						
Cleaning of tank by using fami ly labour	M/day	0.25	600	-	-	150
Slaked lime for cleaning of tan k	Kg	2.5	30	-	-	70
Land Preparation (tractor)	Hours	2.3	728	-	-	1699
Land preparation (manual by family labour)	M/day	0.7	600	-	-	413
Seeds/Seedlings	Rs.	850	-	-	-	850
Sowing by using family labour	M/day	2.25	600	-	-	1350
Fertilizer	Kg	27.5	30	-	-	825
FYM	Trolle	0.5	1500	-	-	750
Family labour for other farm operations (hoeing/weeding, irrigation, FYM &	M/days	15.8	600	-	-	9480
Repair & maintenance hired labour	M/days	0.50	1000	-	-	500
Total						16087 (90.12%)
C. Total cost (A+B) by considering cost of family labour						**18928**
						7535
D. Total cost by excluding opportunity cost of family labour						
E. Revenues Vegetables						
Consumed at home/ gifted	kg	72.0	62.5	-	-	4500
Sold out	kg	228.5	70.0	-	-	15995
Fruits Consumed at home/ gifted	kg	12.5	50			625
Consumed at home/ gifted	dozen	10	80			800
Gross Revenue						**21920**
Net Revenue with the cost of family labour (E-C)						**2992**
Net Revenue without the cost of family labour (E-D)						**14385**
BCR with the cost of family labour (E/C)						**1.16**
BCR without opportunity cost of family labour (E/D)						**2.91**

needed to be sensitized about the water scarcity, the importance of saving rainwater through the adoption of technology, and judicious use of harvested water for household purposes i.e. abolition, bathing, house cleaning, animal watering, kitchen gardening, cloth washing, and toilet use, etc.

4. CONCLUSION AND RECOMMENDATIONS

Findings of the study are useful for key stakeholders as it provides necessary information of the relevant factors and conditions under which the technology performs the best. The technology is very cost-effective and can help decision-makers and water resource planners to meet water scarcity challenges in the region. The study revealed the huge potential of RTWH in hilly areas of Punjab, as these are humid areas with high rainfall and farmers are to face water stress in the summer season, particularly during April to June. Furthermore, this system is very suitable for hilly and scattered houses, where providing water through supply schemes is generally very costly. RTWH can add in household income by reducing food bills especially for

vegetables and fruits which they usually buy from the market at high prices. Adoption of the technology saves the precious time of people which could be used productively elsewhere in other productive income-generating activities. It reduces their fatigue for water fetching for households as well as kitchen gardening. Most importantly use of the water harvested through the system is used to produce contamination-free vegetables and fruits with low use of synthetic fertilizers and hazardous chemicals. That is more fresh, healthy, and readily available to farm families than those supplied at high prices from distant wholesale markets. In the study area, farmers are resource-poor, and mostly take agriculture as a secondary business. Furthermore, great variations in rainfall have been reported from one location to another location. All these factors limit the adoption of the technology. Thus, a participatory promotion approach should be brought to the fore for the promotion of technology and improving subsistence food production. In this regard, increased awareness, capacity building, and collaboration among key stakeholders including subject specialists, researchers, technical experts, development partners, agricultural extension staff, and land users should be pursued. Similarly, farmers' skills regarding system installation, repair & maintenance should be enhanced. The public sector should come forward to up-scale the adoption through policy interventions about subsidies and access to loans. The technology should be given due coverage in the 'National Water Policy' and plans. Furthermore, climate change's impact on rainwater harvesting potential is also required to be researched as farmers reported great variation in rainfall patterns over time.

5. ACKNOWLEDGEMENTS

Authors are highly indebted to USDA for sponsoring the Project called Pakistan 'Water Dialogue-Diffusion and adoption through partnership and action of the best watershed rehabilitation and irrigation practices and technologies to help rural farmers'. The authors are also thankful to the ICARDA-Pakistan office for their constant technical support to conduct this study and Climate Change, Energy and Water Resources Institute (CEWRI) NARC and NRSP to promote the technology and help identify the respondents.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. REFERENCES

1. D. Molden, M. Vithanage C. D. Fraiture, J. M. Faures, L. Gordon, F. Molle and D. Peden. Water availability and its use in agriculture. In: Treatise on Water Science, P. Wilderer (Ed.) T*echnische Universitaet Muenchen, Institute for Advanced Study, Munich, Germany* (2011).
2. J. Rockström, and L. Karlberg. Zooming in on the global hotspots of rainfed agriculture in water-constrained environments. In: Rainfed Agriculture: Unlocking the Potential, S. P. Wani, J. Rockström and T. Oweis (Ed.) *CAB International, UK* (2009).
3. J. Barron. Rainwater harvesting: A lifeline for human well-being. United Nations Environment Programme and Stockholm Environment Institute. Division of Environ- mental Policy Implementation, *United Nations Environment Programme, Nairobi, Kenya* (2009).
4. PBS. Final Results Census-2017. Pakistan Bureau of Statistics. *Government of Pakistan. Islamabad* (2021).
5. M. Ashraf. Importance of water in the light of Quran and Sunnah and ways of its saving. *Pakistan Council of Research in Water Resources (PCRWR), Islamabad,* pp.37 (2015).
6. B. John, and Q. Usman. Pakistan's water economy: running dry. *World Bank, Washington, DC, USA* (2005).
7. D. Aziz. A. Masood, and Z. Hashmi. *Turning the tide,* The News International. https://www.thenews.com.pk/print/328174-turning-the-tide (Accessed 3 July 2021).
8. M. Hassan. Water sector of Pakistan: A situational analysis. Water Security in Pakistan: Issues and Challenges. *Development Advocate of Pakistan.* 3 (4): 2-9 (2015).
9. P. Bitterman, E. Tate, K. J. V. Meter and N. B. Basu. Water security and rainwater harvesting: A conceptual framework and candidate indicators. *Applied Geography.* 76: 75-84 (2016).
10. T. Oweis, Supplemental Irrigation: A highly efficient water-use practice. *International Center for Agricultural Research in the Dry Areas ICARDA, Aleppo, Syria,* pp. 16 (1997).
11. Y. T. Dile, L. Karlberga, M. Temesgenc, and J. Rockströmb. The role of water harvesting to achieve sustainable agricultural intensification and resilience against water related shocks in sub-Saharan Africa. Agric. *Ecosystems Environment.* 181: 69-79 (2013).
12. J. R. Michelcic, J. B. Zimmerman, and A.

Ramaswami. Integrating developed and developing world knowledge into global discussions and strategies for sustainability. Environment *Science and Technology.* 41:10, 3415–3421 (2007).

13. Komariah and M. Senge. The development of water harvesting research for agriculture. *Review of Agricultural Sciences* 1: 31-42 (2013).
14. A. K. Tripathi, and K. U. Pandey. Study of rainwater harvesting potential of Zura Village of Kutch District of Gujarat. *Journal of Human Economics.*18:1, 63–67 (2005).
15. S. N. Ngigi, H. H. G. Savenije, J. Rockstrom and C. K. Gachene. Hydro-economic evaluation of rainwater harvesting and management technologies: Farmers investment options and risks in semi-arid Laikipia district of Kenya. *Physics and Chemistry of the Earth* 30: 772–782 (2005)
16. J. A. Tabor. Improving crop yields in the Sahel by means of water-harvesting. *Journal of Arid Environment.* 30:83-106 (1995).
17. S. Noel, and H. T. Phuong, J. Soussan and J. C. Lovett. The impact of domestic water on household enterprises: Evidence from Vietnam. *Water Policy* 12: 237-247 (2010).
18. M. Zulfiqar, I. Abbasi, H. Khan, A. Nizami, A. Hakeem, J. Ali and M. J. Khan. Agricultural economy of Skardu is based on glaciers and snow melting- A case study of Burgay watershed. *Sarhad Journal of Agriculture.* 35(2): 336-341 (2019).
19. A. Teshome, E. Adgo and B. Mati. Impact of water harvesting ponds on household incomes and rural livelihoods in Minjar Shenkora district of Ethiopia. *Ecohydrology and Hydrobiology.* 10 (2-4): 315-322 (2010).
20. S. I. Oni, E. Ege, C. Asenime and S.A. Oke. Rainwater harvesting potential for domestic water supply in Edo State. *Indus Journal of Management & Social Sciences.* 2 (2):87-98 (2008).
21. D. Prinz, and A. Singh. Technological potential for improvements of water harvesting. A contributing paper prepared for thematic review on Assessment of irrigation Options. *World Commission on Dams. London. UK* (2001).
22. M. W. Kimani, A. N. Gitau, and D. Ndunge. Rainwater Harvesting Technologies in Makueni County, Kenya. *International Journal of Engineering and Science.* 15 (2): 39-49 (2015).
23. S. Z. H. Gardezi. Promotion of rainwater harvesting in Earthquake Affected Areas of KP and AJK. *Earthquake Reconstruction and Rehabilitation Authority (ERRA), Prime Minister's Secretariat, Islamabad, Pakistan* (2012).
24. O. Rashid, F. M. Awan, Z. Ullah and I. Hassan. Rainwater harvesting, a measure to meet domestic water requirement: A case study Islamabad, Pakistan. *Materials Science and Engineering.* 414 (1): 1-9 (2018).
25. R. Siddiqui and S. Siddiqui. Assessing the rooftop rainwater harvesting potential in urban residential areas of Pakistan: A case study of model town, Lahore, Pakistan. *International Journal of Economic and Environment. Geology.* 9 (2): 11-19 (2018).
26. A. Subhani. Rainwater harvesting and efficient uses of water to improve crop productivity in the Pothwar region of Punjab. *Soil and Water Conservation Research Institute, Chakwal, Pakistan* (2017).
27. M. Van-der-Sterren, A. Rahman and G. Dennis. Rainwater harvesting Systems in Australia, ecological water quality - water treatment and reuse. Voudouris (Ed.), Intech Open Limited, *London, United Kingdom* (2012).
28. J. Zhi-yun J, L. Xiao-yan and M. Yu-jun. Water and Energy Conservation of Rainwater Harvesting System in the Loess Plateau of China. *Journal of Integrative Agriculture.* 12(8): 1389-1395 (2013).
29. N. A. Kabbashi, M. S. Jami, N. H. Abdurahman, and N. I. M. Puad. Rainwater harvesting quality assessment and evaluation. *IIUM Engineering Journal.* 21 (1): 12-22 (2020).
30. S.N. Rahmat, A. A. S. Al-Gheethi, S. Ayob, and F. M. Shahli. Development of dual water supply using rooftop rainwater harvesting and groundwater systems. *SN Applied Sciences* 2: 85. (2019).
31. C. C. Nnaji, and C. Aigbavboa. A scenario- driven assessment of the economic feasibility of rainwater harvesting using optimized storage. *Water Resources Management,* 34: 393.
32. J. Gould, and E. P. Nissen. Rainwater catchment systems for domestic supply. *International Technology Publications, London, United Kingdom* (1999).
33. A. Pacey, and A. Cullis. Rainwater harvesting: the collection of rainfall and runoff in rural area. *International Technology Publications, London, United Kingdom* (1989).
34. A. Daniel, H. Smith, E. P. Andrew, P. Hutchinson and S. Homscheid. Rainwater harvesting in the Caribbean. *The Caribbean Environmental Health Institute, Nairobi, Kenya* (2009).
35. GoP. District Census Report. Pakistan Bureau of Statistics, *Government of Pakistan, Islamabad* (2017).
36. A. Hussain, and M. A. Khan. Rapid appraisal of site for establishment of Hill Agricultural Research and

Technology Demonstration Station (HARTDS), Lehtrar, Kotli Sattian. *Social Sciences Division, PARC, Islamabad* (2010).

37. M. Mwangi, and S. Kariuki. Factors determining adoption of new agricultural technology by small farmers in developing countries. *Journal of Economics and Sustainable Development.* 6 (5): 208-216 (2015).
38. E. O. Adio, Y. Abu, S. K. Yusuf and S. Nansoh. Use of agricultural information sources and services by farmers for improve productivity in Kwara State. *University of Nebraska – Lincoln, USA* (2016).
39. P. Fox, J. Rockstrom, and J. Barron. Risk analysis and economic viability of water harvesting for supplemental irrigation in semi-arid Burkina Faso and Kenya. *Agricultural Systems.* 83 (3): 231–250 (2005).
40. X. He, H. Cao, and F. Li. Econometric analysis of the determinants of adoption of rainwater harvesting and supplementary irrigation technology (RHSIT) in the semiarid Loess Plateau of China. *Agricultural Water Management.* 89 (3): 243 – 250 (2005).
41. A. Zingiro. Assessment of adoption and impact of rainwater harvesting technologies on rural farm household income: The case of rainwater harvesting ponds in Rawanda. *Department of Agricultural Economics, Faculty of Agriculture, University of Nairobi, Kenya* (2012).
42. S. Kumar, T. Ramilanb, C. A. Ramaraoc, C. S. Rao, and A. Whitbread. Farm level rainwater harvesting across different agro climatic regions of India: Assessing performance and its determinants. *Agricultural Water Management.*176: 55–66 (2006).
43. A. Berhanu, and B. Fufa. Repayment rate of loans from semi-formal financial institutions among small-scale farmers in Ethiopia: Two-limit Tobit analysis. *The Journal of Socio-Economics.* 37 (6): 2221–2230 (2008).
44. T. Yuan, L. Fengmina, and L. Puhaib. Economic analysis of rainwater harvesting and irrigation methods, with an example from China. *Agricultural Water Management.* 60: 217–226 (2003).
45. E. M. M. Senkondo, A. S. K. Msangi, P. Xavery, E. A. Lazaro and N. Hatibu. Profitability of rainwater harvesting for agricultural production in selected semi-arid areas of Tanzania. *Journal of Applied Irrigation Sciences,* 39. (1): 65 – 81 (2004).
46. R. M. Roebuck, C. Oltean-Dumbrava, and S. Tait. Whole life cost performance of domestic rainwater harvesting systems in the United Kingdom, *Water and Environment Journal.* 25: 355–365 (2011).
47. Y. Zhang, D. Chen, L. Chen, and S. Ashbolt. Potential for rainwater use in high-rise buildings in Australian cities. *Journal of Environment Management.* 91: 222–226 (2009).
48. Y.R. Chiu, C.H. Liaw and L.C. Chen. Optimizing rainwater harvesting systems as an innovative approach to saving energy in hilly communities, *Renewable Energy.* 34: 492–498 (2008).
49. A. Hussain. Adoption and cost-benefit analysis/ feasibility assessment and projection of potential impact of moisture saving technologies in Pakistan. *Social Sciences Research Institute, PARC-National Agricultural Research Center, Islamabad* (2018).
50. P. S. Gotur, S. Devendrppa and S. Sadaqath. Impact of domestic rooftop rainwater water harvesting programmes for rural communities. *Green Farming.* 2 (11): 803-804 (2009).

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 77-88 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)646

Pakistan Academy of Sciences

Research Article

Hydrophobic Drug Release Studies from the Core/Shell Magnetic Mesoporous Silica Nanoparticles and their Anticancer Application

Amina Hussain*

Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi

Abstract: Multiple therapeutic hydrophobic drugs can be delivered simultaneously by inorganic, biocompatible iron core mesoporous silica shell nanoparticles. We synthesized superparamagnetic iron oxide nanocrystals encapsulated within mesostructured silica spheres through the sol-gel process. The dose of hydrophobic drugs Paclitaxel (PTX) and Camptothecin (CPT) loading and released on $Fe_3O_4@SiO_2$ core/shell nanoparticle detected by U.V-visible spectrophotometry using a platform of nanoparticles (NPS). After being subjected to external heating, the drug release efficiency of paclitaxel (PTX) and camptothecin (CPT) $Fe_3O_4@SiO_2$ core/shell nanoparticles is increased. Paclitaxel (PTX) and Camptothecin (CPT) $Fe_3O_4@SiO_2$ core/shell nanoparticles did not heat the solution when an alternating magnetic field (AMF) was applied, and there was only mild drug leakage. When compared to Fe_3O_4@MSNs, the nanoparticles (PTX) and (CPT) Fe_3O_4@MSNs function as cancer-targeting mediators, increasing the killing of PANC-1 cancer cells. Human cancer cells were given these therapeutic anticancer water-insoluble drugs with nanoparticles, which is a valuable vehicle for drug delivery, and induced the inhibition of proliferation. Therefore, the goal of this study to emphasize $Fe_3O_4@SiO_2$ core/shell potential as a superior candidate for hydrophobic drug delivery to the PANC-1 cancer cell.

Keywords: Mesoporous silica, nanoparticles, loading, release, hydrophobic drugs, PANC-1 cells

1. INTRODUCTION

The lack of appropriate biocompatible delivery mechanisms for most hydrophobic anticancer drugs is a big hurdle and threat for cancer treatment. Drugs water insolubility makes it difficult to administer drugs through the intravenous route, so improving aqueous solubility is especially necessary [1]. Although most essential anticancer agents have low water solubility, researchers have concentrated on finding a new delivery system for these molecules that do not rely on organic solvents. Nanoparticles have a lot of potentials and are an excellent means to transport anti-cancer drugs into certain organs or cell types, they've been intensively developed for use in cancer therapy [2]. Paclitaxel (PTX), a plant-derived alkaloid with cytotoxic effects in breast, prostate, and cervical cancers, is an anticancer drug. However, this alkaloid is not fully soluble in water [3, 4]. Camptothecin (CPT) is among the most successful anticancer drugs of the twenty-first century. Even though studies have shown their efficacy against stomach, colon, throat, and prostate cancers, and also breast, lung cancers, and leukemia [5, 6].

Precision medicine, described as "the correct medicine, at the correct dose, at the accurate time, to the correct person," is a rapidly growing and influential cancer treatment practice around the world. Individual variability is taken into account in the design of customized disease care regimens in this new method [7]. The requirement of the correct therapeutic drug distribution at the desired time to the precise site of the disease, as well as the precise therapeutic dosage, poses a trial [8-11]. Over the last decade, many examples of drug-delivery stimuli-responsive platforms have been created, including those that respond to cellular internal stimuli (change in pH or bio compounds), as well as external stimuli such as (ultrasound, heat, and light) [11-15].

Received: January 2021; Accepted: March 2021
*Corresponding Author: Amina Hussain <amina.hussain878@gmail.com>

Progress in using inorganic nanoparticles for biological means has accelerated due to the wide volume of work completed in material modification. These nanoscale materials provide a strong framework that can be used for a variety of purposes [15-16]. Mesoporous magnetic materials have a wide range of uses in industry and research [17]. Magnetic resonance imaging contrast enhancement, drug delivery, heat, or hyperthermia are only a few of the biomedical uses for superparamagnetic nanoparticles that have been studied extensively. Drug delivery triggered by heat produced when magnetic nanoparticles are exposed to the alternating magnetic field. Only a certain amount of drugs can be carried and released by a magnetic nanoparticle [18]. Mesoporous silica has gotten a lot of attention in drug loading and release due to its larger pore volume and size. Mesoporous silica's higher toughness, biocompatibility, surface functionalization, higher efficiency of cellular internalization makes an excellent candidate for use as super magnetic nanoparticles coating in drug distribution and delivery [19]. Furthermore, due to iron oxide composition, such mesostructured is particularly distinctive for clinical applications in terms of toxicity, stability, and biocompatibility [20].

This research work describes the biocompatible inorganic nanoparticle synthesis by the sol-gel method used for the delivery of therapeutic hydrophobic anticancer drugs on the human cancer cell. Magnetic manipulation was achieved by incorporating superparamagnetic iron oxide nanocrystals into mesoporous silica nanoparticles. Chemotherapeutic drug molecules were packed into the mesoporous silicate pores and subsequently delivered into PANC-1 cell lines to improve the therapeutic efficiency, reduced the conventional chemotherapy side effects, and overcoming the insolubility problem of hydrophobic drugs. The anticancer drug-loaded Fe_3O_4@SiO_2 core/shell nanoparticles system developed in this study could pave the way for in vivo selective and controlled cancer therapeutics.

2. MATERIAL AND METHODS

$Fe(acac)_3$, (97%), 1, 2-dodecanediol (92%), oleic acid (90%), oleylamine (70%), benzyl ether (98%), hexane (98.5+%), hexadecyltrimethylammonium bromide (CTAB, 99+%), tetraethyl orthosilicate (TEOS, 98%), phosphate-buffered saline (PBS, 10×), Paclitaxel and Camptothecins purchased from Cayman Chemical, Dimethyl sulfoxide (DMSO, 99.9+%), sodium hydroxide (NaOH, 97+%), hydrochloric acid (HCl, 36.5% 38%, trace metal grade) purchased from Fisher Scientific. Dulbecco's modified Eagle medium (DMEM) with high glucose, fetal bovine serum (FBS), antibiotics (10, 000 U/mL penicillin, 10, 000 μg/ mL streptomycin, and 29.2 mg/ mL L-glutamine), trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (0.05%) were purchased from Gibco and Dulbecco's phosphate-buffered saline (DPBS). Cell-counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Ethanol (200 proof) was purchased from Decon Laboratories, Inc.

2.1 Synthesis of Fe_3O_4 Nanoparticles

Under nitrogen flow, $Fe(acac)_3$ (2 mmol), 1,2 hexadecanediol (10 mmol), oleic acid (6 mmol), oleylamine (6 mmol), and benzyl ether (15 mL) were combined and magnetically stirred. Under a nitrogen blanket, the reaction mixture was heated to reflux (363°C) for another 30 minutes after being heated to 298°C for 30 minutes. The mixture was formulated into black-brown color and allowed to cool to room temperature. 40 mL ethanol was applied at room temperature, and black content was precipitated and centrifugated. In presence of hexane, a black material was dissolved in (0.05 ml) oleylamine and (0.05 ml) oleic acid. For solvent removal, centrifugation at 6000 rpm for 10 minutes was used, followed by dispersion in hexane [21].

2.2 Synthesis of Fe_3O_4@MSNs Nanoparticles

Fe_3O_4 nanoparticles (2.5 mg) were dispersed in 0.2 ml chloroform and added to 2 ml of 40 mg of CTAB in the Fe_3O_4 solutions. The reaction mixture was sonicated for 20 mins with a properly sealed cover to make oil in a water solution. The emulsion was then sonicated for 2 hrs. to evaporate the chloroform. A well-dispersed CTAB Fe_3O_4 aqueous solution (3 ml) was obtained. Then, in 18 ml of water 40 mg of CTAB was dissolved with 120 ul of 2 M NaOH solution in a 100 ml flask. At 70 °C and constant stirring, 2 ml of CTAB Fe_3O_4 aqueous solution was applied to the reaction solution. For mesoporous silica shell coating on CTAB Fe_3O_4, add 1.2 ml ethyl acetate and 200 ul of TEOS dropwise in the solution and allowed it to stir for

2 hrs. After that solution was cooled, centrifuged and thrice times washed with ethanol. Fe_3O_4@ MSNs was distributed properly in ethanol (20 ml) contain 120 mg NH_4NO_3, and to remove surfactants stirred at 60°C for 1hr. To make surfactant-free nanoparticles, the surfactant removal process was repeated two times and Fe_3O_4@MSNs nanoparticles were washed multiple times with distilled water and ethanol [22].

2.3 Characterization

The size and morphology of nanoparticles were determined using transmission electron microscopy (TEM, Tecnai T12). In ethanol, Fe_3O_4 and Fe_3O_4@ MSNs nanoparticles were dispersed. The suspension of nanoparticles was placed onto a carbon-coated grid and allowed to dry at room temperature [23].

2.4 Magnetic Hyperthermia Experiment

Using an alternating magnetic (AM) current hyperthermia experiment with a magnetic field power of H=180 Gauss and a frequency of F= 409 kHz, the magnetic heating efficiency of Fe_3O_4 nanoparticles was evaluated. 2 mg, 4 mg, and 6 mg/ml Fe_3O_4 nanoparticles were distributed in toluene and mounted in the coil center for this experiment. A thermocouple was used to continuously record thermal response data while the magnetic field was applied [24].

2.5 Loading Capacity Analysis of Paclitaxel (PTX) and Camptothecin (CPT) on Fe_3O_4 @ MSNs nanoparticles

Fe_3O_4@MSNs nanoparticles were loaded with 20 mM Paclitaxel (PTX) and 40 mM Camptothecin (CPT) and washed with water five times and PBS 7 times. The supernatant was collected at each washing step and absorption was measured by U.V-visible spectroscopy (Cary 5000). To calculate the amount of drug loading was calculated by loading capacity: (loaded drug mass divided by particles mass) × 100 [25].

2.6 Drug Release Efficiency of Paclitaxel (PTX) and Camptothecin (CPT) Fe_3O_4@ MSNs nanoparticles

Release efficiency of Paclitaxel (PTX) and Camptothecin (CPT) Fe_3O_4@MSNs (0.05 mg) nanoparticles in 1ml of PBS was put in a hot water bath at a different temperature like 23°C, 37°C, 50°C, 80°C with their controls.

Release efficiency of Poclitaxel (PTX) and Camptothecin (CPT) Fe_3O_4@MSNs nanoparticles (0.05 mg/ml) was evaluated by using an alternating magnetic field (AMF) with a magnetic field power of H=180 Gauss and a frequency of F=409 kHz. Centrifugation (7930 rpm, 20 min) was used to separate PTX and CPT released from nanoparticles, which were then measured using UV-visible spectrophotometry (Cary 5000). PTX and CPT release efficiencies are described as the released cargo mass divided by loaded cargo mass multiplied by 100 percent [25].

2.7 Cell Viability Assay

Human cancer cell lines (PANC-1) were supplied by the American Type Culture Collection. Cell culture media were prepared and changed after three days. Cells were passaged using trypsinization until confluence [26].

A cell-counting kit-8 assay was used to find PANC-1 viability after treated with Paclitaxel (PTX) and Camptothecin (CPT) Fe_3O_4@MSNs nanoparticles with their controls. The cells were seeded at a density of 5x 10^3 cells in DMEM for 24 hrs. The medium was removed, and the cells were incubated for 24 hrs in 200 L of fresh DMEM containing Paclitaxel (PTX) and Camptothecin (CPT) Fe_3O_4@MSNs nanoparticles (i.e., 0, 75, and 100 ug/mL). To determine cell viability, each well was filled with DMEM and CCK-8 (10 ml) cellular cytotoxicity reagent and placed in the incubator. Tecan M1000 plate reader was used to test the absorbance at 450 and 650 nm to determine the number of viable cells [26].

3. RESULTS AND DISCUSSIONS

3.1 Production and Characterization of Fe_3O_4 and Fe_3O_4@MSNs Nanoparticles

Fe_3O_4 nanoparticles are formed when $Fe(acac)_3$ reacts with surfactants at high temperatures and is separated from reaction byproducts and high boiling point benzyl ether. Fe_3O_4 nanoparticles of 8-12 nm were formed when benzyl ether was used as the solvent. The large size Fe_3O_4 nanoparticles

obtained from benzyl ether seem to show that a higher reaction temperature would result in larger particles. The key for Fe_3O_4 nanoparticles production is to heat the mixture to 298°C for 30 mins and then reflux at 363°C. A broad range of Fe_3O_4 nanoparticles from 8 to 12 nm are synthesized indicating that Fe_3O_4 nuclei growth is not a quick process under these reaction conditions (Fig 1).

Long-chain alcohols, such as 1,2 hexadecanediol, have been discovered to react well with $Fe(acac)_3$ to form Fe_3O_4 nanoparticles of large size. The reaction produces a red-brown product due to the use of oleic acid. The reaction mixture with only oleylamine produces far fewer Fe_3O_4 nanoparticles than the reaction with both Oleic acid and oleylamine. As particles are oxidized at room temperature by bubbling oxygen by dispersion, they are further precipitated. Increasing the amount of oleylamine results in a redder brown product dispersion. Nanoparticles with a cargo-carrying capacity made with a mesoporous silica shell around a superparamagnetic Fe_3O_4 core [27].

The mechanism for the synthesis of iron oxide nanoparticles is based on the condensation process that occurs during the colloidal growth process. The atomic species that make up an iron oxide particle are stacked on top of each other. The partial reduction and breakdown of $Fe(oleate)_3$ in the presence of oleylamine produces these atomic species. The clustering of the "Fe–O" species produces many nuclei that are saturated in the reaction media and assemble into iron oxide nanoparticles [28]. The solubility of the nuclei in the dispersion determines when nucleation ceases and nuclei aggregation takes control of the growth process. If the nuclei in the dispersion medium are not saturated, the particles cannot develop. The nuclei aggregation becomes impulsive over the saturation threshold till the particles are heated from the dispersion. The reduction in solvent volume in the production of Fe_3O_4 nanoparticles causes early saturation of the oxide-based nucleus, allowing more reactants to participate in the growth cycle, resulting in bigger particles [29]. However, in a greater volume solvent, more nuclei are required to reach saturation, which comes at the expense of the iron salt precursor, leading to reduced Fe_3O_4 nanoparticles. A similar idea can be applied to the surfactant phenomenon. In a high surfactant/metal ratio, more surfactant equates to a larger volume of solvent, and more nuclei are required to reach saturation, resulting in small nanoparticles [30].

Surfactant coating was attained by condensation and hydrolysis of tetraethyl orthosilicate (TEOS) in the presence of hexadecyltrimethylammonium bromide (CTAB). The diameter of Fe_3O_4 @MSN nanoparticles was determined to be 90 nm to 110 nm using Transmission electron microscopy (TEM) (Fig 2).

In a mixture of ethanol, H_2O, CTAB, and ammonium hydroxide, alkoxysilane undergoes hydrolysis and condensation processes, resulting in mesoporous silica. The interaction between the nucleation (hydrolysis) and growth (condensation) processes is critical for efficient encapsulation of the mesoporous silica shell on the Fe_3O_4 core. When the condensation rate exceeds the hydrolysis rate, silica is vulnerable to encapsulating Fe_3O_4 nanoparticles. During the production of the mesoporous silica shell, CTAB works as a surfactant as well as a structure-directing agent. To produce spherical micelles, CTA+ cations first form tight bonds with negative charge Fe_3O_4 nanoparticles. Electrostatic attraction attracts the silicon oligomers produced by TEOS to the spherical micelles [31]. At low concentrations, unbound silicon oligomers primarily deposit on micelle-Fe_3O_4 nanoparticle junctions to conceal the electrostatic repulsion between adjoining head groups in micelles. Furthermore, ethanol diffusion inhibits alkyl tail self-interaction in CTAB micelles, boosting hydrophobic volume and decreasing spherical micelle curvatures. Moreover, using ammonium hydroxide as a basic catalyst can help create hydrogen bonds between neighbouring CTAB micelles and silicon oligomers, promoting the creation of parallel mesoporous channels and lowering micelle curvature energy. Consequently, CTAB-silicon oligomers are structurally transformed from spherical to cylindrical forms. As a consequence, of the above four ingredients, newly hydrolyzed silicon oligomers are adsorbed on the silica/solution interface area and can be further cross-linked via charged head groups. As a result, continuous mesopores form in the axial direction of cylindrical micelles parallel to the nanoparticle surface [28-33].

