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Pakistan Academy of Sciences

Review Article

Bioactive Compounds via *in vitro* Culture Approach and Pharmacological Attributes of Genus *Euphorbia*: A Comprehensive Review

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Abstract: The family *Euphorbiaceae* comprises 2000 species and is listed as third among the largest flowering family. *Euphorbia* is used in traditional treatment for various diseases, including dengue fever, dysentery, diarrhea, and anemia. This review aims to collect data from various published literature sources for quick and effective cultivation of *Euphorbia* species through tissue culture and documentation of potent secondary metabolites obtained from different cultures of *Euphorbia* and its manifold pharmacological activities from various parts extracts. The data for this review were systematically collected from different scientific databases, including Google Scholar, Science Direct, PubMed, and published literature. Different secondary metabolites have been reported from the *in votro* culture of Euphorbia containing anthocyanin, saponins, tannins, sterols, flavonoids, glycosides, diterpenes, and sesquiterpene. The essential oils from extractions of the *Euphorbia* genus embraced about 80 active phytochemical constituents. The extracts and compounds exhibited different pharmacological activities, including hepato-protective, anti-fungal, anti-bacterial, and anti-cancer. Besides the pharmaceutical and importance of the genus *Euphorbia,* this report also described the methodologies of explant cultures, *in-vitro* production of biologically active compounds, and vital phytochemicals extraction from various parts of *Euphorbia*. Therefore, there is great attention for *in-vivo* studies on *Euphorbia* to further investigate and confirm their therapeutic effects for safe and effective medical use. The *in-vitro* cultivation technique needs further development, either in bioreactors or temporary immersions and shakes flasks to obtain vigorous sprouts of *Euphorbia*.

Keywords: Biological Activities, Bioactive Compounds, Pharmacological Relevance, *Euphorbia*, Essential Phytoconstituents, Disease Treated.

1. INTRODUCTION

Family *Euphorbiaceae* contains herbs, shrubs, trees, and succulent plants; both wild and cultivated plants of this family are found all over the globe [1]. Most of the species of this family chiefly existed in tropical regions but also predominantly extended into temperate zones. Most species of the family *Euphorbiaceae* produce milky latex; reported as poisonous in several species (*Euphorbia tirucalli, Euphorbia royleana, Euphorbia lathyris, Euphorbia esula, Euphorbia conitofolia Euphorbia milii, Euphorbia hirta*, *Euphorbia neriifolia*, and *Euphorbia heliscopia*) as described previously

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[2-6]. In Pakistan, the family *Euphorbiaceae* is comprised of 24 genera and 90 species. Besides this, extracts obtained from various parts of the genus *Euphorbia* proved their efficiency in relieving constipation and other gastrointestinal disorders. Moreover, its cytotoxic property bears antineoplastic compounds in the forthcoming time [7]. The traditional uses in medicine and industry of the latex obtained from *Euphorbia* species are well-known. Different members of this family are extensively used for aesthetic and medicinal purposes and cultivation has significant economic importance [8].

Euphorbia hirta has been investigated with diverse medicinal properties; conventionally, used for curing different ailments like dengue fever, diarrhea, dysentery, ulcer, asthma, bronchitis, etc. The latex obtained from *E. hirta* is extensively used in treating jaundice, anemia, and skin disorders. Similar antipyretic potential appeared in *Euphorbia neriifolia* [9]. Moreover, Awaad *et al*. [8] investigated several pharmacological activities from different parts of *E. hirta* extracts, which include antispasmodic, antifungal, antibacterial, anticatarrhal, diuretic, etc. Folk medicine in Australia utilized the latex obtained from *Euphorbia peplus* for curing keratosis and skin cancer [10, 11]. Recently, it is widely used as an essential part of many natural and medicinal products [12]. A huge number of pharmacological attributes have also been reported from *Euphorbia tirucalli* [13].

Tissue culture could be defined as the sterilized culture of cells, tissues, and organs in a controlled condition. Plant tissue culture is also known as sterile culture, in vitro culture, or axenic culture; an essential and fundamental technique in commercial and applied studies [14, 15]. Plant tissue culture media contain vitamins, micronutrients, macronutrients, and all other essential components required for normal growth and development. The pH recommended for the proper culturing of cells and tissues ranges between 5.3 and 5.8 [16]. Auxin's badly affecting the *in-vitro* morphogenesis of *Euphorbia nivulia*. Similarly, Martin *et al.* [17], studied the effect of auxins and cytokinin on hypocotyltyle culture of *Euphorbia esula*. Plant tissue culture is becoming a pressing need of the hour to conserve endangered species through clonal propagation and production of medicinally important plants on large scale in a controlled condition in a well-defined aseptic way [18, 19]**.** Extensive research has been carried out and still improvements need in the *in-vitro* culture technique in boosting the yield of secondary metabolites. In this literature review, we have discussed the efforts made by different phytochemists, botanists, pharmacologists, biochemical engineers, and tissue culturists for the improvement and establishment of the *in-vitro* cultivation approach of *Euphorbia* species. In a parallel review documented only the biological activity and triterpenoids content of the genus *Euphorbia*; however, no review analyzed the procedures for the preparation of different explant cultures, *in-vitro* production of biologically active compounds from various cultures, and enlisting the detailed pharmacological attributes of genus *Euphorbia* [20].

2. DATA COLLECTION STRATEGY

The information regarding this review was systematically collected from different scientific databases including Google Scholar, Science Direct, PubMed, and published literature. The papers selected from the base were most suitable on keywords: biological activities, pharmacological effects, *Euphorbia*, phytochemicals, and disease treated without time limitation. The 149 publications that were chosen, spanning the years 1978 to 2022, displayed remarkable findings that reflected current scientific trends at the time of publication.

3. PHYTOCHEMISTRY AND PHARMACOLOGY OF GENUS *EUPHORBIA*

Conventionally, the genus *Euphorbia* is used in treating some ailments, owing to its astonishing disease-curing properties. Recently the mesmerizing therapeutic potential of the genus *Euphorbia* has startled researchers, several pure compounds were also isolated from extracts of different plant parts [21]. The phytochemical screening of genus *Euphorbia* showed the presence of essential phytochemicals having robust therapeutic influence, other than its conventional uses it has robust medicinal properties [22], such as anti-arthritis, anti-diarrheal, analgesic, hepato-protective and antipyretics (Table 1 in Annexure I). We provided an overview of various parts of the

species belonging to the genus *Euphorbia* (Figures 1 and 2), reflecting the importance of various parts in context to their pharmacological potential.

Whereas, the applications of whole plants or various plant parts on different animals'/cell lines have been documented (Figure 3). In addition, flavonoids, diterpenoids, triterpenoids, tannins, and polyphenols were also being isolated from some species of *Euphorbia* through phytochemical screening. As far as their biological activities are concerned; phytochemical responses vary greatly, in most cases diterpenoids showed anti-cancer and cytotoxic activity; flavonoids and triterpenoids proved effective in treating inflammation and inhibiting pathogenic activities. Many natural products have been derived from *Euphorbia*; mainly including essential oils, pure compounds, and extracts with promising biological activities. About 80 phytochemicals have been reported from essential oils of *Euphorbia* species and prominent secondary metabolites [23]. Furthermore, *E. hirta* leaves extract confirmed the presence of essential vitamins in sufficient amounts, including vitamin B2, vitamin E, and vitamin C [24].

4. *IN VITRO* TISSUE CULTURE APPROACHES IN GENUS *EUPHORBIA*

Family *Euphorbiaceae* contains a large number of species; including many endangered and endemic species. Though, in vitro cultivation is limited to some specific genera having medicinal, aesthetic, food, rubber, and dye-yielding purposes [25]. *In vitro* culture is a conducive and effective technique for the proliferation and conservation of endangered species in a shorter period, mainly for those plants which are difficult to be grown by using conventional methods of cultivation and conservation [26]. Plants regenerated through *in vitro* culture possess some advantageous features over those cultivated in fields via conventional agricultural practices. For instance, the cultured-grown mountain arnica rhizome has a characteristic smell and taste, lacking in the same plant rhizome cultivated in field conditions. Several active phytochemicals were isolated from different plant parts and propagated via cell, tissue, and hair root culture methods [27]. It is immensely important to select a parent plant with a considerable amount of biologically active phytochemicals for callus formation; likewise, selecting those cell lines with higher yield [28]. Succeeding reports on cell culture, callus culture, shoot, leaf, and root culture, somatic embryogenesis, and nodal/inter nodal culture of genus *Euphorbia* (Table 2 in Annexure II).

5. CALLUS CULTURE OF GENUS *EUPHORBIA*

A callus is a mass of tissues having differentiated cells, developed under the influence of determinate hormonal control described previously [29, 30]. Propagated callus and shoot from stem pieces of *Euphorbia esula* HR lines and shoot regeneration from hypocotyls of non-HR lines. Maximum shoot regeneration was observed by inoculating the explants in a growth medium containing Murashige and Skoog (MS) basal salts, MS + vitamins, 1.11 µM 6-benzylaminopurine, 1.97 µM indole-3-butyric acid, and 3.0 % sucrose, pH 5.6–5.8. After 30 days multiple shoots developed from the stem (Figure 4).

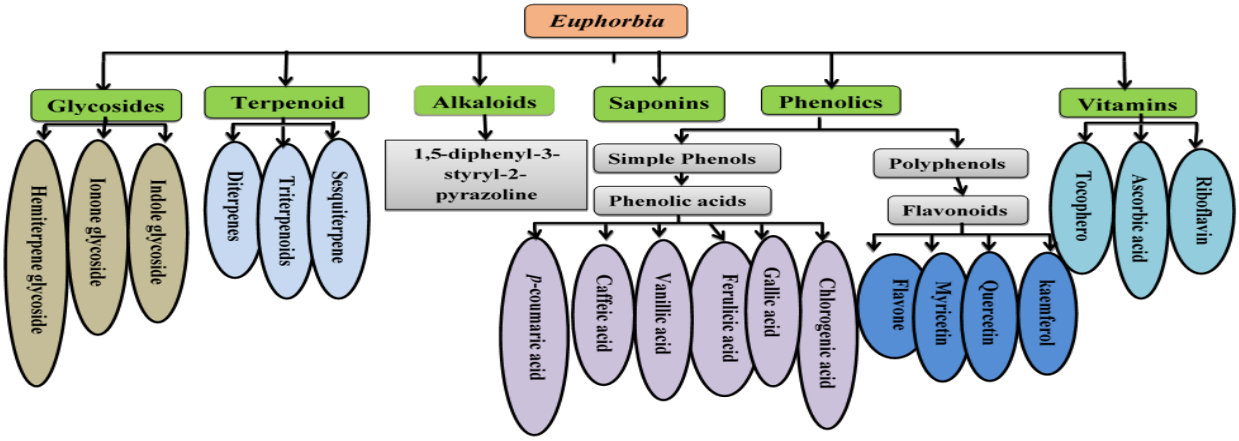


Fig. 1. Major secondary metabolites reported in *Euphorbia* species.

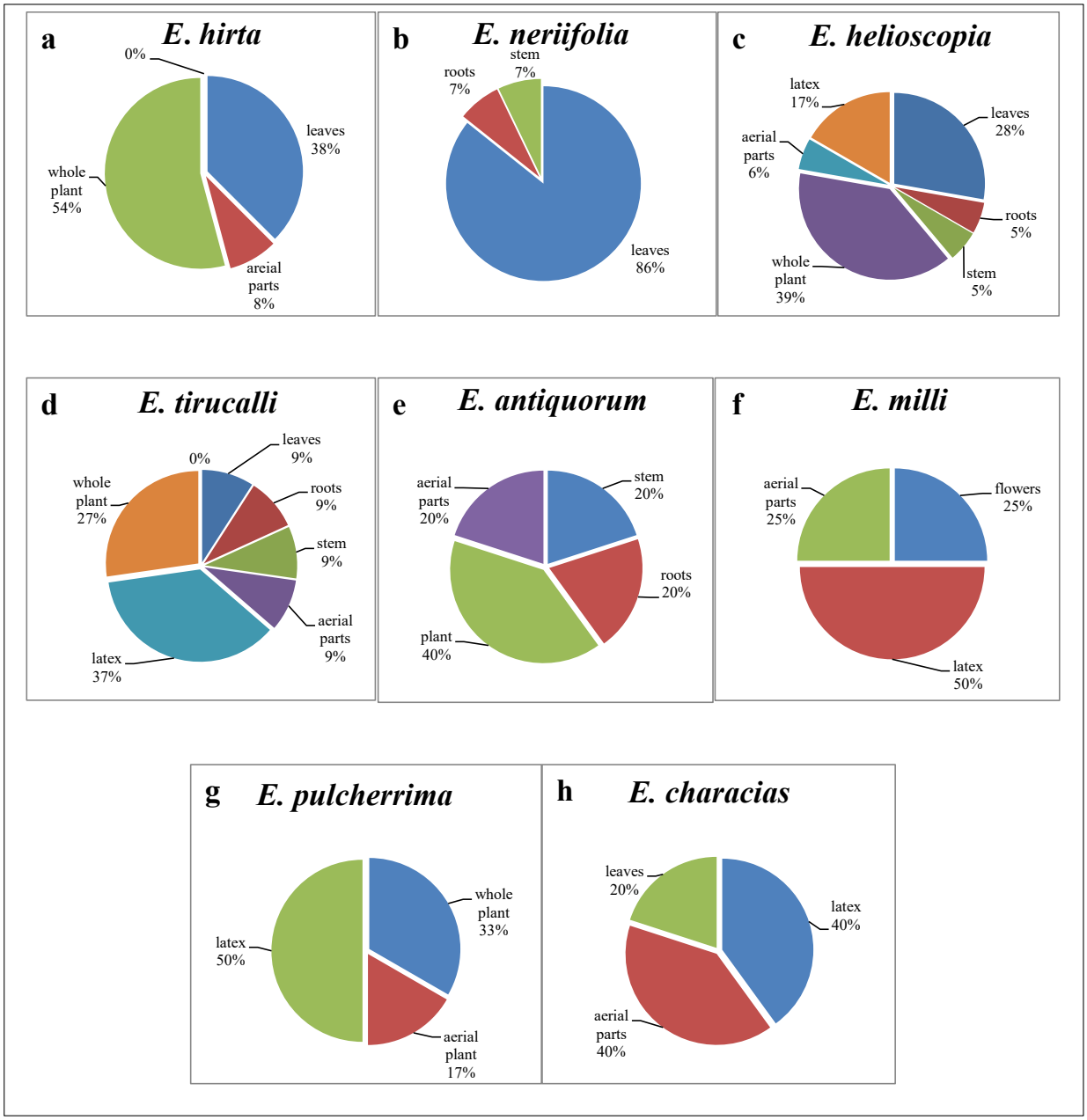
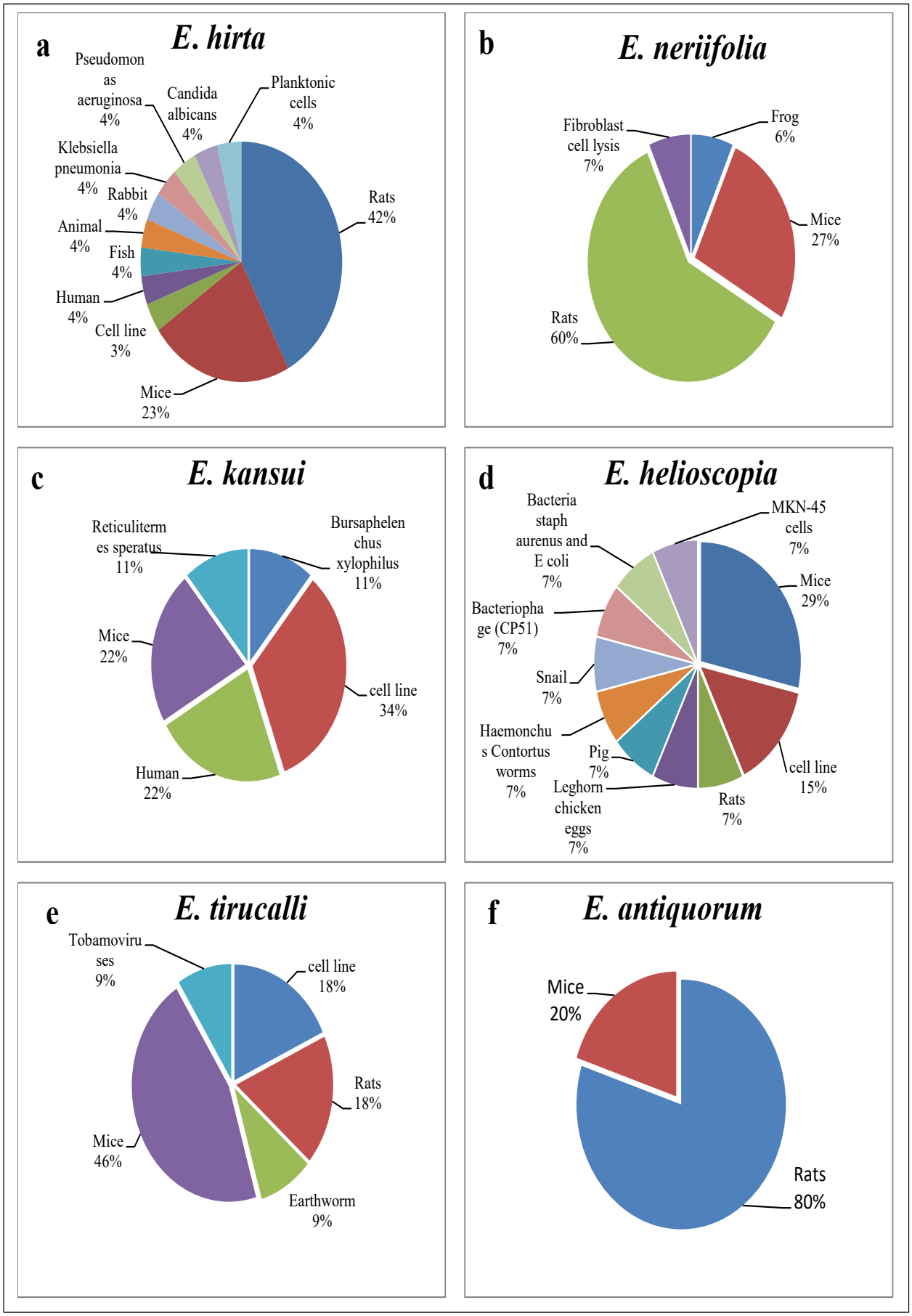


Fig. 2. Percentage of various parts of *Euphorbia* species used in pharmacology (**a**) *Euphorbia hirta* (**b**) *Euphorbia nerrifolia* (**c**) *Euphorbia helioscopia* (**d**) *Euphorbia tirucalli* (**e**) *Euphorbia antiquorum* (**f**) *Euphorbia milli* (**g**) *Euphorbia pulcherrima* (**h**) *Euphorbia characias.*