3.2 Iron oxide Nanoparticles Triggered by Alternating Magnetic field (AMF)

Iron oxide (Fe_3O_4) nanoparticles were distributed

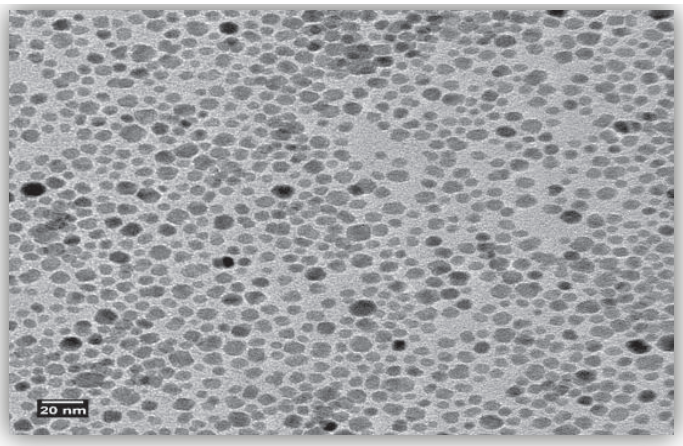


Fig.1. TEM images of Fe_3O_4 produced nanoparticles

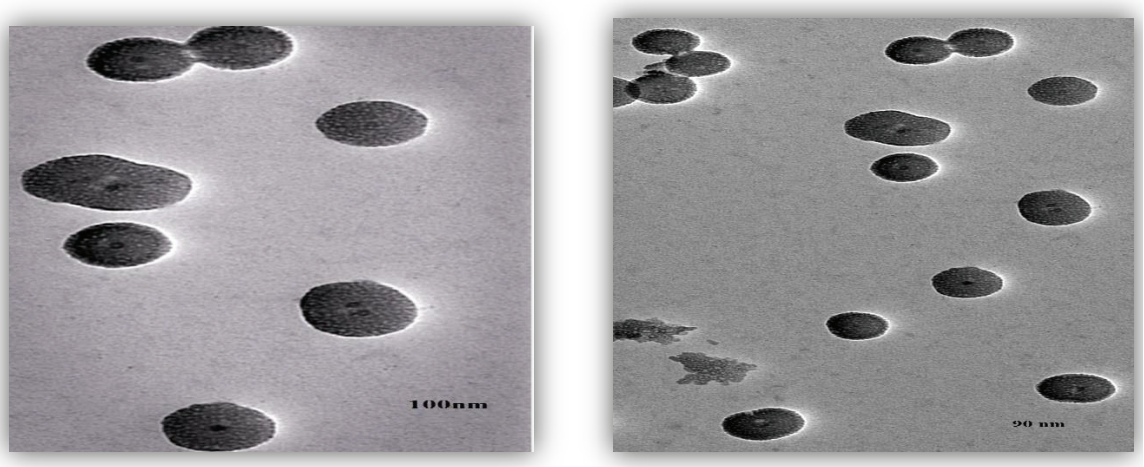


Fig. 2. TEM illustrations of Fe_3O_4@MSNs nanoparticles

in toluene and for heat production, an alternating magnetic field (375 kHz, 5 kW) was used. Fig 3 showed an increase in the temperature profile of time and concentration-dependent when the AMF trigger was applied. The temperature of the toluene solution containing 2mg/ml, 4mg/ml, and 6mg/ml nanoparticles finally achieved 30°C, 33°C, and 35°C after 300 secs.

The ability of Fe_3O_4 nanoparticles to heat up when exposed to AMF shows that they could be used as a heat source for thermally induced drug release. AMF may be responsible for the core and shell mechanism. If movement induced by AMF is confirmed, there is a Brownian heating mechanism of the Fe_3O_4 core in mesoporous silica nanoparticles. In the presence of AMF, there were two probable core moments. 1) Neel relaxation process in which the iron core does not reorient in the AMF, requiring the formation of a dipole atomic structure in the iron core to raise the temperature. 2) The iron core oscillation in the AMF drive silica shell is the other mechanism at work. This resulted in a rise in temperature due to friction-induced by iron mesoporous core-shell nanoparticles, indicating that Brownian relaxation plays a role in the temperature shift of the nano environment [34].

3.3 Loading Capacity Analysis of PTX and CPT Fe_3O_4@MSNs Nanoparticles

To realize the loading of PTX and CPT on Fe_3O_4@MSNs nanoparticles using ethanol and DMSO, widely used solvents for poorly water-soluble drugs. PTX and CPT loading onto Fe_3O_4@MSNs nanoparticles was achieved by soaking overnight and then after 24 hrs loaded nanoparticles were introduced back to the aqueous solution. To calculate the loading capacity by definition as (loading drug mass divided by particles mass) × 100. The drug-loaded nanoparticles were washed with ethanol and DMSO at different times until the supernatant

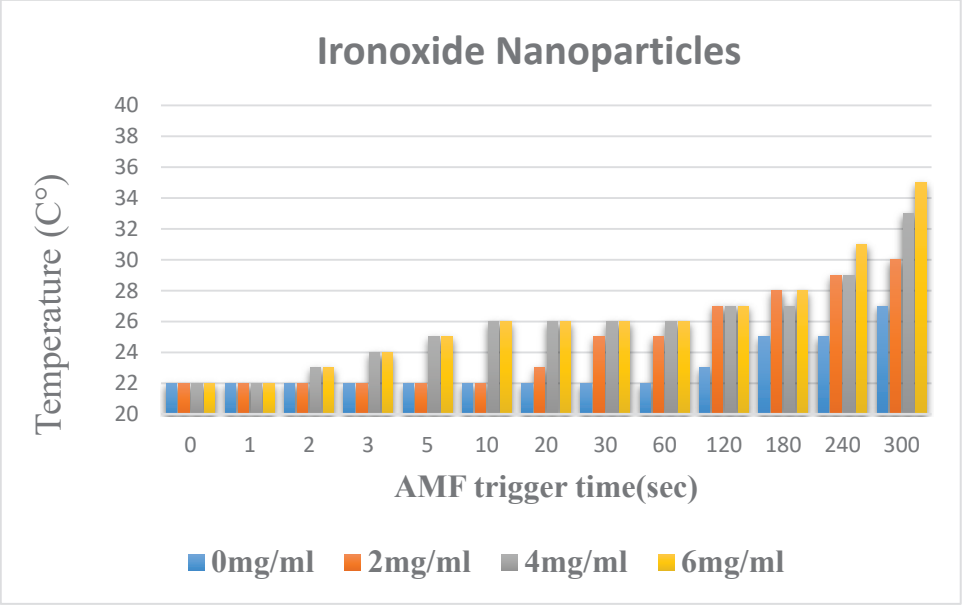


Fig. 3. The temperature increase profile of iron oxide nanoparticles enabled by AMF is time and temperature-dependent.

after centrifugation was clear and collected using U.V-visible spectroscopy. Loading capacity was achieved 20 mM of Paclitaxel was 6.2% and 40 mM of Camptothecin was 8% respectively. Fe_3O_4@MSNs nanoparticles drug loading capacity is due to the mesoporous silica's increased surface area, which provides additional interior spaces and conjugation sites.

The magnetic hollow porous nanocrystal shells have a very high paclitaxel (PTX) loading (20.2 percent) capacity [35]. Sahu and colleagues developed a multimodal theranostic nanoagent based on hollow magnetic mesoporous silica as an effective carrier for high loading and regulated release of Camptothecin (CPT). The synthesized nanomagnetic carrier had a drug-loading capacity of 17.5% [36].

3.4 Influence of External Heating / Oscillating Magnetic Field on PTX and CPT Release from Fe_3O_4@MSNs Nanoparticles

Samples tubes contain PTX and CPT Fe_3O_4@MSNs nanoparticles (0.05 mg/ml) dispersed in deionized water were put in a hotplate of water bath set at 37, 50, and 80°C for 60, 120, 180, and 240 min known as bulk heating. To collect untrapped drug molecules, PTX and CPT loaded Fe_3O_4@MSNs nanoparticles were washed in PBS buffer. The supernatant was collected after the samples were heated and spun down for release calculation. As a control, a non-heated sample at 23 °C was used. The release efficiency is calculated as (the mass of the leaked drug divided by the mass of the loading drug) divided by 100. After 60 mins of heating at a higher temperature (80°C), 70% of the PTX was released with a release efficiency of 70%. Furthermore, 73 and 76% of the drug were released after 120 and 180 min of heating at 80°C, respectively. Nearly 80% of the drug was released at (80°C) after 240 minutes. On the other hand, the release efficiency of the drug at 37°C and 50°C at 240 min is 49% and 62% respectively. The control group at 23 °C drug leakage is only 7.8% at 240 min Fig 4(a). This indicates that the drug was successfully constructed on the surface of Fe_3O_4@MSNs nanoparticles, as shown by the presence of premature leakage at room temperature or physiological temperature. It can simply be inferred that the amount of cargo released is determined by the temperature of the water bath.

Fe_3O_4@MSNs nanoparticles are loaded with the drug Camptothecin. These solid particles showed no drug release at 0 min. When the solution was heated up to 80°C by external hotplate heating at various time intervals for 60, 120, 180, and 240 min there

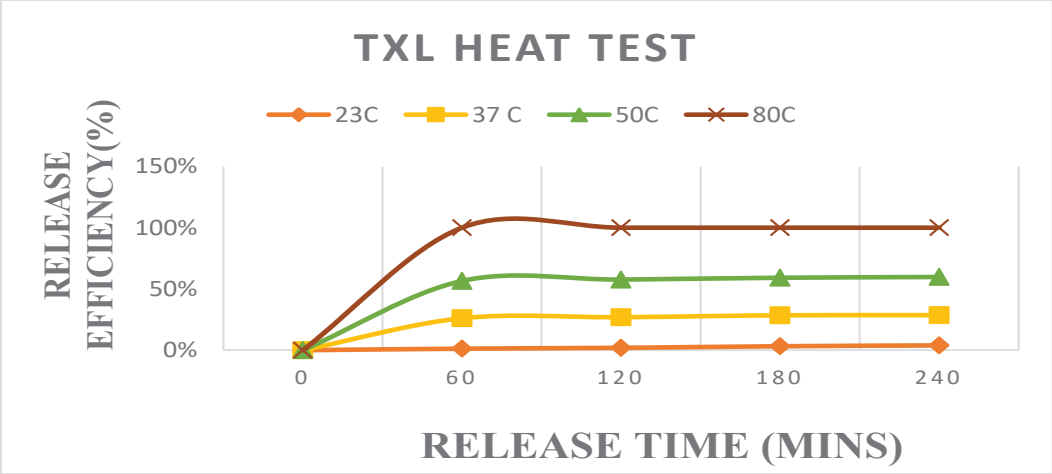


Fig. 4(a). External heating for 1, 2, 3, and 4 hours provided a time dependents leakage profile of Paclitaxel (PTX) from Fe_3O_4@MSNs nanoparticles in PBS buffer.

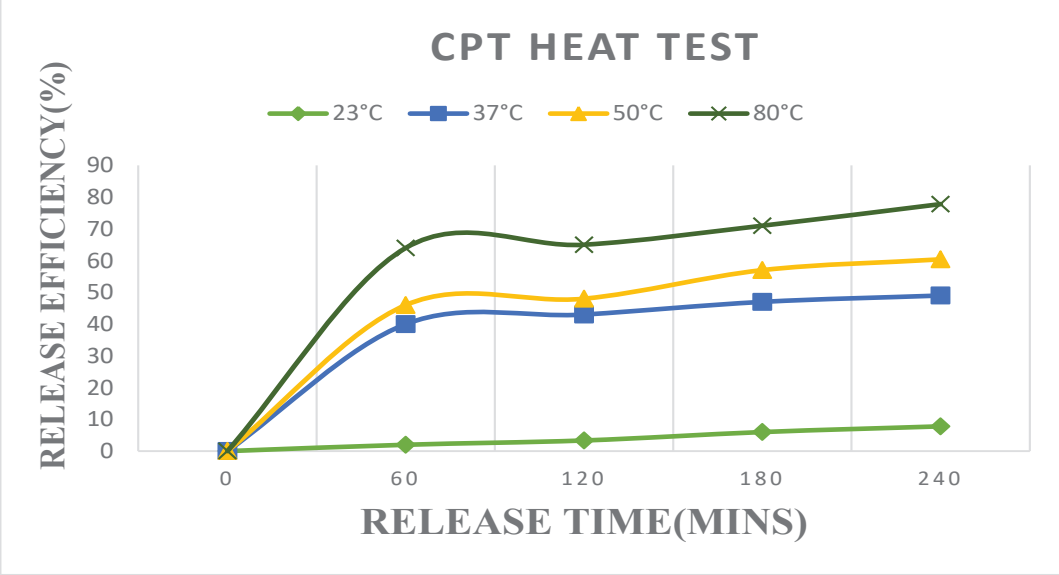


Fig. 4(b). External heating for 1, 2, 3, and 4 hours developed a time dependents leakage profile of Camptothecin (CPT) from Fe_3O_4@MSNs nanoparticles in PBS buffer.

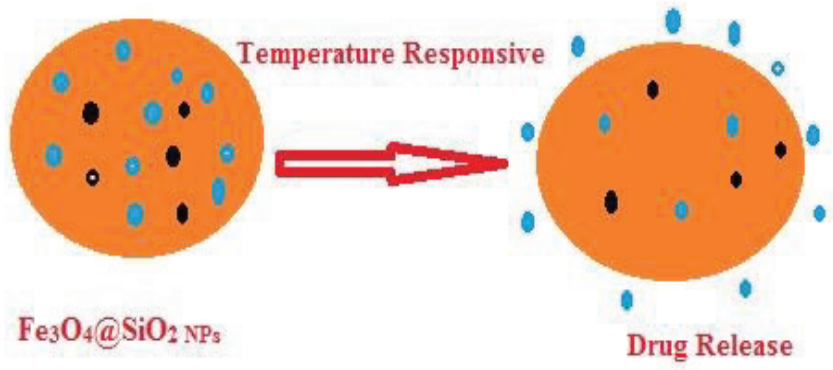


Fig. 4 (c). Scheme illustration of drug release after temperature stimulus-response

was an increase in drug release efficiency was 64%, 65%, 71%, and 77.8% respectively as shown in Fig 4(b). The release efficiency of the drug at 37°C at 240 min is 49%. The control group at 23°C drug leakage is only 7.8% at 240min. The sample heated at (50°C) for 60, 120, 180, and 240 mins and the drug leakage efficiency was 46%, 48%, 57% 60.4% respectively. Both these release results indicate that heating itself can help in drug release (37 - 80℃). Fig 4(c) determines that temperature has a great impact on the molecule's thermodynamic behavior. By increasing the bulk solution temperature to find out that if an increase of surrounding temperature will trigger drug release.

Super magnetic iron oxide nanoparticles were induced at huge temperatures when exposed to the alternating magnetic field. After coated with a mesoporous silica shell, magnetic Fe_3O_4@MSNs increases the local and surrounding temperature to various extents depending upon the alternating magnetic field intensity and as well particle size. For various lengths of time, an alternating magnetic field (AMF) is used as an internal heat resource for drug leakage. PTX and CPT Fe_3O_4@MSNs nanoparticles were distributed at room temperature in PBS (0.05 mg/mL). To confirm the drug leakage before triggering with magnetic heating was monitored by centrifugation of nanoparticles and separate the supernatant observed under U.V- visible spectrophotometer. The sample was mounted in a 3 turn copper coil that was water-cooled and capable of producing an alternating magnetic field (AMF) with a power of 5kW and a frequency of 375 kHz. The exposure time applied was 30 mins, 60 mins, 120 mins, and 180 mins. Only slight leakage was observed. The uptake and as well release efficiency of PTX and CPT Fe_3O_4@ MSNs core/shell nanoparticles are lower because of the radial porous size structure instead of a 2D hexagonal array with a central pore occupied by Fe_3O_4 and oleic acid as the stabilizing agent covers the iron oxide [37].

Comparing this experiment with external heating it was found that nanoparticle local temperature is much lower than doesn't supply enough energy to release the cargo.

3.5 *In vitro* Studies of Cytotoxicity

Many anticancer medications have low water solubility, making intravenous administration difficult. As a result, developing novel delivery mechanisms for these drugs that do not rely on organic solvents is crucial for cancer treatment. In this, we determine the hydrophobic drug-delivery system's potential biological applications, in vitro cytotoxicity tests were performed. Using PTX and CPT Fe_3O_4@MSNs nanoparticles, the cytotoxicity of a pancreatic cancer cell line was assessed using a colorimetric cell counting kit-8 (CCK-8) assay.

To assess PTX Fe_3O_4@MSNs nanoparticles toxicity on PANC-1, cell culture experiments were performed by CCK-8 assay. With each increase in nanoparticle concentration, cell viability decreased in a dose-dependent manner. PTX Fe_3O_4@MSNs nanoparticles at the maximum concentration of 100ug/ml demonstrated substantial cytotoxicity, decreasing cell survival to 25% ± 3.9, whereas the control group had no significant decrease in viable cell numbers. In comparison to the control (Fe_3O_4@ MSNs nanoparticles), low doses of PTX Fe_3O_4@ MSNs nanoparticles (75 ug/ml) tend to demonstrate substantial cytotoxicity 41% ± 2.9. The results showed that PTX Fe_3O_4@MSNs nanoparticles can significantly reduce cell viability Fig 5(a).

CCK8 assay relative percentage viability was determined at selected CPT Fe_3O_4@MSN's nanoparticles concentrations with standard deviations. Among selected in a CCK-8 assay-based cell viability study revealed that CPT Fe_3O_4@MSNs nanoparticles on PANC-1 cells showed viability percentage cytotoxicity at 100 and 75 ug/ml concentration Fig 5(b). At 100 µg/ml concentration CPT Fe_3O_4@MSN's nanoparticles cell viability percentage (25% ± 3.2) after 24 hrs.

PTX and CPT Fe_3O_4@MSNs nanoparticles were uptaken by PANC-1 cells, and the drug was released from nanocarriers in PANC-1 cells, according to these findings. These findings strongly suggest that PANC-1 cells ingested NPs with a high PTX/CPT ratio. Since NPS comes into contact with the acidic and redox microenvironment in tumor cells, hydrophobic drugs are released quickly. Caveolae-mediated endocytosis allowed PTX and CPT Fe_3O_4@MSN nanoparticles to reach PANC-1 cells [38 - 40]. As a result, these findings strongly suggested that PTX and CPT Fe_3O_4@MSN nanoparticles loaded and delivered successfully into pancreatic cancer cells and decreased cancer cell

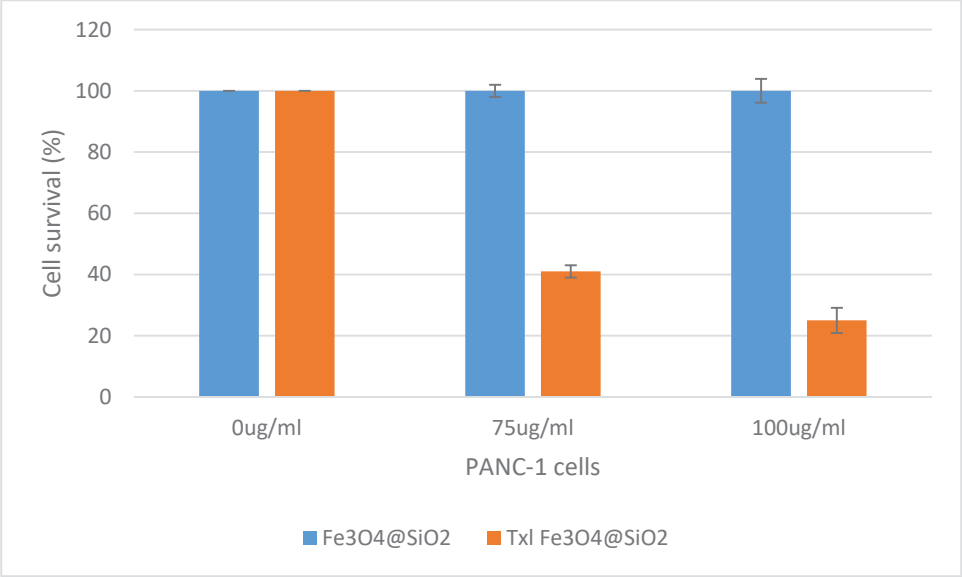


Fig. 5(a). PANC-1 cell viability after exposure to PTX Fe_3O_4@MSN nanoparticles. The data are the averages of three separate experiments plus or minus the standard deviation.

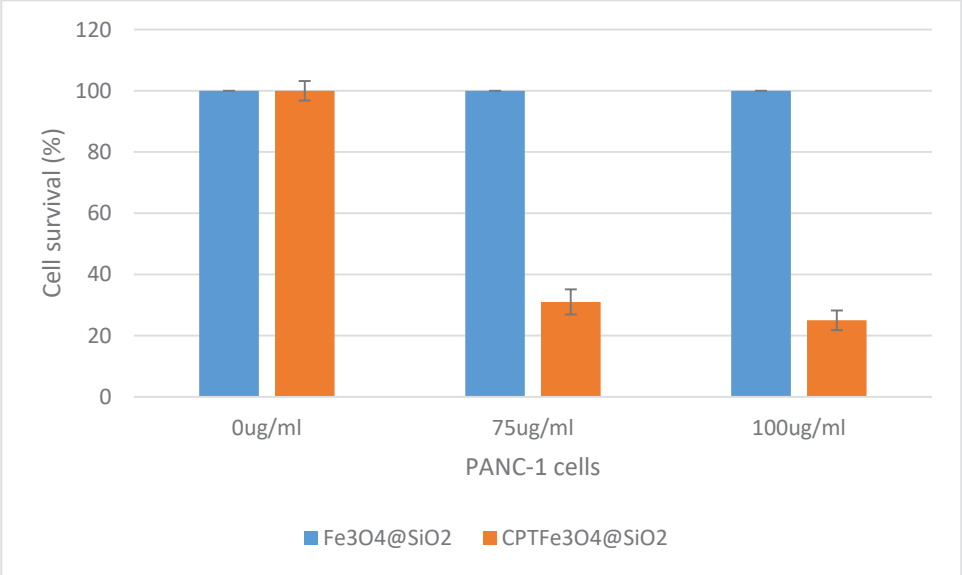


Fig. 5(b). PANC-1 cell viability after being treated with CPT Fe_3O_4@MSN nanoparticles. The results are based on three separate experiments. Standard deviations are represented by error bars.

viability. According to the study, the cytotoxicity of Fe_3O_4@MSN loaded with the chemotherapeutic drug Paclitaxel is significantly higher than that of magnetic nanoparticles containing only Fe_3O_4. The slower drug release of PTX from the porous shell channels of the magnetic hollow porous nanocrystal shells explains the anticancer efficacy of the nanoparticles [35]. A multimodal theranostic nanoagent based on Camptothecin (CPT) loaded magnetic mesoporous silica are not only exceptionally durable in aqueous buffer, but also have a high level of cytotoxicity via apoptosis induction [36]. It would be fascinating to see if this approach can be applied to other anticancer medications. Since water insolubility of therapeutic anticancer drugs is a common issue in successful

cancer therapy development, our research suggests that mesoporous silica nanoparticles are a good solution.

4. CONCLUSION

We successfully synthesized inorganic, biocompatible core/shell structured superparamagnetic iron oxide nanocrystals encased within mesostructured silica spheres as proof of a viable platform for simultaneous hydrophobic drug delivery. To investigate drug loading and release profiles, the pores of Fe_3O_4@MSN nanoparticles are saturated with hydrophobic drugs such as Paclitaxel and Camptothecin. In comparison, to the alternating magnetic field (AMF), which does not generate adequate heat, thermal heating helps to release the hydrophobic drugs from Fe_3O_4@MSN nanoparticles pores. PTX and CPT Fe_3O_4@MSN nanoparticles perform as valuable drug delivery vehicles and cancer-targeting mediators, boosting the death of PANC-1 cancer cells. PTX and CPT) Fe_3O_4@MSNs nanoparticle-based targeted drug delivery may be effective in treating PANC-1 cancer cells while minimizing toxicity to normal tissues in the surrounding region. These nanoparticles may be useful for successful drug delivery vehicles because of the simple procedures and targeting aspect.

5. ACKNOWLEDGEMENTS

The experimental procedures are undertaken in this project were financially supported by the Higher Education Commission of Pakistan under the IRSIP program.

6. REFERENCES

1. O.C. Farokhzad., J. M. Karp., and R. Langer. Nanoparticle-aptamer bioconjugates for cancer targeting. *Expert Opinion on Drug Delivery* 3 :311-24 (2006)
2. F. S. Collins, and H. A. Varmus. New Initiative on Precision Medicine. *The New England Journal of Medicine* 372: 793−795 (2015)
3. E.M. Alexandrino, S. Ritz, F. Marsico, G. Baier, V. Mailänder, K. Landfester. Paclitaxel-loaded polyphosphate nanoparticles: a potential strategy for bone cancer treatment. *Journal of Materials Chemistry B* 2 : 1298-1306 (2014)
4. M. Wu, X.M. Xia, C. Cui, P. Yu, Y. Zhang, and L. Liu,. Highly efficient loading of amorphous paclitaxel in mesoporous hematite nanorods and they are in vitro antitumor activity. *Journal of Materials Chemistry* 1: 1687-1695 (2013)
5. K.S. Cunha, M.L. Reguly, U. Graf, and H. H. Rodrigues de Andrade. Comparison of camptothecin derivatives presently in clinical trials: genotoxic potency and mitotic recombination. *Mutagenesis* 17(2): 141-7 (2002).
6. M. Duphal, and D. Chowdry. Phytochemical-Based Nanomedicine for Advanced Cancer Theranostics: Perspectives on Clinical Trials to Clinical Use. *International Journal of Nanomedicine* 15: 9125-9157 (2020)
7. R.W. Peck, Precision Medicine Is Not Just Genomics: The Right Dose for Every Patient. *Annual Review of Pharmacology and Toxicology* 58: 105−122 (2018)
8. Z . Li, D.L. Clemens, B.Y. Lee, B. J. Dillon, M. A. Horwitz, and J.I. Zink. Mesoporous Silica Nanoparticles with pH-Sensitive Nanovalves for Delivery of Moxifloxacin Provide Improved Treatment of Lethal Pneumonic Tularemia. *American Chemical Society Nano* 9 : 10778−10789 (2015)
9. B. Ruehle, D.L. Clemens, B.Y. Lee, M.A. Horwitz, and J. I . Zink. A Pathogen-Specific Cargo Delivery Platform Based on Mesoporous Silica Nanoparticles. *The Journal of the American Chemical Society* 139 : 6663−6668 (2017)
10. R. Mo, T. Jiang, R. Disanto, W. Tai, and Z. Gu, ATP-Triggered Anticancer Drug Delivery. *Nature Communications* 5: 3364 (2014) DOI: 10.1038/ncomms4364
11. H. Li, L.L. Tan, P. Jia, Q.L. Li, Y.L. Sun, J. Zhang, Y.Q. Ning, J. Yu, and Y.W. Yang. Near-Infrared Light-Responsive Supramolecular Nanovalve Based on Mesoporous Silica-Coated Gold Nanorods. *Chemical Science Journal* 5: 2804−2808 (2014).
12. Y. Zhu and C. Tao. DNA-Capped Fe3O4/SiO2 Magnetic Mesoporous Silica Nanoparticles for Potential Controlled Drug Release and Hyperthermia. *RSC Advances* 5: 22365−22372 (2015)
13. J. L. Paris, M. V. Cabanas, M. Manzano, and M. Vallet-Regí. Polymer-Grafted Mesoporous Silica Nanoparticles as Ultrasound Responsive Drug Carriers. *American Chemical Society Nano* 9: 11023−11033 (2015)
14. M. De. Smet, E. Heijman, S. Langereis, N. M. Hijnen, and H. Grüll. Magnetic Resonance Imaging of High Intensity Focused Ultrasound Mediated Drug Delivery from Temperature-Sensitive Liposomes:

An in Vivo Proof-of-Concept Study. *The Journal of Controlled Release* 150: 102−110 (2011)

15. X. Huang, I. H. El-Sayed, W. Qian, M. and A. El-Sayed, Cancer Cell Imaging and Photothermal Therapy in the Near-Infrared Region by Using Gold Nanorods. *The Journal of the American Chemical Society* 128 : 2115–2120 (2006)
16. R. Nicola, O. Costişor, S. Muntean, M. Nistor, A. Putz, C. Ianăşi, R. Lazău, L. Almásy, and L. Săcărescu. Mesoporous magnetic nanocomposites: a promising adsorbent for the removal of dyes from aqueous solutions. *Journal of Porous Materials* 27: 413–428 (2020)
17. J. F. Liu, B. Jang, D. Issadore, and A. Tsourkas. Use of Magnetic Fields and Nanoparticles to Trigger Drug Release and Improve Tumor Targeting. *Nanomedicine and Nanobiotechnology* 11(6): e1571 (2019) doi:10.1002/wnan.
18. Z. Medarova, W.Pham, C.Farrar, V. Petkova, and A. Moore. In-Vivo Imaging of siRNA Delivery and Silencing in Tumors. *Nature Medicine* 13 : 372–377 (2007)
19. K. Boubbou, R. Ali , S. Al-Humaid , A. Alhallaj, O. M. Lemine, M. Boudjelal , and A. AlKushi. Iron Oxide Mesoporous Magnetic Nanostructures with High Surface Area for Enhanced and Selective Drug Delivery to Metastatic Cancer Cells. *Pharmaceutics* 13: 553 (2021) https://doi.org/10.3390/pharmaceutics13040553
20. F. Ahmadpoor, A. Masood, N. Feliu, W. J. Parak, and S. A. Shojaosadati. The Effect of Surface Coating of Iron Oxide Nanoparticles on Magnetic Resonance Imaging Relaxivity. *Frontier in Nanotechnology* 3:644- 743(2021)
21. T. Xia, M. Kovochich, M. Liong, H. Meng, S. Kabehie, S. George, J. I. Zink, and A. E. Nel, Polyethyleneimine Coating Enhances the Cellular Uptake of Mesoporous Silica Nanoparticles and Allows Safe Delivery of siRNA and DNA Constructs. *American Chemical Society Nano* 3 : 3273−3286 (2009)
22. S. Wu, H. C. Y. Lin, Y. Hung, W. Chen, C. Chang, and C. Y. Mou. PEGylated Silica Nanoparticles Encapsulating Multiple Magnetite Nanocrystals for High-Performance Microscopic Magnetic Resonance Angiography. *Journal of Biomedical Materials Research - Part B Applied Biomaterials* 99B: 81−88 (2011)
23. P. Saint-Crick, S. Deshayes, J. I. Zink, and A. M. Kasko. Magnetic Field Activated Drug Delivery Using Thermodegradable Azo-Functionalised PEG-Coated Core-Shell Mesoporous Silica Nanoparticles. *Nanoscale* 7 (31): 13168– 13172 (2015) DOI: 10.1039/C5NR03777H
24. A. Baeza, E. Guisasola, E. Ruiz-Hernández, and M. Vallet-Regí. Magnetically Triggered Multidrug Release by Hybrid Mesoporous Silica Nanoparticles. *Chemistry of Materials* 24 (3):517– 524 (2012) DOI: 10.1021/cm203000
25. X. Liu, Y. Zhang, Y. Wang, W. Zhu, G. Li, X. Ma, Y. Zhang, S.Chen, S. Tiwari, K. Shi, S. Zhang, H.M. Fan, Y. X. Zhao, and X-J. Liang. Comprehensive Understanding of Magnetic Hyperthermia for Improving Antitumor Therapeutic Efficacy. *Theranostics* 10 (8): 3793– 3815 (2020) DOI: 10.7150/thno.40805
26. A. P. Katsoulidis, D. E. Petrakis, G. S. Armatas, and P. J. Pomonis. Microporosity, pore anisotropy and surface properties of organized mesoporous silicates (OMSi) containing cobalt and cerium. *The Journal of Materials Chemistry* 17: 1518–1528 (2007)
27. X. Yu, P. Kaithal, H. Luo, P. Soman, and S. Ramakrishna, Magnetic Iron Oxide Nanoparticle (IONP) Synthesis to Applications: Present and Future. *Materials,* 13:4644 (2020) doi:10.3390/ma13204644
28. C. Hui, C. Shen, J. Tian, L. Bao, H. Ding, C. Li, Y. Tian, X. Shiab,and H. Gao, Core-shell Fe_3O_4@ SiO_2 nanoparticles synthesized with well-dispersed hydrophilic Fe_3O_4 seeds. *Nanoscale* 3: 701–705 (2011)
29. U. Klekotka, D. Satuła , A. Basa, and B. Kalska-Szostko, Importance of Surfactant Quantity and Quality on Growth Regime of Iron Oxide Nanoparticles. *Materials* 13: 1747 (2020) doi:10.3390/ma13071747
30. C. K. Dixit, S. Bhakta, A. Kumar, S. L. Suib , and J. F. Rusling. Fast nucleation for silica nanoparticle synthesis using a sol-gel method. *Nanoscale* 1;8(47):19662-19667 (2016). doi: 10.1039/c6nr07568a.
31. W. Chen, F. Lu, C.C.V. Chen, K. C. Mo, Y. Hung, Z. X. Guo, C. H. Lin, M. H. Lin, Y. H Lin, C.Chang, and C. Y. Mou. Manganese-Enhanced MRI of Rat Brain Based on Slow Cerebral Delivery of Manganese (II) with Silica-Encapsulated MnxFe1 XO Nanoparticles. *NMR in Biomedicine* 26:1176−1185 (2013).
32. X. Liu, Y. Zhang, Y. Wang, W. Zhu, G.G. Li, X. Ma, Y. Zhang, S.Chen, S.Tiwari, K.Shi, S. Zhang, H.Fan, M. Zhao, and Y. X. Liang. Comprehensive Understanding of Magnetic Hyperthermia for

Improving Antitumor Therapeutic Efficacy. *Theranostics* 10 (8) : 3793– 3815 (2020) DOI: 10.7150/thno.40805

33. J. R. H. Manning, C. Brambila, and S. V. Patwardhan. Unified mechanistic interpretation of amine-assisted silica synthesis methods to enable design of more complex materials. *Molecular Systems Design & Engineering* 6: 170-196 (2021)
34. B. Luo, S. Xu, W.F. Ma, W.-R. Wang, S.L. Wang, J. Guo, W.L. Yang, J.H. Hu and C.C. Wang, Development of redox/pH dual stimuli-responsive MSP@P(MAA-Cy) drug delivery system for programmed release of anticancer drugs in tumour cells. *The Journal of Materials Chemistry* 20:7107–7113 (2010)
35. S. Sahu, N. Sinha, S. K. Bhutia, M. Majhi, and S. Mohapatra, Luminescent magnetic hollow mesoporous silica nanotheranostics for camptothecin delivery and multimodal imaging. *The Journal of Materials Chemistry* 2: 3799–3808 (2014)
36. H. L. Ding, Y. X. Zhang, S. Wang, J. M. Xu, and S. C. Xu, Fe_3O_4@SiO_2Core/Shell Nanoparticles: The Silica Coating Regulations with a Single Core for Different Core Sizes and Shell Thickness. *Chemistry of Materials* 24, 4572-4580 (2012) H.Lidx.doi.org/10.1021/cm302828d
38. J. Voigt, J. Christensen, and V. P . Shastri. Differential uptake of nanoparticles by endothelial cells through polyelectrolytes with an affinity for caveolae. *National Academy of Sciences* (U.S.) 111 (8) : 2942– 7 (2014) DOI: 10.1073/pnas.1322356111
39. H. V. Quang, C. C. Chang, P. Song, E. M. Hauge, and J. Kjems. Caveolae-mediated mesenchymal stem cell labeling by PSS-coated PLGA PFOB nano-contrast agent for MRI. *Theranostics* 8(10): 2657-2671 (2018)
40. M. Mohammed, A. M Jawad, Z. Su, and C.Zhang. Stimuli-responsive Mesoporous silica nanoparticles for on-demand drug release. *International Journal of Advanced Research in Biological Sciences* 5(4): 152-166 (2018).