The callus culture of *Euphorbia hirta* revealed the presence of phenolic and sterol compounds with a substantial amount of chlorogenic acid (79.67 mg/100 g d. m.) syringic acid (32.57 mg/100 g d. m.) and brassicasterol (32.57 mg/100 g d. m.) by Özbilgin et al. [31] and Lone et al. [32]. Euphorbia tirucalli callus culture revealed the presence of euphol, tirucallol, and 4, 4-dimethyl sterols amount [33, 34]. The most prominent secondary metabolites extracted from cultured cells of *Euphorbia* species include cyanidin glycoside from *Euphorbia milli*. Similarly, the sitosterol, palmitic acid, and triterpenoids from cultured cells of *E. esula*, and phytosterol, tirucallol, triterpene, and euphol (Table 3 in Annexure III) extracted from callus cultured cells of *Euphorbia tirucalli* [33, 35]. *Euphorbia characias* callus culture hormonal regulation of triterpinols formation was investigated [36]. Leaf explants of *E. hirta* were cultured on MS+NAA and 6-benzylaminopurine (BAP) medium, at the onset of callus initiation, it was again subcultured on the same media with 1


a
E. hirta
Pseudomonas aeruginosa 4%
Candida albicans 4%
Planktonic cells 4%
Klebsiella pneumonia 4%
Rabbit 4%
Animal 4%
Fish 4%
Human 4%
Cell line 3%
Mice 23%
Rats 42%
b
E. neriifolia
Fibroblast cell lysis 7%
Frog 6%
Mice 27%
Rats 60%
c
E. kansui
Reticuliterm es speratus 11%
Bursaphelen chus xylophilus 11%
Mice 22%
cell line 34%
Human 22%
d
E. helioscopia
Bacteria staph aurenus and E coli 7%
MKN-45 cells 7%
Mice 29%
Bacteriopha ge (CP51) 7%
Snail 7%
Haemonchu s Contortus worms 7%
Pig 7%
Leghorn chicken eggs 7%
Rats 7%
cell line 15%
e
E. tirucalli
Tobamoviru ses 9%
cell line 18%
Rats 18%
Mice 46%
Earthworm 9%
f
E. antiquorum
Mice 20%
Rats 80%

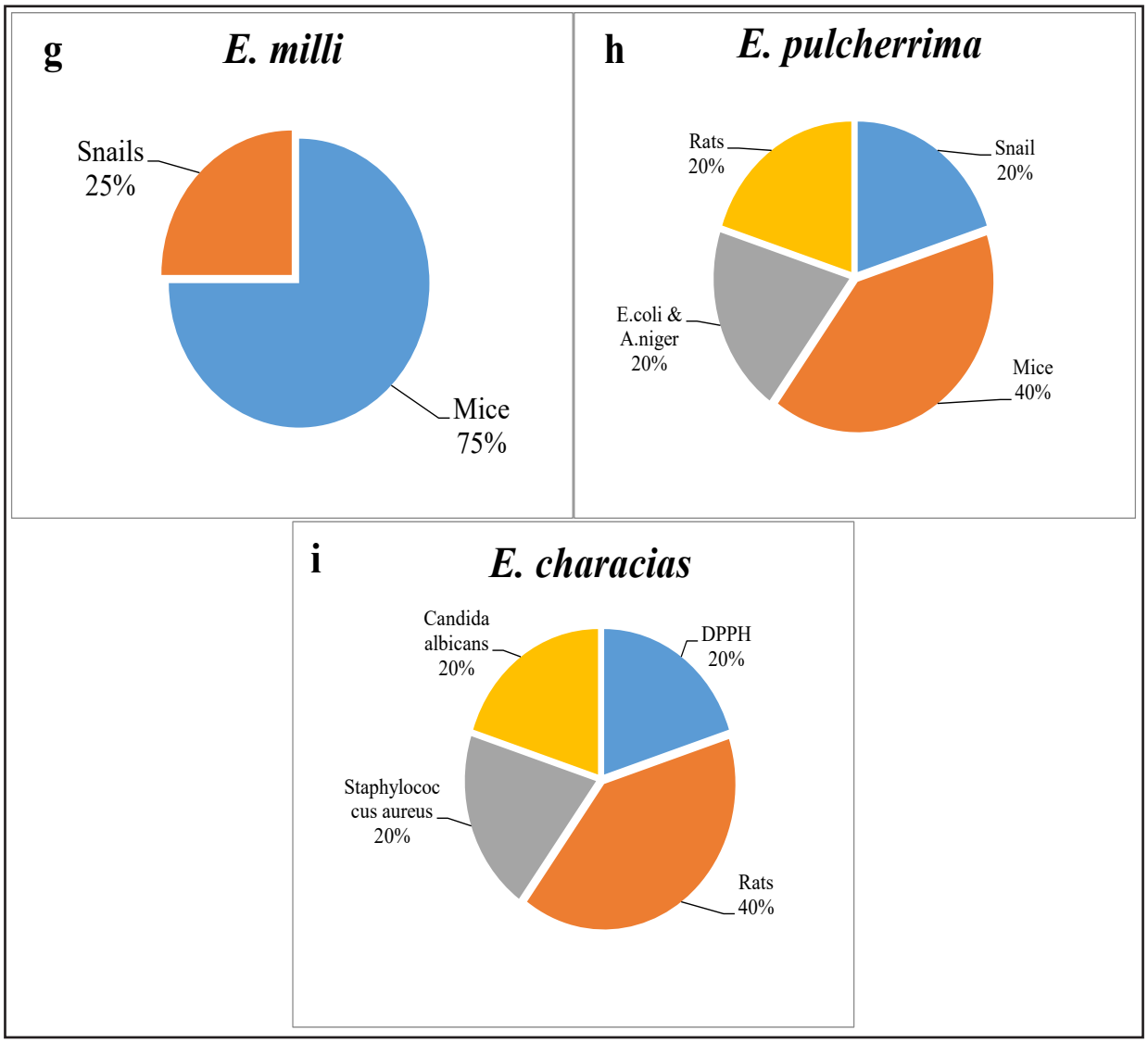


Fig. 3. Percentage of animals/cell lines used for the assessment of various pharmacological activities of euphorbia genus different parts extracts (**a**) *Euphorbia hirta* (**b**) *Euphorbia nerrifolia* (**c**) *Euphorbia kansui* (**d**) *Euphorbia helioscopia* (**e**) *Euphorbia tirucalli* (**f**) *Euphorbia antiquorum* (**g**) *Euphorbia milli* (**h**) *Euphorbia pulcherrima* (**i**) *Euphorbia characias.*

mg/L concentration of NAA (1-naphthaleneacetic acid) and BAP [30]. Red callus was produced from apical and axillary buds of *E. pulcherrima* on MS basal medium containing benzyladenine (BA) and a combination of IAA (indole acetic acid) (IAA) and BA [37]. From leaf explant of *E. helioscopia* callus was induced via Murashige and Skoog's (MS) medium supplemented with 6-benzylaminopurine [38]. Furthermore, secondary metabolite extraction is of utmost importance in culturally grown plants; making it an ideal technique for raising plants for commercial and medicinal purposes [29].

6. SHOOT, LEAF, SEED, AND ROOT REGENERATION VIA DIFFERENT CULTURES

Murashige and Skoog's (MS) + naphthaleneacetic acid (NAA) medium was used to produce roots from *E. tannensis* shoot culture, some of the seedlings were kept under glasshouse in pots containing coarse sand, peat, and perlite with a ratio of 6:3:1, respectively. The plantlets remained healthy and grew well under the glasshouse. On contrary, when placed in peat blocks all seedlings wilted and

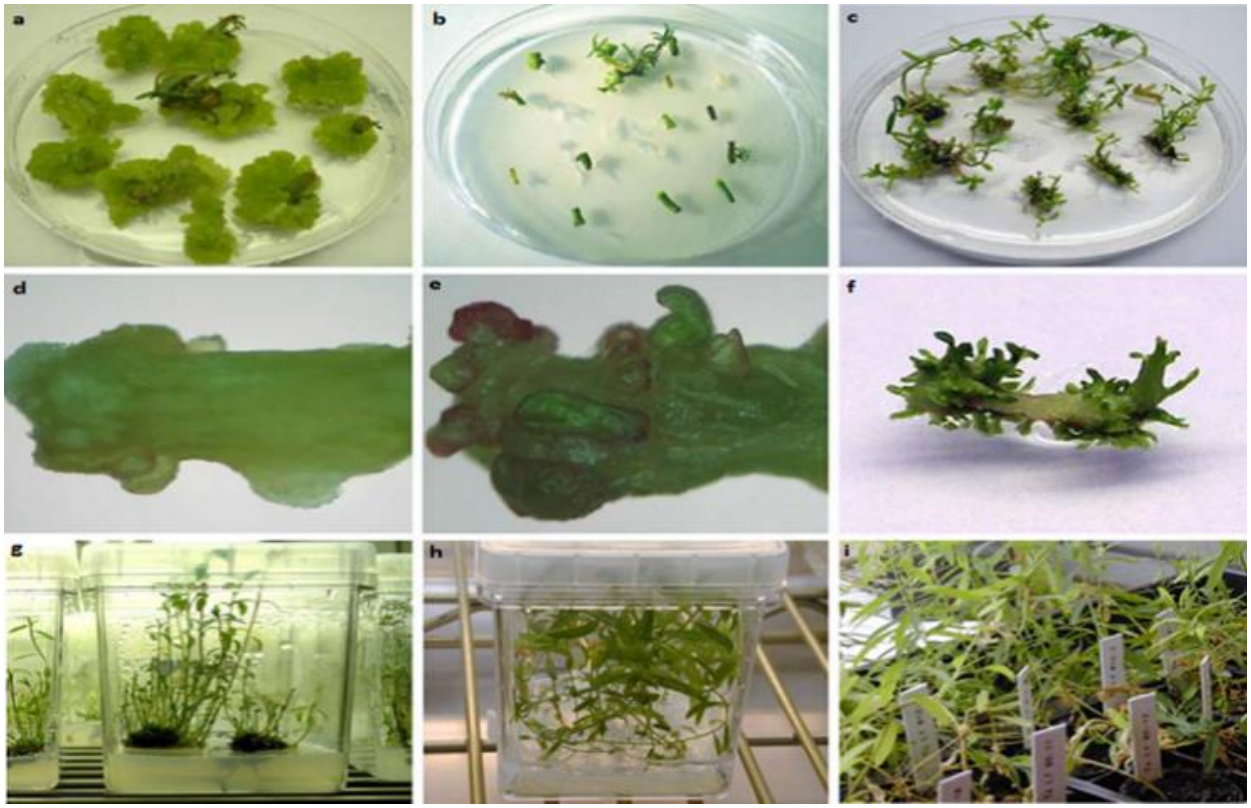


Fig. 4. In vitro regeneration of leafy spurge (*Euphorbia esula* L.) a = Large callus with a loose structure, b = Shoot regeneration from hypocotyls of non-HR lines, c = Shoots regeneration from stem pieces of HR lines, d = Callus, e = Callus and shoot primordium, f = Multiple shoots regenerated from the wounded surface of explants, g = Multiple plantlets growing from calluses, h = Plantlets developing roots in rooting medium. (i) Plantlets growing *In-vitro.*

collapsed. In comparison with *E. tannensis*; *in-vitro* root formation from shoots of *E. lathyris* was quite slow on the same medium used for the formation of roots from *E. tannensis* shoots [39]. *Euphorbia antisyphilitica* shoots were propagated via *in-vitro* culture technique by using BAP (4.44 **µ**M) and MS + NAA (0.13 **µ**M) medium, root progress was satisfactory and when transferred to the field; easily adapted to the natural climatic conditions [31]. *Euphorbia lagascae* shoots were regenerated through tissue culture technique; afterward, dipped in IBA (50 mg/L) for a period of 2 minutes, an increase of 70% to 100% survival rate was observed with the application of benzyladenine (BA) [40]. Tips of *Euphorbia pugniformis* cristate lateral shoots were cultured by using MS + NAA (0.1 mg/L) sucrose 2.0% and IBA in culture media; as a result, both of the normal and cristate types of shoots were produced. 90%-100% of cultured plantlets successfully acclimatized outside the laboratory in field conditions [41]. *In-vitro* propagation of *Euphorbia fulgens* micro shoot cuttings and their adjustment to the natural climatic conditions were established [42]. *Euphorbia pulcherrima* shoot buds were propagated through the tissue culture technique [43]. Shoots were raised from nodal shoot explant of *Euphorbia pulcherrima* (Figure 5) using a medium having 6-benzylaminopurine (BAP) in combination with adenine sulfate and GA3 (Gibberellic acid).

The induction of shoot was optimal by using BAP at 0.5 mg/L in combination with 20 mg/L adenine sulfate. Numerous roots and the highest frequency (77.8%) were observed by using MS media and supplements of 1.0 mg/L indole-acetic acid (IAA) by Sreenika *et al*. [44]. *E. esula* hypocotyl segment was applied as explant and roots were proliferated on culture media containing IAA and IBA [45]. Leaf-cultured cells of *Euphorbia milli* showed the presence of a red colour pigment mainly consisting of anthocyanin [46, 47]. Extract of leaves of *Euphorbia cotinofolia* in streptomycin possessed wide range of flavonoids, terpenoids, and steroids that help remove a pathogenic form of *Bacillus cereus, Bacillus subtilis, Escherichia coli,*

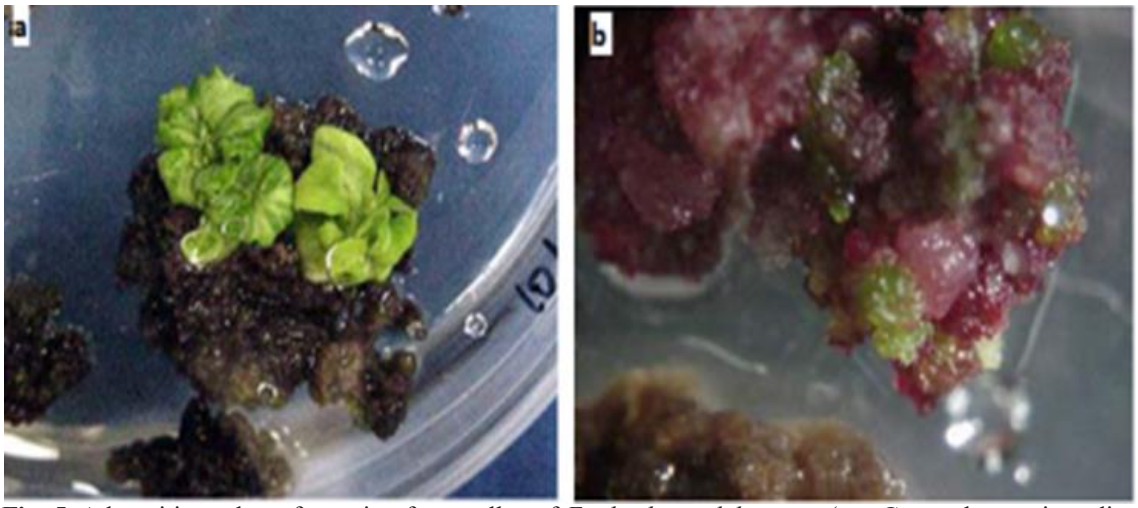


Fig. 5. Adventitious shoot formation from callus of *Euphorbia pulchurrima* (a = Green shoot primordia, b = Elongating Shoots.

Enterobacter aerogenes, Klebsiella pneumoniae, Salmonella typhi, and *Staphylococcus aureus* [48]. Amorphous calcium phosphate nanoparticles (ACP NPs) with coumarin extracts from seeds of *Euphorbia lathyris* possessed strong cytotoxicity against colon cancer [49].

7. SOMATIC EMBRYOGENESIS NODAL AND INTER NODAL CULTURES OF GENUS *EUPHORBIA*

Cultivation through somatic embryogenesis provides a prospect to propagate those lines bearing superior quality in terms of secondary metabolites and yield attributes [43]. To date, propagation through cell suspension culture, callus culture, and somatic embryogenesis was described in several *Euphorbia* species. The somatic embryogenesis of *Euphorbia pulcherrima* was investigated [42]. From the hypocotyl of *Euphorbia pulcherrima* somatic embryo formation was reported by Biesboer *et al*. [50]. *Euphorbia tirucalli* internodal explants (Figure 6) were propagated via *in-vitro* culture technique by using Linsmaier and Skoog's (LS) + TDZ (0.02 mg/L) culture medium [51].

Euphorbia pulcherrima nodal explants were cultured on NAA + MS, isopentenyl adenine (2-iP), and Kin media [52]. *Euphorbia pulcherrima* nodal explant on MS + α-naphthalene acetic acid and isopentenyl adenine (2-iP) medium gave rise to somatic embryos [53]. Internode explant of *E. hirta* gave rise to a higher number of somatic embryos on BAP and Kin media supplemented with indole-acetic acid (IAA) and naphthalene acetic acid (NAA). NAA proved more productive than indole-acetic acid (IAA) with a higher percentage of somatic embryos. 100% response was noticed by using MS in combination with 0.5 mg/L naphthalene acetic acid (NAA), 0.4 mg/L each of BAP, and kinetin (Kin). Medium with IAA in place of NAA gave maximum (92%) somatic embryo induction [54, 55].

8. INFLORESCENCE TISSUE CULTURE

Das *et al.* [56] manipulated the inflorescence of explants (*Euphorbia milli)* for their *in-vitro* propagation. Consequently, vegetative meristems were cultivated from the meristems of an inflorescence of the main axis after one week of inoculation on MS medium supplemented with indole-3-butyric acid (IBA) and benzyl adenine (BA). Among various growth regulators, MS medium with 1.0 mg/L BA and 0.3 mg/L IBA responded better in terms of callus initiation with a maximum percentage of leaf and shoot developed.