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 89-98 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)636

Pakistan Academy of Sciences

Research Article

Evaluation of Soil Fertility and Maize Crop Nutrient Status in Himalayan Region Poonch, Azad Jammu and Kashmir

Abdul Khaliq[1], Aqila Shaheen[1*], Summra Ishaq[1], Mohsin Zafar[1], Majid M. Tahir[1], Tahir Zahoor[2], and Sair Sarwar[3]

[1]Departmentof Soil and Environmental Sciences, University of Poonch, Rawalakot Azad Jammu and Kashmir, Pakistan
[2]Planning and Development Division, Pakistan Agricultural Research Council, Islamabad
[3]Land Resources Research Institute, National Agricultural Research Center, Islamabad

Abstract: The judgment of the fertility status of agricultural soils is very substantial to determine crop sustainability and judicious nutrient management. A field survey of dominant maize growing areas of District Poonch, Azad Jammu, and Kashmir was undertaken in July-August, 2017. The twelve dominant maize growing sites, namely Char, Chak, Dothan, Banjusa, Chamber, Hajira Kelot, Kakuta, Mandhole, Madarpur, Tatrinote, Chattra Abbaspur, and Dawarandi were selected for the collection of soil and associated maize plant samples. A total of 24 composite soil samples (each representative of three individual samples) was separately collected from 0-15 and 15-30 cm depths and were analyzed for physicochemical characteristics, i.e. soil texture, pH, organic matter, soil nitrogen, available P, exchangeable K and micronutrients (Cu, Fe, Mn, and Zn). The maize ear leaves samples were also analyzed for N, P, K, and micronutrients (Cu, Fe, Mn, and Zn). To evaluate the adequacy levels of nutrients for maize growth the critical nutrient levels were used. Soils in the sampled sites were weakly acidic to neutral (pH 6.66-7.29) and had low to medium soil organic matter content (0.57-1.12%). The percent of sites with low nutrient contents were 66.7% for P and K, 8.3% for Cu, and 66.7% for Zn. The 100% sampled sites had plant N, P, and Cu deficiency while percent deficiency for plant K, Mn, and Zn was 1, 50, and 25, respectively. Soil pH had negative and organic matter had a positive significant correlation with all analyzed soil and plant nutrients. These results suggest that having integrated nutrient management strategies can enhance soil fertility and productivity in the study area.

Keywords: Macro-nutrients, Micro-nutrients, Plant nutrient, Soil fertility, Maize

1. INTRODUCTION

The understanding of the fertility status of agricultural soils is of paramount importance for rational soil management and efficient utilization of nutrient resources to achieve crop sustainability [1]. The human population of Pakistan has progressively increased by a factor of 4.5 between 1960 and 2018 (from 45 million to 201 million) whereas currently per capita land availability (0.18 ha head^{-1}) is expected to be reduced by almost 90% by 2050 [2]. Land degradation due to soil salinization, nutrient mining, waterlogging, and growing urbanization is mainly responsible for the progressive reduction in arable land [3 - 5]. Besides, the lands situated on slopes are prone to plant nutrient deficiencies, productivity stagnation, or physical, chemical, and biological deterioration mainly due to water erosion. The micronutrient deficiency at the global scale is one-third of arable soils, mainly in Zn [6] and ultimately this influences human nutrition. In developing countries half of the population is affected by micronutrient deficiencies and globally, approximately 2–3 billion people are suffering [7]. The reason is an unbalanced and lower application of N, P, and K which increases soil degradation too. The micronutrient importance is also being demonstrated [8] but still, its links to nutrition are not well known and understood. Khan *et al*. [9] reported 18.2, 13.8, and 6.7% reductions in the

Received: December 2020; Accepted: March 2021
*Corresponding Author: Aqila Shaheen <aqeela.shaheen@gmail.com>

total arable land area in Pakistan due to deletion of top fertile soil layer by erosion, waterlogging, and salinity/ sodicity hazards, respectively. This ongoing declining trend in land availability has sought the attention of scientists, policy-makers, and other stakeholders in devising sustainable management strategies for restoring the productive potential of these degraded arable lands to ensure feed, food, and fiber requirements of a rapidly growing population.

To develop and adopt appropriate sustainable management strategies for soil fertility restoration and its quality, the updated knowledge on the status of existing soil fertility is very important. Therefore, the evaluation of soil fertility through soil and plant analyses is vital for soil management practices. The soil fertility assessment of an area is a key aspect of the maintenance of soil fertility and sustaining agricultural production [10].

Soil testing and plant analyses are the precise quantitative assessments of nutrients that are frequently used to predict the soil fertility status and confirm visual deficiency symptoms of limiting nutrients [11]. The fertility status of a soil can also be assessed through nutrient indexing by using organic carbon, total soil N, available P, exchangeable K, and micronutrient concentrations as a measure of soil fertility components [12]. Kihara *et al.* [13] stated that micronutrient use efficiency can be improved by applying the type and rate of fertilizers according to soil fertility status and management practices.

Maize is the third significant cereal crop of Pakistan after wheat and rice that requires high amounts of nutrients. The estimates had shown that maize removes nearly 350 kg N, 150 - 220 kg P_2O_5, and 400 - 500 kg K_2O ha^{-1} from the soil over a growing season to produce a maximum yield of 12 tones ha^{-1} [14]. On the other hand, in Pakistan average maize yield of 4.59 tones, ha^{-1} is still below the world's targets of about 5.2 tones ha^{-1} [15]. This difference in yield potential might be attributed to the deficiency of essential plant nutrients.

Keeping in view, the high nutrient demands of maize crop and lack of comprehensive knowledge of the current fertility status of soil from concerned maize growing areas, the present research was planned to evaluate the soil fertility and nutrients status of maize from selected maize growing zones of District Poonch, Azad Jammu, and Kashmir-Pakistan. This data will be valuable for making integrative nutrient management policies and plans suitable for small land-holding farmers.

2. MATERIAL AND METHODS

2.1 Description of Study Area

The study was conducted in maize growing sites of district Poonch (33.8369° N, 73.8889° E) Azad Jammu, and Kashmir-Pakistan. The twelve selected maize growing sites included Char, Chak, Dothan, Banjusa, Chambanar, Hajira-Kelot, Kakuta, Mandhole, Madarpur, Tatrinote, Chatra-Abbaspur, and Dwarandi. The study area ranges at an altitude from 803-1798 m above sea level. The entire area is rain-fed (500 - 2000 mm average annual rainfall), receiving a major portion of rainfall during monsoon and winter months. The parent materials of soils are mainly comprised of colluvial and alluvial sediment depositions. Taxonomically, the soils of the area belong to Thermic Lithic Eutrudepts and Hyperthermic Typic udifluents sub-groups of orders Inceptisols and Entisols with loam to silt loam textures. The area is prone to moderate risk of water erosion with a considerable annual loss of soil, organic matter, and essential plant nutrients. The summer maize followed by winter wheat is the existing cropping sequence traditionally practiced by the farmers in the study area with little or even no applications of recommended fertilizers. Being nutrient exhaustive, both of the crops continuously deplete soil fertility. The weather attributes, i.e. humidity, barometric pressure, air, temperature, altitude, and a heat index of selected sites were noted by using a portable weather tracker (model: Kestrel 4500) is presented in Figure 1.

2.2 Sampling, Processing, and Analyses

A total of 24 composite soil samples (each comprised of three individual samples) were collected by using a soil auger to a 0-15 and 15-30 cm depths randomly, brought to the laboratory of Soil and Environmental Sciences, and were processed for further required determinations. The determinations on soil texture [16], $pH_{1:2}$ by soil water suspension [17] organic matter [18], soil total nitrogen by Kjeldahl method of Bremmer and Mulvaney [19], available P by

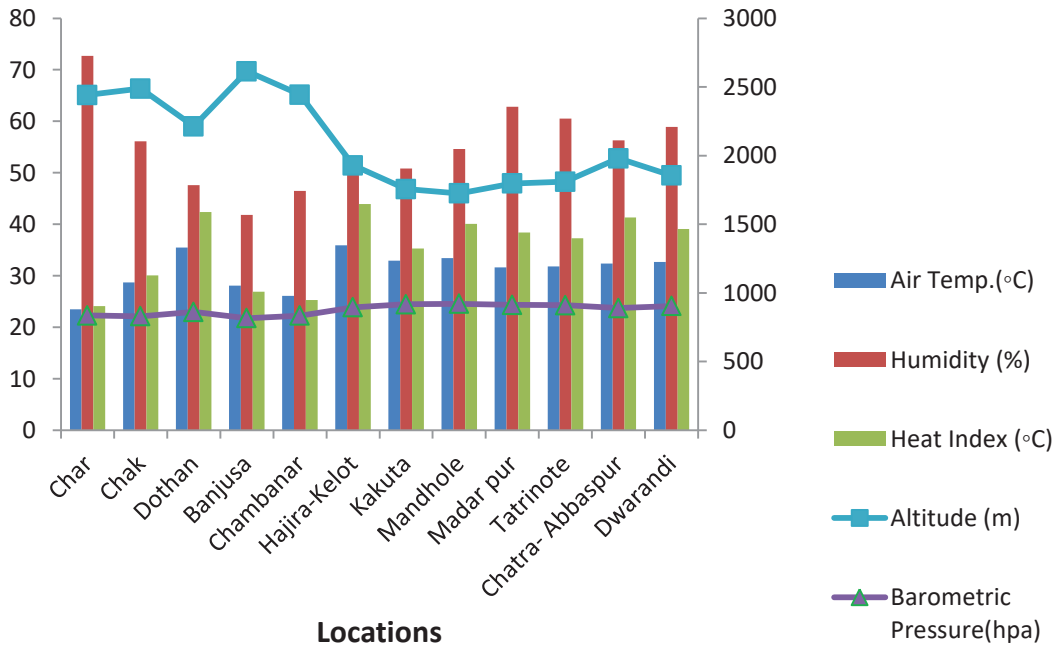


Fig. 1. Weather attributes of selected maize growing sites of District Poonch, Azad Jammu & Kashmir at the time of soil sampling.

Murphy and Riley [20] method, and extractable K [21, 22] were done. The P and K valuation in the the extract was done by spectrophotometer and flame photometer, respectively. The ammonium bicarbonate diethylene triamine penta acetic acid (AB-DTA) extractable iron, zinc, copper, and manganese were determined using the method given by Lindsay and Norvell [23] and then read by atomic absorption. The ear leaves samples of associated maize crops were also obtained from each respective site at silking. The best plant part of maize is the ear leaf and the time of sampling is initial silk [24]. The plant samples were over-dried (at 60°C), processed, and analyzed for total N by Kjeldahl's method described by Bremmer and Mulvaney [19], for P [25], and K [26]. The digestion procedure for plant micronutrients was adopted from Rashid (1986) and concentrations were determined by the atomic absorption spectrometer [27].

2.3 Data Analyses

Illustrative measurable investigations containing means, ranges, and standard deviations were done in MS Excel. Nutrients adequacy ranges (low, sufficient, and abundance) (Table 1) were utilized to bunch the sites relying upon particular adequacy levels. Parker's nutrient index [28] changed by Pathak [29], Kumar *et al.* [30], and Ravikumar and Somashekar [12] based on the percentage of samples in every one of the three classes (for example, low, medium, and high) and multiplied by 1, 2 and 3 individually, was utilized for the assessment of fertility status of soils. Pearson's correlations were also calculated in MS Excel.

3. RESULTS

3.1 Soil Physicochemical Characteristics

The soil pH ranged from 6.66 - 7.29 with an overall mean of 6.99 ± 0.2. The pH showed slightly acidic to neutral conditions (Table 2). The soil organic matter (SOM) ranged between 0.57 - 1.12% and this range is showing low to medium status. Total soil N was 0.037 - 0.06% and it was above the critical value and found in the medium range. Soil available P with the minimum concentration of 5.57 to a maximum of 7.47 mg kg^{-1} was in the low range. The extractable K ranged from 32.7 - 80.25 mg kg^{-1} with an overall mean of 52.64 ± 18.5 mg kg^{-1}. The mean showed that K contents were below a critical value. Among soil micronutrients, the Cu and Zn ranges were below the critical value to medium concentration with a mean of 0.56 to 0.78 mg kg^{-1}, respectively. The Fe and Mn were adequate in both minimum and maximum concentrations. The

Table 1. Soil and tissue nutrient critical levels of macro and micronutrients as an interpretive guide for nutrient ranking

Parameters	Soil nutrient concentration				Plant nutrient concentrations
	Units	Low	Medium	Adequate	Units	
Soil organic matter	(%)	<0.86	0.86-1.29	>1.29	-	-
Total soil nitrogen	(%)	<0.02	0.03-0.40	>0.40	(%)	2.6-3.1
Available P	mg kg^{-1})	4-7	8-11	12-15	(%)	0.22-0.27
Extractable Potassium	(mg kg^{-1})	<60	60-120	>120	(%)	1.2-1.7
Copper	(mg kg^{-1})	<0.2	0.2-0.5	>0.5	(mg kg^{-1})	10-20
Iron	(mg kg^{-1})	<3.0	3.0-5.0	>5.0	(mg kg^{-1})	15
Manganese	(mg kg^{-1})	<0.5	0.5-1.0	>1.0	(mg kg^{-1})	50
Zinc	(mg kg^{-1})	<0.9	0.9-1.5	>1.5	(mg kg^{-1})	25

Source: Maize ear leaf critical values [55], [56] and [57]; Source: Soil critical values [58] and [59]

textural analysis showed a higher percentage of silt followed by sand and clay with means of 40.72, 37.7, and 21.7 respectively.

3.2 Ear Leaf Nutrient Contents

Ear leaf total N had a variation ranged from 0.67 - 1.77% and all sites were below the critical value with 100% deficiency (Table 3). The P concentration was also below critical values in all sites and sites below critical value were 100%. The sampled sites with K values below low were only 1% and 41. 6% were within the range of critical value. The sampled sites with Cu were 100% deficient and Fe was above critical value in all sites with wide variation ranged from 85.6 - 263.2 mg kg^{-1}. Both Mn and Zn also showed variations between the sampled sites averaging 51.61 ± 9.69 mg kg^{-1} and 37.96 ± 10.77 mg kg^{-1}, respectively. About 50% of sampled sites had Mn below a critical value and Zn deficiency was 25% in sampled sites.

3.3 Nutrient Indices for Organic Matter, N, P, K and Micronutrients of Maize

The nutrient index value (NIV) of organic matter was low, i.e. 1.50 and OM was in the low range in 50% sites and was medium again in 50% sites (Table 4). The sampled sites had 100% N in the medium range while 66.7% sites had the P and K deficiency and were in the low category with NIV of 1.33. In sampled sites, the Cu deficiency was 8.3%. The Fe and Mn were higher in all sites with and NIV of 3.00. The sampled sites with Zn were 66.7% at the low range while 33.3% sites were in high nutrient status.

3.4 Correlation between Soil Chemical Properties and Plant Nutrient Contents

The leaf ear TN, P, Mn, and Zn were highly significantly ($P \leq 0.01$) correlated to soil pH and it was a negative correlation (Table 5). A significant negative correlation was also observed between ear leaf Cu and soil pH (-0.78*) and between ear leaf Fe and soil pH (-0.74*). All observed ear leaf nutrients contents positively correlated with soil OM. The ear leaf N and P had a positive significant correlation with Soil N, P, K, Cu, Fe, Mn, and Zn. The ear leaf K had significant positive correlations with soil organic matter (r=0.84), soil N (r=0.81) soil P (r=0.81), soil K (r=0.85), soil Fe (r=0.78) and soil Mn content (r=0.83). The ear leaf Cu significantly correlated with soil TN, Zn, Cu, Fe, and Mn. A positive significant correlation was observed between ear leaf Fe and all analyzed soil nutrients. The ear leaf Mn was significantly and positively correlated with all analyzed soil nutrients

Table 2. Statistics (Mean, SD, Minimum and Maximum) of soil physico-chemical properties and nutrients of maize growing at farmers' fields in District Poonch, Azad Jammu & Kashmir (Data is averaged across 0-30 cm depth)

Variable	Min	Max	Mean	Std. Dev.
pH	6.66	7.29	6.99	0.20
Organic matter (%)	0.57	1.12	0.84	0.19
Total N (%)	0.037	0.06	0.05	0.01
P (mg kg^{-1})	5.57	7.47	6.54	0.73
K (mg kg^{-1})	32.7	80.25	52.64	18.51
Cu (mg kg^{-1})	0.18	1.16	0.56	0.38
Fe (mg kg^{-1})	11.37	21.53	14.10	2.84
Mn (mg kg^{-1})	3.56	10.4	6.53	2.15
Zn (mg kg^{-1})	0.36	1.43	0.78	0.33
Clay (%)	16.9	30.3	21.7	3.65
Silt (%)	33.7	50.1	40.72	4.79
Sand (%)	27.9	42.5	37.7	4.49

Table 3. Statistics (Mean, SD, Minimum and Maximum) of maize plant nutrient content at farmers' fields in District Poonch, Azad Jammu & Kashmir.

Plant nutrients	Min	Max	Mean	Std. Dev.	% sites below a critical value
Total N (%)	0.67	1.77	1.29	0.40	100
P (%)	0.04	0.08	0.06	0.01	100
K (%)	1.07	2.29	1.75	0.39	1
Cu (mg kg^{-1})	2.17	3.95	2.17	0.93	100
Fe (mg kg^{-1})	85.6	263.2	192.32	62.12	0
Mn (mg kg^{-1})	35.2	71.9	51.61	9.69	50
Zn (mg kg^{-1})	21.53	53.9	37.96	10.77	25

Table 4. Nutrient indices for organic matter, N, P, K, and micronutrients of maize growing at farmers' fields in District Poonch, Azad Jammu & Kashmir

Nutrients	No. of Samples	Rating			Nutrient Index value (NIV)
		Low	Medium	High	
Soil organic matter (%)	24	12 (50)	12 (50)	---	1.50
Total Soil Nitrogen (TSN; %)	24	---	24 (100)	---	2.00
Soil Available Phosphorus (mg kg^{-1})	24	16 (66.7)	08 (33.3)	---	1.33
Soil Exchangeable Potassium(mg kg^{-1})	24	16 (66.7)	08 (33.3)	---	1.33
Soil Copper (mg kg^{-1})	24	02 (8.3)	12 (50.0)	10 (41.7)	2.33
Soil Iron (mg kg^{-1})	24	---	---	24 (100)	3.00
Soil Manganese (mg kg^{-1})	24	---	---	24 (100)	3.00
Soil Zinc (mg kg^{-1})	24	16 (66.7)	08 (33.3)	---	1.33

Index value: Low = < 1.67; Medium = 1.67 – 2.33 and High = > 2.33

except AP while Zn was having a significant positive correlation with all analyzed soil nutrients except AP and Cu.

3.5 Interrelations of Soil Chemical Properties

Soil pH had a significant negative correlation (-0.91) with OM and all analyzed soil nutrients while OM had a significant positive correlation with all observed nutrients (Table 6). The N was positively correlated with K (0.95), Zn (0.84), Cu (0.78), Mn (0.82), and Fe (0.93). The K positively and significantly correlated with AP (0.90), Zn

Table 5. Pearson's correlations coefficients (r) between soil and plant nutrient contents of maize growing at farmers' fields in District Poonch, Azad Jammu & Kashmir

Plant nutrients	Soil nutrients								
	pH	OM	TN	AP	K	Zn	Cu	Mn	Fe
N	-0.88**	0.93**	0.85**	0.93**	0.87**	0.74*	0.72*	0.81**	0.71*
P	-0.83**	0.93**	0.91**	0.87**	0.92**	0.77*	0.72*	0.88**	0 .84**
K	-0.73*	0.84**	0.81*	0.81**	0.85**	0.68	0.70*	0.83**	0.78*
Cu	-0.78*	0.79*	0.80*	0.63	0.69	0.90**	0.91**	0.94**	0.85**
Fe	-0.74*	0.85**	0.75*	0.78*	0.79*	0.79*	0.71*	0.82**	0.74*
Mn	- 0.84**	0.84**	0.84**	0.69	0.79*	0.88**	0.83**	0.98**	0.89**
Zn	-0.82**	0.76*	0.76*	0.67	0.73*	0.93**	0.69	0.84**	0.77*

** Correlation is highly significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.01 level (2-tailed)

Table 6. Pearson's correlations coefficients (r) of soil properties at maize growing farmers' fields in District Poonch, Azad Jammu & Kashmir

nutrients	pH	OM	SN	AP	K	Zn	Cu	Mn
OM	-0.91**							
N	-0.90**	0.93**						
AP	-0.81*	0.95**	0.82*					
K	-0.84**	0.94**	0.95**	0.90**				
Zn	-0.87**	0.78*	0.84*	0.64	0.75*			
Cu	-0.80*	0.80*	0.78*	0.68	0.63	0.78*		
Mn	-0.82*	0.84**	0.82*	0.71*	0.76*	0.87**	0.87**	
Fe	-0.80*	0.82*	0.93**	0.64	0.86**	0.86**	0.73*	0.84**

** Correlation is highly significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.01 level (2-tailed)

(0.75), Mn (0.76), and Fe (0.86). The AP had a significant correlation with Mn (0.71) and Zn was significantly correlated to Cu (0.73) and Mn (0.84).

4. DISCUSSION

The sampled sites were slightly acidic to neutral with organic matter concentration from low to medium. Soils at sampled sites were Entisols and Inceptisols which were slightly weathered. The slightly acidic pH could be due to high rainfall that caused the leaching of cations. Low soil pH consequently influences the uptake of plant nutrients by affecting the microbial population and concentration of Al/Fe ions in the solution [31]. The low organic matter in sampled sites may be attributed to the previous cropping system that has depleted it. In sampled sites, the low organic matter contents could be due to exhaustive crop rotation as well. The low diversity of soil microbes, development of host-specific soil-born diseases, and imbalances of soil nutrients resulted due to growing the same crops in the same field continuously [32, 33]. Devkota *et al* [34] reported that cropping systems have an immense effect on plant nutrient dynamics in soil. The medium range of soil total N in sampled sites indicated the low rate of inorganic and organic fertilizer addition. The long-term addition of inorganic and organic fertilizers affects the nutrient concentration and soil organic matter build-up. Where the earlier cropping system has depleted soil organic matter, the soils mostly become acidic

with little capability to retain macro (N, P & K) and some micronutrients. This in addition to the low inorganic and organic fertilizer use, augmented soil degradation. As reported by Bhattacharya [35], in agriculture soil degradation is primarily because of insufficient and imbalanced fertilizer application.

The low N levels in sampled sites could be a result of N leaching, less the amount of N addition, low organic matter, and high nutrient removal due to continuous farming [36]**.** Cobo *et al.* [37] described the results of 57 selected studies and revealed that due to nutrient mining, most systems had a negative balance for N and K while the percent deficiency of P was not severe.

Soil organic matter holds soil N, stops its leaching, and makes it available for plant uptake [38]. Conventional tillage practices can adversely change the soil mechanical behavior, and in turn, it affects the organic compounds, pH, N, C, and micronutrients such as Zn and Mn [39, 40]. It further aggravates water and wind erosion, which ultimately causes soil degradation [41]. The low contents of total N in plant tissue could be attributed to a medium concentration of total N in soil and consequently lesser uptake. The observed low P in the sampled soil could be associated with P leaching, P complexation, and subsequently low P in plant tissues. Phosphorus is distinctive among the anions due to its low mobility and accessibility in soils. It is hard to manage because it makes strong bonds with both soil matrix and solution [42]. In acid soils Al and Fe cause P fixation, whereas Ca compounds dominate P fixation in alkaline soils.

Soil K was found low in the sampled soil while in plant tissue the low concentration of K was only 1% and this is attributed to cation balances. The optimum K concentration in plant tissues could be due to higher mass flow movement [43] and lower soil pH. Low soil pH due to the solubility of Al could be another factor that interferes with the uptake of plant nutrients [31]. The Al becomes soluble at low soil pH and dominates the cation exchange capacity, thereby it affects the soil capacity to retain K [44].

Among micronutrients, Cu and Zn were limited in sampled soil and plant tissues also showed a deficiency. The positive relationship between tissue Cu and Zn and soil Cu and Zn content showed that these nutrients requiring corrective measures.

The protein components of numerous enzymes require Cu [45] and it is an important plant micronutrient. In soil, Cu rarely leaches as it is relatively immobile and binds strongly with OM. The accessibility of Cu to plants relies upon the pH, OM content, and soil type [46]. Copper uptake by the plant is affected by predominant chemical species, soil pH, and its concentration in soil. Zinc is also an important plant growth element and its deficiency reduces the yield [47, 48] The correlation between P and Zn was not significant in sampled sites, however, Murdock and Howe [49] reported that the higher content of soil P and higher soil pH was correlated with lower levels of Zn in the plant. The Zn deficiency is attributed to low OM contents and pH levels above 7.0.

Manganese was found high in the sampled soil, but some sampled sites showed deficiencies in plant tissues. It may be credited to low soil pH and less organic matter addition. Wang et al [33] also reported a similar dependency of Mn on soil organic carbon status. Manganese scarcity has likewise been found in profoundly fertile clayey soils [50]. The studies have demonstrated an antagonism amongst Zn and Mn, a higher content of Zn in the soil diminished the leaf Mn concentration [51]. The concentration of interchangeable metal in soil relies upon their amount in soil solutions, subsequently, soil pH impacts the replaceable Fe, Mn, Cu, and Zn likewise. Mugo *et al.* [52] reported that soil pH strongly correlated with most of the investigated both soil and tissue nutrient contents. The substantial positive association between leaf P and soil P in sampled soils is attributed to low P ions in the soil solution at the root surface. The pH is negatively correlated with P showing its effect on P uptake. The significant association between tissue K contents and soil K in sampled soils, but low K in the plant could be attributed to its negative correlation with pH.

The cropping systems and soil management practices affect soil organic matter concentration and in turn, it influences soil fertility [31]. Goshu *et al.* [53] reported that for sustainable maize production the fertilizer recommendation needs to be soil test-based because due to applied management practices and inherent soil nutrient status the heterogeneity is common in farmer's field.

The low soil nutrient contents of sampled sites caused the lower concentration of nutrients in plant tissues and a strong correlation between plant nutrients and soil nutrients indicates the need for both organic and inorganic inputs. Anthropogenic activities like cropping practices, soil management, intensive land use, and conservation measures impact soil functions [54]. Therefore, to increase productivity and fertility of maize growing areas sustainable and cost-effective measures are required.

5. CONCLUSION

This study shows that N, P, K, Cu, and Zn are restricting nutrients for maize production in significant maize growing zones of District Poonch Azad Jammu & Kasmir. The OM was also lower in sampled sites. The correlation showed the dependence of all plant nutrients on their concentration in soil. The correlation of OM was positive and highly significant with all analyzed soil nutrients. The management practices are required to build up the fertility status and productivity of study sites. The nutrients need to be provided as an integrated nutrient management program where both organic and inorganic sources should be applied.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. REFERENCES

1. C. Li, K. Xiong, and G. Wu. Process of biodiversity research of Karst areas in China, *Acta Ecologica Sinica* 33: 192-200 (2013).
2. R. Lal. Managing agricultural soils of Pakistan for food and climate. *Soil and Environment* 37: 1-10 (2018).
3. P.A. Sanchez. Soil fertility and hunger in Africa. *Science* 295: 2019-2020 (2002).
4. A. Khan, A. Ali, F. Attaurrahman, and R. Shaw. Desertification risk reduction approaches in Pakistan. *Disaster Risk Reduction Approaches in Pakistan* 161-173 (2015).
5. S. Ali, R. Hayat, F. Begum, B. Bohannan, L. Inebert, and K. Meyer. Variation in soil physical, chemical and microbial parameters under different land uses in Bagrot valley, Gilgit, Pakistan. *Journal of the Chemical Society of Pakistan* 39: 97-107 (2017).
6. I. Cakmak, M.J. McLaughlin, and P. White. Zinc for better crop production and human health. *Plant and Soil* 411: 1–4 (2017).
7. B.D. Goudia, and C.T. Hash. Breeding for high grain Fe and Zn levels in cereals. *International Journal of Innovation and Applied Studies* 12(2): 342–354 (2015).
8. J. Kihara, G.W. Sileshi, G. Nziguheba, M. Kinyua, S. Zingore and R. Sommer. Application of secondary nutrients and micronutrients increases crop yields in sub-Saharan Africa. *Agronomy for Sustainable Development* 37: 25 (2017).
9. M.A. Khan, M. Ahmad, and H.S. Hashmi. Review of available knowledge on land degradation in Pakistan. *OASIS Country Report 3, ICARDA, Aleppo, Syria* (2012).
10. R.P. Singh, and S.K. Mishra Available macro nutrients (N, P, K and S) in the soils of Chiraigaon block of district Varanasi (UP) in relation to soil characteristics. *Indian Journal of Scientific Research* 3: 97-100 (2012).
11. B. Lozano-Garcia, L.P. Alcantara, and E.C. Brevik. Impact of topographic aspect and vegetation (native and reforested areas) on soil organic carbon and nitrogen budgets in Mediterranean natural areas. *Science of the Total Environment* 544: 963-970 (2016).
12. P. Ravikumar, and K.R. Somashekar. Evaluation of nutrient index using organic carbon, available P and available K concentrations as a measure of soil fertility in Varahi River basin, India. *Proceedings of the International Academy of Ecology and Environmental Sciences* 3: 330-343 (2013).
13. J. Kihara, P. Bolo, M. Kinyua, J. Rurinda and K. Piikki. Micronutrient deficiencies in African soils and the human nutritional nexus: opportunities with staple crops. *Environmental Geochemistry and Health* 42:3015–3033 (2020).
14. M. Kamidi. D. Cheruiyot, P. Osore and G. Barasa. 1999. Verification of the effect of organic manures and inorganic fertilizers on the yield of maize. 73-77 pp. In: J.S. Tenywa, J.Y.K. Zake, P. Ebanyat, O. Semalulu, S. T. N. kalubo (eds.). Soil Science a Key to Sustainable Land Use. Proceeding of the 17th Conference, 6-10 september 1999 Kampala, Uganda, SSSEA and Makerere University, Kanmpala Uganda.
15. Food and Agriculture Organization. Statistical database. Rome, Italy. Accessed on 21-02-2018.
16. G.W. Gee, and J.W. Bauder. Particle-size analysis. In a Klute (ed.). Methods of soil analysis, Part 1.

Physical and mineralogical methods. Agronomy Monograph No. 9 (2ed.). *American Society of Agronomy/Soil Science Society of America,* Madison WI. 383-41pp (1986).

17. E.O. McLean. Soil pH and lime requirement. In: A. L. Page, (ed.), Methods of soil analysis, part 2: Chemical and microbiological properties. SSSA, Madison, WI.1982, 199-224 pp.
18. A. Walkley, I.A. Black. An examination of Degtjareff method for determining soil organic matter, and proposed modification of the chromic acid titration method. *Soil Science* 37:29-38 (1934).
19. J.M. Bremner, and C.S. Mulvaney. Nitrogen total. In: A. L. Page, (eds.), Methods of soil analysis. Agron. No. 9, Part 2: Chemical and microbiological properties. 2nd edition. *American Society of Agronomy Madison,* WI, USA, 595-624 (1982).
20. J. Murphy, and J.P. Riley. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chemica Acta* 27: 31-36 (1962).
21. L.A. Richard. Diagnosis and improvement of saline and alkali soils. *USDA Agriculture Handbook 60.* Washington. D. C. (1954).
22. R.R. Simard. Ammonium acetate extractable elements. In: M. R. Carter, (ed.), s*oil sampling and methods of analysis.* Lewis Publishers, Boca Raton, FL. 39-42 (1993).
23. W.L. Lindsay, and W.A. Norvell. Development of a DTPA soil test for zinc, iron, manganese and copper. *Soil Science Society American Journal* 42: 421-448 (1978).
24. D.J. Reuter, and J.B. Robinson. Plant Analysis: An Interpretation Manual, 2nd ed., CSIRO Pub. *Commonwealth Scientific and Industrial Research Organization* (1997).
25. S.R. Olsen, and L.E. Sommers. Phosphorus. In methods of soil analysis, Part 2, A.L. Page, R H Miller and DR Keeney (2nd eds.). Agronomy series No.9, *American Society of Agronomy and Soil Science Society of America Madison,* W.I 403-430pp. (1982).
26. R.J. Wright, and T. Stuczynski. Atomic Absorption and Flame Emission Spectrometry. In: (ed.). Sparks, D. L. Methods of Soil Analysis: Part 3. *Chemical Methods,* Soil Science Society of America Book Series 5. SSSA-ASA, Madison WI 63-65pp. (1996).
27. A. Rashid. Mapping zinc fertility of soils using indicator plants and soils-analyses. Ph.D. Dissertation. University of Hawaii, HI, USA. (1986).
28. F.W. Parker, W.L. Nelson, E. Winters, and I.E Miles. The broad interpretation and application of soil test Information. *Agronomy Journal* 43: 105-112. (1951).
29. Pathak H. Trend of fertility status of Indian soils. *Current Advances in Agricultural Sciences* 2: 10-12. (2010).
30. P. Kumar, A. Kumar, B.P. Dyani, P. Kumar, U.P. Shahi, S.P. Singh, R. Kumar, Y. Kumar, and R. Sumith. Soil fertility status in some soils of Muzaffarnagar District of Uttar Pradesh, India, along with Ganga canal command area. *African Journal of Agricultural Research* 8: 1209-1217 (2013).
31. J.J. Burke. Growing the Potato crop. *Vita* (2016).
32. T. Bai, S. Xu, F. Rupp. H. Fan, K. Yin, Z. Guo, L. Zhang, B. Yang, Y. Huang, Y. Li, X. Li, L. Zeng, S.J. Zheng. Temporal variations of Fusarium oxysporum f. sp. cubense tropical race 4 population in a heavily infected banana field in Southwest China. *Acta Agriculturae Scandinavica Scand. Section B Soil Plant Science* 69: 641-648 (2019).
33. F. Wang, S. Chen, Y. Wang, Y. Zhang, C. Hu, and B. Liu. Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying microbial communities in an upland soil: implications for nitrogen loss from intensive agricultural systems. *Frontiers in Microbiology* 9: 2424 (2018).
34. P. Devkota, D. Aryal, and B. Khanal. Assessment of Soil Nutrient Status under Different Cropping Systems in Khotang, *Nepal International Journal of Applied Sciences and Biotechnolog*y 7(3): 341-346 (2019).
35. A. Bhattacharya. Chapter 1 - global climate change and its impact on agriculture. In: Bhattacharya, A. (Ed.), *Changing Climate and Resource Use Efficiency in Plants*. Academic Press pp. 1e50. (2019).
36. A. Burton. Influence of Solution Management Techniques on Nutrient Use Efficiency in Hydroponically Grown Salad-type Plants. *ProQuest* (2018).
37. J.G. Cobo, G. Dercon, and G. Cadisch. Nutrient balances in African land use systems across different spatial scales: A review of approaches, challenges and progress. *Agriculture, Ecosystems & Environment* 136: 1–15 (2010).
38. A.H. Bingham, and M.F. Cotrufo. Organic nitrogen storage in mineral soil: Implications for policy and management. *Science of the Total Environment* 551–552, 116–126 (2016).

39. K.A. Congreves, A. Hayes, E.A. Verhallen, and L.L. Van Eerd. Long-term impact of tillage and crop rotation on soil health at four temperate agroecosystems. *Soil Tillage and Research* 152: 17e28 (2015).
40. K. Grahmann, R. Dellepiane, V. Terra, and J.A. Quincke. Long-term observations in contrasting crop-pasture rotations over half a century:statistical analysis of chemical soil properties and implications for soil sampling frequency.*Agriculture, Ecosystems & Environment* 287: 106710 (2020).
41. P. Carr. Guest Editorial: Conservation Tillage for Organic Farming. Multidisciplinary Digital Publishing Institute. (2017).
42. S.C. Hodges. Soil Fertility Basics. Soil Science Extension, North Carolina State Univ. (2010)
43. IPNI. Potassium availability and uptake. *Better Crop* 82: 14–15 (1998).
44. J.L. Havlin. Fertility. in Reference Module in Earth Systems and Environmental Sciences, https://doi.org/10.1016/B978-0-12-409548- 9.05162-9 (2013).
45. H. Marschner. Mineral Nutrition of Higher Plants. 2nd ed., Academic Press, Inc. (1995)
46. J.L. Burkhead, K.A. Gogolin Reynolds, S.E. Abdel-Ghany, C.M. Cohu, and N. Pilon. Copper homeostasis. *New Phytologist* 182: 799-816 (2009).
47. W.J. Gangloff, D.G. Westfall, G.A. Peterson, and J.J. Mortvedt. Relative availability coefficients of organic and inorganic Zn fertilizers. *Journal of Plant Nutrition* 25: 259-273 (2002).
48. N.K. Fageria, V.C. Baligar, and R.B. Clark. Micronutrients in crop production. *Advances in Agronomy* 77: 185-268 (2002).
49. L. Murdock, and P. Howe. Zinc fertilizer rates and Mehlich III soil test levels for corn. *Agronomy.* Notes, 33: p. 1. (2001).
50. F. Aref. Influence of zinc and boron interaction on residual available iron and manganese in the soil after corn harvest. *American-Eurasian Journal of Agricultural & Environmental Sciences* 8(6): 767-772 (2010).
51. F. Aref. Zinc and boron content by maize leaves from soil and foliar application of zinc sulfate and boric acid in zinc and boron deficient soils. *Middle-East Journal of Scientific Research* 7(4): 610-618. (2011).
52. J.N. Mugo, N. Nancy. Karanja, K. Charles. Gachene, K. Dittert, O. Shadrack. Nyawade and Elmar Schulte-Geldermann. Assessment of soil fertility and potato crop nutrient status in central and eastern highlands of Kenya. *Scientific Reports* 10: 7779 (2020).
53. T.A. Goshu, E. Semu, T. Debele, D. Wegary, and H. Kim. Nutrient Status of Soils from Farmers' Maize Fields in Mid Altitude Areas of Western Ethiopia. *Merit Research Journal of Agricultural Science and Soil Sciences* 3(8): 113-121 (2015).
54. T. Yang, H.M. Kadambot, Siddique, and K. Liu. Cropping systems in agriculture and their impact on soil health-A review. *Global Ecology and Conservation* 23: e01118. (2020)
55. D.J. Reuter, D.G. Edwards, and N.S. Wilhelm. Temperate and tropical crops. In: Reuter DJ, Robinson JB (eds) Plant analysis: an interpretation manual. CSIRO, Australia pp 83–284 (1997).
56. G.O. Adeoye, and A.A. Agboola. 1985. Critical levels for soil pH, available P, K, Zn and Mn and maize ear-leaf content of P, Cu and Mn in sedimentary soils of *South-Western Nigeria Fertilizer Research* 6: 65-71. (1985).
57. C.R. Campbell. Reference sufficiency Ranges for Plant Analysis in the Southern Region of the United States. Southern Cooperative Series Bulletin No. 394, North Carolina Department of Agriculture and Consumer Services Agronomic Division, USA. (2009).
58. A. Rashid, and N. Ahmad. Soil testing in Pakistan: Country Report. Proceedings of the FADINAP Regional workshop on co-operation in soil Testing for Asia and the Pacific, August 16-18, 1993, Bangkok, Thiland, United Nations, New York,; pp: 39-53 (1994).
59. T.T. Cochrane, L.G. Sanckez, L.G. deAzevedo, J.A. Porras, and C.L. Graver. Land in Tropical America. CIAT/EMBRAPA-CPAC., Colombia. (1984).

Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 58(1): 99-110 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)639

Pakistan Academy of Sciences

Research Article

A Method for Soil Samples Collection during Site Assessment for Aquaculture

Javairia Shafi*, Kashifa N. Waheed, Zahid S. Mirza, and Muhammad Zafarullah

Fisheries Research and Training Institute, Manawan, Lahore, Pakistan

Abstract: Assessment of soil quality is one of the crucial steps during the assessment of a site for aquaculture. However, no clear guidelines are available in literature to guide fish farmers about soil sample collection resulting in a waste of their time and energy. The present study was, therefore, designed to determine the variability of soil characteristics at different sites and give recommendations for sample collection during soil assessment. Two hundred and eighty-six (286) soil samples collected from different subsites of seven sampling sites were analyzed for particle size distribution and chemical parameters. Results showed significant variation in soil separate content at different subsites of a sampling site. At Moza Bahak Maken in district Sargodha, the soil was found to be sandy at one subsite and clayey on the other within 35 acres of land area. Moreover, significant differences in soil quality parameters were also found with varying sampling depths. pH of soil indicated the calcareous nature of the soil in Punjab and outruled the necessity to lime soil. Electrical conductivity measurements showed that soil in the Sargodha division can be characterized as very strongly saline. The study led to the conclusion that sample collection for soil analysis in aquaculture should be based on stratified sampling selecting at least three sampling points from each stratum. Soil samples should be collected in 1 ft. increment from the surface up to the depth that should be 1 ft. deep than the soil depth that will be dug in excavated ponds. Culturable fish/ shrimp species should be selected based on the salinity of soil at the proposed fish pond site.

Keywords: Soil quality, aquaculture, stratified sampling, saline soil

1. INTRODUCTION

World population is estimated to be 9.3 billion in 2050 [1] which indicates that a sustainable supply of food fish as a source of high-quality protein in the human diet is becoming essential. Deterioration of capture fisheries at the global level has stimulated the tremendous development of aquaculture. In the Asia Pacific region, annual growth rate of aquaculture has been reported to be 6.1% compared to 1.6% recorded for capture fisheries during 2004-2014 [2]. It has been estimated that aquaculture contribution to total production of fish and fish products will outpace the capture fisheries, increasing its share from 44% in 2013-2015 to 52% in 2025 [3]. Subjected to sustainable improvement and continuous progression, it can be anticipated that the aquaculture industry will play a key role in coping with the challenges of rising food fish requirements resulting from the population growth in developing countries. The world's top aquaculture producers are focussing on the ecosystem approach for aquaculture development. The approach translates that in order to fully utilize the potential of this sector to reduce hunger and help in achieving sustainable development goals, the adoption of schematic spatial planning and management are the key factors. Lack of strategic planning and selection of inappropriate sites can lead to economic losses and financial risks during seafood production cycles.

In Pakistan, a high population growth rate (3% on annual basis), malnutrition, and increasing rates for poultry/ red meat drive the demand for increasing fish consumption in the human diet.

Received: January 2021; Accepted: March 2021
*Corresponding Author: Javairia Shafi <javairiamalik@gmail.com>

These conditions urge the need to ensure fast and sustainable development of aquaculture. However, despite of huge water resources in the country [4], aquaculture development is not progressing at a high pace. Lack of high-quality economical fish feed, fast-growing fish seed, as well as poor management of fish ponds during the production cycle are the major constraints faced by the sectors that impede its fast development.

To address the issues associated with poor fish pond management, suitable site selection with high-quality soil is one of the key factors [5]. In the ecosystem approach for aquaculture development, soil chemistry and its texture has been considered as one of the essential features to be considered at the time of site selection. A suitable distribution of different-sized particles is essential for pond bottom soil. It is, therefore, mandatory to assess the mechanical and chemical properties of the soil before the use of a site for pond construction. Due to the unavailability of any published guideline, there is a lack of awareness among the general public for soil sample collection to analyze its suitability for use as fish pond bottoms and embankment. Most often potential farmers have large areas of land that they want to use for aquaculture. Due to a lack of appropriate information, they collect one or two soil samples from the surface and transfer them to the soil testing laboratory. The net result is waste of time & effort and a delay in soil analysis for the site. It is, therefore, essential to provide a method for the collection of soil samples site assessment for aquaculture. The present work is based on the study of variation in soil quality parameters at different depths and sampling units within a site to propose a suitable method for sample collection during soil suitability assessment.

2. MATERIAL AND METHODS

The study was carried out from February 2019 to February 2020. Sample analysis was carried out in Water and Soil Analysis Laboratory at Fisheries Research and Training Institute, Lahore, Pakistan.

2.1 Sampling Sites

Sampling sites selected for the present study were the locations proposed to be used for fish pond construction. All sampling sites were located in different districts of the Punjab province of Pakistan. Following is a brief description of each site and the soil sample collection method adopted thereof.

2.1.1 Site A

Site A was a 35 acre land area situated in Moza Behak Maken in the Sargodha district. The area was divided into seven subsites of five acres each. Soil samples were collected from five equidistant locations of each subsite covering four corners and one center. From each location two samples were collected; one from the surface and the other from 1 ft. depth. Due to the presence of a water table at 2 ft. depth, samples from deeper soil layers could not be collected. Seventy soil samples were collected from this site.

2.1.2 Site B

Site B was situated in Moza Nari, Khushab district, and comprised of 50 acre land. The site was divided into 10 subsites each of 5 acres. Soil samples were collected from 5 locations of each subsite as in the case of Site A. Due to the presence of a water table at 3 ft., soil samples were collected from the surface, 1 ft. and 2 ft. depth from each location of every subsite. One hundred and fifty soil samples were collected from Site B.

2.1.3 Site C

Site C was a 5-acre land area in Khushab. Samples were collected from five locations starting from the surface up to the depth of 3 ft with a 1 ft. increment. A total of fifteen soil samples was collected from this site.

2.1.4 Site D

Site D is comprised of an area of 2 acres situated in Chistian, Bahawalnagar. Samples were collected from three equidistant locations at this site, covering soil depth up to 3 ft. at each location. Twelve soil samples were collected from this site.

2.1.5 Site E

A land of 5 acres located in the Sargodha district was marked as Site E. Soil samples were collected

from surface up to the depth of 3 ft from five (5) equidistant locations. Fifteen soil samples were collected from this site.

2.1.6 Site F

Site F was situated at Bediyan Road, Lahore, and consisted of an area of 2 acres. Soil samples were collected from the surface up to the depth of 3 ft. from three locations. Twelve soil samples were collected from this site.

2.1.7 Site G

Site G comprised of the land of 1 acre in Karor Paka, Lodhran District. Samples were collected from the surface up to the depth of 3 ft. with one ft. increment from thee equidistant locations. Twelve soil samples were collected from this sampling site.

2.2 Sample Collection

Soil samples collected with the help of an auger from each sampling location were stored in properly labeled air-tight polyethylene bags and transported to the laboratory.

2.3 Sample Preparation

In the laboratory, soil samples were air-dried to reduce their moisture content. Air-dried soil samples were ground to pass a 2 mm mesh size screen. Homogenized and sieved soil samples were stored in air-tight bags till further analysis. All the soil samples were subjected to analysis of physical and chemical parameters viz particle size distribution, pH, and electrical conductivity.

2.4 Soil Particle Size Distribution

Soil particle size distribution was determined by the hydrometer method following the method of Bentone [6]. Calgon solution (5%) was prepared using sodium hexametaphosphate and sodium carbonate. An accurately weighed portion of the soil sample (50 g ± 0.05 g) was mixed with 100 mL of Calgon solution and the soil: Calgon solution suspension was allowed to stand overnight. Then the suspension was quantitatively transferred to a 1000 ml glass cylinder and volume was made up to the mark with distilled water. The soil suspension in the cylinder was mixed thoroughly and a hydrometer meter reading was recorded after 40 s and 120 s of mixing. The content of sand, silt, and clay were calculated as follows.

Silt and clay content (%) = (Temperature corrected hydrometer reading recorded at 40 second/ soil sample weight) x 100

Clay content (%) = (Temperature corrected hydrometer reading recorded at 120 s / soil sample weight) x 100

Silt content (%) = (Silt and clay content) – (Clay content)

Sand content (%) =100- (Silt and clay content)

2.5 Soil Chemical Analysis

For chemical analysis, the soil sample was mixed with distilled water in a 1:2 ratio and the supernatant was analyzed for pH and electrical conductivity [7].

2.6 Statistical Analysis

For Site A, an independent t-test was used to find significant differences in soil parameters measured at two soil depths for 7 subsites. For Site B-G, a one-way analysis of variance was used to identify significant differences in soil characteristics measured at varying depths of each sampling site. In the case of Site A and Site B consisting of 7 and 10 subsites respectively, a one-way analysis of variance was used to study any significant variation in soil quality with varying sampling subsites at each soil depth. All the analysis was carried out using SPSS version 22 using two-tailed significance at 0.05 significance level [8].

3. RESULTS

The particle size distribution of soil collected from different sampling sites is presented in Table 1. Physico-chemical parameters of soil are shown in Table 2.

3.1 Site A

The highest sand content in the surface and 1 ft. deep

soil layer was found at subsite SA7 where it was 77.21 ± 6.54% and 74.01 ± 7.67% respectively. The soil at subsite SA5 showed the lowest sand content; 9.63 ± 0.02% and 12.02 ± 1.68% at the surface and 1 ft. depth. The highest clay content was found to be 45.99 ± 9.53% and 53.88 ± 2.28% in surface and 1 ft. deep soil of SA5 and SA4 respectively. The lowest clay content was 9.59 ± 6.42% and 12.79 ± 10.39% in the surface and deeper soil layer of SA2 and SA1 respectively. The lowest silt content was 12.40 ± 4.33% (surface soil) and 12.00 ± 3.46% (1 ft.) found at SA7. The soil at SA4 (surface) and SA3 (1 ft.) showed the highest content of silt i.e. 47.17 ± 18.68% and 35.19 ± 5.40%. pH at all the subsites of site A was higher than 8.00 at the surface and deeper soil. Highest soil EC was found to be 16788.00 ± 3136.44 μScm^{-1} (surface; SA4) and 13262.00 ± 695.68 μScm^{-1} (1ft., SA6). On the other hand, the lowest soil EC was 10828.00 ± 2711.16 μScm^{-1} (Surface, SA3) and 3446.00 ± 4458.76 μScm^{-1} (1 ft., SA1). In general, soil EC at the deeper soil layer was lower than that of surface soil.

Independent t-test showed no significant difference in any parameter measured in surface and 1 ft. deep soil layer at SA1-SA3 and SA7. At SA4, EC was significantly different in two soil layers ($p < 0.05$). The difference in soil sand content at the surface and 1 ft. was statistically significant ($p < 0.05$) at SA5 and SA6.

One-way analysis of variance showed significant differences ($p < 0.05$) in soil parameters at a similar depth of different subsites of Site A. Sand content at surface soil of SA1, SA2 and SA7 was significantly higher than that of the soil of SA3-SA6. The difference in sand content of surface soil at SA3 - SA6 was not statistically significant. A similar trend was observed in the soil at 1 ft. depth of subsites and the sand content of soil at SA1, SA2, and SA7 was significantly higher than SA3 - SA6. The silt content of surface soil at SA1 was significantly lower than that of SA3 - SA6. There was no statistically significant difference in clay content of surface soil at SA1, SA2, and SA7. Surface soil at all these three sites contained lower clay content than that of SA3 - SA6. There was no difference in the clay content of soil at SA3 - SA6. A similar pattern in soil clay content was found at 1 ft. depth. Also, there were significant differences among pH of soil at different subsites of Site A (surface and 1 ft. Table 2).

3.2 Site B

Sand content at subsites of Site B varied from 13.23 ± 4.61% (SB 9) to 43.23 ± 5.58% (SB1), 12.90 ± 2.22% (SB8) to 43.61 ± 10.72% (SB1) and 12.50 ± 2.90% (SB8) to 36.05 ± 3.12% (SB1) at surface, 1 ft. and 2 ft. depth respectively. Highest silt content was found to be 63.86 ± 15.52% at surface (SB9), 53.94 ± 4.41% at 1 ft. (SB8) and 57.14 ± 7.17% at 2 ft. depth (SB8). Clay content ranged from 17.59 ± 6.38% (SB1) to 47.75 ± 13.41% (SB6) at surface, 25.19 ± 6.41% (SB1) to 47.13 ± 3.10% (SB4) at 1 ft. and 29.18 ± 13.01% (SB3) to 40.77 ± 4.56% (SB5) at 2 ft. soil depth. pH at surface, 1 ft. and 2 ft. depth varied from 7.68 ± 1.11 to 8.19 ± 0.14, 7.87 ± 0.05 to 8.18 ± 0.08 and 7.71 ± 0.09 to 8.35 ± 0.15 respectively. Highest soil EC was 19390.00 ± 2204.81 μScm^{-1} (SB8), 18384.00 ± 2886.44 μScm^{-1} (SB6) and 18150.00 ± 2611.62 μScm^{-1} (SB6) at surface, 1 ft. and 2 ft. respectively.

One-way analysis of variance showed significant differences ($p < 0.05$) in soil parameters measured at different depths of each subsite. Clay content in surface soil of SB1 was significantly low than that of soil at 2 ft. depth. The difference in soil clay content at 1 ft. and 2 ft. was, however, not significant. At SB4, the clay content of soil at 1 ft. was significantly higher than that of soil at 2 ft. depth. Sand content of surface soil was significantly high than deeper soil layers at SB3 and SB8. Surface soil EC was significantly high than that of soil at deeper layers at SB5 and SB7 - SB10.

The use of one way Anova to assess soil quality at similar depths of different subsites of Sites B showed interesting results that have been shown in Table 1. Surface soil sand content at SB1 was significantly higher than that of soil at SB2-SB6 and SB8-SB9. There were significant differences in soil silt content at various subsites (Table 1). The clay content of surface soil at SB1, SB3, and SB8 - SB10 was significantly lower than that of other subsites. For SB4, SB7, soil clay content was significantly lower than that of soil at SB6. At 1 ft. soil depth and content found at SB1 was significantly higher than that of SB2 - SB6, SB8, and SB9. The clay content of 1 ft. deep soil layer at SB1 was significantly lower than that of SB2-SB6. At SB4, clay content was significantly higher than that of SB3 - SB10. At 2 ft. depth, soil sand content found at SB1 was significantly higher than that of SB2-SB9. For pH,

no significant difference was found in surface soil at various subsites. However, soil pH at 1 ft. and 2 ft. showed significant differences among subsites (Table 2). Unlike Site A, significant differences in soil EC were found at various depths of different subsites of Site B. Surface soil EC at SB2 was significantly lower than that of SB1 and SB3.

3.3 Site C

Significant differences in soil parameters at different depths were identified. At Site C, sand, silt and clay content varied from 6.62 ± 1.57% to 10.39 ± 1.22%, 21.10 ± 0.62% to 33.24 ± 3.23% and 58.65 ± 1.95% to 68.94 ± 1.72% at varying soil depths respectively. Sand content of soil at 1 ft. depth was significantly lower than that of soil at 2 ft. The silt content of the surface and 1 ft. soil layer was significantly higher than deeper soil layers. Surface clay content was significantly lower than soil at deeper layers. pH was 7.5-8.0 at all soil depths while soil EC ranged from 12740.00 ± 1166.81 μScm^{-1} (3 ft.) to 18376.67 ± 782.33 μScm^{-1} (surface). Soil pH at the surface was significantly lower than deeper layers. Soil EC at all studied soil layers was statistically different from each other and there was a gradual decrease in EC as one moved from the surface toward deeper layers.

3.4 Site D

The highest sand content at Site D was 29.64 ± 3.91% found at 2 ft. soil depth. Sand content of 1 ft. soil layer was significantly lower than that of surface and deeper soil layers. The highest silt and clay content at this site was 53.95 ± 3.48% (3 ft.) and 43.01 ± 2.31% (1 ft.) respectively. The clay content of soil at the surface, 2 ft. and 3 ft. depth was significantly lower than that of soil at 1 ft. Soil pH was higher than 9.5 at all studied soil depths. the pH of the soil of 1 ft. deep layer was significantly higher than that of soil at 3 ft. Soil EC ranged from 1870.33 ± 54.50 μScm^{-1} (1 ft.) to 998.67 ± 32.08 μScm^{-1} (3 ft.). Surface & 1 ft. layer soil EC was significantly higher than that of soil at 3 ft.

3.5 Site E

Sand, silt and clay content at this site ranged from 27.25 ± 13.54% to 38.03 ± 4.38%, 43.17 ± 7.01% to 50.77 ± 7.81% and 16.79 ± 3.74% to 26.38 ± 14.15% respectively. Soil pH varied from 7.79 ± 0.67 to 8.18 ± 0.21. The highest EC was found at surface soil (4881.20 ± 4965.73 μScm^{-1}) and the lowest was shown by soil at 3 ft. depth (222.80 ± 1664.5 μScm^{-1}). There was no significant difference in soil parameters at different depths.

3.6 Site F

The highest sand content at site F was found at 3 ft. depth (48.28 ± 8.08%). The highest silt and clay content was 36.66 ± 11.37% (3 ft.) and 28.39 ± 4.0% (3 ft.) respectively. The clay content of the surface and 3 ft. was significantly lower than that of soil at 1 and 2 ft. Soil pH varied from 8.46 ± 0.75 at the surface to 9.74 ± 0.65 at 2 ft. depth. Surface soil pH was significantly lower than that of deeper layers. The highest EC was found in surface soil where it was 2960 ± 2343.61 μScm^{-1} while the lowest EC was 796.0 ± 156.79 μScm^{-1} found at 3 ft.

3.7 Site G

Sand content varied from 13.74 ± 1.19% (at 2 ft.) to 17.70 ± 2.32% (at the surface) at this site. The highest silt content was 72.94 ± 5.28% found in soil at 3 ft. Soil clay content varied from 12.66 ± 6.43% (at 2 ft.) to 21.99 ± 2.01% (at surface). Soil pH ranged from 9.82 ± 0.12 at the surface to 8.89 ± 0.44 at 3 ft. pH of soil at the surface and 1 ft. layer was significantly higher than that of soil at 2 ft. and 3 ft. There was no significant difference in other soil parameters at varying soil depth. The highest soil EC was found at the surface where it was 14662.00 ± 10977.74 μScm^{-1}. The lowest EC was found at 1 ft. depth (2968.00 ± 2754.09 μScm^{-1}).

4. DISCUSSION

Soil quality is a crucial factor in determining the success of an aquaculture project. It is the material that forms the base and embankments of ponds and holds water over it. In addition to several natural pedogenic aspects including the nature of parent rock, climate, and activity of plants & other soil-dwelling animals, anthropogenic factors also remarkably influence the soil properties [9].

The soil quality of any site is assessed through its texture class and physicochemical properties. Soil texture class refers to the relative distribution of soil particles of a defined size range and can be determined through soil particle size distribution analysis. According to the United States Department of Agriculture (USDA), soil

particles with a diameter of 0.05 mm – 2.00 mm are considered as sand, those with a diameter of 0.05 mm - 0.002 mm are named as silt, and those with a diameter of < 0.002 mm are classified as clay [7]. There is, however, slight variation among different classification systems and the International Society for Soil Science (ISSS) considers particles with 0.02 mm - 2.00 mm as silt [10].

It is important to note that any method for collection of soil samples during aquaculture site assessment is not suggested earlier in literature according to our knowledge. Boyd [11] has recommended a method to collect soil samples from prepared ponds. According to the author, several soil samples can be collected from pond bottom randomly and combined to form a composite sample. In the present study, results of soil analysis at various sites have been used to recommend a method for soil sample collection before the construction of a pond.