9. CONCLUSIONS

In this review, pharmacological activities, methodologies of preparing different explant cultures, *in-vitro* production of biologically active compounds, and vital phytochemicals extracted from various parts of the genus *Euphorbia* has been documented. For successful *in-vitro* propagation of seedlings; media composition, plant growth regulators (PGRs) selection, and other vital requirements such as temperature, light, and pH are immensely important to be determined. Moreover,

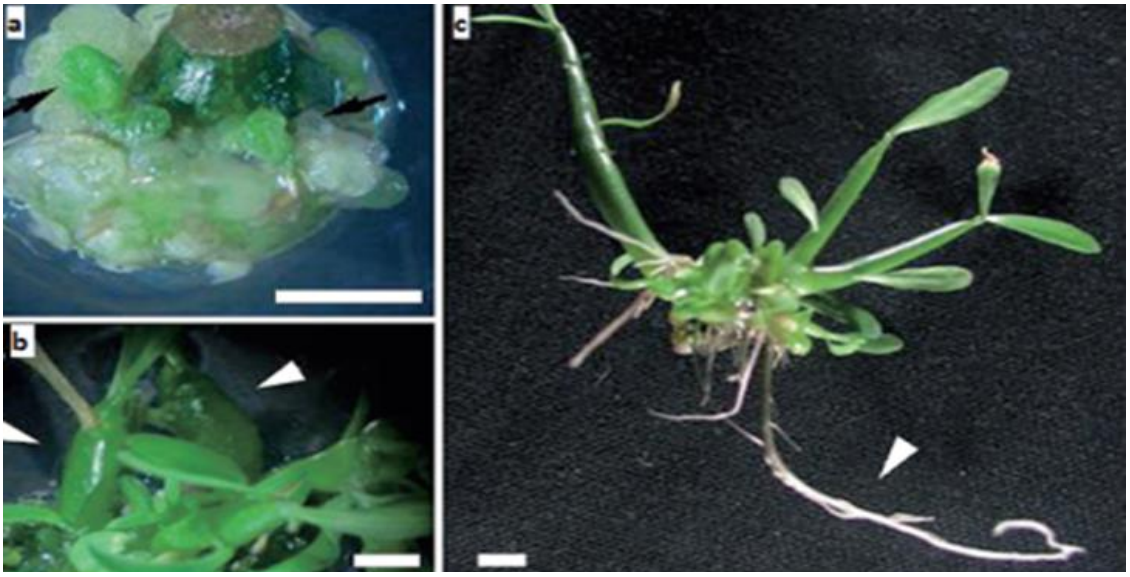


Fig. 6. Plant regeneration from internode segments of E. tirucalli. (a = Adventitious buds, b = Trunk-shaped shoots c = whole plantlet.

the plants regenerated through tissue culture need to be assessed for their safety in living organisms is also necessary for higher marketability and significance. Besides this, investigating the potential of preventing diseases by applying *Euphorbia* should be a relevant issue. Disease prevention methods and modern cultivation practices for *Euphorbia* need to be further elucidated with the application of advanced techniques like hydroponic, aeroponic, and plugged seedling culture methods that should be adopted for rapid growth and better quality of *Euphorbia* species. Still, there is a great need for an imperative propagative method and an immense desire for the swift proliferation of superior qualities of *Euphorbia* species.

10. FUTURE PERSPECTIVES AND RECOMMENDATIONS

The identification and isolation of novel bioactive chemicals from the genus *Euphorbia* should be the focus of future study. Analytical advances such as mass spectrometry and nuclear magnetic resonance can help with the isolation and structural elucidation of previously unknown substances. Further optimisation of *in vitro* culture conditions is required to maximise bioactive chemical synthesis. This includes optimising nutrient formulas, growth regulators, and culture medium components to mimic the natural environment and increase target compound yield. Exploring biotechnological technologies for large-scale synthesis of bioactive substances, such as plant cell and tissue culture, can provide a sustainable and controlled supply. This could include the development of bioreactor systems and commercial scale-up initiatives. More thorough pharmacological investigations on isolated chemicals from *Euphorbia* species are required to fully grasp their medicinal potential. Following clinical trials, their efficacy, safety, and prospective applicability in treating various diseases can be validated.

Promote interdisciplinary collaborations between geneticists, pharmacologists, biotechnologists, and other experts. Such collaborations have the potential to speed research and bring varied perspectives to bear on the issues connected with the study of *Euphorbia* species as well as establish standardized protocols for the extraction of bioactive compounds from *Euphorbia* plants. This will assure uniformity in research outputs and make it easier to compare results across investigations. Finally, the possibilities for future study on bioactive substances from the genus *Euphorbia* are encouraging, with opportunities for scientific improvements, biotechnological innovations, and sustainable practices. Implementing the recommended procedures can aid in the discovery of novel treatments, the conservation of plant species, and the promotion of responsible resource use.

11. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ANNEXURE I

Table 1. Various pharmacological attributes of the genus *Euphorbia.*

Species	Part used	Extract type	Pharmacological activities	Study type	Dose	Animal model/Cell line	References
E. hirta	Whole plant	AqE	Anti-arthritis	*In-vitro*	50 mg/kg	Rats	[57]
	Leaves	AqE	Immunomodulatory		50 g/kg	Fish	[58]
	Whole plant	HAE	Anxiolytic		200 mg/kg	Rats	[59]
	Whole plant	EtOH	Anti-nephrotoxicity		400 mg/kg	Rats	[60]
	Leaves	AqE	Anti- gastrointestinal motility		100 mg/kg to 1000 mg/kg	Rats	[61]
	Whole plant	EtOH	Wound Healing		2% w/w	Rats	[62]
	Whole plant	EtOH	Bronchodilator		200 mg/kg	Animal	[31]
	Leaves	MeOH	Acute oral toxicity		100 mg/mL to 0.07 mg/mL	Mice	[34]
	Whole Plant	EqOH	Anti-dengue		12.5 µg/mL	Human	[63]
	Whole Plant	EtOH	Anti-anaphylaxis		100 mg/kg to 1000 mg/kg	Mice	[64]
	Leaves	AqE	Anti-diabetic		300 mg/kg	Rats	[65]
	Whole Plant	AqE	Sedative		100 mg/kg	Mice	[66]
	Whole Plant	MeOH	Anti-biofilm		0.25 mg/mL	Planktonic cells	[67]
	Whole plant	EtOH	Wound healing		2% W/W cream.	Albino rats	[62]
	Aerial parts	MeOH	Anti-bacterial		0.25 mg/mL to 0.5 mg/mL	*Pseudomonas aeruginosa*	[67]
	Leaves	EtOH	Anti-bacterial		1.25 µg/mL to 200 µg/mL	*Klebsiella pneumoniae*	[68]
	Whole plant	EtOH	Anti-cancer		200 µg/mL	Cell lines	[69]
	Whole plant	Aq	Anti-inflammatory		10 µg/mL	Rabbit	[70]
	Whole plant	EtOH	Anti-allergic		0.3 mg/g	Mice	[71]
	Aerial parts	MeXOH	Antidiarrheal		50 mg/kg	Mice	[5]
	Leaves	MeOH	Anti-tumors		200 mg/kg	Rats	[72]
E. neriifolia	Leaves	HAE	Analgesic	*In-vitro*	400 mg/kg	Frog	[73]
	Stem	MeOH	Hepatoprotective	Laboratory	2.0 g/kg	Mice	[74]
	Leaves	DMSOE	Anti-cancer	*In-vitro*	500 µg/mL	Mice	[75]
	Leaves	HAE	Immunomodulatory		400 mg/kg	Rats	[76]
	Leaves	HAE	Anti-ulcer		400 mg/kg	Rats	[77]
	Leaves	EtOH	Anti-diabetic		400 mg/kg	Rats	[78]
	Leaves	EtOH	Anti-anxiety		400 mg/kg	Mice	[79]
	Leaves	HAE	Hematological		400 mg/kg	Rats	[51]
	Leaves	HAE	analgesic	Laboratory	400 mg/kg	Rats	[80]
	Latex	EF	Anti-inflammatory	-	500 mg/mL	Rats	[81]
	Roots & Leaves	EtOH	Anti-thrombotic	*In-vitro*	2.0 mg/kg	Rats	[82]
	Leaves	EtOH	Wound healing	Laboratory	200 mg/kg and 400 mg/kg	Rats	[83]
	Leaves	AqE	Anti-scorpion venom		0.706 mg/mL	Fibroblast cell lysis	[84]
	Leaves	HAE	Immunomodulatory		400 mg/kg	Rats	[85]
	Leaves	HEE	Anti-DENA-Induced Renal Carcinogenesis	*In-vivo*	50 mg/kg	Mice	[86]

Species	Part used	Extract type	Pharmacological activities	Study type	Dose	Animal model/Cell line	References
E. kansui	Roots	EtOH	Anti-nematode	*In-vitro*	5 µg	*Bursaphelenchus xylophilus*	[87]
	Roots	EtOH	Anti-proliferative		8.7 µg/mL	Cell lines	[88]
	Roots	EtOH	Hepatotoxic		8 µg/mL	Cell lines	[89]
	Roots	CdE	Anti-HIV		500 µg/mL	Human	[90]
	Roots	EtOH	Anti-cancer		50 µg/mL	Human	[91]
	Roots	DCME	Anti-cancer		2.0 µmol	Mice	[92]
	Roots	EtOH,	Anti-termites		50 *µ*g/mL	*Reticulitermes speratus*	[93]
	Roots	EtOH	Anti-obesity		100 mg/kg	Mice	[94]
	Roots	EtOH	Cytotoxic	.....	30.67 µg/mL	Cell lines	[95]
E. helioscopia	Whole plant	EAE	Anti-cancer	*In-vivo*	200 µg/mL	Mice	[88]
	Whole plant	EAE	Anti-cancer	*In-vitro*	200 µg/mL	Cell lines	[96]
	Whole plant	EAE	Anti-asthmatic		30 mg/kg	Pig	[97]
	Leaves and latex	MeOH	Anti-angiogenic	*In-vivo*	200 µg/L	Leghorn chicken eggs	[98]
	Plant	MeOH & AqE	Anthelmintic		50mg/mL	*Haemonchus vontortus*	[99]
	Leaves and stem	MeOH	Molluscicidal	Laboratory	10 ppm to 100 ppm	Snail	[100]
	Whole plant	MeOH	Anti-viral	.....	0.125 mg/mL	Bacteriophage (CP51)	[101]
	Aerial parts	MeOH	Vasodepressor	.....	2.0 mg/kg	Rats	[102]
	Leaves	MeOH	Anti-pyretic	*In-vitro*	300 mg/kg	Mice	[103]
	Leaves and latex	MeOH	Anti-bacterial		250 mg/mL	Bacteria (*S. aureus* and *E. coli*)	[104]
	Leaves and latex	MeOH	Anti-oxidant		1200 mg/kg	Mice	[96]
	Whole plant	EAE	Anti-cancer		200 mg/mL	Cell lines	[105]
	Roots	AqE	Anti-tumor		4.0 mg/mL	MKN-45 cells	[106]
	Whole plant	EtOH	Insulin secretagogue		10 µg/mL	Mice	[107]
E. tirucalli	Stem	EtOH	Anti-cancer		300.70 µg/mL	Cell lines	[108]
	Leaves	AqE	Anti-cancer		200 µg/mL	Cell lines	[109]
	Whole plant	EAE	Anti-inflammatory	*In-vivo*	10 mg/kg	Mice	[110]
	Latex	AqE	Analgesic	.....	300 mg/kg	Mice	[111]
	Whole plant	BET	Anti-arthritic	*In-vivo*	2000 mg/kg	Mice and rats	[112]
	Aerial parts	AqE	Hepatoprotective		150 mg/kg and 250 mg/kg	Rats	[113]
	Latex	PEE & DCME	Antiviral	.....	150 ppm	Tobamoviruses	[114]
	Whole plant	MeOH	Antinociceptive	*In-vivo*	30 mg/kg	Mice	[115]
E. antiquorum	Roots	AqE	Anti-diabetes	*In-vitro*	400 mg	Rats	[116]
	Whole plant	A-EtOH	Analgesic	Laboratory	500 mg/kg	Mice	[117]
	Whole plant	AEA & AqE	Anti-arthritic	.....	400 mg/kg	Rats	[118]

Species	Part used	Extract type	Pharmacological activities	Study type	Dose	Animal model/Cell line	References
E. millli	Flower	EAE	Anti-cancer	*In-vivo*	200 mg/kg and 400 mg/kg	Mice	[119]
	Aerial parts	MeOH	Sedative	.....	10 ppm and 20 mg/kg	Mice	[120]
	Aerial parts	MeOH	Antinociceptive	.....	50 mg/kg, 100 mg/kg and 150 mg/kg	Mice	[121]
	Latex	AqE	Molluscicidal	.....	22 ppm (mg/L)	Snail	[122]
E. pulcherrima	Latex	AqE	Molluscicidal	.....	0.02 mg/kg and 0.09 mg/L	Snail	[123]
	Latex	AqE	Anti-convulsive	Laboratory	250 mg/kg, 500 mg/kg and 1000 mg/kg	Mice	[124]
	Aerial parts	MeOH	Analgesic effect	.....	50 mg/kg,100 mg/kg and 150 mg/kg	Mice	[125]
	Whole plant	AqE and EtOH	Antimicrobial	*In-vitro*	2000 µg to 5000 µg	*E. coli* and *A. niger*	[126]
	Latex	AqE	Anxiolytic effect	.....	250 mg/kg, 500 mg/kg and 1000 mg/kg	Mice	[127]
	Whole plant	MeOH	Hepatoprotective	.....	1000 mg/kg	Rats	[67]
E. characias	Latex	MeOH	Antioxidant	.....	25 µL	DPPH	[128]
	Aerial parts	MeOH	Anti-inflammatory	*In-vivo*	100 mg/kg	Rats	[32]
	Leaves	EtOH	Anti-bacterial	....	1250 *µg*/mL	*Staphylococcus aureus*	[36]
	Aerial parts	MeOH	Wound healing	*In-vivo*	.....	Rats	[129]
	Latex		Antifungal	*In-vitro*	62.5 *µg* protein/mL	*Candida albicans*	[130]

ANNEXURE II

Table 2. A list of explants used growth regulators and reported basal media for *in-vitro* propagation of *Euphorbia* species.

Species	Explant	PGRs	Culture Medium	Responses	References
E. esula	Stem explant	BA + IBA + 3% sucrose + vitamins	MS	Shoot	[29]
	Hypocotyl segment	IAA + 2,4-D	B5	Shoots and Roots	[131]
	Root tissue	NAA + KIN + IAA	MS	Callus	[35]
	Cell suspension culture	2,4-D+NR	B5	Roots	[132]
	Hypocotyl segments.	IAA + polyamines	B5	Roots	[133]
	Hypocotyl segments.	Putrescine	B5	Roots and shoots	[45]
	Stem callus	Fluorescent light	MS	Cell suspension	[131]
E. lagascae	Axillary shoots	IBA or NAA	MS	Roots	[40]
E. tirucalli	Stem	BA, NAA, 2,4-D	MS	Callus	[134]
	Internode	TDZ, NAA	LS medium	AB	[135]
	Internode	TDZ	LS	AB	[54]
E. milli	Apical bud	Paclobutrazol + Sucrose + LEDs	MS	Inflorescence	[56]
	Buds	BA+ IAA+ Sucrose + vitamins	MS	Roots	[136]
E. pulcherrima	Nodal shoot segments	BAP+ GA3+AS	MS	Shoot	[44]
	Apical buds and axillary buds	IAA+BA	MS	Callus	[137]
	Shoot tips	BA + sucrose (3%) + agar (75%)	MS	Shoot	[138]
	Nodal explant	2ip + NAA	MS	SE	[139]
	Shoot	BA+ sucrose (3%) + agar (75%)	MS	Shoot	[46]
	Terminal buds and Leaf tissue	IAA + BA	MS	Callus and Shoot	[53]
	Stem nodes	NAA +2-Ip	MS	SE	[26]
	Hypocotyle segment	IAA	MS	SE	[140]
	CS	2,4-D + BA	MS	SE	[141]
	Stem nodal explants	BAP + NAA	MS	Shoot	[142]
	Petiole explants	NAA + BA+ KIN +2,4-D+IBA+IAA	MS	Buds	[142]
E. pugniformis	Tip explants	NAA + BA + sucrose	MS	Shoot	[41]
E. antisyphilitiea	Shoot	BA + NAA	MS	Axillary shoot	[143]
E. nivulia	Mesophyll cell	NAA	MS	Shoot	[17]
E. hitra	Stem explants	BAP + KIN + NAA	MS	SE	[55]
	Leaf bits	NAA+ BAP	MS	Callus	[30]
E. helioscopa	Leaf discs	2,4-D	MS	Callus	[38]
E. lathyris	Apical shoot	NAA	MS	AS	[39]
	Nodes and internodes	NAA+ BA	MS	Shoot and callus	[144]

ANNEXURE III

Table 3. *In-vitro* production of secondary metabolites from *Euphorbia* species via using different approaches.

Secondary Metabolites	Species	Culture Types	PGRs	Medium	References
Phytosterols	E. milli	LC	2,4-D, NAA, ME	MS	[145]
Triterpenol			Auxin, 2,4-D, NAA, YE		
Anthocyanin			2,4-D, NAA, CH		
		CC	2,4-D, NAA		[146]
		LC	2% sucrose, 0.8% agar, 2,4-D		[146]
		SC	MS, LS, GA, NN, HE, ME, sucrose,		[147]
Phytosterols	E. triucalli		ME, 2,4-D		[63]
Fatty Acids			YE, 2,4-D		
Anthocyanin			ME, 2,4-D		[50]
Triterpenol			ME, 2,4-D		
Triterpenol	E. characias	CC	KIN, BA, ZEA, 2,4-D		[52]
Taraxerol	E. hirta		NAA, BAP		[34]
Triterpenoid		CSC	2,4-D+BAP+NAA		[148]

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Review Article

Antimicrobial Resistance: An Emerging Concern for Humans

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Abstract: Most of the pathogens have developed the ability to combat advanced antimicrobial agents due to which bacterial infections have become complicated to treat. It may occur when microorganisms such as fungi, parasites, and bacteria change their behavior against conventional antimicrobial agents. Some bacteria are intrinsically resistant to some of the antimicrobial agents, if not, they may become resistant by de novo mutations or acquiring some resistant genes. Antimicrobial resistance (AMR) has become a global issue because it may spread worldwide through trade, travel, migration, and healthcare facilities. Antimicrobial resistance has been associated with adverse consequences in the context of invasive infections, including escalated hospital costs, heightened mortality rates, and prolonged hospital stays. In the case of the disturbing normal flora of the intestine, serious and incurable health problems and sometimes death also occur. To combat this rising havoc many emerging approaches are being considered to combat AMR. Some of these include one health approach, phage therapy, nanoparticles, medicinal plants and metals. This review article discusses the leading causes of antimicrobial resistance, its economic impact, and employing emerging approaches as an effective way to treat antimicrobial resistance.

Keywords: Antimicrobial Resistance, One Health Approach, Pathogens, Phage Therapy, Public Health, Vaccines.