In the present study, soil texture class was found to be sand, loamy sand, sandy loam at SA1, SA2, and SA7, and clay or clay loam at SA3-SA6, based on soil separates found at Site A. It is noteworthy that within the 35 acre land area, the soil was sandy at one subsite and clayey at the other. At Site B, the texture class was classified as either clay or clay loam, silty clay loam, silt loam & loam at SB1 - SB10. Particle size distribution analysis is of utmost importance in the assessment of soil suitability of aquaculture [12]. Unsuitable distribution of different soil separates can result in economic losses and even complete failure of an aquaculture project. It does not mean that soil with inappropriate particle size distribution cannot be used for fish pond construction. It only emphasizes the need to determine the soil quality before pond construction and adopt suitable soil management techniques to maintain the soil efficacy during aquaculture activities. A soil with low water seepage, fast mineralization of organic matter, and capability of adsorbing and releasing nutrients is considered suitable for aquaculture [11]. These qualities specify a soil with low sand content, optimum clay content, neutral pH, and low salinity (for freshwater aquaculture). The presence of an optimum amount of clay particles is considered vital in pond bottom soil due to two reasons. Owing to their small size, they perfectly interlock with each other, reducing the pore size and consequently reducing water seepage. Secondly, their enormous surface area enables them to adsorb nutrients and slowly release them to the overlying water [13, 14]. Hajek and Boyd [15] suggested clay content of > 35% as suitable for pond soil as well as embankments and dikes. However, it was suggested later by Boyd and coworkers [17] that such a high amount of clay particles is undesirable for pond soil. The highly plastic nature of clay particles causes soil engineering problems during the construction of ponds and the compaction of soil between crops. The moreover high content of clay particles in ponds' bottom can cause clay turbidity in pond water that influences fish growth and production directly by depositing on fish gills thereby producing respiratory ailments and indirectly by interfering with sunlight penetration that in turn reduces the pond's primary production [18]. The level of clay turbidity in pond water should be less than 100 mgL^{-1} for optimum fish production [17]. Uzukwu *et al*. [19] conducted the case study of aquaculture ponds in Nigeria and found the bottom soil to be sandy in most of the ponds. The authors suggested adopting a suitable soil lining technique or mixing additional clay from allochthonous sources to control water seepage. Ahmad *et al.* [20] compared physical, chemical, and biological methods to reduce water seepage in earthen ponds with silt loam calcareous soil. The authors found physical and biological methods as effective means to reduce water seepage.

Differences in soil characteristics with depth can be visualized in Figure 1. Figure 1a shows the distribution of soil particles of Site A that was a 35 acre land area. There seems to be no appreciable difference in soil sand content at the surface and 1 ft. depth. Silt content, however, appeared to range from 40-60% at the surface, and 30% - 50% at 1 ft. in 25% of soil samples. Average clay content at 1 ft. depth (33.57%) was slightly higher than that of surface (29.20%). The distribution of sand, silt, and clay in the soil at Site B has been shown in Figure 1b. There was a slight variation in soil sand and silt content at various depths. In the case of clay, the difference from upper quartile to upper whisker ranged from 38% to 55% at the surface, 41% to 50% at 1 ft. and 40% to 48% at 2 ft. depth. The clay content of 50% of soil samples varied from 16% to 37% at the surface, 27% to 41% at 1 ft. and 30% to 40% at 2 ft. depth.

Table 1. Particle size distribution of soil at different sites (Mean ± SD)

Site	Subsite	Sand (%)				Silt (%)				Clay (%)			
		Surface	1 ft.	2 ft.	3 ft.	Surface	1 ft.	2 ft.	3 ft.	Surface	1 ft.	2 ft.	3 ft.
A	1	69.62 ± 20.80 a, A	72.82 ± 20.26 a, A, B	***	***	19.52 ± 10.53 a, a, C	14.39 ± 10.52 a, A, B	***	***	10.72 ± 10.49 a, A	10.79 ± 10.49 a, B	***	***
	2	64.43 ± 8.78 a, A	57.22 ± 27.97 a, A, B, C	***	***	25.97 ± 8.92 a, , B, C	26.79 ± 14.88 a, A, B	***	***	9.59 ± 6.42 a, A	15.99 ± 13.16 a, B	***	***
	3	14.65 ± 5.51 a, B	18.02 ± 11.97 a, C	***	***	39.99 ± 7.35 a, , A, B	35.19 ± 5.40 a, A	***	***	45.35 ± 6.95 a, B	46.79 ± 8.72 a, A	***	***
	4	14.54 ± 2.16 a, B	14.14 ± 3.56 a, C	***	***	47.17 ± 18.68 a, A	31.98 ± 4.46 a, A	***	***	38.29 ± 17.71 a, B	53.88 ± 2.28 a, A, B	***	***
	5	9.63 ± 0.02 a, B	12.02 ± 1.68 b, C	***	***	44.38 ± 9.52 a, A	38.39 ± 16.58 a, A, B	***	***	45.99 ± 9.53 a, B	49.59 ± 17.58 a, A	***	***
	6	16.82 ± 5.76 a, B	30.83 ± 10.35 b, B, C	***	***	39.19 ± 12.38 a, A, B	27.19 ± 3.03 a, A	***	***	43.99 ± 10.13 a, B	41.98 ± 10.52 a, A	***	***
	7	77.21 ± 6.54 a, A	74.01 ± 7.67 a, A	***	***	12.40 ± 4.33 a, C	12.00 ± 3.46 a, B	***	***	10.40 ± 3.16 a, A	14.00 ± 4.33 a, B	***	***
B	1	43.23 ± 5.58 a, A	43.61 ± 10.72 a, A	36.05 ± 3.12 a, A	***	39.18 ± 11.36 a, C, D	31.19 ± 5.94 a, D	33.97 ± 4.45 a, D	***	17.59 ± 6.38 a, C	25.19 ± 6.41 a, b, C	29.98 ± 4.00 b, A	***
	2	33.63 ± 9.54 a, B, C	26.04 ± 6.17 a, A, B	26.02 ± 5.81 a, B, C	***	43.18 ± 9.34 a, D	35.18 ± 4.15 a, A, B, C, D	35.59 ± 4.77 a, C, D	***	23.19 ± 13.61 a, A, B	38.78 ± 5.21 a, B	38.39 ± 1.67 a, A	***
	3	24.86 ± 4.60 a, B, C	18.46 ± 3.31 b, A, B	15.67 ± 2.20 b, D, E	***	55.95 ± 9.34 a, A, B	44.77 ± 13.45 a, A, B, C	55.16 ± 12.37 a, A	***	19.19 ± 11.88 a, C	36.77 ± 13.89 a, B	29.18 ± 13.01 a, A	***
	4	18.23 ± 4.66 a, C	16.68 ± 3.90 a, B	18.81 ± 7.04 a, C, D, E	***	52.23 ± 12.88 a, A, B, C	36.19 ± 4.76 a, B, C, D	42.43 ± 6.19 a, B, C, D	***	29.55 ± 13.15 a, b, B, C	47.13 ± 3.10 a, A, B	38.76 ± 2.99 b, A	***
	5	22.46 ± 8.31 a, B, C	22.11 ± 8.89 a, A, B	23.26 ± 8.43 a, B, C, D	***	35.77 ± 9.41 a, D	40.74 ± 8.92 a, B, C, D	35.97 ± 5.41 a, C, D	***	41.77 ± 13.41 a, A, B	37.15 ± 9.01 a, A, B	40.77 ± 4.56 a, A	***
	6	15.03 ± 3.24 a, C	15.80 ± 5.41 a, B	15.36 ± 5.83 a, D, E	***	37.21 ± 6.93 a, D	46.81 ± 12.33 a, A, B, C	44.84 ± 10.42 a, B, C	***	47.75 ± 4.45 a, A	37.39 ± 8.23 a, A	39.80 ± 5.83 a, A	***
	7	27.31 ± 14.38 a, A, B, C	30.93 ± 17.51 a, A, B	26.55 ± 11.02 a, B, C	***	43.94 ± 7.05 a, B, C, D	37.52 ± 12.39 a, B, C, D	39.12 ± 6.99 a, C, D	***	28.75 ± 10.41 a, B, C	31.54 ± 6.21 a, B, C	34.33 ± 4.53 a, A	***
	8	20.05 ± 6.30 a, B, C	12.90 ± 2.22 b, B	12.50 ± 2.90 b, E	***	57.12 ± 13.02 a, A, B	53.94 ± 4.41 a, A	57.14 ± 7.17 a, A	***	22.84 ± 12.27 a, C	33.17 ± 4.16 a, B, C	30.36 ± 8.74 a, A	***
	9	13.23 ± 4.61 a, C	22.74 ± 9.57 a, A, B	14.42 ± 6.07 a, E	***	63.86 ± 15.52 a, A	48.74 ± 15.21 a, A, B	51.54 ± 6.08 a, A, B	***	22.91 ± 16.68 a, C	28.52 ± 7.40 a, B, C	34.04 ± 4.24 a, A	***
	10	35.67 ± 7.40 a, A, B	36.87 ± 7.54 a, B	30.86 ± 6.71 a, A, B	***	41.56 ± 13.51 a, C, D	33.96 ± 7.06 a, C, D	38.77 ± 4.62 a, C, D	***	22.78 ± 8.32 a, C	29.17 ± 5.58 a, B, C	30.37 ± 6.21 a, A	***
C	1	8.11 ± 2.40 a, b	6.62 ± 1.57 a	10.39 ± 1.22 b	9.21 ± 2.26 b	33.24 ± 3.23 a	26.98 ± 4.40 b	21.10 ± 0.62 c	21.85 ± 2.28 c	58.65 ± 1.95 a	66.40 ± 3.08 b	68.51 ± 1.55 b	68.94 ± 1.72 b
D	1	25.67 ± 3.94 a	14.37 ± 1.15 b	29.64 ± 3.91 a	24.34 ± 3.07 a	45.95 ± 3.97 a	42.62 ± 1.15 a	47.31 ± 4.97 a, b	53.95 ± 3.48 b	28.37 ± 0.02 a	43.01 ± 2.31 b	23.06 ± 1.19 c	21.71 ± 1.14 c
E	1	38.03 ± 4.38 a	34.46 ± 10.61 a	27.25 ± 13.54 a	30.45 ± 15.30 a	45.18 ± 5.76 a	45.96 ± 8.70 a	48.77 ± 11.97 a	43.17 ± 7.01 a	16.79 ± 3.74 a	19.58 ± 3.62 a	23.98 ± 9.94 a	26.38 ± 14.15 a
F	1	38.28 ± 6.42 a	39.62 ± 11.13 a	38.98 ± 3.06 a	48.28 ± 8.08 a	41.32 ± 6.10 a	31.99 ± 7.21 a	34.64 ± 3.06 a	36.66 ± 11.37 a	20.40 ± 5.30 a	28.39 ± 4.00 b	26.38 ± 0.01 b	15.07 ± 4.16 a
G	1	17.70 ± 2.32 a	15.74 ± 2.27 a	13.74 ± 1.19 a	14.40 ± 1.16 a	60.31 ± 3.04 a	64.94 ± 6.92 a	68.28 ± 6.08 a	72.94 ± 5.28 a	21.99 ± 2.01 a	19.32 ± 8.32 a	17.99 ± 7.21 a	12.66 ± 6.43 a

***: Samples were not collected up to this depth either due to the presence of a shallow water table or due to farmer's requirements. Non-Identical small letters within the same row for each parameter represent significant differences among soil parameters with varying soil depth. For Site A and B; Non-identical capital letters within the same column for each sampling site represent significant differences among soil parameters with varying sampling location

Table 2. Physico-chemical parameters of soil at different sites (Mean ± SD)

Site	Subsite	pH				EC (µScm^{-1})			
		Surface	1 ft.	2 ft.	3 ft.	Surface	1 ft.	2 ft.	3 ft.
A	1	8.932 ± 0.28$^{a, A}$	8.73 ± 0.15$^{a, A}$	***	***	6336 ± 8308.8$^{a, B}$	3446.00 ± 4458.76$^{a, A}$	***	***
	2	8.38 ± 0.38$^{a, C, D}$	8.32 ± 0.40$^{a, A}$	***	***	11046 ± 6995.64$^{a, A, B}$	5996.00 ± 3482.82$^{a, A}$	***	***
	3	8.45 ± 0.25$^{a, B, C, D}$	8.57 ± 0.34$^{a, A}$	***	***	10828.00 ± 2711.16$^{a, A, B}$	9992.00 ± 3078.29$^{a, A}$	***	***
	4	8.24 ± 0.28$^{a, D}$	8.55 ± 0.30$^{a, A}$	***	***	16788.00 ± 3136.44$^{a, A}$	10612.00 ± 2808.07$^{b, A}$	***	***
	5	8.40 ± 0.30$^{a, C, D}$	8.26 ± 0.38$^{a, A}$	***	***	15290.00 ± 3407.34$^{a, A}$	12474.00 ± 2292.19$^{a, A}$	***	***
	6	8.77 ± 0.17$^{a, A, B}$	8.99 ± 0.06$^{a, A}$	***	***	16617.50 ± 4273.69$^{a, A}$	17294 ± 3998.3$^{a, A}$	***	***
	7	8.70 ± 0.25$^{a, A, B, C}$	8.81 ± 0.20$^{a, A}$	***	***	10836.80 ± 6692.63$^{a, A, B}$	6813.00 ± 5003.61$^{a, A}$	***	***
B	1	7.75 ± 0.15$^{a, A}$	7.82 ± 0.14$^{a, A}$	7.71 ± 0.09$^{a, D}$	***	13646 ± 4566.14$^{a, C, D}$	12324.00 ± 2926.93$^{a, C, D, E}$	13680.00 ± 4947.62$^{a, A, B}$	***
	2	7.96 ± 0.21$^{a, A}$	7.85 ± 0.15$^{a, A}$	7.90 ± 0.16$^{a, B, C}$	***	7398.00 ± 1362.05$^{a, A, B}$	9538.00 ± 2398.41$^{a, A}$	8736.00 ± 3372.67$^{b, B}$	***
	3	7.90 ± 0.17$^{a, A}$	7.96 ± 0.20$^{a, A, B}$	7.90 ± 0.23$^{a, B, C}$	***	10266.00 ± 3959.39$^{a, D}$	9116.00 ± 1396.74$^{a, E}$	12678.00 ± 2565.17$^{a, A, B}$	***
	4	8.01 ± 0.20$^{a, A}$	8.07 ± 0.19$^{a, A, B}$	8.06 ± 0.14$^{a, B}$	***	18500.00 ± 3989.40$^{a, A, B, C}$	17216.00 ± 6205.80$^{a, A, B}$	15304.00 ± 5193.20$^{a, A, B}$	***
	5	7.88 ± 0.16$^{a, A}$	7.88 ± 0.19$^{a, A, B}$	7.92 ± 0.24$^{a, B, C}$	***	18357.40 ± 2612.80$^{a, A, B, C}$	15948.00 ± 3443.29$^{a, b, A, B, C}$	12482.00 ± 2413.18$^{b, A, B}$	***
	6	7.99 ± 0.16$^{a, A}$	7.87 ± 0.05$^{a, A}$	7.84 ± 0.07$^{a, C, D}$	***	14190.00 ± 4665.48$^{a, B, C, D}$	18384.00 ± 2886.44$^{a, A}$	18150.00 ± 2611.62$^{a, A}$	***
	7	7.90 ± 0.16$^{a, A}$	8.03 ± 0.19$^{a, A, B}$	8.03 ± 0.08$^{a, B}$	***	18826.00 ± 4207.47$^{a, A, B}$	13054.00 ± 2180.67$^{b, B, C, D, E}$	11488.00 ± 2476.88$^{b, A, B}$	***
	8	7.86 ± 0.19$^{a, A}$	7.94 ± 0.10$^{a, A, B}$	7.94 ± 0.09$^{a, B, C}$	***	19390.00 ± 2204.81$^{a, A}$	15368.00 ± 1571.82$^{b, A, B, C, D}$	13978.00 ± 1325.28$^{b, A, B}$	***
	9	7.68 ± 1.11$^{a, A}$	8.18 ± 0.08$^{a, B}$	8.26 ± 0.07$^{a, A}$	***	16490.00 ± 5389.46$^{a, A, B, C}$	11282.00 ± 3578.01$^{a, b, D, E}$	9394.00 ± 2931.94$^{b, B}$	***
	10	8.19 ± 0.14$^{a, A}$	8.21 ± 0.17$^{a, A, B}$	8.35 ± 0.15$^{a, A}$	***	17584.00 ± 3649.81$^{a, A, B, C}$	10864.00 ± 3676.58$^{b, E}$	8962.00 ± 1977.60$^{b, B}$	***
C	1	7.76 ± 0.08^{a}	7.91 ± 0.08^{b}	7.93 ± 0.15^{b}	7.98 ± 0.10^{b}	19426 ± 1539.7$^{a, A, B, C}$	17440.00 ± 1292.05^{b}	14352.00 ± 530.63^{c}	12740.00 ± 1166.81^{d}
D	1	9.82 ± 0.11$^{a, b, c}$	9.94 ± 0.12^{b}	9.75 ± 0.02$^{a, b, c}$	9.70 ± 0.14^{c}	1663.00 ± 47.29^{a}	1870.33 ± 54.50^{b}	1157.00 ± 307.22$^{a, b, c}$	998.67 ± 32.08^{c}
E	1	7.79 ± 0.67^{a}	8.13 ± 0.18^{a}	8.18 ± 0.21^{a}	8.16 ± 0.13^{a}	4881.20 ± 4965.73^{a}	3024.40 ± 2102.73^{a}	2519.00 ± 1737.90^{a}	2222.80 ± 1664.50^{a}
F	1	8.46 ± 0.75^{a}	9.58 ± 0.71^{b}	9.74 ± 0.65^{b}	9.48 ± 0.71^{b}	2960.00 ± 2343.61^{a}	1152.00 ± 360.53^{a}	1014.00 ± 93.06^{a}	796.00 ± 156.79^{a}
G	1	9.82 ± 0.04^{a}	9.51 ± 0.12^{a}	8.90 ± 0.31^{b}	8.89 ± 0.44^{b}	14662.00 ± 10977.74^{a}	2968.00 ± 2754.09^{a}	6526.67 ± 1666.92^{a}	5840.00 ± 858.60^{a}

***: Samples were not collected up to this depth either due to the presence of a shallow water table or due to farmer's requirements.

Non-Identical small letters within the same row for each parameter represent significant differences among soil parameters with varying soil depth. For Site A and B; Non-identical capital letters within the same column for each sampling site represent significant differences among soil parameters with varying sampling location

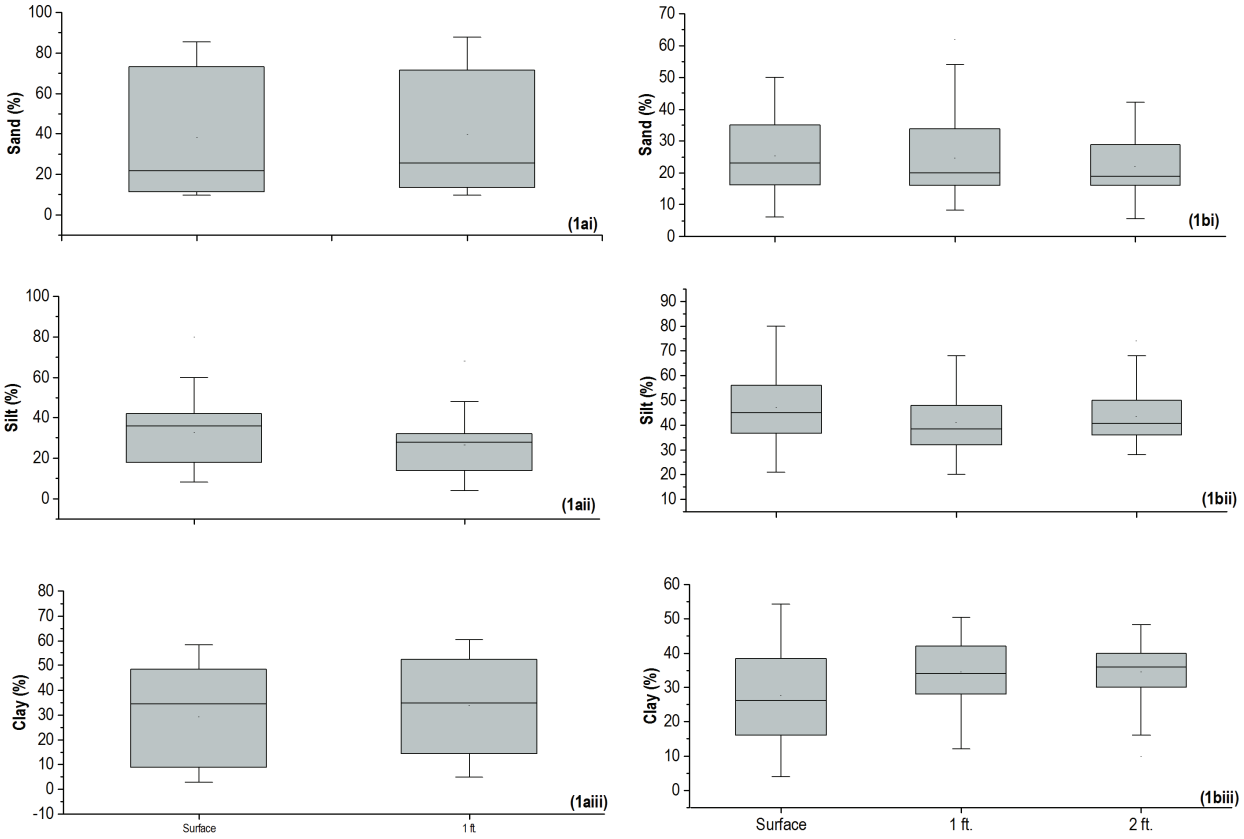


Fig. 1. Variation in soil separates with depth at a) Site A, b) Site B

Variation in soil quality within the different subsites of the same sampling site has been shown in Figures 2a (Site A) and 2b (Site B). Sand content was less than 30% in SA3-SA6 while it was greater than 50% at SA2 and greater than 70% at SA1 and SA7 (Figure 2ai). Silt content of SA1 and SA7 was less than 20% while at all other subsites it was greater than 20% (Figure 2aii). Likewise, clay content at SA1, SA2, and SA7 was less than 20% while at SA3-SA6, it was greater than 60% (Figure 2aiii). Figure 2bi-biii shows the variation in soil separates at different subsites of Site B. It can be clearly shown that soil particulates showed remarkable differences at different subsites of the same sampling sites.

The pH of the soil was greater than 7.5 at all studied sites. The use of lime to improve soil pH is a common practice in Punjab. In the present study, the pH of soil indicates its calcareous nature and out rules the necessity to lime the soil [21, 22]. The calcareous nature of the soil in Punjab was also found in one of our earlier studies [23] based on the assessment of soil quality in four divisions of Punjab. Optimum soil pH for fish ponds bottom has been recommended to be 7.5-8.0 to maintain the optimum activity of soil microbial community and macroflora [22, 24-25]. Ghobadi *et al.* [26] used GIS-DANP based multicriteria approach for aquaculture land suitability assessment. They considered soil pH; 7.00 to 8.5 as most suitable and 5.5 to 6.5 and 8.5 to 9.0 as least suitable. At site A and site B, soil EC was greater than 7500 μScm^{-1} in 75% of soil samples at surface and 1 ft. It was higher than 15000 μScm^{-1} in 50% of soil samples at the surface and 25% of soil samples at 1 ft. and 2 ft. depth. As both of the sites were located in the Sargodha division, Punjab, high soil EC shows the saline nature of the soil in Sargodha. These results are in agreement with those of Siddiq and Raza, who also reported the saline nature of the soil in the Sargodha division [27]. According to the classification system of Dellavalle [28], the soil at most of the subsites of Site A and B fell into the category of very strongly saline.

Based on observations of the present study, we suggest the following method for soil sample

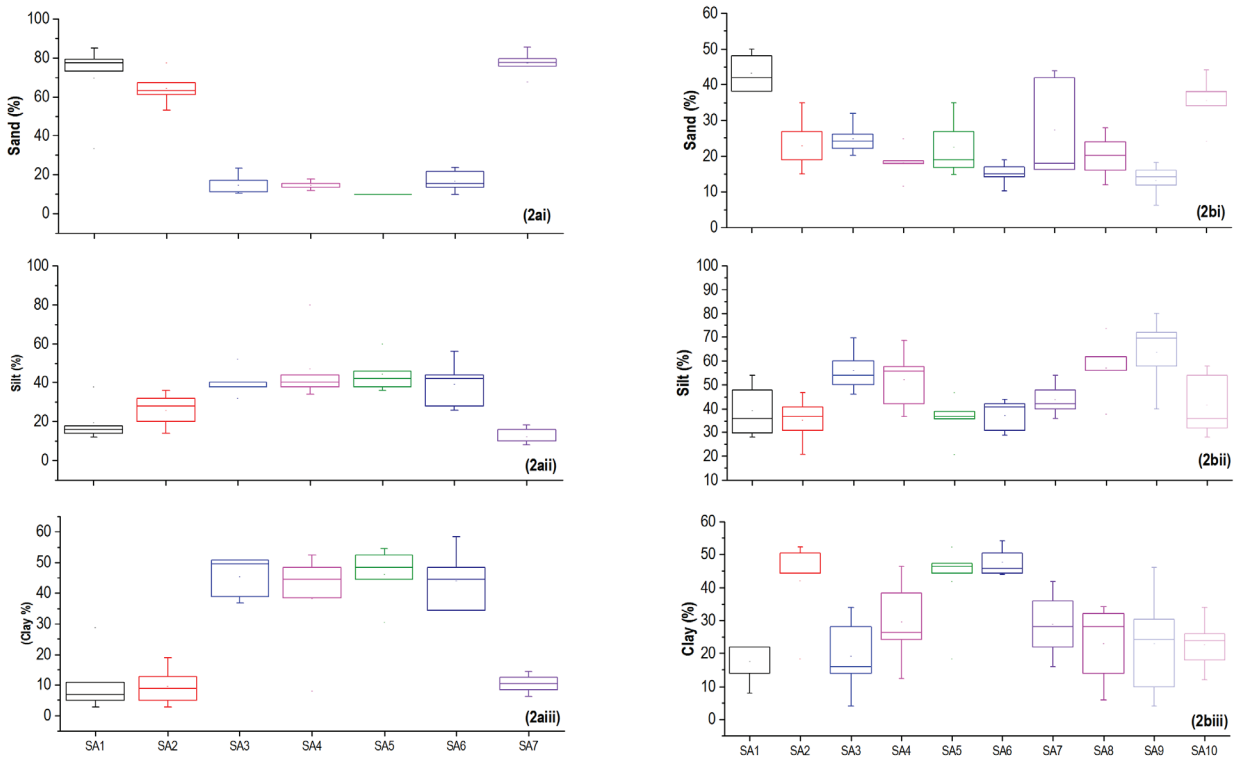


Fig. 2. Variation in soil separates at surface soil of different subsites of a) Site A, b) Site B

collection for aquaculture site assessment. Significant differences in the soil at different subsites of Site A and Site B indicate that the site must be divided into subsites (or strata) to determine its suitability. Soil samples should be collected from various locations of subsites to thoroughly determine the soil quality within that specified area. Moreover, results of the present soil survey have also shown that soil particle size distribution and physicochemical parameters can vary significantly with soil depth. Earlier investigations have also reported variability in soil quality with varying depth [29, 30]. Soil assessment of each subsite, therefore, must be based on a vertical segment of soil covering the depth that would be finally dug to construct a fish pond. Evaluation of vertical segments of soil is also important as the soil dug from the pond will be used to build embankments.

Soil with a wide range of particle size distribution can be used as pond bottom and embankments, however, it is necessary to set certain limits for soil separates to notify the farmers about the potential problems that can arise if the soil contains unsuitable particle size distribution. In general, soil with high sand content and high clay content is not suitable for aquaculture and suitable soil management technique must be employed before using such soils. Soil with very low clay content is also rendered unsuitable. Based on recommendations of Boyd *et al.* [16] and observations about issues faced by fish farmers in Punjab, less than 40% sand content and 10-20% clay content have been set as threshold values for suitable fish pond soil. These values have been arbitrarily used as benchmarks in soil assessment in Punjab, Pakistan. A soil with less than 40% sand content did not mean that there will be no seepage in such soil, instead, these values have been set to inform the farmers about critical conditions that they may face in case of unsuitable particle size distribution.

5. CONCLUSION

Field sampling is one of the most crucial steps in assessing a soil's suitability for aquaculture. The reliability of analytical tests performed on soil depends on the accuracy of the sampling procedure. If the collected sample is not representative of the soil of that particular area, analytical results cannot specify the true characteristic of the soil leading

to erroneous decisions. Soil composition and its quality parameters vary within short distances at the same site. The common practice used in agriculture soil analysis is to collect a composite sample from each 5 acre area or other as defined by total soil area. Analysis of this combined sample will only give an average value of the soil characteristics and this method, therefore, is not suitable to study variation in different areas within the same site. In the present study, we presented a method for soil sample collection before pond construction. In aquaculture, the farmer must know the soil parameters at different subsites within proposed farm sites so that appropriate soil management techniques can be suggested for each soil type within an area. This recommendation is supported by the results of the present study where the soil was found to be sandy or clayey (the two extremes in soil separates content) at different subsites of the same sampling site. Therefore, the determination of variability in soil characteristics within an area is vital for deciding the recommendations about soil management techniques. Analysis of a site's soil for aquaculture should be based on stratified sampling from several subsites based on the total land area. Moreover, as excavated ponds are the most commonly used form of ponds, samples should be analyzed at various soil depths. The following recommendations for soil sampling and analysis have been presented based on the present investigation.