1. INTRODUCTION

Antimicrobial resistance (AMR) is a major worldwide health threat [1]. AMR caused 4.95 million deaths worldwide in 2019, according to estimates [2]. The predominant etiological agents responsible for this condition primarily include six pathogens, namely *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [3]. AMR results when microorganisms including bacteria, fungi, parasites and viruses evolve to the extent that they eventually become resistant to antimicrobial medications, such as antibiotics, which are used to treat such conditions [4]. Most of the pathogens have developed the ability to combat advanced antimicrobial agents due to which bacterial infections have become complicated to treat [1]. In history, humans become reconciled to many bacterial outbreaks. With the unearthing of penicillin midway through the 20th century, scientists found a way out by developing agents against pathogens to fight these maladies [2]. During the era (1930-1960) a vast variety of antimicrobials were discovered and made available [5]. Globally the death rate increased due to resistance in pathogens against traditional medications and treatments, the leading cause of which was overuse/misuse of antimicrobials [2]. Multi-drug resistance was spotted initially in the late 1950s and early 1960s in some members of the family *Enterobacteriaceae* [5]. According to the facts given by United States Pharmacopeia (USP), it is evaluated that 0.7 million deaths per year are the result of antimicrobial resistance and if this issue is not properly addressed and resolved death rate can increase up to 10 million per annum till the year 2050 [6].

2. DATA COLLECTION STRATEGY

Through an extensive examination of relevant scholarly works, essential data was gathered, evaluated, and employed to comprehend the

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ramifications and financial burdens associated with antimicrobial resistance on a global scale. Information was obtained by utilizing various search engines and databases such as Google, Google Scholar, PubMed, Microsoft Academic, and Scopus. Additionally, searches were conducted within reputable health organizations and websites including the CDC, WHO and other comparable sources. The review exclusively encompassed published articles authored in the English language. The present study employed a range of search terms, namely "AMR and global burden," "Antimicrobial resistance implications," and "AMR and disease burden," to collect pertinent information.

3. GLOBAL BURDEN OF ANTIMICROBIAL RESISTANCE

The emergence of antimicrobial resistance (AMR) has become a huge concern, prompting the constant monitoring of infection and mortality rates associated with this phenomenon. According to data from the United Kingdom, the estimated incidence of antimicrobial resistance (AMR) infections was 65,162 individuals diagnosed in 2019, representing an increase from the 61,946 patients recorded in the previous year. In contrast, the European Centre for Disease Prevention and Control (ECDC) has documented that within the European Union (EU) exclusively, the incidence of AMR has escalated to exceed 670,000 cases on an annual basis. Based on the findings of a previous investigation [7], it has been determined that in the year 2019, a total of 4.95 million fatalities across the globe were associated with bacterial antimicrobial resistance. Furthermore, it was observed that 1.27 million of these deaths were directly attributable to bacterial AMR. It has been previously documented that the yearly mortality rate attributable to AMR is anticipated to escalate to 10 million by the year 2050. Asia and Africa have been identified as the regions with the highest estimated mortality rates attributed to antimicrobial resistance. This can be primarily attributed to their large populations and the lack of regulatory measures in place for AMR prevention. Based on prior scholarly investigations, it has been established that Sub-Saharan Africa exhibits the most elevated all-age mortality rate within the Global Burden of Diseases (GBD) region. It has been specifically associated with or connected to AMR. In stark contrast, Australasia recorded the lowest rate of mortality attributed to AMR in the year 2019 [7].

4. CAUSES OF ANTIMICROBIAL RESISTANCE

Those drugs which were formerly called the saviour of life on earth stopped working lately owing to the emergence of bacterial resistance to multiple antimicrobials [8]. Due to the unregulated use of antibiotics and incomplete treatment of bacterial infections it has become a global issue. Overuse of antibiotics closely correlates with the disclosure of the resistance against them. Inappropriate antimicrobials can trigger spontaneous mutations in microbial genes leading to resistance. The use of antibiotics is unregulated as these medicines are available everywhere without prescription [9]. The US Food and Drug Administration (FDA) highlights the major point that majority of the doctors prescribe antibiotics to patients suffering from sore throat and flu [10]. Antimicrobial resistance is also caused by the extensive use of broad-spectrum antibiotics. Most of the time physicians avoid proper testing. Rather than targeting the main pathogen after proper clinical investigations by using narrow-spectrum drugs doctors prescribe broad-spectrum antibiotics to avoid laboratory analysis [8]. Using an old or someone else prescription is also a wrong practice because each prescribed antibiotic is for a specific infection and hence cannot be used for every infection or in any other condition. Using old prescriptions can make the bacteria resist [10]. The resistant bacteria are not killed by antibiotics they multiply rapidly and dominate the entire microbial community. The vigorous use of antibiotics in agriculture and livestock is also a major hub of the production of resistant bacterial strains. Antimicrobials are extensively used in fodder to make sure animals do not get infected. The bacterial strains in livestock become resistant and are passed on to humans after the consumption of meat by them. Resistant strains also merge with the environment as they are part of animal waste and thus resistance continues to spread [11]. The availability of new antimicrobials (which can kill resistant strains) is reduced because pharmaceutical industries are no longer interested in funding for them as they are of no profit value [12]. Secondly, if new antimicrobials become available easily at every pharmacy, then, they will again prescribe repeatedly thus spreading resistance against themselves [13].

5. DRIVERS OF ANTIMICROBIAL RESISTANCE

Antimicrobial resistance is a complex phenomenon that is influenced by a multitude of factors, including intrinsic characteristics of the microorganisms and a range of environmental factors that are influenced by both prescribers and consumers. In a comprehensive analysis, the factors that contribute to AMR can be classified into four distinct categories. Firstly, environmental factors play a significant role, encompassing aspects such as population density and overcrowding, rapid transmission facilitated by mass travel, inadequate sanitation practices, ineffective infection control programs, and the widespread use of antimicrobials in agriculture. Secondly, drug-related factors contribute to AMR, including the presence of counterfeit or substandard drugs, as well as the unrestricted availability of antimicrobials without prescription. Thirdly, patient-related factors also contribute to the development of AMR, such as poor adherence to prescribed treatment regimens, poverty, limited education, self-medication practices, and misconceptions about the appropriate use of antimicrobials. Lastly, physician-related factors, such as inappropriate prescription practices, inadequate dosing, and a lack of up-to-date knowledge and training, also contribute to the emergence and spread of AMR [14]. The emergence of resistance is a multifaceted phenomenon that not only poses a threat to human health but also has significant implications for the environment. This is particularly evident in the context of global travel and trade, where the movement of food products can serve as a vector for the dissemination of resistant organisms. As depicted in Figure 1, the impact of resistance development extends beyond the realm of human health and underscores the need for a comprehensive approach to address this complex issue [15].

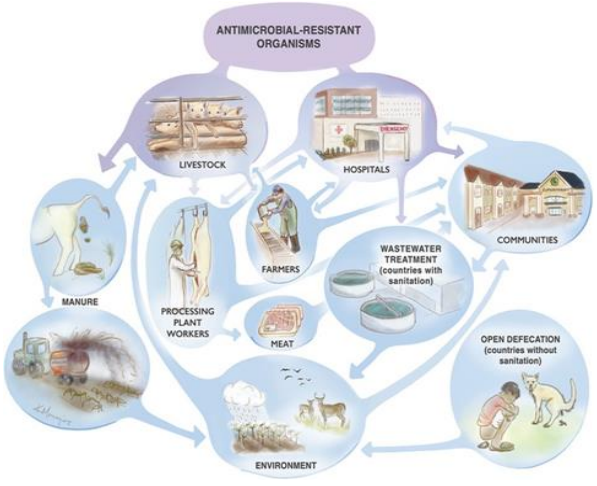

Fig. 1. Dissemination of antimicrobial-resistant organisms [13].

6. CLINICAL OUTCOMES AND ANTIMICROBIAL RESISTANCE

Antimicrobial resistance has been linked to negative outcomes in invasive infections, such as increased hospital expenditures, mortality, and stay duration. This finding has been attributed to a variety of factors, including a delay in implementing effective treatments, less effective definitive therapy compared to that available for susceptible bacteria, and the virulence of some resistant species (Table 1) [16]. Lambert *et al.* [17] investigated in a study between January 1, 2005, and December 31, 2008, they gathered information on 119 699 patients who had spent more than two days in 537 intensive care units across 10 different nations. The higher risk of death (hazard ratio) for pneumonia is present in the completely modified model ranging from 17 (95 percent CI 14–19) for S aureus is drug-sensitive to 35 (29–42) for *P. aeruginosa* is drug-resistant. The excess risk for bloodstream illnesses varied from 21 (16–26) for *S. aureus* which was drug-sensitive to 40 (27–58) for *P. aeruginosa* which was drug-resistant. For a combination of pneumonia and bloodstream infections, antimicrobial resistance increased the probability of death from infections by 12 (111–14) in the case of pneumonia and by 12 (09–15) in the case of septicemia. They concluded that pneumonia dramatically lengthens hospital stays in intensive care units, and increases mortality from healthcare-associated bloodstream infections, and death overall; however, the most prevalent antimicrobial resistance patterns have only a small additional impact [17]. So, antimicrobial resistance can affect the fitness of microorganisms as it can boost their strength to survive in critical conditions and enhance their pathogenicity. One of the most important elements that can cause proper medication to be delayed is a discrepancy between the analytical therapeutic agent and subsequent susceptibility results for a specific organism [18].

Table 1. Representative reports of attributable costs, excess lengths of stay, and risks of mortality associated with various anti-microbial resistance pathogens.

Type of antimicrobial-resistant infection	Increased risk of death	Attributable (days)	Attributable costs
MRSA bacteremia	1.9	2.2	US$6,916
MRSA surgical infection	3.4	2.6	US$13,901
VRE infection	2.1	6.2	US$12,766
Resistant *Pseudomonas*	1.8–5.4	5.7–6.5	US$11,981–32,949
Resistant *Enterobacter* infection	5.0	9	US$29,379
Resistant *Acinetobacter* infection	2.4–6.2	5–13	US$3,758
ESBL or KPC-producing *Escherichia coli* or *Klebsiella* infection	3.6	1.6-fold increase	1.7-fold increase

* ESBL: Extended-spectrum β lactamase; KPC: *Klebsiella pneumonia carbapenemase*; VRE: Vancomycin-resistant *enterococci*; MRSA: Methicillin-resistant *staphylococcus aureus*

7. EVALUATION OF THE FINANCIAL IMPACT OF ANTIMICROBIAL RESISTANCE

Poverty and the gap in knowledge between developed and developing countries are going to increase due to antimicrobial resistance. Due to an increase in the resistance of livestock, treatments become ineffective and increase people's death rate due to the severity of infection [19]. This may result in a decrease in livestock trade and an increased price rate of protein, milk, eggs, and meat; all this exerts pressure on the economy [20]. Even in the greatest institutions around the world, the rising incidence of resistant infections is raising healthcare costs, making infection management more challenging, and harming patient outcomes. Therapy becomes more challenging, requiring less tried-and-true procedures and therapies as well as more awareness among healthcare professionals. Reduced income can cause families to experience financial losses as long-term illness and premature death wreak havoc. In addition, using antibiotics inappropriately, such as taking them for a cold, results in needless out-of-pocket expenses for a medication. Instead, this money may be applied to the cost of necessary medications or the cost of education. AMR has a major influence on healthcare prices; an estimated $700 will be added to the cost of treatment for a resistant bacterial illness [21].

8. EMERGING APPROACHES TO LIMIT ANTIMICROBIAL RESISTANCE

8.1 One Health Approach

Antimicrobial resistance (AMR) is a burgeoning concern that necessitates a cohesive global strategy. "One Health" embraces the concept that there is a clear connection between the health of both humans and animals and the shared surrounding environment. After careful consideration of all relevant factors, it is evident that AMR has emerged as a highly significant concern within the framework of the "One Health" approach. This is primarily due to its capacity to rapidly disseminate throughout populations, as well as infiltrate the food chain, healthcare facilities, and the environment; consequently, the management of numerous infectious diseases in both human and animal populations becomes considerably more complex. The "One Health Concept" (Figure 2) embodies a multidisciplinary approach that necessitates the collaborative involvement of all relevant stakeholders to effectively participate in this initiative. The World Health Organization (WHO) has engaged in a close partnership with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization of Animal Health (OIE) to ensure that comprehensive measures are implemented across all sectors to mitigate the risks of AMR stemming from this approach [22]. One of the approaches employed to enhance recognition of the issue of AMR was the initiation of the "Global Antimicrobial Awareness Week". Since the year 2020, the designated term "World Antimicrobial Awareness Week" has been employed to encompass a comprehensive range of antimicrobial agents, including antibiotics, antifungals, antiparasitic, and antivirals. This global initiative endeavors to enhance global consciousness regarding AMR and promote optimal approaches among the general populace, healthcare professionals, and policymakers to mitigate the

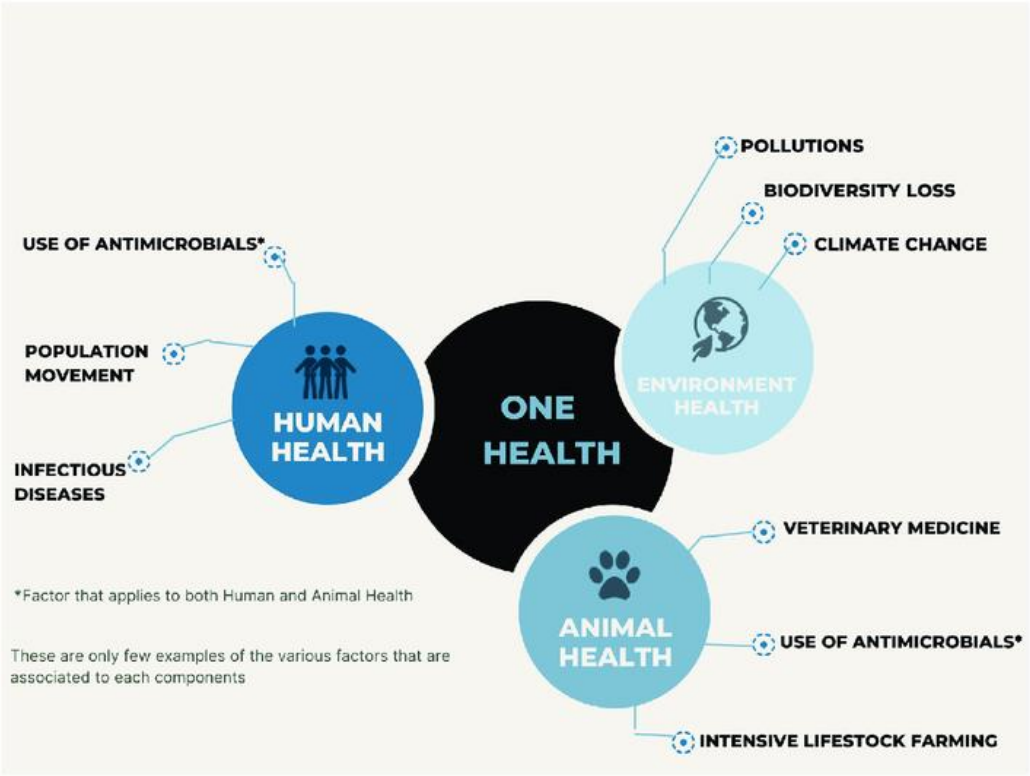

Fig. 2. Illustration representing the concept of One Health Approach [7].

progression and dissemination of drug-resistant infections [23].

8.2 Phage therapy

Phage therapy employs bacteriophages to (1) specifically target a bacterial population, (2) transport drug molecules to a bacterial target, and (3) introduce enzymes capable of deactivating or reversing resistance genes. Due to their widespread prevalence and remarkable selectivity for bacterial receptors, bacteriophages can be genetically modified to specifically target a particular pathogenic bacterial population, while leaving the surrounding microbiota unharmed [23].

8.3 Nanoparticles

Nanoparticles, consisting of metals or metal oxides, exhibit a multifaceted approach to combating microbes. This is achieved through various simultaneous mechanisms, such as the disruption of the cell membrane, the generation of reactive oxygen species, and the infliction of damage to intracellular contents. Consequently, nanoparticles possess a reduced risk of resistance development, rendering them highly appealing in the fight against multidrug-resistant (MDR) pathogens. Nanoparticles exhibit significant promise as protective coatings, particularly in the context of wound dressings or implants, intending to mitigate the occurrence of biofilm-related infections. Despite the theoretical and initial investigational potential of innovative strategies, the majority of these approaches are still in their nascent stages of development. Further investigations are required to establish their safety, in vivo efficacy, interactions with human immunity, and pharmacokinetics before they can be employed clinically to address antimicrobial resistance [24].

8.4 Vaccines

Vaccines have a very minimal impact on developing resistant microbes because they affect the immune system. Vaccination has long-lasting impacts due to which they are less frequently used. Contrary to vaccination, antimicrobial agents must be used regularly, and they have short-term influence. Vaccines can be produced by using more harmful virulence factors such as a vaccine against Streptococcus pneumoniae has been made by using this technique [25].

8.5 Medicinal Plants and Phytochemicals

Plants have developed distinctive mechanisms to safeguard themselves against microorganisms through the presence of natural phytochemicals, also known as secondary metabolites, which are present in various parts of the plant such as seeds, roots, leaves, stems, flowers, and fruits. Moreover, plants can synthesize a wide array of chemically diverse compounds that play a crucial role in their defense mechanisms against microbial invasion. Hence, the pharmaceutical and scientific communities have shown considerable interest in exploring the potential effectiveness of plant-derived compounds as viable drug candidates. In this pursuit, numerous plant extracts and oils have been thoroughly assessed for their potential as antibacterial agents and agents capable of modifying antibiotic resistance. The novel drug discovery screening programs are comprised of three distinct approaches: random, computational, and ethnopharmacological. The most potent antimicrobial activity is exhibited by a selection of plant-derived substances (PDSs) that hold significant medical relevance. These substances encompass alkaloids, organosulfur compounds, phenolic compounds, coumarin, and terpenes [26].

8.6 Metals

The utilization of unbound metal ions as a means to eradicate bacteria and fungi has a well-established historical background. Bacteria, analogous to the majority of organisms, exhibit a complex association with metals, characterized by a simultaneous dependence and aversion: while a particular metal may be indispensable for their survival. It can also become toxic under specific forms and concentrations. Metal ions have been recognized for their antimicrobial properties for a considerable time and have garnered significant attention in contemporary times due to the emergence of antimicrobial resistance. The host immune system employs various strategies to disrupt the equilibrium of transition metals to counteract the intrusion of pathogens. One example of a host defense mechanism against bacterial infection is the inhibition of bacterial iron acquisition by the host protein lipocalin. This protein binds to siderophores, which are molecules produced by bacteria to sequester iron from their environment. By binding to siderophores, lipocalin prevents bacterial access to iron, thereby limiting their growth and survival. Calprotectin, an additional protein host, exhibits the ability to sequester manganese at locations where infection is present. Zinc plays a crucial role in maintaining the normal immune function of the host. At the cellular level, macrophages possess the ability to employ distinct strategies, namely zinc starvation and zinc toxicity, to effectively eliminate the bacteria, they engulf [27].