1. Sample collection for soil analysis in aquaculture should be carried out using stratified sampling. As soil properties show remarkable differences within the subsites of the same site, it is suitable to divide the site into smaller subunits that consist of homogenous soil types. These subunits that can be referred to as strata can be as small as 0.2 acres or as large as 5 acres. It will be advantageous if farmers may specify the subsite area that will be used for the construction of one fish pond and consider it as one stratum.
2. From each stratum, at least three points should be identified covering two corners and the center of the area diagonally.
3. Farmers must decide before sample collection that how deep they will dig the soil during pond construction
4. Soil samples should be collected from each sampling location starting from the surface up to the depth that is 1 ft. deep than the soil depth that will be dug during the construction of ponds.
5. Soil samples from each sampling location should be collected with a 1 ft. increment.
6. If soil analysis indicates the sand content to be less than 40% and clay content as 10-20%, the specific location of the site can be used for aquaculture although water seepage is still likely to happen in such soil. However, as water loss through seepage cannot be completely avoided, these values should be used as threshold values during pond construction.
7. If the sand content of an area is found to be greater than ≈40%, it is advised to use suitable soil lining techniques using polymeric membranes. As an alternative to soil layering, clay minerals (bentonite or kaolin, etc.) may be compacted with the pond bottom soil to reduce sand content below the threshold values.
8. In the case, a soil contains higher than 30% clay content, the farmer should be aware that there can be difficulties in working with such soil during the construction of ponds and embankments. Moreover, once the pond is operative, the farmer may have to use additional measures to reduce clay turbidity in pond's water.
9. In general, the nature of the soil is calcareous in Punjab, Pakistan. Once a pond is constructed, farmers are advised to use lime only if the soil analysis revealed soil pH to be less than 6.5. The unnecessary use of lime on calcareous soil can raise soil pH to critical levels that may interfere with the activity of soil microbes and benthic organisms.
10. While deciding the species to be cultured in the fish farm, the farmers must keep in view, the salinity of soil and source water. If either or both of them are saline, salt tolerable species must be cultured to get optimum fish production.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. REFERENCES

1. United Nations. World population to 2300. Vol. 236. *Department of International Economic Social Affairs. Population Division. United Nations*

Publications, (2004).
2. FAO. Regional overview of food insecurity in Asia and the Pacific; Investing in a zero hunger generation. *Food and Agriculture Organization, Rome* (2016).
3. A. Mathiesen. The state of the world fisheries and aquaculture 2014. F*ood and Agriculture Organization, Rome* (2016).
4. State Bank of Pakistan. Aquaculture & inland fishery value chain in Pakistan. Islamabad (2015)
5. M. Siddique, P. Barua and M. Ghani. Comparative study of physico-chemical properties of soil according to the age of aquaculture pond of Bangladesh. *Mesopotamian Journal of Marine Science* 27(1): 29-38 (2012).
6. J. J Bentone. Agronomic Handbook: Management of crops, soils, and their fertility. *CRC Press, Boca Raton, Fl. USA* (2003).
7. E. Gregorich. Soil Sampling and Methods of Analysis. *Taylor & Francis Ltd., Hoboken, NJ. USA* (2007).
8. A.P. Field. Discovering Statistics using IBM SPSS Statistics. 4th ed., *Sage, Los Angeles, California, USA* (2013).
9. S.W. Buol, F.D. Hole, and R.J. McCracken. Soil Genesis and Classification. *Wiley-Blackwell, Ames, Iowa, USA* (2011).
10. V. NováK, and H. HlaváČIková. Applied Soil Hydrology. *Springer, Cham, Switzerland* (2018).
11. C.E. Boyd. Bottom Soils, Sediment, and Pond Aquaculture. *Chapman & Hall, New York, USA* (1995).
12. C.C. Mischke. Aquaculture Pond Fertilization : Impacts of Nutrient Input on Production. *Wiley-Blackwell, Chichester, UK* (2012).
13. R.A. Schoonheydt, and C.T. Johnston. Surface and interface chemistry of clay minerals. Vol. 1. *Elsvier Science, Amsterdam, Netherlands* (2018).
14. R.R. Weil, and N.C. Brady. The nature and properties of soils. *Pearson, Columbus, Ohio, USA* (2016).
15. B. Hajek, and C.E Boyd. Rating soil and water information for aquaculture. *Aquaculture Engineering* 31: 115-128 (1994).
16. C.E. Boyd, C. Wood and T. Thunjai. Aquaculture pond bottom soil quality management. *Pond Dynamics/ Aquaculture Collaborative Research Support Program, Oregon, USA* (2002).
17. I. Ardjosoediro, and I.W. Ramnarine. The influence of turbidity on growth, feed conversion and survivorship of the jamaica red tilapia strain. *Aquaculture* 212(1-4): 159-165 (2002).
18. A.F.M. El-Sayed. Tilapia culture. *Academic Press, Texas, USA* (2019).
19. P. Uzukwu, O. George, and N. Jamabo. The problem of water seepage in aquaculture: A preliminary study of the soils of Arac fish farm, Omuihuechi-Aluu, Rivers State, Nigeria. *Asian Journal of Agricultural Sciences* 3(2): 63-69 (2011).
20. S. Ahmad, M. Aslam, and M. Shafiq. Reducing water seepage from earthen ponds. *Agricultural Water Management* 30(1): 69-76 (1996).
21. C.E. Boyd, and De Queiroz. The role and management of bottom soils in aquaculture ponds. *Infofish International* 2: 22-27 (2014).
22. C.E. Boyd, and S. Pippopinyo. Factors affecting respiration in dry pond bottom soils. *Aquaculture* 120(3-4): 283-293 (1994).
23. J. Shafi, K. N. Waheed, Z.S. Mirza, and M. Zafarullah. Assessment of soil quality for aquaculture activities from four divisions of Punjab, Pakistan. *Jounral of Animal and Plant Sciences* 31(2): 556-566 (2021).
24. J. Cornut, H. Clivot, E. Chauvet, A. Elger, C. Pagnout, and F. Guérold. Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams. *Water Research* 46(19): 6430-6444 (2012).
25. M. Dokulil. Comparative primary production. *Encyclopedia of Inland Waters:* 130-137 (2009).
26. M. Ghobadi, M. Nasri, and M. Ahmadipari. Land suitability assessment (LSA) for aquaculture site selection via an integrated GIS-DAMP multi-criteria method; A case study of Lorestan province, Ian. *Aquaculture* 530: 735776 (2021).
27. S. Siddiq, and A. Raza. An insight into prominent soil characters of Sargodha areas for the establishment of healthy citrus orchards. *Journal of Agricultural Research* 49(1): 27-36 (2011).
28. N. Dellavalle. Determination of specific conductance in supernatant 1: 2 soil: sater solution, In: Handbook on Reference Methods for Soil Analysis. *Soil and Plant Analysis Council, Inc., Athens, GA* ,p. 44-50 (1992).
29. C.E. Boyd. Chemical and textural properties of muds from different depths in ponds. *Hydrobiologia* 48(2): 141-144 (1976).
30. A. Mustafa, and M.C. Undu. Study on determination of categories of soil quality variable concentrations in brackish water ponds of Java island, Indonesia. *Journal of Fisheries Sciences.com* 11(3): (2017).

Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 58(1): 111-128 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(58-1)583

Pakistan Academy of Sciences

Research Article

Preparation and Quality Assessment of Fruit Yoghurt with Persimmon *(Diospyros kaki)*

Nafeesa Khatoon[1*] Sartaj Ali[2], Nan Liu[3], and Hafeez S. Muzammil[1]

[1]Department of National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan
[2]Department of Agriculture & Food Technology, Karakoram International University, Gilgit, Gilgit-Baltistan
[3]School of Public Health, Zhengzhou University, Zhongyuan District, Zhengzhou, Henan, China

Abstract: Yoghurt is a fermented dairy product developed by fermentation of milk with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Assimilation of fruit pulp can boost the nutritional and health benefits of the yoghurt to many folds. The prime objective of preparing fruit yoghurt with the addition of persimmon pulp was to enhance the nutritional value and to improve overall acceptance. Yoghurt was prepared with the incorporation of persimmon in the concentration of 5%, 10% & 15% to assess the best combination. Evaluation of physico-chemical and organoleptic properties was performed during storage. The product was stored for 28 days at 4°C and quality parameters were assessed at an interval of 7 days. Acidity, total solids, and moisture content of fruit yoghurt increased while pH, lactose, protein, fat and textural profile values decreased significantly ($p < 0.01$) during storage. The phenolic content of the controlled sample was 175 mg GAE/g, which increased to 180, 214 and 250 mg GAE/g with 5%, 10% & 15% persimmon combinations respectively. Syneresis value for controlled sample was 2.49% which decreased to 2.45%, 1.94% & 1.10% in 5, 10 & 15% persimmon blends. Additionally, the sensorial properties of yoghurt blended with 10% fruit pulp gained higher acceptability during storage at 4°C.

Keywords: Fruit yoghurt, persimmon, syneresis, health benefits, organoleptic properties

1. INTRODUCTION

Changing lifestyles and increasing consumer's demands make it necessary to formulate new food products which have a better nutritional profile and have more consumer acceptability [1]. In this era, demand for milk-based fermented products and yoghurt has been increased due to its beneficial health consequence on consumers [2]. Yoghurt starts from countries around the Mediterranean Sea and Bulkan. According to FAO/WHO yoghurt is a coagulated dairy product developed from lactose fermentation due to the activity of starter culture such as *Lactobacillus dulbruicki* subsp. *bulgaricus* and *Streptococcus thermophillus* obtained from milk and milk products [3]. The lactic acid produced during the fermentation process changes the milk protein which provides a unique taste and pleasant flavour to the yoghurt [4]. During the formation of yoghurt, whey powder, skim milk powder and milk powder are incorporated into the milk to achieve desire characteristics [5]. The cultured organism in the finished product must be sustainable and should be in sufficient amount to perform its function [6]. Yoghurt has a huge amount of vitamins, minerals, proteins along with viable beneficial bacteria [6, 7]. A 100 g of yoghurt gave 257 KJ energy, carbohydrate (4.7 g), vitamin A (27 µg), fat content (3.3 g), protein (3.5 g) and riboflavin (0.14 mg) and also some proportions of vitamin B6 and vitamin B12. Yoghurt contained a high amount of salt, vitamins and major nutrients as compared to milk [8].

Received: March 2020; Accepted: March 2021
*Corresponding Author: Nafeesa Khatoon <nafeesaali.ft1354@gmail.com>

Yoghurt improves the immune system, decreases cancer, prevent diarrhoea, help in absorptions of iron, protein and calcium [9]. Yoghurt lowers the blood cholesterol level and imparts many other health benefits owing to the presence of protein. Yoghurt strengthens the abdomen and provides assistance to the nervous system and decreases bowel cancer and inflammation effectively due to the availability of vitamins. Yoghurt also helps to reduce various disorders such as intestinal illnesses. Yoghurt promotes gut and vaginal fitness by providing probiotics (beneficial bacteria) [10]. Regular consumption of yoghurt suppressed the pathogenic bacteria like *Helicobacter pylori* which cause stomach ulcers [11]. The culturing organisms in yoghurt convert lactose into lactic acid that is easily digestible by lactose-intolerant persons [12]. Fruits contain a large number of minerals, vitamins, sugar content, dietary fibre and a smaller amount of fat content [13]. The bioactive substances required for the proper functioning of the body are provided by fruits because fruits are a rich supplier of natural antioxidants and dietary fibre [14].

Persimmon (*Diospyros kaki*) is an edible fruit that belongs to Ebenaceafamilywith a sweet/ slightly tangy taste and fibrous to the soft texture, originated from China and Japan [15]. Persimmon contains total lipid (0.19%), total carbohydrate (18.6%) water (80.3%), protein (0.58%) and several minerals such as copper, magnesium, manganese zinc and iron [16]. Total dietary fibre is 1.48% and ascorbic acid comprises 7.5mg. Persimmon contains a high amount of sugar 12.5 g/100 g than other highly consume fruit such as apple, peach, pear and orange [17, 18]. Persimmon fruit has several health benefits against diabetes, blood pressure, diuretics, cough, and a variety of viral and bacterial transmittable diseases, including dental caries [19]. The beneficial properties of persimmon are due to the availability of several bioactive compounds like carotenoid and polyphenol which play a vital role in anticipation of degenerative diseases [15]. Persimmon fruit furthermore utilizes in the development of numerous products because of its functional characteristics [19].

The yoghurt prepared with fruit is a fastidious variety of yoghurt which is mostly liked by all people around the world is fruit yoghurt [20]. Assimilation of different fruits makes the fruit yoghurt good-looking [21, 22]. Incorporation of fruit in yoghurt development improves formulation and increases the taste and results in a good flavour product which accompanied the refreshing and health-promoting effects [23, 24]. FAO/WHO recommended percentage of fruit in yoghurt should be in the range of 5 to 15% [25, 26]. In Pakistan Persimmon is also consumed extensively but unfortunately, it was not used in different dairy products. So in the current study fruit yoghurt was prepared with the following objectives: 1) To develop fruit yoghurt with supplementation of persimmon, and 2) To evaluate the acceptability, textural properties and shelf life of persimmon fruit yoghurt.

2. MATERIALS AND METHODS

2.1 Materials

Fresh whole buffalo milk was collected from the Dairy Farm of the University of Agriculture Faisalabad. Fresh Persimmon (*Diospyros kaki*, oriental persimmon) collected from Gilgit-Baltistan region, stabilizer and skim milk powder was purchased from local market of Faisalabad and starter culture was obtained from SAAF International Pvt. Lt.

2.2 Preparation of Persimmon Pulp

Fresh Persimmon (*Diospyros kaki*, oriental persimmon) which is non-astringent (sweet) collected from the Gilgit-Baltistan region. Fresh fruit was washed in tap water to remove dust and residue. Peel of persimmon was removed with a sharp knife, after peeling fruit pulp was extracted. Then fruit pulp was homogenized and blanched at 70^{o}C for 5 minutes to kill possible spores of pathogenic microorganisms. Then fruit pulp was stored at refrigeration temperature (4^{o}C) for further use.

2.3 Preparation of Yoghurt

Raw milk was standardized to 3.5% fat and 11.0% solid, not fat by adding skim milk powder and stabilizer 0.5% was added and 2% starter culture was inoculated. The yoghurt mixture was pasteurized for 10 minutes at 90^{o}C then cool to 45^{o}C. After that different concentration of fruit pulp such

as 5%, 10% and 15% was added into the yoghurt mixture and incubated at 42°C for four hours in the incubator. After that yoghurt was placed at 4°C in refrigeration temperature to evaluate physico-chemical parameters of fruit yoghurt during the storage period. The yoghurt was prepared with slight modification followed by the procedure of Arslan *et al.* [15]. The schematic diagram of fruit yoghurt was presented in figure 1 below.

2.4 Physico-chemical Analysis of Raw Milk

The physico-chemical analysis of raw milk was performed for pH, total solids, crude protein, lactose, titratable acidity, total solid, solid not fat, moisture content and fat according to AOAC [26].

2.5 Physico-chemical Analysis of Fruit Yoghurt

Physico-chemical analysis for crude protein, pH, total solids, ash, moisture, titratable acidity, fat, lactose content was carried out according to AOAC [26]. Data obtained from all parameters were assessed by statistic 8.1 under CRD design [27].

2.5.1 Measurement of syneresis

Syneresis of yoghurt was determined by following the method of Amatayakul *et al.* [28]. The yoghurt sample of 5 ml was taken in the conical tube to measure the syneresis value and placed the sample in a centrifuge machine at 36000 rpm for 30 minutes. After that, the whey appeared on the surface of the conical tube which was removed with help of the syringe carefully to avoid mixing. Then measured the volume of that removed whey and recorded the value of syneresis in percentage.

2.5.2 Determination of total phenolic content

Total phenolic content in the yoghurt sample was determined by spectrophotometer through

Table 1. Physico-chemical analysis of raw milk

Parameter	Quantity (%)*
Ash	0.76%
Fat	7.6%
Protein	4.14%
pH	6.62
Acidity	0.14%
Lactose	5.02%
TS	16.70%
SNF	9.87%
Moisture content	84.16%

*The values are the means of three replications for each parameter

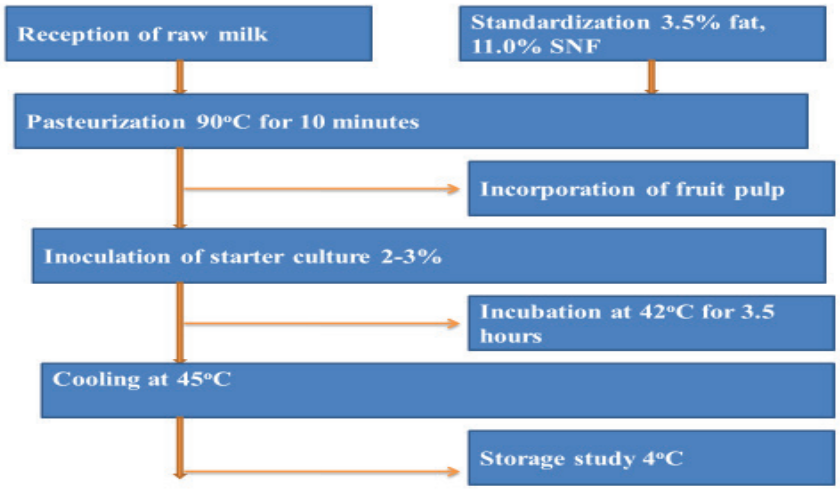


Fig. 1. Schematic diagram of yoghurt supplemented with persimmon

Ciocalteu reagent [29]. An amount of 10 ml of yoghurt sample was diluted with 20 ml of distilled water and mixed with 10 ml of 6% ethanol solution. The mixture was poured into a beaker and kept for 30 minutes at room temperature. The mixture was then stirred for 15 minutes at 15000 rpm in an electrical stirrer. After stirring the extracted sample was placed at room temperature at 25oC and 1.5 ml of 0.2 N Folin-Ciocalteu reagent was mixed with the sample. After 5 minutes, 1.2 ml of 0.7 N Sodium Carbonate was mixed with 0.3 ml of extracted yoghurt sample and poured into a beaker. The prepared sample was placed in the incubator for 2 hours at room temperature. After incubation, the sample was placed in a spectrophotometer to check the absorbance of the total phenolic content at 765 nm by using Gallic acid as standard and the values were expressed as GAE/g of yoghurt.

2.5.3 Determination of textural profile

The texture of prepared yoghurt was determined by a texture analyzer (Version 6.0, Hamilton, MA, USA) according to [26]. For this purpose, a cylinder probe, made of aluminium having a diameter of 35 mm was inserted into yoghurt at a speed of 5 mm/s to measure the body texture and noted reading in triplicates.

2.5.4 Analysis of organoleptic properties

The sensory characteristic of prepared yoghurt was determined by the method of Meilgaard *et al*. [30] by using 9 points hedonic score system. The parameters colour, aroma, taste, mouthfeel and overall acceptability was measured. The score contains numbers from 1 (dislike extremely) to 9 (like extremely).

3. RESULTS AND DISCUSSION

From table 1, it is revealed that fat content in whole raw buffalo milk was in the range of 6 - 7% [31 - 33]. Buffalo milk is considered healthier and more economical as compared to cow milk because of the higher concentration of unsaturated fatty content [34, 35]. Khedkar *et al.* also stated that the fat content (6.7%) of buffalo whole milk is the richest than other cattle milk [36]. Persimmon restrains total lipid (0.19%), total carbohydrate (18.6%), water (80.3%), protein (0.58%) and several minerals such as copper, magnesium, manganese zinc and iron [16]. Persimmon contains a high amount of sugar (12.5 g/100 g) than other highly consumed fruits such as apple, peach, pear and orange. Total dietary fibre is 1.48% and ascorbic acid comprises 7.5 mg [17, 18].

Persimmon contains a large number of antioxidants that provide shelter to various diseases. Among antioxidants; carotenoids, tocopherol, polyphenol is most abundant [37]. The leave and fruit of persimmon hold components as proanthocyanidin, flavonoid, tannin, catechin, phenolic acid etc and mainly exacting portions are carotenoid and tannin components [38]. The titratable acidity, pH and total soluble solids (TSS) were set up in the range of 0.12 g / 100 g, 5.52 and 11.5% respectively. Celik and Ersisli [39] stated the average content of titratable acidity, pH and total soluble solids were 2.06%, 5.40 and 17.1. Candir *et al.* reported that the content of TSS, pH and titratable acidity were like the results of previous experiments [40]. The overall sugar content of persimmon was 16.3 g / 100 g, while glucose and fructose contents were 10.2 and 6.13 g / 100 g respectively. Chen *et al.* reported that total phenolic content 32.3 mg / 100 g [41].

Table 2. Treatment plan of fruit supplemented yoghurt

Treatments	Yoghurt Mixture %	Fruit Pulp %
T_{0i}	100	-t
T_{1i}	95	5t
T_{2l}	90	10t
T_{3l}	85	15t

3.2 Physico-chemical Analysis of Yoghurt

The yoghurt mixture was incorporated with persimmon fruit to formulate nutritiously rich fruit yoghurt to fulfil the dietary requirements of consumers in the present study (Table 2). The purpose of this experiment was to find out the organoleptic as well as physico-chemical characteristics of fruit yoghurt which make it possible to meet the increasing market demand. The findings of all parameters from treatments were discussed below.

3.2.1 Total Acidity (%)

The distinct acidic flavour in yoghurt is attributed to the action of lactic acid bacteria which breaks down lactose into lactic acid. The mean values regarding the acidity of yoghurt fortified with *Diospyros kaki* have shown a non-significant ($p < 0.01$) increasing trend during storage (Table 3). The acidity for the control sample (T_0) was noted as 1.10% which was lower than the treated samples with different concentrations of persimmon at zero days. The incorporation of fruit leads to a rise in the acidity level at different study intervals of 7 days in fruit yoghurt. From the present findings, higher acidity of 1.69% was found in sample T_3 with a higher amount of fruit at the end day of the storage study.

The acidity of fortified yoghurt was also low but the water holding capacity of fortified yoghurt was increased due to the presence of fibres and pectin in apple pulp, as compared to control. Anuyahong *et al.* reported that the accumulation of red rice into yoghurt did not influence pH and titratable acidity when compared to the control yoghurt [42]. Similar results were also found in other reports of yoghurt supplemented with grape, green coffee powder, and blueberry flower pulp [43 - 45]. Nguyen also demonstrated similar findings [46]. The level of titratable acidity of yoghurt blended with apple and tulsi leaf extract also meets the required criteria established by the Codex Alimentarius which defines that the titratable acidity (express as % lactic acid) of yoghurt should not be less than 0.6%. Comparable findings were revealed that the acidity content of fruit yoghurt increased with the increasing amount of fruit [47].

The higher acidity values of persimmon yoghurt in our study might be because of the function of lactic acid bacteria and the development of lactic acid during storage [6, 48]. Petridis *et al.* demonstrated that the acidity of the yoghurt prepared with honey and pomegranate juice increased along the storage period [49]. The lactic acid bacteria convert the lactose content into lactic acid which increased the acidity of fruit yoghurt. The incorporation of fruit and the amount of pulp increased the acid content of fruit yoghurt. The higher acidity values of persimmon yoghurt in our study might be because of the function of lactic acid bacteria and more production of lactic acid during storage. The varying results of the experiment indicated that the addition of different constituents and prolonged storage time increased the acidity of the fruit yoghurt and thus resulted in poor quality with sore taste.

3.2.2 pH

The pH of yoghurt indicates the shelf life of the product throughout storage. Results from mean values revealed that the addition of persimmon non-significantly influenced ($p < 0.01$) the characteristics of fruit yoghurt (Table 3). The mean value of the controlled sample was higher (4.66) than samples incorporated with fruit pulp. The mean values of pH decreased in all samples during storage at 4 °C, the lowest pH value was observed in sample T_3 with 15% fruit pulp followed by T_2, T_1 and T_0 respectively. Similar trends have been reported by previous studies that the addition of different fruits significantly lowers the pH during storage study [6, 50, 51].

De Moura *et al.* investigated that the pH value of fruit smoothies declined during storage intervals [52]. Buchilina *et al.* also demonstrated that the pH content of yoghurt declined with camel milk with monk fruit sweetener [53]. pH is the most imperative physicochemical parameter in yoghurt manufacturing, since the yoghurt production process is complete, by definition, when a pH (4.7) is reached (corresponding to the average isoelectric point for caseins) [54]. The main reason for the decrease in pH and increase in acidity during fermentation of yoghurt was the lactic acid produced by yoghurt starter culture [55]. Michael *et al.* reported that the pH of yoghurt ranged between 4.3 and 4.6 [56]. Supplementation of *H. sabdariffa*

calyx extract into the reconstituted low-fat milk yoghurt resulted in a significant reduction of pH 4.4-4.16 vs.4.54-4.31 as control [57].

Gunawardhana *et al.* observed higher acidity and lower pH of yoghurt incorporated with apple juice such that an increase in apple juice concentration in yoghurt leads to increased acidity [58]. It was noticed that the acid content and pH value of fruit yoghurt has an inverse relationship with each other, increasing acidic content leads to a lower pH value of fruit yoghurt. Yoghurt is an acidic dairy product with natural keeping quality. However, the quality deteriorates quickly with time as the acidity increases and pH value decreased over the storage period.

3.2.3 Ash (%)

Ash is the remaining substance comprised of minerals after charring the fruit yoghurt. In other words, ash content is the measure of the total amount of minerals present within the food. Minerals are the amount of specific inorganic components present within food such as Ca, Na, K and Cl. Results have shown a highly significant ($p < 0.01$) interaction between treatments and storage. The mean values (Fig. 2) for ash content of fruit yoghurt supplemented with persimmon pulp was recorded considerably in the range of 1.67%, 1.65%, 1.84% and 1.77% respectively within treatments at zero days. These values decreased along with the treatment with an increasing amount of fruit pulp as 5, 10 & 15%. The present outcomes indicated a reduction in ash content of yoghurt samples (T_0, T_1, T_2 and T_3) which were recorded as 0.47%, 0.20%, 0.14% and 0.08% respectively during the 28th day of storage. The present work was found in agreement with the previous finding of Roy *et al.* and Bakirci *et al*. stating that the ash content decreases with the addition of fruit pulp [51, 59].

According to the study conducted by Ismail *et al*. who reported that the ash content increased in yoghurt developed with the fortification of apricot juice than red grape juice [60]. These reports propose that the ash content in fruit fortified yoghurt is affected by the type of fruits. Those blended fruits having higher ash content will result in increased values of ash while lower ash containing fruits will have fewer values in the end product. Thus, the current results show that the ash content of fruit yoghurt blended with persimmon is low due to the lower ash contents of persimmon fruit. The declining trend in ash content of fruit yoghurt was possibly due to the profound influence of persimmon pulp during the development of fruit yoghurt because persimmon has a low amount of ash content. Ash content reduced could be attributed to loss, water carries off the mineral during refrigeration storage. During storage and processing operations, inorganic salt leach off due to inappropriate storage condition.

3.2.4 Protein

It was revealed from the results (Fig. 3) that the persimmon had a noteworthy effect ($p < 0.01$) on the protein content of yoghurt due to different concentrations of fruit pulp along with treatments. The protein content showed a declining trend non-significantly along with the storage at 4^0C. The mean value of protein of the control yoghurt sample was recorded maximum of 4.78% while the yoghurt sample with fruit pulp has a low content of protein

Table 3. Effect of persimmon on acidity and pH of fruit yoghurt

Parameters	Samples	Storage (days)				
		0	7	14	21	28
pH	T_0	1.10±0.04	1.31±0.04	1.45±0.04	1.57±0.06	1.60±0.06
	T_1	1.11±0.03	1.37±0.05	1.42±0.06	1.48±0.07	1.53±0.08
	T_2	1.14±0.05	1.41±0.07	1.46±0.07	1.51±0.05	1.56±0.07
	T_3	1.19±0.06	1.47±0.05	1.56±0.05	1.59±0.05	1.69±0.05
	T_0	4.66±0.16	4.62±0.14	4.46±0.14	4.38±0.18	4.32±0.1
	T_1	4.64±0.14	4.61±0.19	4.54±0.18	4.38±0.22	4.34±0.22
	T_2	4.52±0.18	4.50±0.22	4.38±0.22	4.26±0.13	4.31±0.12
	T_3	4.44±0.22	4.42±0.15	4.37±0.13	4.35±0.14	4.30±0.17

within treatments at zero-day of storage. On the 28th day of the storage study lowest protein content was recorded as 0.08% in the yoghurt sample with 15% of fruit pulp due to the low content of protein in fruit. The current study was supported by Bakirci *et al.* [59] and Mohamed *et al*. [61]. Brodziak *et al.* stated that the protein content of fruit yoghurt reduces during the storage study [62].

Ismail *et al.* researched fruit yoghurt and evaluated that the protein content decline during cold storage [60]. Similar results were reported by Roy *et al.* [51], Mbaeyi-Nwaoha *et al.* [63] and Desouky *et al.* [64] that protein content was decreased in the fruit-flavoured treatment with the accumulation of fruit juices because fruit juices are full of lower protein than milk. Escalating the amount of fruit juices significantly decreased the protein percentage. The declining trend in protein content was might be due to the proteolytic activity of microorganisms which degrades the protein content due to the high amount of acid content of fruit yoghurt during storage as demonstrated by [33, 50]. Scientists investigated that the addition of fruit pulp significantly reduced ($p < 0.01$) the protein content of yoghurt due to less content of protein in fruit [65].

3.2.5 Fat

Fat content is considered an important component of milk, it builds up organoleptic characteristics

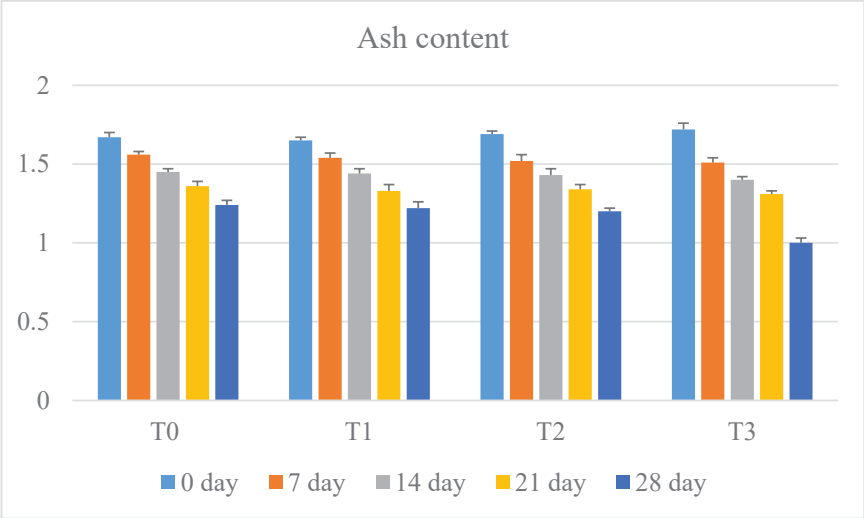


Fig. 2. Effect of persimmon on the ash of yoghurt

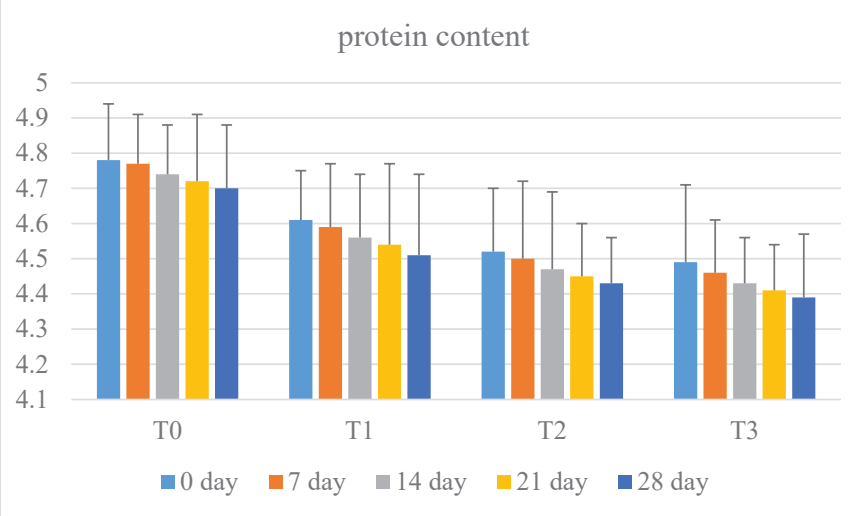


Fig. 3. Effectof persimmon on the protein content of yoghurt

like appearance and texture of milk as well as it plays a major role in products development. It was evaluated from the finding (Fig. 4) that persimmon had a considerable effect on yoghurt quality due to the difference in concentration of fruit. The storage interval revealed a decline trend non-significantly ($p < 0.01$) in the fat content of fruit yoghurt. The mean value of fat of control yoghurt was a maximum of 3.48% and the mean values of the fat content of yoghurt samples with fruit pulp decreased along with treatments at 0 days of storage. The lowest fat content 2.95% was recorded in the yoghurt sample with 15% fruit pulp at 28 days of storage study.

These results agree with previous studies which confirmed these outcomes [66 - 69]. Increasing the number of fruit juices were significantly declined the fat percent for all yoghurt treatments because the fruit juices contain low-fat content than milk [60]. Palka and flis [70] stated that yoghurt formulated with various vegetable and fruit extracts and studied the storage period which shows lower fat content throughout the period.

The researcher reported that the addition of pumpkin reduced the fat content [59] while on the other hand, another study observed that supplementation of apricot (3, 6 & 9%) reduced the fat content in fruit yoghurt [6]. Brodziak *et al.* analyses that the fat content of fruit yoghurt gradually declines during the storage interval of 21 days [61]. The declining trend in fat content of fruit yoghurt might be due to the formation of volatile fatty acids as a result of the breakdown of fat content which caused to significantly reduce the fat content of fruit yoghurt, thus the fat is split due to the function of lactic acid bacteria in yoghurt. Another reason behind the low amount of fat content in fruit yoghurt might be due to less amount of fat in fruit.

3.2.6 Syneresis

Syneresis is the collection of whey on the surface of the yoghurt. It was observed from the results (Fig. 5) that the addition of persimmon fruit had the potential effect ($p < 0.01$) on syneresis due to different concentrations of fruit. The syneresis for the control sample was noted as 2.49% which was greater as compared to yoghurt prepared with the addition of fruit as 2.45, 1.90 and 1.10%. The addition of persimmon fruit reduced the syneresis value along with the treatments and mean values gradually reduced during storage intervals. The lowest value 3.00% for syneresis of yoghurt was found in sample (T_3) after the 28th day of storage study because of the high concentration of fruit pulp.

Literature showed that supplementation of *H. sabdariffa* Calyx extract into the probiotic yoghurt resulted in syneresis 18.85 to 24.90 ml / 50 gm of the sample [71]. Yoghurts enriched by *H. polyrhizus* resulted in a higher syneresis percentage

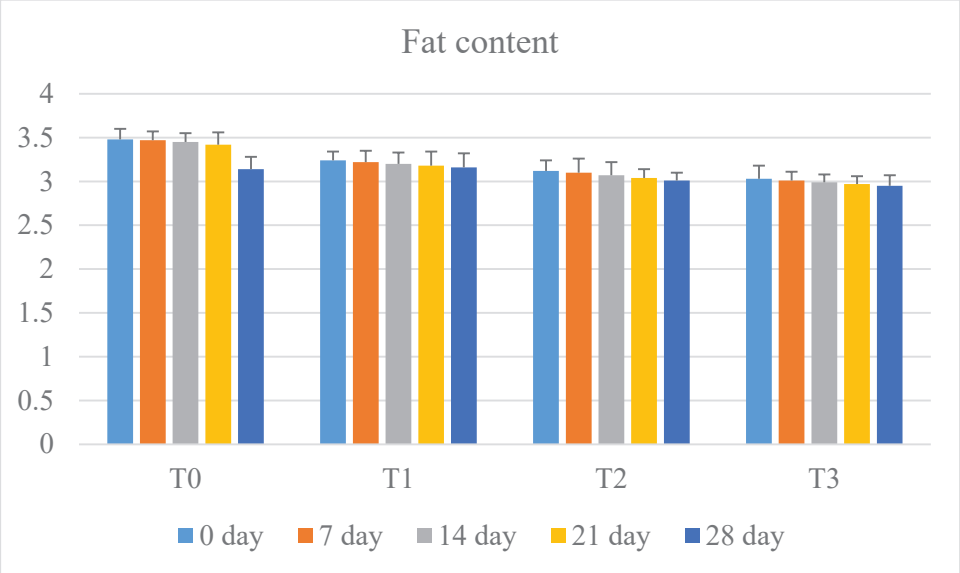


Fig. 4. Effect of persimmon on the fat content of yoghurt

(57.19 - 70.32%) compared to plain yoghurt (52.93%) [56]. Nguyen stated yoghurt developed with *H. sabdariffa, H.polyrhizus* and *P. bivalvis* extract that syneresis value increase during the storage time [57]. Similar results were found that the addition of fruit considerably reduced the syneresis value during the storage periods [51, 59, 61]. While a study reported that the addition of concentrated grape juice in yoghurt caused to increase in the syneresis value at 4 °C after 15 days of storage period [72]. The increasing trend might be due to inappropriate storage condition that leads to increase the syneresis value of yoghurt during the storage period. The low pH and high acid content lose the water holding capacity in yoghurt with storage which possibly be the reason to increase the syneresis value in refrigeration conditions.

3.2.7 Total phenolic content

The menace of diseases such as cardiovascular diseases and cancer is reduced because of consumption of fruit and vegetables regularly because they are comprised of natural antioxidants like ascorbic acid, phenolic content. The natural antioxidant has proved to reduce the chances of occurrence of coronary heart diseases. The current findings (Fig. 6) revealed that the persimmon had a significant effect ($p < 0.01$) on the total phenolic content of yoghurt due to variation in concentration of fruit within treatments. The total phenolic content for the control yoghurt sample (T_0) was lowest (157%) as compared to the yoghurt sample containing persimmon fruit (180, 214 and 250%). The addition of persimmon fruit caused to increase in the total phenolic content of yoghurt along the storage period at refrigeration temperature 4°C. The yoghurt sample with a high concentration of fruit pulp (T_3) gained higher total phenolic content because of the higher amount of total phenolic content of persimmon fruit.

Yoghurt supplemented with *H. sabdariffa* had the highest total phenolic content (9.4 - 9.7 mg GAE / 100 gm). Meanwhile, the control yoghurt had the lowest total phenolic content (3.0 - 3.4 mg GAE / 100 gm). *H. sabdariffa* L. flowers marmalade significantly increased total phenolic content (5.57 - 14.69 mg GAE / 100 gm) of formulated yoghurt [73]. Dimitra *et al.* conducted a similar study and find out the results which show resemblance [74]. The addition of different fruit leads to an increase in the total phenolic content of fruit yoghurt during the period [15, 75]. The high concentration of phenolic content in persimmon fruit could be the possible reason for the increasing amount of phenolic content in fruit yoghurt as well as the higher antioxidant potential caused to raise the total phenolic content of fruit yoghurt. The antioxidant potential is attributed to phytochemicals that scavenge the free radicals. Any fruit high in phytochemicals will show high activity.

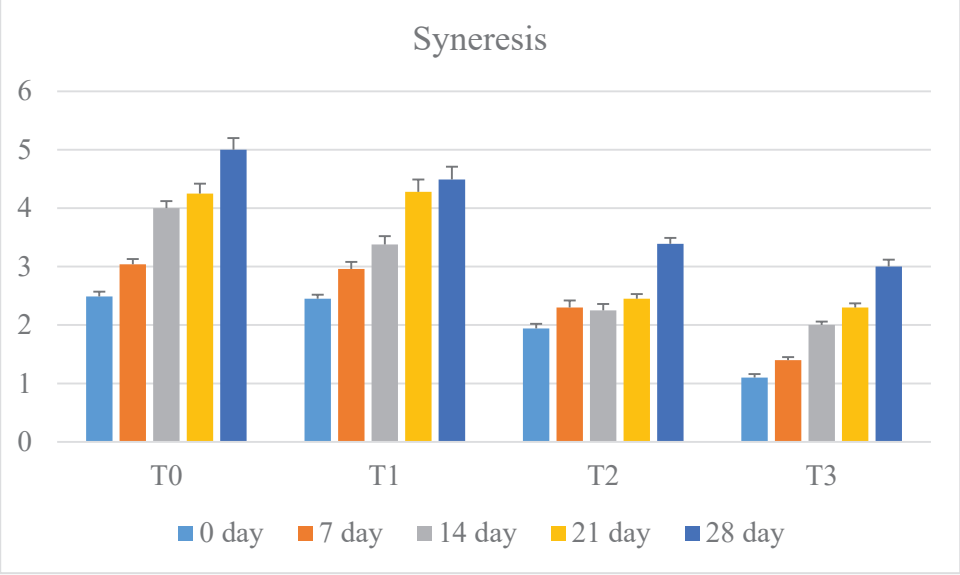


Fig. 5. Effect of persimmon on syneresis of yoghurt

3.2.8 Total solid content

The mean values for total solids of fruit yoghurt have shown that the addition of persimmon fruit highly affects ($p < 0.01$) the total solid content of yoghurt because of various concentrations of fruit within treatment (Fig. 7). The mean value of total solid content of the control sample (T_0) was found lowest at 15.70% than other yoghurt samples supplemented with persimmon fruit. The yoghurt samples were evaluated at 7 day's intervals to check the effect of persimmon on the total solid content of yoghurt. The finding revealed that total solid content increased within treatments due to increasing amounts of persimmon fruit as 5%, 10% and 15% respectively. During the storage study, the total solid content was increased to 16.60%, 16.75% and 16.80% respectively. From current results (Fig. 7), it was concluded that yoghurt containing 15% persimmon pulp had a maximum concentration of total solids (16.80%) at the end day of storage.

Addition of apricot fruit in concentration (3, 6 and 9%) caused to raise total solid of yoghurt as 16.15% to 16.55%, 16.29% to 16.06% and 16.44% to 16.88% [6]. These findings found a resemblance with the literature [58,71, 76, 77, 78]. It was analyzed that addition of fruit juice caused to increase in the total solid content of yoghurt. The researcher examined that total solid content was significantly increased ($p < 0.01$) by the treatments due to variation in quantities of fruit [15]. Another study supported the present study that the addition of banana caused to significantly increase ($p < 0.01$) in the total solid contents of yoghurt during storage [47]. Ritu *et al.* observed that the addition of 5% and 15% pineapple juice increased the total solid content of yoghurt as compared to plain yoghurt [79]. The total solid content was the increase in yoghurt developed with the fortification of red grape juice [60]. It was concluded that the addition of sweetened fruit might be one of the majors caused to increase total solid of fruit yoghurt. The reduction in the pH value of fruit yoghurt might be a possible reason to upraise the total solids contents of fruit yoghurt.

3.2.9 Lactose content

Mean values have shown the lactose content of yoghurt prepared with persimmon fruit (Fig. 8). The lactose content of simple yoghurt was 5.17% which was highest than other mean values of fruit yoghurt samples T_1, T_2 and T_3 such as 5.11 5.03 and 4.86%. The lactose contents of the yoghurt sample fortified with persimmon fruit as 5%, 10% and 15% were noted as 5.11%, 5.03% and 4.86% respectively at 0 days. Results have shown that the lactose content reduced considerably with the increasing amount of persimmon fruit in yoghurt.

On the 28th day of the storage study, the

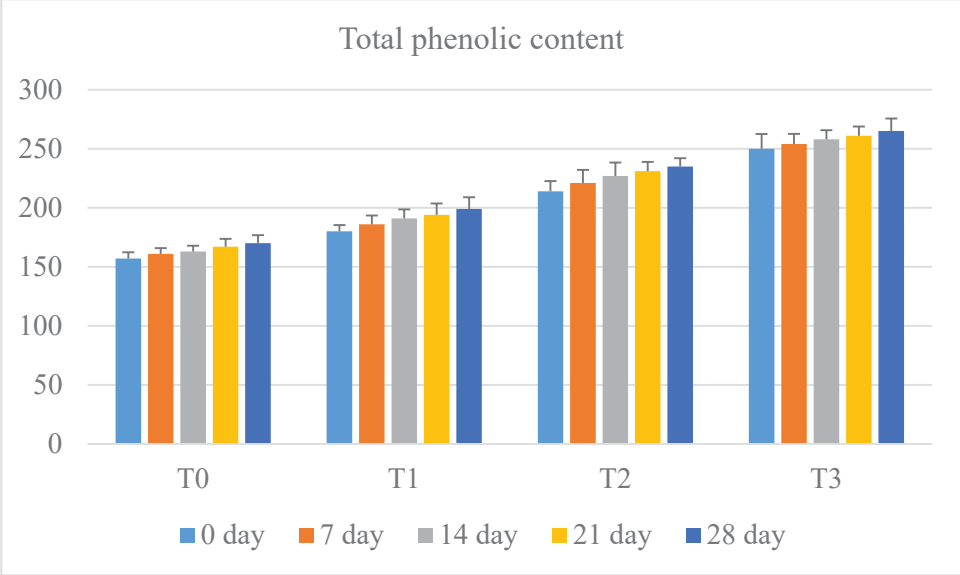


Fig. 6. Effect of persimmon on total phenolic of yoghurt

lactose contents were recorded in the range of 5.02%, 4.89% and 4.80% noticeably. A prominent reduction was observed in the yoghurt sample with 15% of persimmon fruit which was 4.80% (Fig. 8). The fruit yoghurt developed with 5% and 15% pineapple juice has the maximum amount of carbohydrate along the storage period [79]. Carbohydrates content (CHO) was significantly diminished within the evolution of the cold storage period of all yoghurt treatments reaching the lowest values at the end of the storage period (21 days). This decrease is due to the carbohydrate's hydrolysis, which is attributed to the growth and activity of lactic acid bacteria and acid development that increased greatly throughout storage. These findings agree with the findings obtained by Kauser *et al* [6].

The lactose content of the sample with 3%, 6% and 9% apricot pulp was reduced from 5.12%, 5.04% and 4.96% to 5.04%, 4.92% and 4.82% respectively after 22 days of storage study [6]. The fruit yoghurt mostly has a negligible amount of lactose which was might be due to breaks down of lactose into lactic acid by the function of lactic acid bacteria during storage. The fermentation process and production of lactic acid bacteria minimized the amount of lactose content.

3.2.10 Moisture content

The addition of fruit pulp has a highly significant effect on the moisture content of yoghurt during storage (Fig. 9). The incorporation of fruit pulp profoundly affects ($p < 0.01$) the moisture contents of yoghurt because of various concentrations of persimmon fruit pulp. The moisture content of the control sample (T_0) was (52.32%) the lowest as compared to other treatments prepared with the addition of persimmon. The present finding revealed that the moisture content of fruit yoghurt increased with the increasing amount of persimmon fruit within treatments along with the storage at refrigeration temperature 4°C. The moisture content of yoghurt samples (T_1, T_2 and T_3) with 5%, 10% and 15% were recorded as 54.36%, 56.41% and 58.46% respectively at 0 days of study. During storage periods moisture contents of fruit yoghurt significantly increased to 88.61%, 89.48% and 89.76% on the 28th day of storage respectively. Higher moisture content was found in the yoghurt sample with 15% persimmon pulp because of the high concentration of fruit in yoghurt (Fig. 9). The yoghurt prepared with sweet lemon and apple juice (5%, 10% and 15%) had a higher concentration of moisture than the yoghurt prepared with pineapple juice 10%.

The moisture content was slightly high because of fibres present in the yoghurt which holds the water and the setting time was similar to that of control, however, the setting time was less and it might be due to fast metabolizing culture [58]. The researcher explained a similar trend that the addition of fruit pulp significantly increased ($p < 0.01$) the moisture content of yoghurt and concluded that the

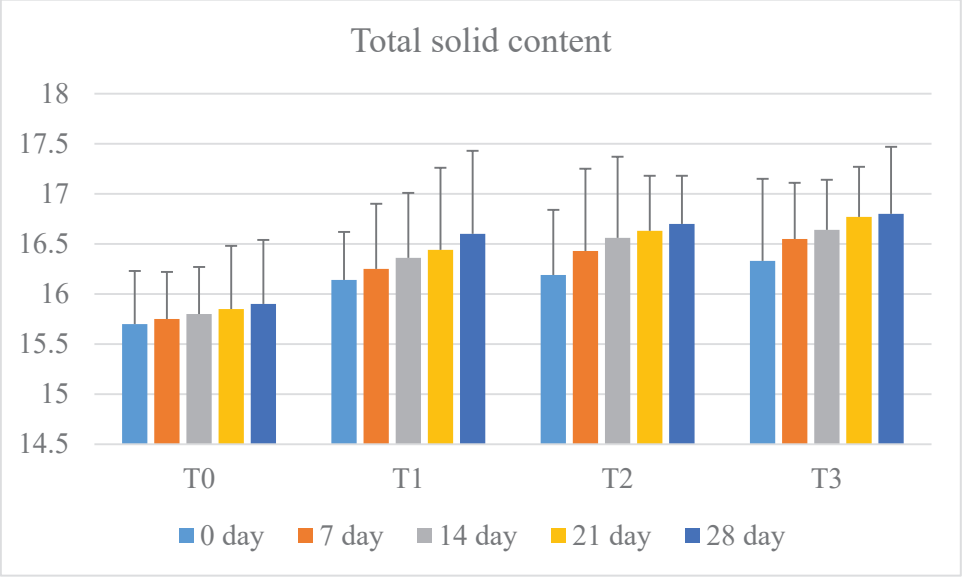


Fig. 7. Effect of persimmon on total solid content

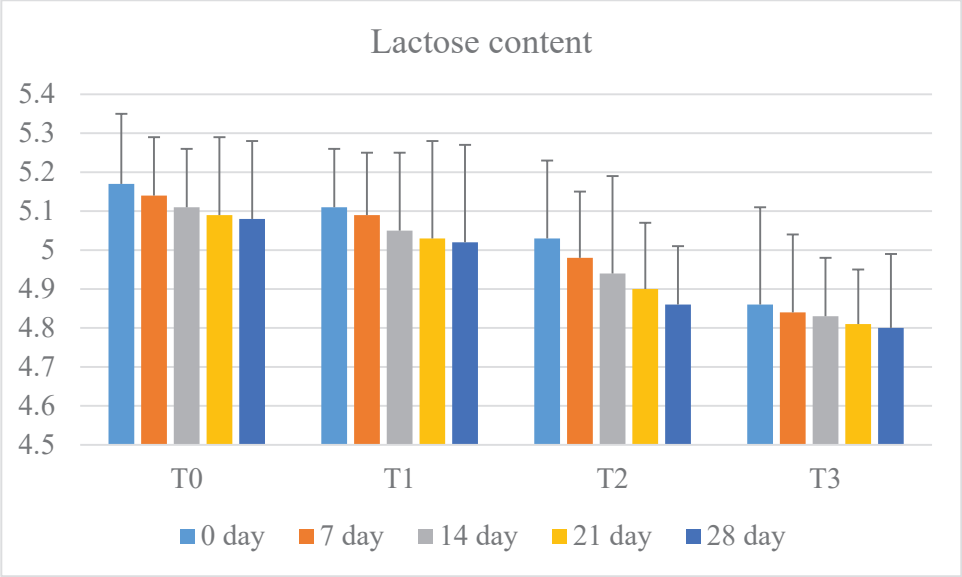


Fig. 8. Effect of persimmon on lactose content

moisture content of fruit yoghurt increased because of the high moisture content in fruit pulp [51].

3.2.11 Textural profile

The texture is related to the appearance and consistency of the surface of any product. Hardness, or firmness, is the most important characteristic in determining yoghurt texture. It is regarded as the force required to attain a certain deformation and is considered as a measure of the hardness of the yoghurt [80]. The addition of fruit non-significantly affects ($p > 0.05$) the textural profile of yoghurt (Fig. 10). The mean value of textural profile for control sample was lowest 5.87 N than other yoghurt sample prepared with incorporation of persimmon fruit as 5.88, 5.89 and 5.90 N respectively at zero days of storage. During the storage study, the mean values of fruit yoghurt decreased to 5.74, 5.77 and 5.75 N respectively at refrigeration temperature.

The same trend was analyzed that dadih prepared with papaya and pineapple has a noticeable effect on the textural properties of yoghurt [81]. They investigated those values for hardness was greater for papaya dadih as compared to dadih made from pineapple. Another research conducted by Mohammad *et al.* [82] was also following the present study that strawberry yoghurt developed with the fortification of red beet extract 1.25%, 2.5% and 4%.

Jaglan *et al.* [2] also reported that the mango pulp incorporated at 15% concentration was best due to sensory acceptability and the pH at that level was slightly acidic as compared to the market sample and moisture content was greater than that of the market sample. The researcher concluded the possible reasons that fruit pulp plays a crucial role in the maintenance of textural characteristics of finish product [51]. The fruit pulp has higher solids and fibre content which results to increase viscosity thus improve the textural profile of fruit yoghurt.

3.2.12 Sensory characteristics of yoghurt

The consumer's response in form of feedback can be gained by sensory or organoleptic characteristics such as aroma, colour, taste, the mouthfeel of the product which is an important factor for product acceptability. The mean value for the mouthfeel of fruit yoghurt was observed at different intervals to check the effect of persimmon fruit on yoghurt. The score of mouthfeel for the controlled sample was found highest compare to the yoghurt sample treated with fruit pulp at the storage study (Table 4). The incorporation of different fruit during storage influenced the characteristics of fruit yoghurt for mouthfeel [15, 16, 51].

The findings revealed that the addition of fruit has a considerable effect on the colour of fruit yoghurt. The various concentrations of

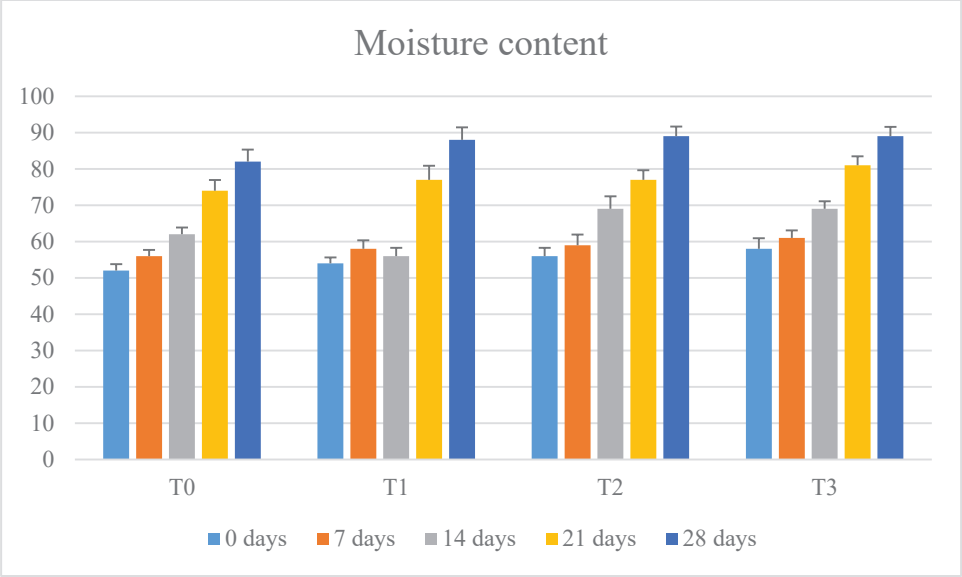


Fig. 9. Effect of persimmon on moisture content

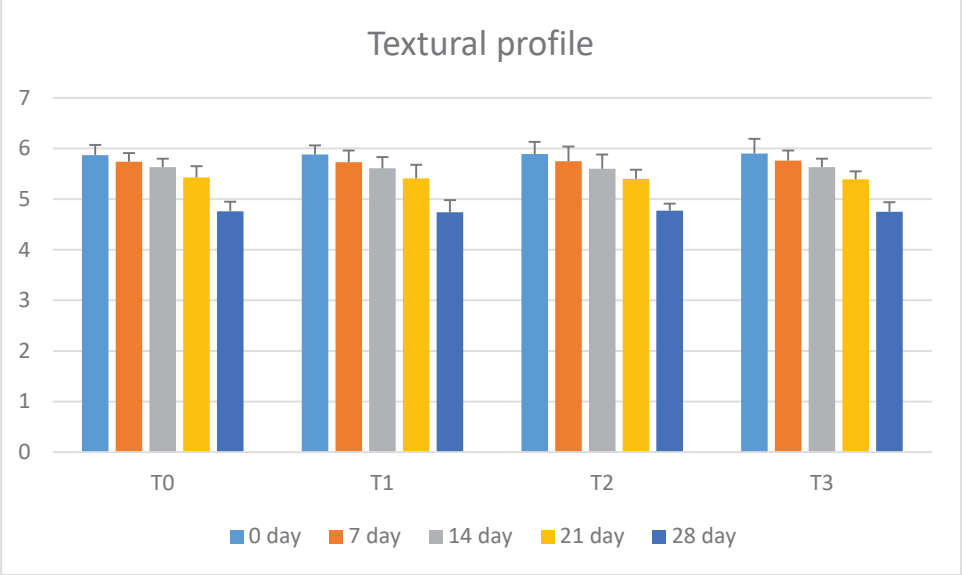


Fig. 10. Effect of persimmon on the textural profile of yoghurt

fruit in yoghurt effectively influence the colour of yoghurt within treatments ($p < 0.01$). During storage conditions the colour of fruit yoghurt. it was revealed that the reduction in the colour value of fruit yoghurt might be due to activities of bacteria with low pH and higher syneresis value of fruit yoghurt during the research period. The addition of fruit in various concentrations (T_1, T_2 and T_3) during yoghurt development considerably effect ($p < 0.01$) on the aroma of fruit yoghurt within treatments. The best score was found for treatment T_2 as compared to other treatments. Best score gained by the treatment T_2 while least score gained by T_3 with the maximum concentration of fruit (15%). The same results were presented that the mean values of yoghurt with cactus pear in the concentration of (5%, 10%, 15%) for aroma was decreased during storage [40, 75]. The lowest mean value for aroma was might be due to the reduction of pH value within the storage period which might be most likely leads to a lower flavour and aroma of fruit yoghurt.

The present study revealed that the addition of persimmon fruit on yoghurt considerably effect ($p < 0.01$) the taste of developed yoghurt because of various concentrations of fruit along with treatments (Table 4). The taste of the yoghurt sample reduced

Table 4. Effect of persimmon on sensory characteristics of yoghurt

Parameters	Samples	Storage (days)				
		0	7 14 21	28		
Appearance	T_0	7.54±0.26	7.39±0.22	7.34±0.22	6.94±0.28	6.44±0.26
	T_1	6.54±0.20	6.49±0.26	6.24±0.25	6.09±0.30	5.94±0.30
	T_2	8.24±0.33	8.20±0.41	7.93±0.40	7.43±0.25	7.12±0.21
	T_3	4.39±0.22	4.84±0.16	4.69±0.14	3.29±0.10	3.53±0.14
Taste	T_0	7.4±0.25	7.39±0.13	7.34±0.22	6.84±0.27	5.79±0.23
	T_1	6.44±0.19	6.39±0.26	6.29±0.25	5.89±0.29	4.79±0.24
	T_2	8.29±0.33	824 ±0.41	8.09±0.40	7.79±0.26	6.89±0.21
	T_3	4.79±0.24	4.74±0.16	4.69±0.14	3.64±0.11	2.44±0.10
Overall acceptability	T_0	7.51±0.26	7.46±0.22	7.41±0.22	6.91±0.28	5.86±0.23
	T_1	6.66±0.20	6.51±0.26	6.36±0.25	5.96±0.30	4.86±0.24
	T_2	8.46±0.34	8.41±0.42	8.16±0.41	7.86±0.27	6.96±0.21
	T_3	4.86±0.24	4.81±0.16	4.66±0.14	3.76±0.23	2.51±0.10

considerably during storage conditions. Most excellent scores were observed for treatment T_2 with 10% of persimmon fruit supplementation. The reduction in values of taste for fruit yoghurt possibly due to the purification of milk protein because of the proteolytic activities of bacteria. The present finding revealed that the addition of persimmon has a highly significant influence ($p < 0.01$) on the overall acceptability of yoghurt due to various concentrations of persimmon during storage. It was noticed from the mean value that the yoghurt sample with 10% fruit pulp has the best overall acceptability than other samples with 5% and 15% persimmon. These current results were following Arslan and Bayrakci (2016) [15] that mean value of yoghurt with persimmon puree with (10, 12%) reduced from 6.30% to 6.00% and 6.10% to 5.50%, while yoghurt with persimmon marmalade with (10, 12%) reduced from 7.60% to 7.30% and 7.70% to 7.80% respectively. The declining trend in the overall acceptability of fruit yoghurt might be due to the low values of colour, aroma, and taste of fruit yoghurt considerably.

4. CONCLUSION

The current study supported the conclusion that supplementation of persimmon *Diospyros kaki* fruit leads to develop enriched yoghurt with the most desired features in comparison with yoghurt prepared without the addition of fruit. It was analyzed from the present study that the syneresis value declined effectively and total phenolic content increased with the increasing amount of persimmon fruit along with treatments. The total solid content also increased significantly with the increased amount of persimmon fruit. Furthermore, the organoleptic characteristics of fruit yoghurt (T_2) prepared with 10% persimmon fruit have gained better acceptability as compared to other fruit yoghurt prepared with 5% and 15% fruit

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

6. REFERENCES

1. S. Sarkar. Innovations in Indian Fermented Milk Products-A Review. *Food Biotechnology.* 22:78-97 (2008).
2. V. Jaglan., A. Ojha., A. Singh, R. Singh, and S. Gaur. Development of novel herb supplemented soymilk fortified fruit based dairy yoghurt. *Journal of Pharmacognosy Phytochemistry*. 7: 1621-1625 (2018).
3. H. Xue., Z. Yang., X. Jing, P. Yu, Y. Zhang, H. Yi, and L. Zhang. Improvement of the Texture of Yogurt by Use of Exopolysaccharide Producing Lactic Acid Bacteria. *BioMedical Research International.* 4: 7-14 (2016).
4. A. Armuzzi., J. Cremonini., F. Bartolozzi, M. Candelli, and O. Jetti. The effect of oral administration of Lactobacillus GG on antibiotic-associated gastrointestinal side effects. Aliment. Pharmacological Ther. 15: 163-169 (2001).
5. H. Kiani., M. E. Mosari., H. Razavi, and E.R. Morris. Effect of Gelatin, alone and in combination with HM- pectin, on the structure and stability of doogh,

a yogurt based Iranian drink. *Food Hydrocolloid.* 24: 74-75 (2010).