9. CONCLUSIONS

The significant morbidity and mortality caused by antimicrobial resistance in bacterial infections is a matter of great concern. Both gram-positive and gram-negative bacteria with multidrug resistance patterns pose challenges in terms of treatment, and may even exhibit resistance to currently available standard medications. Given the lack of successful treatments, effective preventive measures, and new antibiotics, bacterial infections and the associated diseases present formidable challenges that necessitate the development of novel strategies and alternative antimicrobial medicines for a more promising future.

10. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

In Vitro Screening of Tomato Cultivars against Cadmium Tolerance in Iraq

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Abstract: Cadmium (Cd) is a heavy metal, highly toxic and soluble in water easily taken up by the plants, produces abnormal growth, and disturbs the metabolism of plants. The present study consists of an *in vitro* study of nine tomato cultivars in comparison to the application of Cd to know their effect on different growth parameters of tomato cultivars including germination, mean generation time, shoot, and length (cm). All cultivars were scored based on a systematic procedure. Results showed that the Cd treatment influenced the germination percentage of tomato cultivars. After Cd treatment, a maximum and a minimum germination percentage of 62.67±2.89 and 25.00±5.00 were observed for T-59 and Tmt-9, respectively. However, the most susceptible cultivars were recorded for Tmt-9. The shortest mean generation time (MGT) was recorded for T-59 (4.04±2.57 days) and the longest for Tmt-9 (10.12±2.61 days). Heavy impact of Cd was recorded on shoot height; meanwhile, T-59 produced a maximum shoot height of 7.47±0.26 cm and showed the lowest Cd inhibition. The root height in Tmt-9 was 6.00±1.01 cm to 1.85±0.16 cm; thus, showed high influence with Cd application. After Cd application, for roots less inhibition of 7.10±0.62 cm was recorded. Based on the ranking score, T-59 ranked first with 16.00 points for seed germination, mean generation time (MGT), shoot length, and root length. Based on the results, it is recommended to sow the T-59 tomato variety that proved less influenced by Cd.

Keywords: Tomato Cultivars, Cadmium, Growth Evaluation, Iraq.

1. INTRODUCTION

Since cadmium (Cd) is a non-essential element that negatively affects plant growth and development. Cd is also a heavy metal toxic to all organisms [1]. Human activities giving rise to the Cd accumulation in biotic systems is becoming a major problem for environment. Cd content in the soils is increasing with the application of Cd containing fertilizers, city waste, and sewage sludge [2]. The existence of carbon based or inorganic impurities in our biospheres produce its deterioration, and which further leads to threatening issues to the global ecosystem [3]. The presence of soil with toxic heavy metals at low concentrations in the environment, for instance, chromium (Cr), arsenic (As), nickel (Ni), mercury (Hg), lead (Pb) and cadmium (Cd), which results in serious risks to life of plant and directly or indirectly also effects the human health [4, 5]. Unsustainable urbanization, boost of industrialization and less judicious ways of enhancing the agricultural practices are beings reasoned for affecting the environment. Among these, Cadmium (Cd) contamination of soil and food crops is highly addressed issues nowadays as it is a serious extent of bioaccumulation [6]. It is a silver white heavy metal, toxic in nature, quickly soluble in water thus easily translocated and taken

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by higher plants [7]. Furthermore, Cd can be swiftly absorbed by roots of many plants due to its fluidity. After accumulation in roots, Cd disturb the functional and structural properties of plants most particularly delay or inhibit the germination process and roots penetration [8]. Moreover, Cd is also recorded for its worse impact on physiological processes of many plants for instance exchange of gas, photosynthesis, respiration, and water movement. All these greatly elicit the weak metabolism of plant and consequently resulted in loss more or less [9]. The effects of Cd on plants increase by rising Cd concentration in the soil that further lead to uptake by human body through the food chain system (soil-plant-human) resulting in severe chronic diseases thus threating to the human health [10] It is therefore essential to explore a judicious method to overcome this problem by remediate Cd-contaminated soils.

In such regards, it has been documented well to observe the effects of Cd on various crops including wheat *Triticum aestivum* L. [11], rice *Oryza sativa* L., oat *Avena sativa*, mustard *Barsica juncea*, even in commonly grown most of green leafy vegetables [12] including spinach *Spinach oleracea* L., fenugreek *Trigonella foenum-graecum* L., coriander *Corainder satium* L.) and on more importantly Tomato *Solanum lycopersicum* [13] that showed the root browning in many plants under Cd exposure. Furthermore, root length and dry mass decreased, and root diameter increased with Cd toxicity [10]. The stunting, chlorosis, necrosis, and desiccation, typical toxic symptoms of Cd stress in the foliage of plants were also noted in most of the plants. Latif *et al*. [12] further noted that Cd importantly create an impact on photosynthetic process that further leads to biochemical changes at different growth stages of plants.

Tomatoes are major source of nutrients namely iron, lycopene, vitamin C and potassium and also provide so many antioxidants that contribute essentially to human health [9, 14-16]. It is one of the unique vegetable fruits in the world for their high nutritive value, with leading producers like China, USA and Turkey. It is estimated that more than 80% of Cd contamination occurs by ingestion of vegetables and cereals [17]. In most of the vegetables, which are taken on daily basis, the accumulation of Cd in such case increases the health risk index with increasing concentration of it because of its direct toxic effect on human health. Nowadays, vegetables are one of a key food component and without this life is almost impossible but the increasing concentration of cadmium (Cd) in the food chain vegetable continuum is posing a threat to their growth as well as human life thus creating a real threat in present scenario and needs more progressive research to explore for possible solutions. Keeping all this in mind, this study has been conducted focusing the first time in Iraq showing effect of Cd on tomato cultivars as to screen the best varieties for health and productivity for local people.

2. MATERIALS AND METHODS

2.1 Experimental Design

The nine tomato seeds/cultivators (i.e., T59, T0-9, TM-1, Red-T, Chr-t, Dr-Tmto, Redone-T, Bgt-10 and Tmt-9) were purchased from local seed market at Baghdad, Iraq. The seeds were kept 25-27 °C until to be used. Pitgrow ready soil media in small plastic pots for *in vitro* experiment having all essential micro and macro nutrients was used for seed germination and seedling growth. In such manner two types of media were used for culture experiment. One is the control media that only contain Pitgrow media and other with Cd treatment media. All the seeds were sterilized well with 60% ethanol for 2 min followed washed by distilled water for various times. We inoculated three seeds of each variety into each cultured bottle which further repeated thrice. All cultures were maintained at 25-27 °C at 14:10 photoperiods at University of Misan, College of Agriculture, Iraq. Germinated seeds were counted daily according to the seedling evaluation procedure. The seeds were considered as germinated when the radical size was 2 mm. The number of germinated seeds was recorded every 24 h. In physical characteristics, 20 days after inoculating, the germination percentage using the formula (Germinated seeds number/total seeds × 100) for each replication of the treatment. After growth of seedling, the parameters included mean germination time (MGT = Σ (n × d)/N, where *n* is number of seeds germinated on day *d* and N is the total number of germinated seeds at 10^{th} day, seedling shoot height and root length were measured in cm. In cadmium treatment for growth evaluation of tomato varieties, 50 seeds were sown in each replicate. $CdCl_2$ solutions at 15 mg/kg (based upon our literature and lab trails)

concentration were applied at the time of sowing respectively. Seed germination was recorded as total number after 15 days according to whether the planetules came up obviously from soil. The comprehensive assessment of tomato cultivars expressed a total score obtained through evaluating four parameters after Cd treatment from high to low (the only exception was in MGT from low to high) with the score from the highest 5.00 points to the lowest 0.50 point. Overall, all the experiments were laid out in a Complete Randomized Design (CRD) with nine treatments (replicated thrice) for varietal comparison and two treatments (replicated thrice) for comparing tomato plants treated with cadmium and control.

2.2 Data Analysis

All the obtained data were analyzed using Student T-test for comparison of the significance of difference between the control and Cd treatment at $P < 0.05$. The data for tomato varietal studies were analyzed using Analysis of Variance (Anova) and the significance of differences were further determined using least significant difference (LSD) test at $p < 0.05$ through SAS (ver. 8.1) software. The results were presented in Mean ± S.E/S/D.

3. RESULTS AND DISCUSSION

3.1 Effect of Cd on Tomato Seed Germination

The Cd treatment influenced on germination percentage of nine tomato cultivars as presented in Table 1 and Figure 1. The analyzed results showed overall significant difference in germinations percentage of tomato cultivars ($P < 0.05$). However, in comparison to control, only three cultivars such as T-59, T0-9 and Chr-t showed more prominent difference and other cultivars were with non-significant ($P > 0.05$). After treatment of Cd, only two cultivars showed an increase in germination percentage such as Tmt-9 and Chr-t; meanwhile other seven cultivars showed decreased in germinations percentage. The maximum percentage of 81.33 ± 11.67 was recorded in T-59 ($p = 0.0238$, $t = 3.5011$) at control and the lowest germination percentage of 13.10 ± 3.48 in Chr-t at control treatment ($p = 0.0215$; $t = 4.3558$).

After Cd treatment, the maximum and the minimum percentages of 62.67 ± 2.89 and 25.00 ± 5.00 were respectively observed on T-59 and Tmt-9. These findings clearly pointed out the effect of Cd on germination percentage of tomato cultivars; however, the most susceptible cultivars were recorded for Tmt-9. It has been well reported that germination is the most vulnerable stage of higher plants and seedling development [18]. Thus, when the seed surrounding is contaminated with Cd, indiscretion in seed germination may be often noticed [19, 20]. Similar findings were noticed in the present study when in comparison to control treatment, most of the tomato cultivars germinated less. These findings are in line with those reported earlier in which it was reported that Cd stress decreased seed germination, germination index and vigour index of different crops [21].

3.2 Effect of Cd on Mean Germination Time (MGT)

After germination percentage, the effect of Cd on mean germination time (MGT) of selected tomato

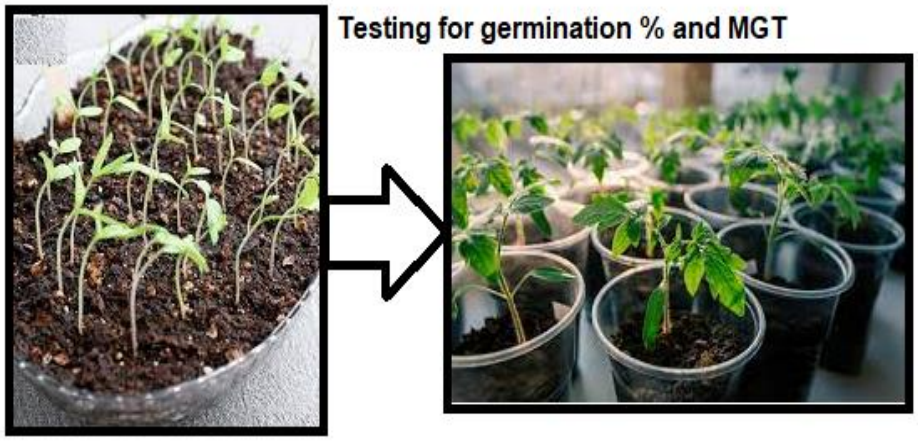

Fig. 1. Tomato seedling for testing germination percentage and calculation of Mean Generation Percentage (MGT) *in vitro* conditions.

Table 1. Effect of Cd on tomato seed germination percentage under in vitro culture.

Tomato cultivars	Control	Cd treatment	t value	p valve
T-59	81.33±11.67	62.67±2.89 a	3.5011	0.0238
T0-9	79.23±12.51	42.00±10.00 b	5.0071	0.0142
TM-1	49.47±10.72	38.00±5.08 b	1.6281	0.5055
Red-T	39.77±24.28	32.33±5.77 bc	1.2074	0.7721
Chr-t	13.10±3.48	31.33±7.64 cd	4.3558	0.0215
Dr-Tmto	36.47±23.60	29.33±7.64 cd	1.2281	0.6916
Redone-T	33.43±5.87	28.33±2.89 cd	2.4151	0.1811
Bgt-10	28.80±12.07	28.01±5.77 cde	1.2465	0.8919
Tmt-9	17.53±11.09	25.00±5.00 cde	2.1481	0.2422

cultivars was assessed. The results overall indicated a significant difference ($P < 0.05$) in MGT of tomato cultivars. Though, out of nine, six cultivars (TM-1, Red-T, Chr-t, Dr-Tmto, Redone-T and Bgt-10) took almost similar time to grow with non-significant difference ($P > 0.05$) in MGT between the control and the Cd treatment. In comparison to control treatment, Cd treatment increased the MGT in four tomato cultivars and decreased in six tomato cultivars (Table 2). However, the shortest MGT was recorded in T-59 (4.04 ± 2.57 days) than control (3.11 ± 1.01 days) and the longest in Tmt-9 (10.12 ± 2.61 days) than control (7.62 ± 4.48 days). The findings of this experiment show that T-59 had the lowest MGT than rest of other cultivars which showed less delayed in germination time meanwhile Tmt-9 found much influence with application of Cd as it delayed maximum with the highest MGT. In response to Cd treatment, the prolonged MGT may be due to inhibitory effect of Cd on germination ability of tomato seed as the Cd stress might confer reduced tolerance at the time of in vitro seed germination of tomato cultivars. Similarly, it has been observed that the higher Cd concentration in the Vigna unguiculata seeds seemed to prevent water uptake and water movement in the embryo axis which resulted in the delayed development with higher germination time of seeds [22].

3.3 Effect of Cd on Tomato Shoot Height

The results on the effect of Cd on shoot height of selected tomato cultivars under in vitro culture (Figure 2) revealed that there was a significant difference in shoot height between the control and the treatments (Table 3). The shoot height in comparison to the control decreased in response to Cd treatment in overall for all tomato cultivars. The highly significant difference between the control and the treatment ($p < 0.05$) was found in Tmt-9 as it looks heavy impact of Cd on shoot height (7.02 cm decreased to 1.61 cm). Meanwhile, T-59 produced maximum shoot height of 9.10 ± 2.03 cm in control and later after treatment of Cd remained higher 7.47 ± 0.26 cm as compared to rest of tomato cultivars and showed the lowest Cd

Table 2. Effect of Cd on Mean germination time (MGT) of tomato in vitro culture.

Tomato cultivars	Control	Cd treatment	t value	p value
T-59	3.11±1.01	4.04±2.57 d	0.4604	0.0149
T0-9	5.15±1.62	4.47±0.95 d	0.3392	0.0226
TM-1	5.32±0.51	5.37±1.42 bc	0.2392	0.5959
Red-T	7.94±2.11	6.33±2.14 bc	0.5021	0.4181
Chr-t	7.72±2.56	7.41±2.82 abc	0.2571	0.4025
Dr-Tmto	5.32±0.41	7.54±1.77 abc	2.2072	0.0623
Redone-T	7.62±1.62	8.10±0.12 abc	0.4101	0.6033
Bgt-10	8.01±3.11	10.40±5.41 abc	0.4341	0.6212
Tmt-9	7.62±4.48	10.12±2.61 a	0.6984	0.4695

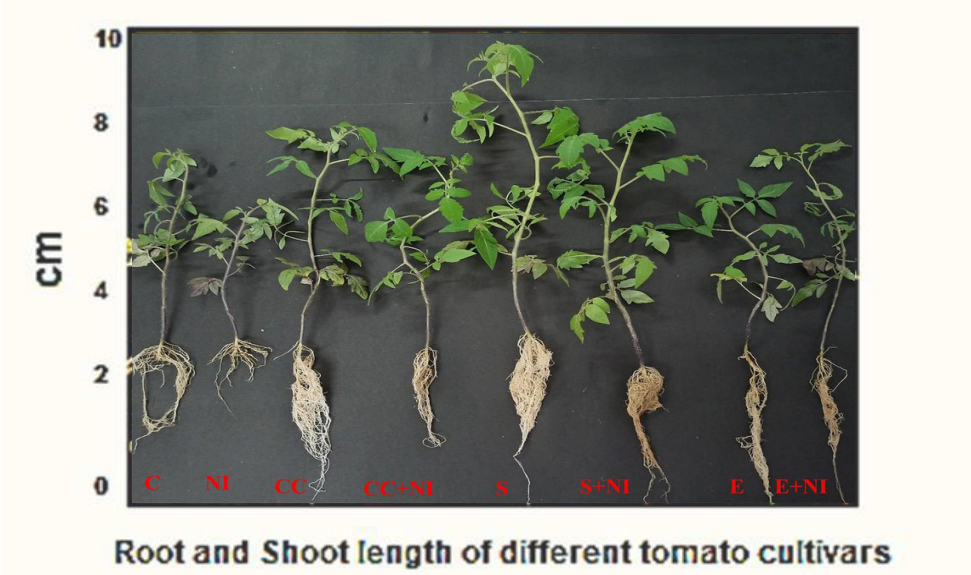

Fig. 2. Measurement of root and shoot height of tomato cultivars.

inhibition in term of shoot height. Similarly, these results also found non-significant differences in shoot height in some tomato cultivars ($P > 0.05$) such as between T-59 and T0-9 and among TM-1, Red-T and Chr-t likewise. There are so many instances of Cd absorption by plant in quite smooth way by roots and then transported to shoots [23]. Such transportation of Cd, results in physiochemical changes and then badly affects the plant growth [24]. Roots are likely to be affected by heavy metals since much more metal ions are accumulated in roots than shoots [23].

3.4 Effect of Cd on Tomato Root Length

A similar trend was found in the results regarding effects of Cd on root height of selected tomato cultivars under in vitro culture (Table 4). It is observed that there was a significant difference in root height between the control and the treatments. The root height in comparison to the control decreased in response to Cd treatment in overall all tomato cultivars. The highly significant difference between the control and the treatment ($p < 0.05$) was found in Tmt-9 with 6.00 ± 1.01 cm to 1.85 ± 0.16 (maximum inhibition in overall all tomato cultivars) and showed highly influenced with Cd application. On the contrary, the maximum root height of 3.41 ± 0.11 cm in T-59 after Cd application was recorded which showed less inhibition as the root length as in control the root height for T-59 was 7.10 ± 0.62 cm. Similarly, these results also found a non-significant differences in shoot height in some tomato cultivars ($P > 0.05$) such as between T-59 and T0-9 and among TM-1 and Red-T. These results are consistent with the findings of other authors who also reported the influence of Cd on different physiological and biochemical traits of

Table 3. Effect of Cd on shoot length (cm) of tomato in vitro culture.

Tomato cultivars	Control	Cd treatment	t value	p value
T-59	9.10±2.03	7.47±0.26 a	6.9845	0.0012
T0-9	9.64±0.97	7.32±0.54 a	8.9723	0.0601
TM-1	8.21±0.31	4.64±0.71 b	6.9531	0.0011
Red-T	8.31±0.67	3.91±0.82 b	4.7421	0.0230
Chr-t	9.32±0.82	5.14±0.94 bc	5.8574	0.0014
Dr-Tmto	7.44±1.51	3.23±0.64 c	2.3741	0.0221
Redone-T	8.11±1.32	3.05±0.41 cd	6.8828	0.0011
Bgt-10	7.47±0.56	2.24±0.23 de	12.6824	0.0002
Tmt-9	7.02±0.25	1.61±0.22 f	18.1010	0.0001

plants [25, 26]. Similarly, a significant reduction in root and shoot length was recorded in Phyllanthus amarus with higher Cd stress [27].

3.5 Comprehensive Assessment of Selected Tomato Cultivars

According to the multiple comparison results (Table 5) in which tomato cultivars with the same small letters ($p < 0.05$) from ANOVA statistical analysis were thought as the same ranking and given with same score from weighted average of their deserved total scores. Based on this, T-59 displayed the highest score with 16.00 points as it performed well in seed germination, mean generation time (MGT), shoot length and root length (ranked at the first). T0-9 showed the second comprehensive score with 14.75 points and the third highest score was observed in Red-T with 10.00 points. It was obtained with an equivalent level weight coefficient of four parameters, the tomato cultivars comparatively better than others under Cd treatment particularly, T-59, T0-9 and Red-T established on growth index and index including Cd absorption, translocation, tolerance as well as some physiological and biochemical responses to Cd by tomato germinated seeds and growing seedlings. Thus, it is obvious from all our findings that Cd toxicity obviously inhibited the observed parameters of tomato cultivars and similarly previously reported for plant root growth [21]. Furthermore, root, shoot and seedling length are more crucial for last accumulation site of Cd and considered as good indicators for metal toxicity [20].

Table 4. Effect of Cd on tomato root length under in vitro culture.

Tomato cultivars	Control	Cd treatment	t value	p value
T-59	7.10±0.62	3.41±0.11 a	9.0672	0.0002
T0-9	6.91±2.15	3.01±0.08 a	4.3535	0.0624
TM-1	7.28±0.73	2.01±0.14 ab	10.2843	0.0007
Red-T	6.35±0.32	2.11±0.12 ab	19.3212	0.0003
Chr-t	6.42±0.21	2.21±0.11 bc	35.5961	0.0003
Dr-Tmto	6.62±0.52	1.84±0.04 bc	15.7413	0.0032
Redone-T	6.71±0.51	1.71±0.13 bcd	16.8314	0.0002
Bgt-10	6.11±1.14	1.09±0.15 bcd	9.7974	0.0006
Tmt-9	6.00±1.01	1.85±0.16 cde	11.8948	0.0003

Table 5. Comprehensive assessment of selected tomato cultivars.

Cultivar name	Seed germination rate			Mean germination time			Shoot height			Root length			
	MCSD	Seq.	Score	MCSD	Seq.	Score	MCSD	Seq.	Score	MCSD	Seq.	Score	Aggregate score
T-59	A	1	5.00	ab	5	2.50	ab	3	3.75	a	1	4.75	16 .00
T0-9	Bc	8	3.00	A	1	4.00	ab	3	3.75	ab	3	4.00	14.75
TM-1	Cd	5	2.50	D	11	0.00	c	7	2.00	ab	4	3.50	8.000
Red-T	D	12	0.00	C	10	0.50	a	1	4.75	a	1	4.75	10.00
Chr-t	Cd	8	0.75	Ab	5	2.50	bc	5	3.00	cd	9	1.00	7.250
Dr-Tmto	B	2	2.25	Ab	2	3.50	cd	9	1.00	bc	5	2.75	9.500
Redone-T	Cd	8	0.75	A	3	3.75	bc	6	2.50	E	12	0.00	7.000
Bgt-10	Cd	8	0.75	abc	9	1.00	d	11	0.00	de	10	0.25	2.000
Tmt-9	D	14	0.00	abc	5	1.50	d	14	0.00	e	13	0.00	1.500

MCSD: Multiple comparison of significance of difference among the average of Cd treatment Seq.: The sequence of the average of Cd treatment from high to low

Score: List the first as 5 points, the second as 4.5 points, the third as 4 points, and so on until the tenth as 0.5 point. If several cultivars have it sequence, their score is same by calculating the average of total aggregate scores deserved.

4. CONCLUSIONS

The Cd treatment greatly influenced all observed parameters of each tomato cultivars more or less. But T-59 tomato cultivar was observed less affected by Cd treatment as it showed the shortest MGT, maximum shoot and root height and with the highest score ranking thus recommend for cultivation.

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Knowledge of Medical Students Regarding Antimicrobial Resistance

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Abstract: The discovery of antibiotics has enhanced the treatment outcomes of infectious diseases. Nevertheless, the injudicious use of antibiotics has triggered a global public health crisis and caused a worldwide spread of antimicrobial-resistant microorganisms. Antimicrobial resistance is slowly becoming a major health problem all around the world, especially in Iraq, and this might be due to the incorrect, unwise prescription of antimicrobial agents among some doctors, which gives rise to this problem. The present study aimed to estimate the knowledge of medical students in Baghdad regarding antimicrobial usage and resistance and to find the association of sociodemographic factors with knowledge scores. A descriptive cross-sectional study was conducted in six medical colleges in Baghdad. An online-based questionnaire was published and used in collecting the data. The data were reviewed and entered to be statistically analyzed in SPSS using the Chi-square test and were presented as frequencies, percentages, graphs and tables. The results showed that 44.1% of the studied sample had a fair knowledge of antimicrobial resistance. There was a statistical significance between gender and knowledge about antimicrobial resistance as the P-value was 0.006. In addition, there was a statistically significant association between the stage and the knowledge about antimicrobial resistance, where the P-value was (0.000). It was concluded that most of the participants had fair to good knowledge regarding antimicrobial resistance that was significantly associated with gender and stage.

Keywords: Antibiotic Resistance, Knowledge, Medical Students.

1. INTRODUCTION

The development of antibiotics has led to improvements in the diagnosis, management, and outcomes of infectious diseases. On the other hand, the irresponsible use of antibiotics has led to a crisis in public health on a global scale and contributed to the proliferation of microorganisms that are resistant to antimicrobials [1]. Antimicrobial stewardship refers to the process of selecting the most effective antimicrobial medication, as well as determining the appropriate dosage and length of time to take it, so as to achieve the best possible clinical outcome for the treatment or prevention of infection, while causing the patient the least amount of harm and having the least influence on the development of antibiotic resistance [2]. There are a variety of factors that can affect antibiotic prescribing and use. These include patients' knowledge and attitudes toward antibiotic use, doctors' knowledge and experiences, patients' interactions with prescribers, and the availability of unregulated drugs and lax health policies regarding regulations on antibiotic use [3, 4].

Antimicrobial resistance is a growing problem that poses a danger to the efficacy of antimicrobial drugs used to treat infectious illnesses. It happens when bacteria, viruses, fungi, or parasites undergo conformational changes that prevent them from responding to treatments. This complicates infection management and heightens the danger of spreading disease, experiencing severe illness, and dying [5]. Since it causes severe infections and extended hospital admissions, increases in healthcare expenses, higher prices of second-line treatments, and treatment failures, antimicrobial resistance is seen as a major danger to public health systems worldwide, not only in poor nations [6]. Around 700,000 deaths a year may be attributed to drug resistance, making it one of the leading causes of mortality worldwide. If current trends

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continue, antimicrobial resistance is projected to cause over 10 million deaths annually and over 100 trillion US dollars in lost productivity throughout the world by the year 2050 [7]. Without an effective tool for prevention, available efficient treatment of resistant infections, and improvement of already existing antimicrobials, the number of patients with treatment failure will increase, and medical operations such as cesareans section, joint replacements, chemotherapy, transplantations will be more insecure [8].

There are a number of approaches that have been proposed for the administration of antibiotics. These include the replacement or restriction of formularies, the education of health care providers, the implementation of response activities, the requirement of approval from an infectious disease specialist for the prescription of drugs, and a more rational application of antimicrobial agents in every region of the world [1]. Students at medical schools are being trained to become primary care doctors who will serve the community. These future medical professionals are on the front lines of the battle against antimicrobial resistance, because they prescribe antibiotics responsibly and educate patients about the issues. There is ample evidence to support the contention that freshly licensed physicians and prescribers do not have the proper training necessary to administer drugs in a safe manner. It's possible that one of the causes for this is inadequate training received by students throughout their time in medical school [1]. The World Health Organization has placed a strong emphasis on the need to providing medical students with appropriate and effective training in the prudent prescription of antibiotics. In addition, antimicrobial stewardship has been recognized as a discipline in medicine that is quickly expanding and has the objective of making reasonable use of antibiotics in terms of dose, length of treatment, and method of administration. It is essential that students in the healthcare field be made aware of the dangers presented by antimicrobial resistance, and that they get enough instruction on the themes that are pertinent to the appropriate administration of antibiotics in their respective fields of practice [9]. Thus, the aim of the current study is to estimate knowledge of medical students in Baghdad regarding antimicrobial use and resistance. Moreover, to find the association of sociodemographic factors with knowledge score.

2. MATERIALS AND METHODS

A descriptive cross-sectional study was conducted at Al-Kindy College of Medicine during the period from the 1st of November 2021 to the 30th of January 2022. A convenient sample of 365 medical students from various universities in Baghdad was enrolled in the current study. These universities included: University of Baghdad/College of Medicine, University of Baghdad/Al-Kindy College of Medicine, Al-Mustansiriyah University/College of Medicine, Al-Nahrain University/College of Medicine, Al-Iraqia University/College of Medicine, University of Ibn-Sina for Medical and Pharmaceutical Sciences/College of Medicine.

Pilot study: A pilot study was conducted among 20 medical students, to assess the compliance and response of students, to find out any difficulty of any unclear question, and to find any other questions or aspects that may affect students that were not included in the questionnaire. Fortunately, after this pilot study, no significant changes were made to the questionnaire. For that reason, the twenty recruited students were included in the study.

Data collection: An online questionnaire by Google forms was used to collect the data. The questionnaire was adopted from previous studies measuring the same studied variables, the supervisor and panel of experts revised the questionnaire in Al-Kindy College of Medicine (Two Community Medicine, two Family Medicine, and one Pharmacology) and their modification and advice regarding the proposed questionnaire were considered.

Ethical and official approval: The conduction of the study was approved by the Ethical and Scientific Committee at Al Kindy College of Medicine/Family and Community Medicine department. All participants were informed that their responses would remain confidential, and permission to participate in the study was obtained during the data collection.

Statistical Analysis: Collected data were reviewed, entered into Microsoft Excel Sheet 2016 and loaded into the SPSS software version for statistical analysis. Descriptive statistics were presented as frequencies and percentages. The Chi-square test was used in inferential statistics to find

the significance of related variables. A P-value < 0.05 was considered as the discrimination point of significance.

Scoring: The Knowledge score was calculated by dividing the total number of correct answers in each Knowledge item by the total number of questions in that item, and the results were multiplied by 100%. As in the following example:

Knowledge score = (Number of knowledge questions answered correctly)/ (Total number of knowledge questions) × 100

A score of < 50 was considered 'poor', a score of 50-75 was considered 'fair', while a score of >75 was considered 'good'.

3. RESULTS AND DISCUSSION

A total number of 365 medical students were included in the current study. The majority of the participants (60%) were females, 68.8% aged 20 years and older. Most of the participants (44.1%) were from Al-Kindy College of Medicine. In addition, 81.9% of the participant were in the preclinical stage (Table 1). Regarding the responses to knowledge questions, the following questions (5, 24, and 3) had the highest percentage of corrected answers as 88.5%, 83.4%, and 82.1% of the participants correctly answered these questions, respectively. At the same time, the lowest percentage of corrected answers was regarding question 6 questions (13%),

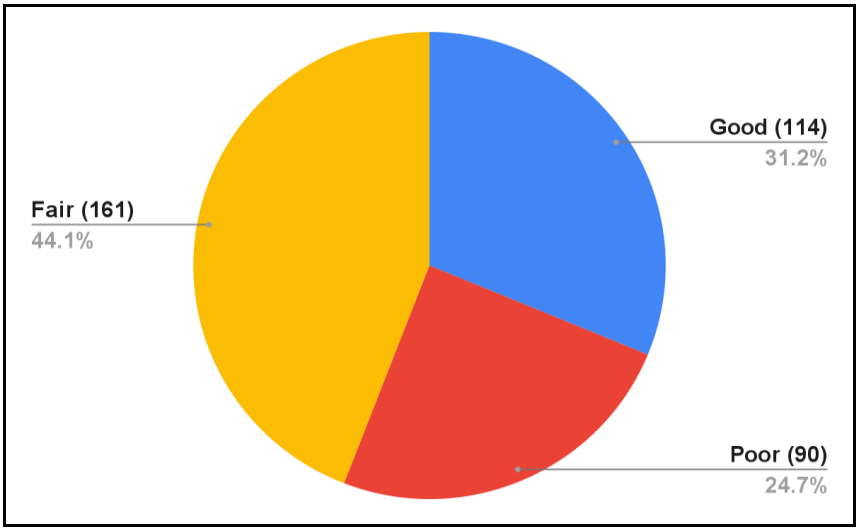

Fig. 1. Distribution of the studied sample regarding Knowledge score.

Table 1. General characteristics of the participants.

	Categories	No. of Participants	Percentage (%)
Gender	Male	146	40.0
	Female	219	60.0
Age	< 20 years	114	31.2
	≥ 20 years	251	68.8
College	Baghdad	52	14.2
	Al-Kindy	161	44.1
	Al-Iraqia	19	5.2
	Al-Nahrain	61	16.7
	Al-Mustansiriya	44	12.1
	Ibn-Sina	28	7.7
Stage	Preclinical	299	81.9
	Clinical	66	18.1
Residency	Baghdad	233	63.8
	Other than Baghdad	132	36.2

Table 2. Knowledge of the studied sample.

Sr. No.	Question	True		False		Uncertain	
		No.	%	No.	%	No.	%
1	Overuse of antibiotics reduces their effectiveness in the long run, a phenomenon known as antimicrobial resistance.	290	77.3	38	10.1	47	12.5
2	Flu and colds are caused by bacteria.	72	19.2	262	69.8	41	10.9
3	When it comes to public health, antibiotic resistance is a major and urgent problem throughout the world.	308	82.1	14	3.7	53	14.1
4	Improper and unnecessary use of antibiotics might compromise therapy efficacy.	280	74.6	28	7.4	67	17.8
5	Penicillin or amoxicillin are antibiotics.	333	88.8	13	3.4	29	7.7
6	Aspirin is an antibiotic.	50	13.3	273	72.8	52	13.8
7	Infections become resistant to antibiotics, including paracetamol, if you take it too often.	72	19.2	144	38.4	159	42.4
8	To treat bacterial infections, antibiotics are helpful (e.g., tuberculosis).	291	77.6	30	8.0	54	14.4
9	For viral infections, antibiotics might be helpful (e.g., common cold, influenza).	85	22.6	241	64.2	49	13.0
10	The use of antibiotics is recommended for the treatment of any condition characterized by pain or inflammation.	101	26.9	189	50.4	85	22.6
11	Side-effects of the so-called “good bacteria” in our bodies may be eliminated by antibiotics.	291	77.6	24	6.4	60	16.0
12	When antibiotics kill out good bacteria in our body, they might leave us vulnerable to other illnesses.	239	63.7	32	8.5	104	27.7
13	Some antibiotics have been linked to severe allergic responses.	292	77.8	16	4.2	67	17.8
14	Infections caused by strains of Staphylococcus aureus resistant to methicillin may be successfully treated with ampicillin.	107	28.5	73	19.4	195	52.0
15	When used to treat infections caused by methicillin-resistant Staphylococcus aureus, clindamycin has been shown to be very successful.	114	30.4	33	8.8	228	60.8
16	Antibiotic resistance occurs when germs stop being treated by an antibiotic.	286	76.2	37	9.8	52	13.8
17	If your condition improves while taking antibiotics, you may stop taking them before the prescribed number of days has passed.	74	19.7	247	65.8	54	14.4
18	Resistance often arises because of inadequate or nonexistent infection control procedures.	231	61.6	51	13.6	93	24.8
19	Antibiotic resistance increases the perilousness of medical treatments such as surgery, transplants, etc.	257	68.5	33	8.8	85	22.6
20	The transmission of antibiotic-resistant germs from one individual to another is a real health concern.	172	45.8	127	33.8	76	20.2
21	Regular antibiotic users are the only population at risk for developing resistance.	92	24.5	221	58.9	62	16.5
22	Antimicrobial resistance is an international problem.	129	34.4	127	33.8	119	31.7
23	Antibiotic resistance is an issue that can affect my family or me.	295	78.6	33	8.8	47	12.5

24	Infections brought on by germs that have developed resistance are notoriously difficult to cure.	313	83.4	21	5.6	41	10.9
25	Antibiotic resistance is on the rise, making it harder and harder to treat common diseases.	288	76.8	24	6.4	63	16.8
26	When the body develops a tolerance to antibiotics, this is known as antibiotic resistance.	202	53.8	134	35.7	39	10.4

Table 3. Association between essential studied variables and the knowledge level.

General characteristics		Knowledge score						P-Value
		Good		Fair		Poor		
		No.	%	No.	%	No.	%	
Age	<20 years	29	25.4	51	44.7	34	29.8	0.166
	≥20 years	85	33.9	110	43.8	56	22.3	
Gender	Male	58	39.7	62	42.5	26	17.8	**0.006**
	Female	56	25.6	99	45.2	64	29.2	
Stage	Pre-clinical	78	26.1	141	47.2	80	26.8	**< 0.01**
	Clinical	36	54.5	20	30.3	10	15.2	
College	Baghdad	22	42.3	21	40.4	9	17.3	0.149
	Al-Kindy	46	28.6	71	44.1	44	27.3	
	Mustansiriyah	10	22.7	25	56.8	9	20.5	
	Nahrain	25	41.0	19	31.1	17	27.9	
	Iraqiya	4	21.1	9	47.4	6	31.6	
	Ibn-Sina	7	25.0	16	57.1	5	17.9	
Residency	Baghdad	68	29.2	108	46.4	57	24.5	0.447
	Others	46	34.8	53	40.2	33	25.0	

as shown in Table 2. Regarding the distribution of the knowledge among the participants, 31.2 % had good knowledge, 44.1% had fair knowledge, and 24.7% of them had poor knowledge, as shown in Figure 1.

As shown in Table 3, the percentage of the participants who had good knowledge was significantly higher in males than in females (P-value = 0.006). In addition, there was a significant difference between the medical students in the clinical stages and those in the preclinical stages (P-value = 0.001).