6. S. Kauser., A. Saeed., I. Kalim, A. M. Salariya, and M. Iqbal. Studies on the development and nutritional evaluation of apricot based yoghurt. *Pakistan Journal Biochemistry Molecular Biology.* 44:156-159 (2011).
7. P.J.Sanchez-Segarra., M. Garcia-Martinez., M.J. Gordillo- Otero, A. Diaz-Valverde, M.A. Amaro-Lopez, and R. Moreno-Rojas. Influence of the addition of fruit on mineral content of yoghurts: nutritional assessment. *Food Chemistry*. 70: 85-89 (2000).
8. P. Ariaii., M. Mahmoudi, and R. I. Amoli. The production of fruity yoghurt with banana flavor. Food Science and Technology Department of Islamic Azad University. 6: 368-370 (2011).
9. R.Agarwal. probiotics: An emerging food supplement with health benefits. *Food Biotechnology.* 19: 227-246 (2009).
10. A. Livngh, and T. Wadstrom. Lactic acid bacteria as probiotics. *Current Interest in Microbiology.* 7: 73-89 (2006).
11. P. Aswal., A. Shukla, and S. Priyadarshi. Yoghurt: Preparation, characteristics and recent advancements.*Center for Injury Biomechanics Technology Journal of Bio-Protocols.* 1:32-44 (2012).
12. J. N. Khedkar., D. M. Choudhari., B. K. Pawar, and V. S. Kadam. Development of fruit based yoghurt. *Research Journal of Animal Husbandry and Dairy Science.* 6: 72-75 (2015).
13. R. H. Liu. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *Journal of Nutrition*. 134:3479-3485 (2004).
14. M. A. Njoya., C. W. Nain., P. Y. Mahbou, S. D. Mendi, and H. Imele. Physicochemical and sensory properties of pawpaw (*Carica papaya Linn*) flavoured yoghurt. *African Journal of Food,Agriculture, Nutrition and Development*. 17: 23-24 (2017).
15. S. Arslan, and S. Bayrakci. Physicochemical, functional, and sensory properties of yogurts containing persimmon. *Turkish Journel of Agriculture*. 40:68-74 (2016).
16. S. Karaman., O. S. Toker., M. Çam, M. Hayta, M. Doğan, and A. Kayacier. Bioactive and physicochemical properties of persimmon as affected by drying methods. *Drying Technology*. 32: 258-267 (2014).
17. S. Ercisli., M. Akbulut., O. Ozdemir, M. Sengul, and E. Orhan. Phenolic and antioxidant diversity among persimmon (*Diospyrus kaki* L.) genotypes in Turkey. *International Journal of Food Science and Nutrition*. 59: 77-82 (2007).
18. S. B. Masood., M.T. Sultan., M. Aziza, A. Nazc†, W. Ahmeda, N. Kumard, and M. Imrane. Persimmon (*diospyros kaki*) fruit: hidden phytochemicals and health claims. *Experimental Clinic Journal.* 14:542-561 (2015).
19. S. Yaqub., U. Farooq., A. Shafi, K. Akram, M. A. Murtaza, T. Kausar, and F. Siddique. Chemistry and functionality of bioactive compounds present in persimmon. *Journal of Chemistry.* 65: 19-26 (2016).
20. T. Getenesh., A. Keba., Z. Assefa, B. Agza, and F. Kassa. Development of Fruit Flavored Yoghurt with Mango (*Mangifera indica* L.) and Papaya (*Carica papaya* L.) Fruits Juices. *Food Science and Quality Management.* 67: 6-9 (2017).
21. P. Nazni, and K. Komathi. Quality characteristics and acceptability of papaya pulp incorporated yoghurt. *International Journal of Food and Nutrition Science.* 3: 76–78 (2014).
22. S. T. S. K.Warakaulle., W. A. D. V. Weerathilake, and N. R. Abeynayake. Production and evaluation of set type yoghurt incorporated with watermelon (*Citralluslanatus*). *International Journal of Scientific and Research Publications.* 4: 1-4 (2014).
23. S. Arslan, and S.Ozel. Some properties of stirred yoghurt made with processed grape seed powder, carrot juice or a mixture of grape seed powder and carrot juice. *Milchwissenschaft.* 67:281–285 (2012).
24. K. Singh., R. Chandra, and S. Shukla. Studies on chemical properties of low-fat frozen yoghurt prepared with mixing of varying pulp level of different fruits. *The Pharmacological Innovation*. 5: 6-12 (2016).
25. A. M. Farahat, and O. I. El-Batawy. Proteolytic activity and some properties of stirried fruit yoghurt made using some fruits containing proteolytic enzymes. *World Journal of Dairy & Food Sciences.* 8: 38-44 (2013).
26. AOAC. Official Methods of Analysis. The Association of Official Analytical Chemists. 18th Ed. Arlington, VA, USA. 1-48 (2006).
27. D. C. Montgomery. Design and Analysis of Experiments. 7th Ed. John Wiley and Sons. Inc. Hoboken, NJ, USA. 162-264 (2008).
28. T. Amatayakul., F. Sherkat, and N. P. Shah. Syneresis in set yogurt as affected by EPS starter cultures and levels of solids. *International Journal of Dairy Technology*. 59: 216-221 (2006).

29. E. A. Guz., E. G. Novitskaya., T.K. Kalenik, L.V. Levochkina, and W. Piekoszewski. The influence of vegetable puree containing carotenoids on the nutrient composition and structure of milk yoghurt. *International Journal of Dairy Technology*. 70:1-7 (2017).
30. M. C. Meilgaard., G. V. Civile, and B. T. Carr. Sensory evaluation techniques. 4th Ed. CRC Press. Boca Raton, FL, USA. 1-464 (2007).
31. I. T. Khan., M. Nadeem., M. Imran, M. Asif, M. K. Khan, A. Din, and R. Ullah. Triglyceride, fatty acid profile and antioxidant characteristics of low melting point fractions of Buffalo Milk fat. *Lipids in health and disease*. 18:1-11 (2019).
32. V. Thakore, and N. K. Jain. Protein and fat examination from the raw milk of different mammalian species (Cow, buffalo, goat, and human) with successive lactation days. P*harmacology Innovation*. 7: 506-510 (2018).
33. X. Han., F. L. Lee., L. Zhang, and M. R. Guo. Chemical composition of water buffalo milk and its low-fat symbiotic yogurt development. *Functional Foods in Health and Disease,* 2: 86-106 (2012).
34. F. N. Talpur., N. N. Memon, and M.I. Bhanger. Comparison of fatty acid and cholesterol content of pakistan water buffalo breeds. *Pakistan Journal of Analytical and Environmental Chemistry.* 8: 15-20 (2007).
35. S. Ahmad., F. M. Anjum., N. Huma, A. Sameen, and T. Zahoor. Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. *Journal of Animal Plant Science*. 23:62-74 (2013).
36. C. D. Khedkar, S. D. Kalyankar and, S. S. Deosarkar. Buffalo milk. In: B. Caballero, P. Finglas, and F. Toldra, (Eds). The Encyclopedia of Food and Health. 1: 522-528 (2016).
37. S. Sakanaka, Y. Tachibana, and Y. Okada. Preparation and antioxidant properties of extract of Japanese persimmon leaf tea (kakinoha-cha). *Food Chemistry*. 89:569-575 (2005).
38. J. H. Lee, Y. B. Lee, W. D. Seo, S. T. Kang, J. W. Lim, and K. M. cho. Comparative studies of antioxidant activities and nutritional condtituents of persimmon juice (*Diospyros kaki*). *Preventive Nutrition and Food Science.* 17:141 (2012).
39. A. Celik, and S. Ercisli. Persimmon cv. Hachiya (*Diospyros kaki Thunb*.) fruit: some physical, chemical and nutrional properties. I*nternational Journal Food Science Nutrition.* 59: 599-606 (2008).
40. E. E. Candir, A. E. Özdemir, and M. Kaplankýran. Physicochemical changes during growth of persimmon fruits in the East Mediterranean climate region. *Science Horticulture*. 121: 42-48 (2009).
41. X. N. Chen., J. F. Fan., X. Yue, X. R. Wu, and L. T. Li. Radical scavenging activity and phenolic compounds in persimmon (*Diospyros kaki* L. cv. Mopan). *Journal of Food Science.* 73:24-28 (2008).
42. T. Anuyahong., C. Chusak,and S. Adisakwattana. Incorporation of anthocyanin-rich riceberry rice in yogurts: Effect on physicochemical properties, antioxidant activity and in vitro gastrointestinal digestion. *Lwt—food Science and Technology.* 129: 109571 (2020).
43. D. F.da Silva., N. N. T.Junior., R. G. Gomes, M. S.dos Santos Pozza, M.Britten, and P. T. Matumoto-Pintro. Physical, microbiological and rheological properties of probiotic yogurt supplemented with grape extract. *Journal of Food Science & Technology.* 54: 1608– 1615(2017).
44. D. Liu, and X. X. Lv. Effect of blueberry flower pulp on sensory, physicochemical properties, lactic acid bacteria, and antioxidant activity of set-type yogurt during refrigeration. *Journal of Food Processing and Preservation.* 43:13856(2019).
45. Ö. Dönmez., B. A.Mogol,and V.Gökmen. Syneresis and rheological behaviors of set yogurt containing green tea and green coffee powders. *Journal of Dairy Science.* 100: 901– 907(2017).
46. M. P. N. Nguyen. Physicochemical characteristics, viability of starters, total phenolics and antioxidant activities of functional yoghurt supplemented with extracts from Hylocereuspolyrhizus, Hibiscus sabdariffa and Peristrophebivalvis. *Plant Science Today.* 8:149-154 (2021).
47. Codex Alimentarius. *Codex standard for fermented milks (CODEX STAN 243–2003). Adopted in 2003, Revision* 2008. FAO (2010).
48. E. P. Madora., T. K. Takalani, and M. E. Mashau. Physico-chemical, microbiological and sensory properties of low fat yoghurt fortified with carrot powder. *International Journal of Agriculture and Biological Engineering.* 9: 118-124 (2016).
49. A. Petridis., D. Petridis, and G.Dimitreli. A novel dairy fermented frozen dessert with honey and pomegranate juice: physicochemical, rheological and sensory properties. *Journal of Food Research.* 9:52-66 (2020).
50. G. Teshome., A. Kebal., Z. Assefa, B. Agza, and F. Kassal. Development of fruit flavored yoghurt with

mango (*Mangifera indica* L.) and papaya (*Carica papaya* L.) fruits juices. *Food Science and Quality Management.* 67: 2224-6088 (2017).

51. D. K. D. Roy., T. Saha., M. Akter, M. Hosain, H. Khatun, and M.C. Roy. Quality Evaluation of Yogurt Supplemented with Fruit Pulp (Banana, Papaya, and Water Melon. *International Journal of Nutrition and Food Sciences*. 4:695-699 (2015).
52. S. C. S. R. de Moura., F. Z. Vissotto., S. A.G. Berbari, E. D. C. G. Souza, F. G. P. Toti, and P. A. Júnior. Stability of bioactive compounds in fruit smoothies. (2021).
53. A. Buchilina, and K. Aryana. Physicochemical and microbiological characteristics of camel milk yogurt as influenced by monk fruit sweetener. *Journal of Dairy Science.* 104:1484-1493 (2021).
54. Y. HHui., L. M. L. Nollet., F.Toldrá, S.Benjakul, and G. Paliyath. In B. K. Simpson (Ed.). *Food biochemistry and food processing. John Wiley & Sons Inc.* (2012).
55. K. Kailasapathy. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. *LWT-Food Science and Technology*. 39: 1221– 1227 (2006).
56. M. Michael., R.K. Phebus, and K.A. Schmidt. Plant extract enhances the viability of Lactobacillus delbrueckii subsp. bulgaricus and Lactobacillus acidophilus in probiotic nonfat yogurt. *Food science & nutrition.* 3:48-55 (2015).
57. M. P. N. Nguyen. Physicochemical characteristics, viability of starters, total phenolics and antioxidant activities of functional yoghurt supplemented with extracts from Hylocereuspolyrhizus, Hibiscus sabdariffa and Peristrophebivalvis. *Plant Science Today.* 8:149-154 (2021).
58. W. A. D. C. Gunawardhana, and H. N. N. Dilrukshi. Development of yoghurt drink enriched with avocado pulp (Perseaamericana). *International Journal of Advance Scientific Research and Management.* 1: 97-102 (2016).
59. S. Bakirci., E. Dagdemir., O. S. Boran, and A.A. Hayaloglu. The effect of pumpkin fiber on quality and storage stability of reduced-fat set type yoghurt. *International Journal of Food Science and Technology.* 52: 180-187 (2017).
60. E. Ismail., M. Shenana., M. Elalfy, E. Essawy, and S. Abdelhahim.. Novel Probiotic Adjunct Cultures for the Production of Fruit-Flavoured Drinkable Yoghurt. *Egyptian Journal of Food Science.* 48: 213-228 (2020).
61. O. M. Mohamed., M.O. Abdallai., N.M. Fawi, S.O. Ahmed, E.G. Mohamed, and G.E. Ahmed. Quality evaluation of stirred yoghurt flavoured with Guddaim (*Grewia tenax*) fruit. A*sian Journal of Agriculture and Food Science.* 3: 27-33 (2017).
62. A. Brodziak., J. Król., A. Matwijczuk, T. Czernecki, P. Glibowski, Ł. Wlazło, and A. Litwińczuk. Effect of Sea Buckthorn (*Hippophaerhamnoides* L.) Mousse on Properties of Probiotic Yoghurt. *Applied Sciences.* 11:545 (2021).
63. I. E. Mbaeyi-Nwaoha., L. C. Umeh., C. J. Igbokwe, C. M. Obodoechi, and N. C. Okoronkwo. Production and quality evaluation of flavoured yoghurt from graded levels of sweet variety of African bush mango "Ugiri" (Irvingiagabonensis) juice and pulp. *Food Science and Technology.* 5: 56-69 (2017).
64. M. M. Desouky. Effect of using cactus pear pulp on the properties of goats' milk bioyoghurt drinks. *Egypt. Journal of Food Science*. 46: 25-41 (2018).
65. N. I. E. Mbaeyi., L. C. Umeh., C. J. Igbokwe, C. M. Obodoechi, and N. C. Okoronkwo. Production and quality evaluation of flavoured yoghurt from graded levels of sweet variety of african bush mango" Ugiri"(Irvingiagabonensis) juice and pulp. *Food Science and Technology.* 5: 56-69 (2017).
66. K. C. Dey., R. Begum., M.R.T. Rahman, A. Sultana, S. Akter, and R.J. Janny. Development of fruit juice yoghurt by utilization of jackfruit juice: A preliminary study on sensory evaluation, chemical composition, and microbial analysis. *International Journal of Engineering Research and Technology.*3: 1074-1079 (2014).
67. V. Raut., P. Sawant., D. Sawant, and A.S. Ingole. Studies on preparation of mango yoghurt drink. *Asian Journal of Dairy and Food Research*.34: 13-17 (2015).
68. A. A. Matter., E. A. M. Mahmoud, and N. S. Zidan. Fruit flavored yoghurt: chemical, functional and rheological properties. *International Journal Environment and Agriculture Research.*2: 57-66 (2016).
69. S. Sengupta., A. Chakraborty, and J. Bhowal. Production and evaluation of yoghurt with watermelon (Citrulluslanatus) juice. *Journal of International Academic Research. Multidisciplinary,* 2: 249-257 (2014).
70. A. Palka, and A. Flis-Kaczykowska. Evaluation of extract and fat content in selected fruit and fruit-vegetable yogurts (available on the market). *Scientific Journal of Gdynia Maritime University* (2019).
71. S. Subhashini., D. Baskaran., B. Dhanalakshmi, B.

Murugan, and A.P. Manoharan. Physicochemical properties of grape (VitisviniferaL.) pomace fortified drinkable yoghurt. *International Journal Current Microbiology Applied Science.* 7:2875-2880 (2018).

72. B. A. Öztürk, and M. D. Öner. Production and evaluation of yogurt with concentrated grape juice. *Journal of Food Science.* 64: 530-532 (1999).
73. A. Ayla., A. S. Mehmet, and B. Ihsan. The effects of adding *Hibiscussabdariffa* L. flowers marmalade on some quality properties,mineral content and antioxidant activities of yogurt. *Journal of Food Science Technology*.5:1-20 (2020).
74. D. Dimitra., S. Nikoletta., K. Evangelos, and K. Panagiotis. Yoghurtssupplemented with juices from grapes and berries. *Foods*.9:1158 (2020).
75. A. Amal., A. M. Eman, and S. Z. Nahla. Fruit flavored yoghurt. Chemical, functional and rheological properties. *International Journal of Environmental and Agriculture Research.* 2: 57-66 (2016).
76. E. A. M. Ahmed. Production of functional drinking yoghurt supplemented with strawberry and berry fruits. *Journal of Food and Dairy Science. Mansoura University.*8: 263-266 (2017).
77. M. M. Ismail., M.F. Hamad, and E.M. Elraghy. Rheological, physicochemical, microbial, and sensory properties of bio-rayeb milk fortified with pulp. *International Journal of Food Science and Biotechnology.*1: 8-18(2016)
78. M. N. A. Hammad. Studies on producing some therapeutic dairy products. Ph.D. Thesis, Fac. Agric., Moshtohor, Benha University Egypt (2019).
79. G. Ritu., H. A. P. K. Hai, and K. S. Naveen. Development and Quality Evaluation of Yoghurt Fortified with Pineapple, Apple and Sweet Lemon Juice (Fruit Yoghurt). *International Journal of Engineering and Research Technology.* 5: 2-5 (2016).
80. E. K. Yagmur., V.Y. Yil., I. Itlun. Determination of Texture Profile Analysis of Yogurt Produced by Industrial and Traditional Method. *International Journal of Scientific and Technological Research.* 4: 2422-8702 (2018).
81. S. J. Chye., R. Ahmad, and A. A. Noor Aziah.Studies on the physicochemical and sensory characteristics of goat's milk dadih incorporated with tropical-fruit purees. *International Food Research Journal.* 19:1387-1392 (2012).
82. S. S. Mohammad., A. M. Sani., M. Hojjatoleslamy. Physicochemical, microbiological and sensory characteristics of strawberry yoghurt fortified with Red beet extract. *Indian Journal.* 9: 187-193 (2013).

Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 58(1): 129 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X

Pakistan Academy of Sciences

Obituary

Prof. Dr. Syed Irtifaq Ali (1930-2021)

Prof. Dr. Syed Irtifaq Ali was a renowned botanist, Fellow PAS, former Vice Chancellor of Karachi University (KU), and Professor Emeritus. Prof. Ali passed away on February 18, 2021, after a brief illness. He was born in Lucknow, India, in 1930, completed his matriculation from Allahabad in 1946. In 1950, he received his Bachelor of Science degree and Master's degree in botany in 1952 from Allahabad University and later migrated to Pakistan. He completed his Ph.D. in botany in 1958 and DSc in 1979 from the University of London.

Dr. Irtifaq Ali also worked as a Professor of botany at the University of Tripoli, Libya from 1972 to 1976. He was also the pioneer and co-editor of Flora of Libya. The distinguished professor joined KU in 1958 as a lecturer at its botany department and later served as its chairman. He continued to work at KU's Plant Conser¬vation Centre till his death. The professor also served as the Director General of Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering and the dean of KU's science faculty (1981 to 1990).

He was a renowned top-ranking (plant) taxonomist in the region and considered an authority on the plants of Southeast Asia in general and Pakistan in particular. One of Prof Ali's greatest contributions as a researcher was The Flora of Pakistan — the most comprehensive and authentic scientific work ever on Pakistan's flora. Initiated over four decades ago and completed in 2020, the book has 224 volumes containing details of over 6,000 indigenous and commonly cultivated plants. He has contributed to the field of plant sciences at the national and international level and provided basic information on the plant wealth of Pakistan, which is widely used by phytochemists, pharmacists, ecologists, agriculturists, and foresters.

For his contribution and distinguished career, he was conferred with Sitara-e-Imtiaz in 1988 and honored with the Emeritus status in 2011. He was also awarded the title of the Higher Education Commission Distinguished National Professor in 2004. He will be missed by the PAS Fellows.

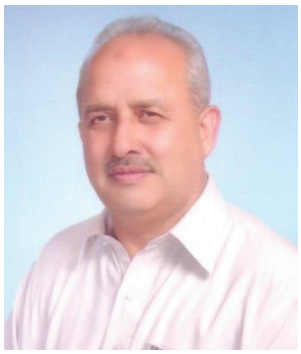
May the departed soul rest in eternal peace. Aameen!

Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 58(1): 131-132 (2021)
Copyright © Pakistan Academy of Sciences
ISSN (Print): 2518-4261; ISSN (Online): 2518-427X

Pakistan Academy of Sciences

Obituary

Prof. Dr. Habib Ahmad
(1959-2021)



The remarkable Prof. Dr. Habib Ahmad left this mortal world on 7th April 2021 for his journey to the hereafter. He was an Eminent Scientist, Distinct Teacher, great Cyto-geneticist, eminent Plant Taxonomist, Fellow of the Pakistan Academy of Sciences, and above all a best Human Being. He was serving as Professor Emeritus of Genetics in the Department of Genetics, Hazara University Mansehra Pakistan. He was born on 13th July 1959 in Matta Swat in the home of his father Muhammad Saeed (known as Serae Mulvi Seb)– the first teacher of the Swat State and a pronounced philanthropist and educationist in the era of Swat State. He passed his SSC & FSc from Matta Swat and BSc from the historical Post Graduate Jahanzeb College Saidu Sharif Swat in 1982. He got his MSc and MPhil degrees from the Department of Botany, University of Peshawar in the years 1985 and 1991, respectively. He did his Ph.D. from the University of the Punjab Lahore in 2003. He remained on various positions including Scientific Officer, Cytogenetics Program NARC, Islamabad(1986 - 1990), Lecturer Botany Higher Education Department, Khyber Pakhtunkhwa (1990 - 2002), Technical Coordinator WWF-Pakistan (2002 - 2005), Professor of Botany, Hazara University (2005 - 2010), Tenured Professor of Genetics, Hazara University (2010 - 2019), and Professor Emeritus of Genetics, Hazara University (2019 - 2021). He also remained Vice Chancellor, Islamia College Peshawar (2016 to 2020), Vice Chancellor, Hazara University (2015 - 2016), Dean of Sciences Hazara University (2006 - 2010 & 2013 – 2016), and Chairman Botany & Genetics (2006-2010).

Based on his MPhil and Ph.D. studies and the initial job at NARC, he was able to introduce a Rapeseed Variety 'Hasnain-2013' that was approved by the Seed Council Government of KPK on March 5, 2013. This variety is being sown widely in Pakistan and is one of the popular rapeseed variety in Pakistan. He completed 28 research projects, supervised 47 Ph.D., 148 MPhil & 84 MSc/BS students, published 325 journal research articles, 51 Book chapters/monographs & more than 100 abstracts, and explored 35 New Genes/ Nucleotide Sequences in his career. He visited more than 15 different countries in the world for various academic purposes. One of his collaborative research projects with US researchers 'Ethnogenetic elaboration of NWFP through dental morphology and DNA analysis' gave rise to one of his prominent research articles in the prestigious journal of Science (IF = 41.84) titled "The first horse herders and the impact of early Bronze Age steppe expansions into Asia".

For his splendid research career, he was awarded the Presidential award Tamgha-e-Imtiaz (2010), KP R&D efforts award (2015), PAS Gold Medal (2019), HEC Best Teacher Award (2015), Productive Scientist of Pakistan Award (2011, 2012, 2013, 2014, 2015 & 2016), IBC Cambridge Leading Scientist Award 2011, UNESCO Teacher of the Year Award 2007, and Best Researcher of Hazara University Award 2006. He remained Fellow of the Pakistan Academy of Sciences, President Pakistan Botanical Society, President Society Conservation Biology of Pakistan, Chief Patron Crying Sky Ambassadors Kennesaw State University (CSA-Pakistan Chapter), Honorary Fellow Zoological Society of Pakistan, member of many national as well as international scientific societies including International Association for Plant Biotechnology (UK), Pak-US Working Group for Science and Technology (Biology/Biotech), Biotech Advisory Committee, DoST Khyber Pakhtunkhwa, National Core Group in Genetics, Government of Pakistan, National Testing Services, Government of Pakistan, Member: Board of Directors Society of

Conservation Biology (USA), Genetics Society of America (USA), International Association of Ecology (USA), Society of Conservation Biology (Asian Group), Society of Conservation Biology (North America), Genetics Society of Pakistan, Ethnobotanical Association of Pakistan, Pakistan Botanical Society, Weed Science Society of Pakistan, Environmental Protection Society Swat, Board of Advisors Ethnobotany Project, WWF.

People like Prof. Habib Ahmad him born once in centuries. It will not be an exaggeration if we claim him as one of the perfect human beings. He was candlelight for many in the field of education and research, especially, the deprived masses of society. We are sure that all his students and followers will take his noble cause ahead in society to educate people and work for the welfare of people.

We pray to the almighty ALLAH that best blessings may shower upon his gallant soul and may he is in the eternal Peace of the best of paradises. Aameen summa Aameen!

Khan Bahadar Marwat (PhD)
Fellow, Pakistan Academy of Sciences

Shujaul Mulk Khan (PhD)
Quaid-i-Azam University Islamabad
Member Pakistan Academy of Sciences

Instructions for Authors

Manuscript Format

The manuscript may contain Abstract, Keywords, INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION (or RESULTS AND DISCUSSION), CONCLUSIONS, ACKNOWLEDGEMENTS, CONFLICT OF INTEREST and REFERENCES, *and any other information that the author(s) may consider necessary*.

Abstract (font size 10; max 250 words)**:** Must be self-explanatory**,** stating the rationale, objective(s), methodology, main results, and conclusions of the study. Abbreviations, if used, must be defined on the first mention in the Abstract as well as in the main text. Abstract of review articles may have a variable format.

Keywords (font size 10)**:** Three to eight keywords**,** depicting the article.

INTRODUCTION: Provide a clear and concise statement of the problem, citing relevant recent literature, and objectives of the investigation.

MATERIALS AND METHODS: Provide an adequate account of the procedures or experimental details, including statistical tests (if any), concisely but sufficient enough to replicate the study.

RESULTS: Be clear and concise with the help of appropriate Tables, Figures, and other illustrations. Data should not be repeated in Tables and Figures, but must be supported with statistics.

DISCUSSION: Provide interpretation of the RESULTS in the light of previous relevant studies, citing published references.

ACKNOWLEDGEMENTS: (font size 10)**:** In a brief statement, acknowledge the financial support and other assistance.

CONFLICT OF INTEREST: State if there is any conflict of interest.

REFERENCES (font size 10)**:** Cite references in the text **by number only** in **square brackets**, e.g. "Brown et al [2] reported ..." or "... as previously described [3, 6–8]", and list them in the REFERENCES section, in the order of citation in the text, Tables and Figures (not alphabetically). Only published (and accepted for publication) journal articles, books, and book chapters qualify for REFERENCES.

Declaration: Provide a declaration that: (i) the results are original; (ii) the same material is neither published nor under consideration elsewhere; (iii) approval of all authors have been obtained; and (iv) in case the article is accepted for publication, its copyright will be assigned to *Pakistan Academy of Sciences*. Authors must obtain permission to reproduce, where needed, copyrighted material from other sources and ensure that no copyrights are infringed upon.

Manuscript Formatting

Manuscripts must be submitted in Microsoft Word (2007 Version .doc or .docx format); **pdf** files not acceptable. Figures can be submitted in Word format, TIFF, GIF, JPEG, EPS, PPT. Manuscripts, in *Times New Roman*, 1.15spaced (but use single-space for Tables, long headings, and long captions of tables & figures). The text must be typed in a double-column across the paper width. The Manuscript sections must be numbered, i.e., **1. INTRODUCTION, 2. MATERIALS AND METHODS,** and so on… (a) **Title** of the article **(**Capitalize initial letter of each main word; font-size 16; **bold**), max 160 characters (no abbreviations or acronyms), depicting article's contents; (b) Author' first name, middle initial, and last name (font size 12, **bold**), and professional affiliation (i.e., each author's Department, Institution, Mailing address and Email; but no position titles) (font size 12); (c) Indicate the corresponding author with *; (d) **Short running title**, max 50 characters (font size 10).

Headings and Subheadings (font size 11): All flush left

LEVEL-1: **ALL CAPITAL LETTERS; Bold**

Level-2: **Capitalize Each Main Word (**Except prepositions**); Bold**

Level-3: Capitalize each main word (Except prepositions)***; Bold, Italic***

Level-4: *Run-in head*; *Italics*, *in the normal paragraph position. Capitalize the initial word only and end in a colon (i.e., :)*

List of REFERENCES must be prepared as under:

a. Journal Articles *(Name of journals must be stated in full)*

1. I. Golding, J. Paulsson, S.M. Zawilski, and E.C. Cox. Real time kinetics of gene activity in individual bacteria. *Cell* 123: 1025–1036 (2005).
2. W. Bialek, and S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100: 258–263 (2008).
3. R.K. Robert, and C.R.L.Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1(6) (2009).
4. D. Fravel. Commercialization and implementation of biocontrol. *Annual Reviews of Phytopathology* 43: 337359 (2005).

b. Books

5. W.R. Luellen. Fine-Tuning Your Writing. *Wise Owl Publishing Company, Madison, WI, USA* (2001).
6. U. Alon, and D.N. Wegner (Ed.). An Introduction to Systems Biology: Design Principles of Biological Circuits. *Chapman & Hall/CRC, Boca Raton, FL, USA* (2006).

c. Book Chapters

7. M.S. Sarnthein, and J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: The Northern North Atlantic: A Changing Environment. P.R. Schafer, & W. Schluter (Ed.), *Springer, Berlin, Germany*, pp. 365–410 (2000).
8. J.E. Smolen, and L.A. Boxer. Functions of Europhiles. In: Hematology, 4th ed. W.J. Williams., E. Butler and M.A. Litchman (Ed.), *McGraw Hill, New York, USA*, pp. 103–101 (1991).

d. Reports

9. M.D. Sobsey, and F.K. Pfaender. Evaluation of the H2S method for Detection of Fecal Contamination of Drinking Water, Report WHO/SDE/WSH/02.08, *Water Sanitation and Health Programme, WHO, Geneva, Switzerland* (2002).

e. Online references

These should specify the full URL for reference and give the date on which it was consulted. Please check again to confirm that the work you are citing is still accessible:

10. L. Branston. SENSPOL: Sensors for Monitoring Water Pollution from Contaminated Land, Landfills and Sediment (2000). http://www.cranfield.ac.uk/biotech/senspol/ (accessed 22 July 2005)

Tables and Figures

Insert all tables as editable text, not as images. Number tables consecutively following their appearance in the text, Figures should appear in numerical order, be described in the body of the text, and be positioned close to where they are first cited. Each figure should have a caption that describes the illustration, and that can be understood independently of the main text (Caption Table 1. and Fig 1. font size 10; Bold; Captions should be in sentence case; left-aligned). All Figures should have sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher) to enhance the readability. Figures may be printed in two sizes: column width of 8.0 cm or page width of 16.5 cm; number them as **Fig. 1**, **Fig. 2**, … in the order of citation in the text. Parts in a figure can be identified by A, B, C, D, ... and cited as Figure 2A, Figure 2B, Figure 2C. Captions to Figures must be concise but self-explanatory. Laser printed line drawings are acceptable. Do not use lettering smaller than 9 points or unnecessarily large. Photographs must be of high quality. A scale bar should be provided on all photomicrographs.

Tables: with concise but self-explanatory headings must be numbered according to the order of citation (like **Table 1.**, **Table 2.**). *Do not* abbreviate the word "Table" to "Tab.". Round off data to the nearest three significant digits. Provide essential explanatory footnotes, with superscript letters or symbols keyed to the data. Do not use vertical or horizontal lines, except for separating column heads from the data and at end of the Table.

Figures: Figures may be printed in two sizes: column width of 8.0 cm or page width of 16.5 cm; number them as **Fig. 1**, **Fig. 2**, … in the order of citation in the text. Captions to Figures must be concise but self-explanatory. Laser printed line drawings are acceptable. Do not use lettering smaller than 9 points or unnecessarily large. Photographs must be of high quality. A scale bar should be provided on all photomicrographs.

Note: The template of the manuscript is available at http://www.paspk.org/proceedings/; http://ppaspk.org/

Reviewers: Authors may suggest four relevant reviewers, two National and two International (with their **institutional E-mail** addresses).

SUBMISSION CHECKLIST

The following list will be useful during the final checking of an article before sending it to the journal for review.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address (Correct and valid)

• Full address of Institute/organization

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for the use of copyrighted material from other sources (including the Internet)

In case of any difficulty while submitting your manuscript, please get in touch with:

Editor
Pakistan Academy of Sciences
3-Constitution Avenue, Sector G-5/2
Islamabad, Pakistan
Email: editor@paspk.org
Tel: +92-51-920 7140
Websites: http://www.paspk.org/proceedings/; http://ppaspk.org/

PROCEEDINGS
OF THE PAKISTAN ACADEMY OF SCIENCES:
B. Life and Environmental Sciences

CONTENTS

Volume 58, No. 1, March 2021 **Page**

Research Articles

PAKISTAN ACADEMY OF SCIENCES, ISLAMABAD, PAKISTAN

HEC Recognized, Category Y

Websites: http://www.paspk.org/proceedings/; http://ppaspk.org