Our results revealed that there is no significant difference in students' knowledge between different Iraqi colleges. This reflects uniformity in the syllabus and way of teaching practiced by Iraqi institutions. The main finding of the current study was that about one-third of participants had good knowledge regarding antimicrobial resistance. In comparison, it was better than the knowledge score obtained in other studies, as in Ethiopia, a study was done there in 2018 revealed that only 12% of participants had good knowledge regarding antimicrobial resistance [10]. While in Nigeria, a good knowledge score was achieved by 10.8% of the participants [7].

In contrast, other studies revealed a better knowledge score than what was obtained in the current study. A study was done in India in 2015 revealed that 98% of the participated medical student had good knowledge [11]. In Zambia, about 87% of the medical student had good knowledge regarding antimicrobial resistance, as revealed by a study done there [4].

Regarding gender, the males showed better knowledge regarding antimicrobial resistance than females. In another research conducted in Nigeria in 2019, males showed more knowledge about antimicrobial resistance than females, although females had better usage of antibiotics [12]. In

addition, the gender correlation to the knowledge about antimicrobial resistance is also affected by other factors: socioeconomic status and level of education [12]. The same findings were obtained by another study that was done by Wang *et al*. [13] in China in 2020. This might be due to males visit to healthcare facilities more often than females and seeking information about the subjects (including antimicrobial resistance) that they may be interested.

In the present research, participants younger than 20 years demonstrated a high level of understanding on the development of antibiotic resistance. In contrast, different research that was carried out in 2019 found that students who were between the ages of 22 and 26 years had four times the likelihood of having sufficient knowledge on antibiotic resistance compared to students in other age groups [14]. Students in this age range are most likely to have completed the foundational years of their education and may currently be in the para-clinical (year four) or clinical (years five and six) years of their education, during which they are exposed to pharmacology, microbiology, and other related fields that may influence their awareness of antimicrobial resistance [14]. The current study revealed that the students in the clinical stage had significantly better knowledge than others. The same results were obtained by another study in Malaysia in 2019, as most respondents possessed a good level of knowledge and practice regarding antimicrobials [15]. This reflected into the students' own behaviors towards the use of antimicrobials, since they utilize antimicrobials only when an official prescription is given to them. The closer the students were to graduation (clinical years), the better their knowledge and abilities were [15]. In China, a study was done there revealed that medical students with clinical experience had significantly better knowledge regarding than those without clinical experience [13].

Limitations: The study is restricted to medical students in Baghdad. It didn't include students from other Iraq provinces. Inclusion of such areas may give different picture.

4. CONCLUSIONS

The main finding of the current study was that most participants had fair to good knowledge regarding antimicrobial use and resistance, more precisely, about one-third of the participants showed good knowledge. Participants younger than 20 years of age, exhibited a high level of understanding about the development of antibiotic resistance. There was a statistically significant association between knowledge score and gender and stage. The students in the clinical stage had significantly better knowledge than others. Moreover, there was no significant difference in students' knowledge between different Iraqi colleges, which reflects uniformity in the syllabus and way of teaching practiced by Iraqi institutions.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Risk Factors and Clinical Patterns of Infertility in Couples: A Hospital-based Cross-sectional Study in Southern Khyber Pakhtunkhwa, Pakistan

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Abstract: Infertility is ranked as the fifth-leading cause of disability in the world's population under 60 years old, according to the World Health Organization, affecting an estimated 80 million people worldwide. This reproductive health disorder can be caused by various factors, including structural, biological, and congenital issues, as well as acquired and environmental variables. To investigate the prevalence and potential risk factors of infertility, a hospital-based cross-sectional study was conducted in district Kohat, Pakistan, from January to May 2021. A total of 120 infertile couples were recruited from the outpatient center at Liaquat Memorial Hospital, and their medical files were reviewed, followed by face-to-face interviews with both partners of each couple. Of the participants, 47.5% were suffering from primary infertility, while 52.5% were affected by secondary infertility. Female infertility was the most common form of infertility, affecting 49.16% of couples, followed by male infertility (15.83%), couples with both partners facing infertility issues (8.33%), and unspecified infertility (26.66%). The most frequent causes of male and female infertility were erection issues (48.27%) and menstrual disorders (44.92%), respectively. Additionally, 46.34% of couples reported stress and anxiety, and 14.16% had a history of assisted reproductive technology. Furthermore, 18.3% of couples had a family history of infertility, with 77.27% of those affected being infertile females. Infertile couples were also assessed based on potential risk factors, including age difference, age at menarche, occupation, smoking, and Body Mass Index (BMI). These findings may aid in identifying the factors contributing to infertility among the population.

Keywords: Cross-sectional Study, Infertility, Risk Factors, Clinical Patterns, Reproductive Health Disorder.

1. INTRODUCTION

Infertility is the incapability to conceive after trying for one year without the use of contraceptive methods while having normal sexual intercourse [1]. Infertility is the fifth-leading cause of disability in the world's population under 60 years old, according to the World Health Organization. An estimated 80 million people worldwide are affected severely by the reproductive health disorder of infertility [2]. The frequency of infertility in Pakistan is 22% where, primary infertility is 4% and secondary infertility is 18.0% [3]. Primary infertility is a condition where a couple cannot get pregnancy after 1 year of unprotected sexual intercourse. Secondary infertility is the condition where a couple has perceived previously but became

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unable then [4]. Numerous causes of infertility have been reported in studies on different populations, including structural, biological, and congenital factors. Additionally, a number of acquired and environmental variables can affect fertility and cause infertility. The most frequent causes of reproductive impairment include uterine problems, menstrual and ovulation disorders, and irregular menses. The causes of infertility and their patterns of occurrence vary greatly across geographical areas. This discrepancy results from the presence of changing environmental factors that are connected to reproductive activities, such as altering lifestyle and dietary patterns, environmental pollution, age at marriage, and smoking and drinking habits [5]. Deficits in spermatogenesis or sperm transport are the main causes of male infertility. Analysis can be confirmed by doing a complete assessment of semen analyses, gonadotropin and other tests [6]. Infections like gonorrhea and sexually transmitted illnesses used to be the main reasons of infertility, but today stress, male factor, etc. have taken their place. Additionally, it has been demonstrated that the problem of infertility is exacerbated by the rising frequency of lifestyle problems like obesity and addiction in young people as well as medical conditions like diabetes, hypertension, and hypothyroidism [7].

While infertility encompasses various factors, male infertility, particularly in the form of azoospermia, emerges as a significant concern, with non-obstructive azoospermia (NOA) representing the most severe manifestation of spermatogenic failure. Recent genetic investigations have identified specific variants in KCTD19, a gene associated with potassium channel tetramerization, in both Chinese and Pakistani populations experiencing NOA [8]. Couples who struggle with infertility often feel distressed because of societal norms and religious beliefs that may view infertility as a sign of failure on a social, emotional, interpersonal, or personal level [9]. As a result, infertility is not only a medical problem but also has a significant psychological impact and social shame [10]. To date, no studies have provided local estimates of primary and secondary infertility prevalence in District Kohat of the Khyber Pakhtunkhwa province. Therefore, the current study aims to evaluate the clinical patterns and distribution of infertile couples based on potential risk factors in this region.

2. MATERIALS AND METHODS

2.1 Study Area and Study Design

A cross-sectional study was conducted between November 2020 and April 2021, on a conveniently selected adult population, recruited from the outpatient center at Liaquat Memorial Hospital, a tertiary care hospital in district Kohat, of southern Khyber Pakhtunkhwa. Kohat is a medium sized district located at a distance of about 47 km from Peshawar, the capital of Khyber Pakhtunkhawa, located at 33°35′13N 71°26′29E with an altitude of 489 m (1607 feet) with total area is 2973 km^2 with total population of 7, 82,070 and annual growth rate of 3.25% [11]. Using a convenience sampling technique, 120 participants were enlisted after providing written, reliable, and informed permission. The study included married couples who had been together for more than a year and who did not take contraceptives in situations of primary or secondary infertility. The couple's ages ranged from 19 to 59. Participants from lower socioeconomic classes are more likely to use public hospitals for treatment than couples in middle- to higher-socioeconomic classes, who often choose private hospitals. The information was evaluated and retrieved from the medical records of infertile couples. Age, infertility type, duration, and factors thought to be contributing to infertility were among the details taken from the records. The online questionnaire was sent to infertile couples, and researchers also visited their homes to ensure complete questionnaire responses. Couples in which one partner was reluctant to undergo clinical assessment were excluded from the study.

2.2 Questionnaire Designing and Data Collection

In the study, data was gathered through face-to-face interview, using self-generated standardized questionnaire. To ensure conceptual coherence, the questionnaire was first written in English, then translated into the regional tongue (Pashto), and then back into English. Data for this study were gathered using a standardized four-section questionnaire. The participants' age, gender, occupation, and Body Mass Index (BMI) were all covered in the first section's socio-demographic questions. The patients' menstrual histories are covered in the second section. The third section

includes the patients' medical histories, including information on endometriosis, polycystic ovary syndrome (PCOS), hormonal imbalance, pelvic infection, semen analysis, undescended testicles, issues related to infertility, and treatment guidelines. The fourth part consist of social history and family history of infertile couple such as Cystic Fibrosis and hormone Imbalance.

2.3 Data Analysis

Data were managed in Microsoft Excel and descriptive statistics were performed using IBM SPSS Statistics (Version 23). The results were presented in form of tables and graphs to illustrate the distribution through numerical counts and percentages.

3. RESULTS

A cross-sectional study was conducted between November 2020 and April 2021 on a conveniently selected adult population of District Kohat in the Khyber Pakhtunkhwa province of Pakistan, with a sample of 120 infertile couples. Questionnaires were filled out by the infertile couples, which included questions related to various risk factors. For convenience, the results were divided into six sections. The first section consists of the types of infertility. The second section consists the demographic and socio-economic factors including age, BMI, family status and occupation. The third section of results is about the distribution of affected individuals on the basis of potential risk factors. The fourth section consist of disease associated with infertility. The fifth section consist of tendency of couple for assisted reproductive technology. Finally, the sixth section of the results is about the family history of infertility and disease associated with infertility.

3.1 Types of Infertility in Couples

A total of 120 couples were analyzed, and they were split into four standard groups: (a) Female affected (b) Male affected (c) Both affected (d) Unexplained infertility. Of the 120 couples studied, 19 (15.83%) were 'male affected' couples, bearing primary (63.1%) and secondary (36.8%) type of infertility, 59 (49.16%) were 'female affected' couples, bearing primary (28.8%) and secondary (71.1%) type of infertility. 10 (8.33%) were 'Both', both partners were affected, with 60% and 40% cases of primary and secondary types of infertility, respectively. Similarly, out of 32 (26.66%) Unexplained couples, no partner specified as infertile, 22 (68.75%) cases were affected with primary and 10 (31.25%) and were affected with secondary infertility (Figure 1).

3.2 Demographic and Socio-economic Factors of Infertile Couples

The socio-demographic characteristics of the participants are presented in Table 1. Among 69 affected females, women under age of 25 were 10 (14.49%), ranging 25-35 were 40 (57.97%) and ranging 36-45 were 19 (27.53%). Among 29

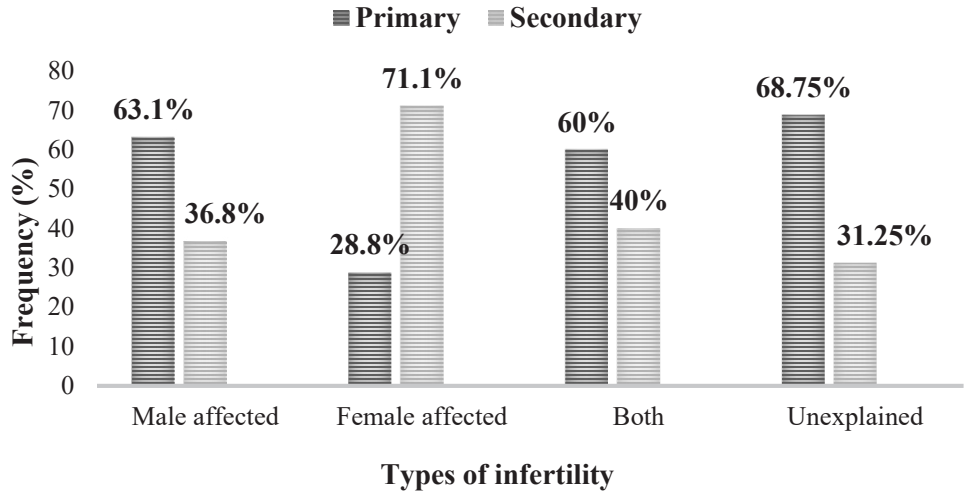

Fig. 1. Types of infertility in couples participated in the study (N = 120).

Table 1. Demographic and socio-economic factors of infertile couples in the study (N = 120).

S. No.	Demographic variables	Categories		N	(%)
1	Women's age	<25 years old		10	14.49
		25-35 years old		40	57.97
		36-45 years old		19	27.53
2	Husband's age	< 25 years old		4	13.79
		25-35 years old		10	34.48
		36-45 years old		6	20.68
		46-56 years old		9	31.03
3	Couple's age difference	< 1 year		2	1.66
		1-5 years		73	60.83
		6-10 years		26	21.66
		>10 years		19	15.83
4	'Female Affected' Occupation	Housewife		45	76.27
		Teachers		5	8.47
		Medical profession		5	8.47
		Any other		4	6.77
5	'Male Affected' Occupation	Govt. employee		2	10.52
		Teachers		2	10.52
		Labor		5	26.31
		Any other		10	52.63
6	'Both' Occupation	Male	Teacher	2	20.00
			Any other	8	80.00
		Female	Teacher	3	30.00
			Housewife	7	70.00
7	'Unexplained' Occupation	Male	Medical	7	21.87
			Farmer	4	12.5
			Labor	3	9.37
			Teachers	3	9.37
			Govt. employee	3	9.37
			Others	2	6.25
			Unemployed	10	31.2
		Female	Housewife	26	81.2
			Teachers	4	12.5
			Medical	2	6.25
8	Family status (Per month income)	Poor (< Rs. 5000/adult) Low (Rs. 5000 – 10000/adult) Middle (Rs. 10000-20000/adult) High (>Rs. 20000/adult)		14 24 70 12	11.6 20.0 58.3 10.0

affected males, men under the age of 25 years were 4 (13.79%), between 25-35 years were 10 (34.48%), between 36-45 years were 6 (20.68%) and between 45-56 years of age were 9 (31.03%). On the basis of age difference between partners in a couple, the infertile couples (N=120) were categorized into four groups with age less than one year (1.66%), one to five years (60.83%), and six to ten years (21.66%) and more than 10 years of age were 15.83%. On the basis of occupation, the infertile couples were categorized into four groups, i.e., females affected, 45 (76.27%) women were housewives, 5 (8.47%) were teachers, 5 (8.47%) were having medical profession, and 4 (6.77%) were belonging to other profession. In male affected couples 2 (10.52%) were government employee, 2 (10.52%) were teachers, 5 (26.31%) labor, 11 (57.89%) were having other profession. In couples with both partners affected, 2 (20%) man were teachers, 8 (80%) were having others profession and in 3 (30%) women were teachers and 7 (70%) were housewives. In Unexplained infertile couples, 7 (21.87%) males were associated with medical profession, farmers 4 (12.5%), labors 3 (9.37%), teachers 3 (9.37%), government employees 3 (9.37%), other professions 2 (6.25%), and unemployed 10 (31.2%). In unexplained infertile females 26 (81.2%) were housewives, 4 (12.5%) teachers and 2 (6.25%) were having medical profession. The financial status of the infertile couples was categorized into four groups: poor, low, middle, and high. The majority of cases belonged to the middle-class category in terms of family income.

3.3 Distribution of Affected Individuals on the basis of Potential Risk Factors

The female BMI index or status, was determined in which 11 (15.94%) are normal females, 26 (37.68%) were over weighted and 22 (31.88%) were obese. In males 4 (21.05%) are normal, 10 (52.63%) were over weighted and 5 (26.31%) were obese. In Both cases 2 (20%) are normal couples, 5 (50%) were over weighted and 3 (30%) were obese. In unexplained 5 (15.62 %) are normal couples 17 (53.12%) were over weighted and 10 (31.25%) were obese. 7.5% of the respondents had a history of smoking as well. The history of addiction was also reported by male, a total of 16 respondents (13.3%), including Naswar and Hashish, which was identified as the most popular substance utilized whereas little to no drug usage was observed in women. (Table 2).

3.4 Midwifery Variables in Couples

Table 3 shows the duration of infertility and the age at menarche. The majority (43.8%) of couples had primary infertility of 3-5 years' duration and 34.9% of couples had secondary infertility of 4-5 years' duration.

3.5 Distribution of Couples with respect to Clinical Factors Associated with Infertility

Figure 2 shows the distributions of infertile females (N = 69) with respect to female factors. Analyzing the female factors in detail where it was responsible for infertility, it was found that the main causes originate in our study population were the Menstrual disorders (44.92%) and Hormonal imbalance (40.57%). Other causes of female infertility included endometriosis (5.79%), Ovarian cyst (1.44%), Uterine cyst (4.34%), PCOS (24.63%), and Fallopian tube blockage (8.69%). Figure 3 shows the occurrence of menstrual disorders in affected females (N = 69) among the infertile couples. The occurrence of menstrual diseases in affected females were as follow: Menorrhagia (18.44%), Dysmenorrhea (51.43%), Oligomenorrhea (21.35%), and Metrorrhagia (8.73%). Figure 4 shows the distributions of infertile males (N = 29) with respect to male specific clinical factors. Male infertility was most frequently caused by erection problems (48.27%) and ejaculatory problems (27.58%). The most often diagnosed instances, according to the findings of the semen analysis, were Azoospermia (17.24%), Asthenospermia (3.44%), Payospermia (6.89%), Hematospermia (3.44%), Oligospermia (10.34%), Teratozoospermia (3.44%) respectively. There were two cases of undescended testicle (10.34%).

3.6 Associated Diseases, may Influence Fertility, in the Infertile Couples

Data were collected from infertile couples regarding various chronic diseases. Out of 120 affected couples, 82 reported having a chronic medical condition in either male, female or both partners. The results showed highest frequency of

Table 2. Potential risk factors in infertile individuals in couples (N = 120).

S. No.	Variables	Categories	N	(%)
1	'Male affected' BMI status	Normal	4	21.05
		Overweight	10	52.63
		Obese	5	26.31
2	'Female affected' BMI Status	Normal	11	15.94
		Overweight	26	37.68
		Obese	22	31.88
3	'Both' BMI status	Normal	2	20.0
		Overweight	5	50.0
		Obese	3	30.0
4	'Unexplained' BMI status	Normal	5	15.62
		Overweight	17	53.12
		Obese	10	31.25
5	History of Miscarriage	Yes	31	25.8
		No	89	74.2
6	Number of miscarriages (n=31)	Once	20	64.5
		Twice	4	3.33
		3 times and more	7	5.83
7	History of smoking	Yes	9	7.5
		No	111	92.5
8	History of drug addiction	Yes	16	13.3
		No	104	86.6
9	Tea or coffee per day	1 cup	16	13.3
		2 cup	45	37.5
		3 cup	35	29.1
		4 cup and more	24	20.0

Table 3. Midwifery variables of infertile couples (N = 120).

S. No.	Variables	Categories	N	(%)
1	Duration of primary infertility	2 years	13	22.8
		3-5 years	25	43.8
		5-10 years	14	24.5
		>10 years	5	8.7
2	Duration of secondary infertility	2 years	3	4.7
		3 years	14	22.2
		4-5 years	22	34.9
		5-10 years	19	30.1
		>10 years	5	7.9
3	Age at menarche	Early (≤11 years)	25	20.83
		Normal (12-14years)	87	72.5
		Late (≥15 years)	8	6.66

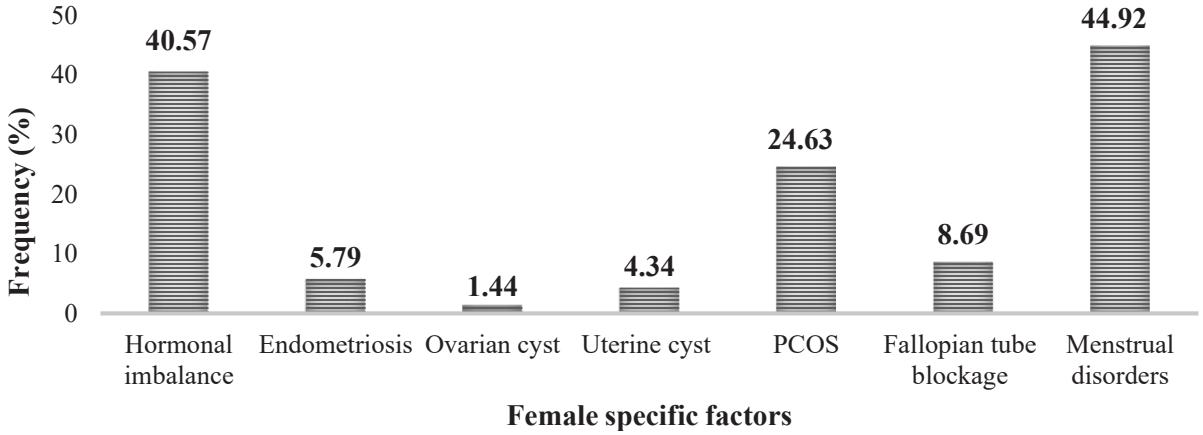

Fig. 2. Distribution of infertile females with respect to occurrence of clinical conditions (N = 69).

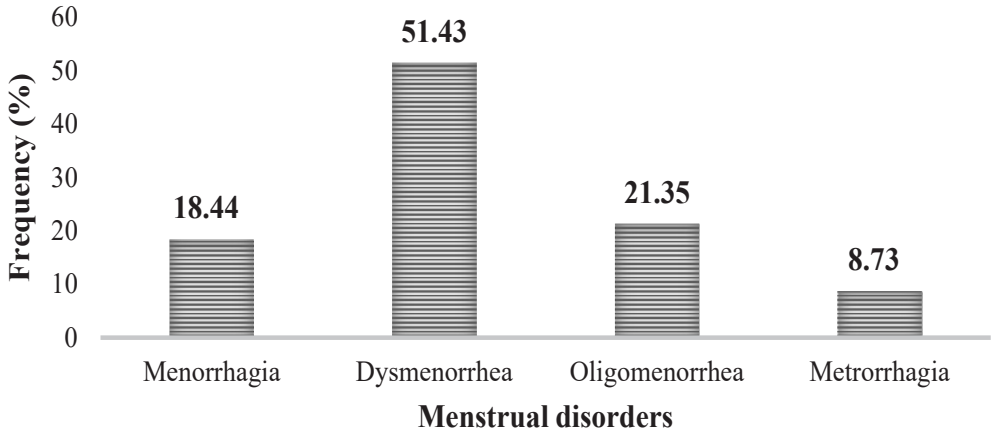

Fig. 3. Distribution of menstrual disorders on the basis of its type (N = 31).

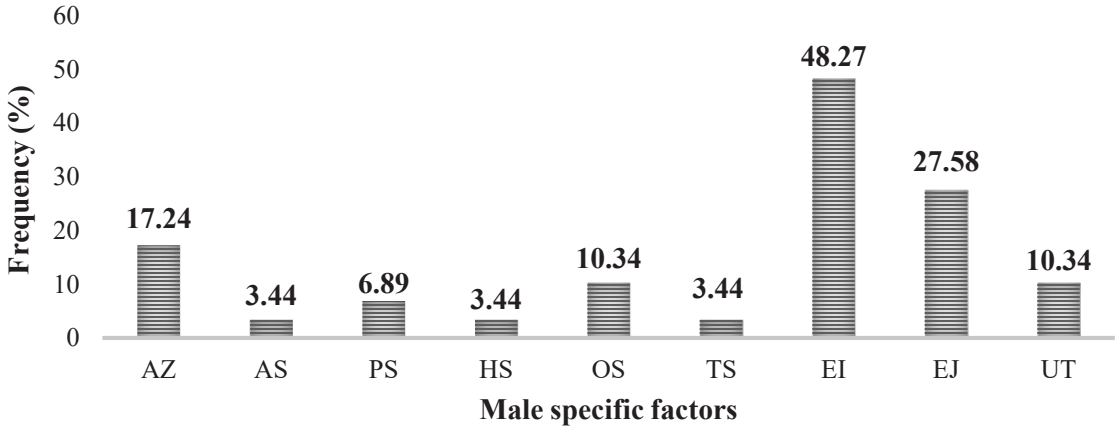

Fig. 4. Male specific clinical factors in infertile males (N = 29).
AZ= Azoospermia, AS=Asthenospermia, PS=Payospermia, HS=Hematospermia, OS=Oligospermia, TS=Teratozoospermia, EI=Erection Issue, EJ=Ejaculatory Issue, UT=Undescended testicle.

stress in infertile couples (46.34%), followed by high blood pressure (14.63%), Diabetes (12.19%), Thyroid disease (9.75%), Hepatitis (2.43%), and Tuberculosis (1.21%). It is significant to note that stress is usually observed in infertile couples due to social causes (Figure 5).

3.7 Attitude of Infertile Couples towards Assisted Reproductive Technology (ART)

Table 4 shows the tendency of couples for assisted reproductive technology. A total of 14.16% of the subjects had a history of ART and 29.16% were interested in ART. Figure 6 shows frequency of ART procedure in N = 17 infertile couples with positive history of ART. In 70.58% couples Ovulation induction was performed followed by Intra uterine insemination (IUI) in 17.6% infertile couples and *In vitro* fertilization (IVF) in 11.76% infertile couples.

3.8 Family History of Infertile Couples Participated in the Study (N = 120)

This bar graph shows two areas i.e., vertical lines show frequency of participants and horizontal lines shows familial/sporadic nature of the disease. The percentage of the sporadic infertility was 81.67% while 18.33% were having positive family history of infertility at least in one partner, familial cases (Figure 7). Among the N = 22 familial cases, 77.27% were in 'Female affected' couples while 22.72% were 'Male affected' couples. While no case with family history was found in 'Both' and 'Unexplained' categories of infertile couples. The details are shown in Figure 8.

3.9 Family History of Cystic Fibrosis and Hormonal Imbalance

Results about the family history of infertile females (N = 69) showed 12.5% of infertile females had a family history of cystic fibrosis and 9.16% shows hormonal imbalance (Figure 9).

4. DISCUSSION

Infertility is defined as the inability to conceive after attempting to do so for one year without using any contraceptive methods while having regular

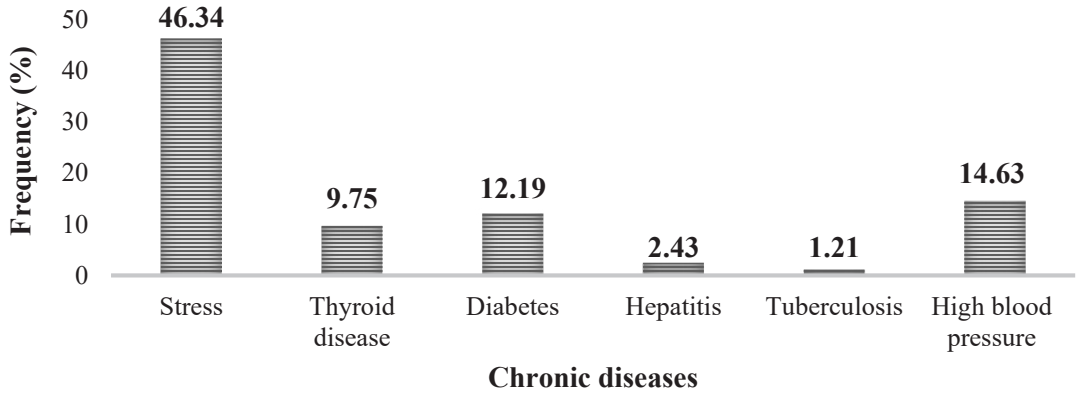

Fig. 5. Association of chronic disease in infertile subjects (N = 82).

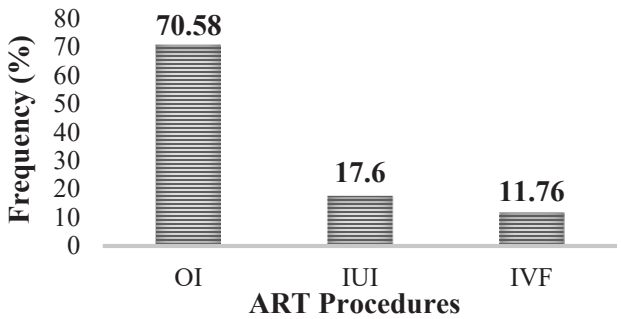

Fig. 6. History of ART procedures in infertile couples (N = 17).

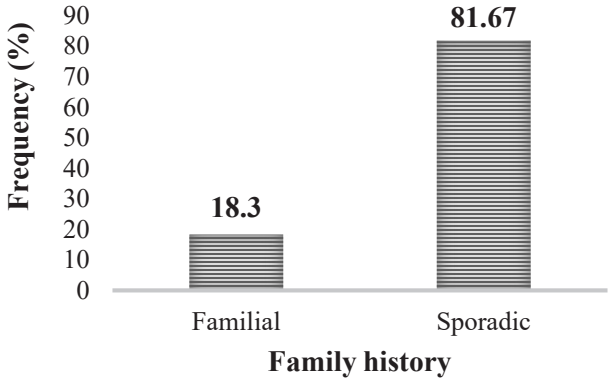

Fig. 7. Family history of infertile couples.

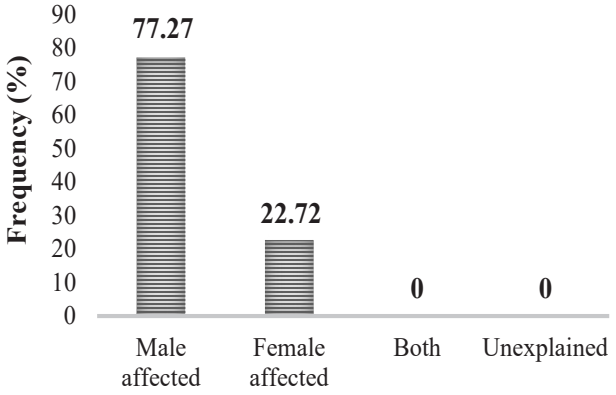

Fig. 8. Gender wise distribution of familial cases.

sexual intercourse [1]. Data analysis showed among 120 couples, 32 were with unexplained infertility. In their assessment of the literature from 1950 to 2013, Gelbaya *et al*. [12] discovered that 15% to 30% of couples would still experience infertility after having routine fertility testing performed. While 88 couples were with either single or both partners identified as infertile. In the N = 120 females, women age >35 years were 27 (22.5%). The age of the woman and her age of marriage are

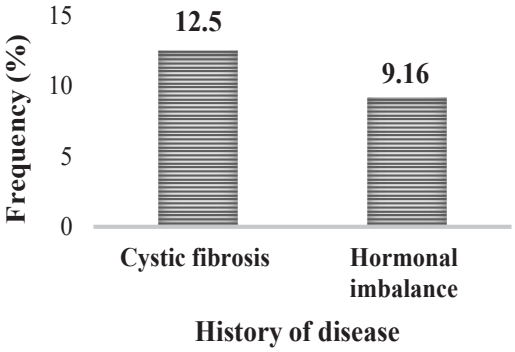

Fig. 9. Family history of CF and HI among infertile females (N = 69).

Table 4. Tendency of couples for assisted reproductive technology (ART).

S. No.	ART		N	(%)
1	History of ART (N=120)	Yes	17	14.16
		No	103	85.83
2	Interested in ART (N=103)	Yes	35	29.16
		No	85	70.83

factors that cause infertility, according to the current study and another Iranian study [4]. According to reports, one of the key factors contributing to female infertility is age [13]. The woman's fertility peaks between the ages of 18 and 24. After that, it progressively drops until the age of 27, when it suddenly declines around the age of 35. In other words, ovarian reserves get smaller as you get older [14]. According to the current study, the majority of affected males are in the middle of their ages and have been diagnosed as infertile. An earlier study revealed that the age factor has no effect on male infertility [15].

In the present study, 21.66% of infertile women were employed and 78.33% women were unemployed. In contrast to the current study, the two studies performed in Finland, maternal occupation in the field of agriculture, and in the second case in fur farming, was found to be associated with a significantly increased risk of spontaneous abortion [16]. The findings of the present study did not show a significant correlation between female occupation and infertility. However, it is worth mentioning that most females in Pakistani population tend avoid working outside their homes. Further research with larger sample size of individuals who are directly exposed to chemicals for longer periods is recommended. In this study, 80.83% of husbands were employed and 19.16% were unemployed. Previous studies revealed that employed men's odd ratios for male factor infertility were 1.55 times greater than those of unemployed men [17].

In the present study, infertility in overweight women 69 (57.5%) were higher compared to normal women. Another study found that the likelihood of obesity and overweight was, respectively, 4.8 and 3.8 times higher in infertile women than in fertile ones [18]. Furthermore, Cong *et al.* [19] indicated a substantial connection between the female infertility factor and BMI more than 30. In males 38 (31.66%) were over weighted. In present study,

among the 120 infertile couples, primary infertility was 47.5% versus 52.5% of secondary infertility. A similar study performed by Sami and Ali [3] in Pakistan, showed that the frequency of secondary infertility surpasses the occurrence of primary infertility. Contrarily, data from India's National Family Health Survey revealed that primary infertility is more common than secondary infertility on average [20]. The results of other research conducted in Iran were consistent with the findings of the present study, which found that the female-specific variables linked to infertility were present in (49.16%) of infertile couples. The frequency of both male and female variables, both of them, as well as other unidentified causes, was typically 20–40%, 30–35%, and 5–15%, respectively [4, 21]. In the current study the occurrence of female specific infertility was 49.16%, male infertility was observed as 15.83%, in 8.33% infertile couples, both partners were affected, and 26.66% of the 120 couples had unexplained infertility. Result analysis manifested majority of the infertile couples were with multiple causative factors where just few couples were observed with a single cause of infertility. A comparable study carried out in Iran revealed that most infertile couples gave more than one explanation for their infertility, with only a small minority giving only one [4].

According to the findings of the current study, erection problems accounted for 48.27% of all 29 male infertiles, making them the most common cause of male infertility. The most often identified reasons, as determined by the results of the semen analysis, were azoospermia (17.24%) and oligospermia (10.34%). According to a prior Chinese study, varicocele is the most common cause of male infertility [22]. The high incidence of erectile dysfunction causing infertility can be attributed to the high frequency of males in older age groups, as the severity and occurrence of erectile dysfunction tend to increase consistently with age in males. In the present study it has been noted that hormonal imbalance had a significant role in ovulation disorders. Hormonal imbalances lower the quantity of eggs in the ovary and even lower the quality of the egg cell. Low gonadotropin levels cause infertility in 5–10% of women, while decreased hypothalamic GnRH secretion causes secondary estradiol. According to one study, tubal factors and ovarian causes were the two most common reasons of female infertility [23].

Several studies from Pakistan have identified several genetic factors responsible for infertility in consanguine. For instance, frameshift mutation in KCTD19 and SPATA22 has been reported to be associated with infertility in Pakistan [8, 24]. Additionally, MTHFR gene plays a major role in regulation of spermatogenesis with its C667T allele variants can disturb the process ensuing male infertility. However, a study by Fatima *et al.* [25] reported that MTHFR C677T polymorphism is not significantly associated with increased risk of male infertility in Pakistan. The present study showed that the underlying chronic disease may influence fertility. Glazer *et al.* [26] observed that infertile males had lower testosterone levels and higher levels of anxiety and stress. This increased the production of stress hormones and increased the risk of cardiovascular disease, diabetes, and death. The results of the current study indicated a connection between drug addiction and male infertility. According to another study, 60% of smokers were infertile [14]. Smoking alters a number of sperm characteristics, including quantity, motility, and antioxidant properties. This has an impact on the sperm normal morphology. In studies conducted by Penzias *et al.* [27] and Caserta *et al.* [28], it was found that cigarette smoking had a significant correlation with oligospermia (low sperm count) in men, as well as with progressive motility and reduced sperm volume. However, these studies did not find a significant association between cigarette smoking and alterations in sperm morphology.

5. CONCLUSIONS

In conclusion, our study identified the primary causes of infertility, with a higher prevalence observed in female-related cases. The most common causes of female infertility were menstrual disorders, hormonal imbalances, Polycystic Ovary Syndrome (PCOS), and endometriosis. These conditions, which disrupt the normal reproductive processes, were found to significantly contribute to the high rate of infertility among women. On the other hand, male-related infertility was predominantly caused by oligospermia (low sperm count), azoospermia (absence of sperm), and erectile dysfunction. These conditions, which affect sperm production and delivery, were the leading factors contributing to male infertility.

6. ETHICAL STATEMENT

The Undergraduate Research Ethical Committee, Department of Zoology, Kohat University of Science and Technology (KUST), Kohat, granted approval for the current study wide Ref. No. ZO-220192049, dated: August 17, 2021.

7. CONFLICT OF INTEREST

The authors declared no conflicts of interest.

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Research Article

Deficiency of Iron: A Risk Factor in Pregnant Women in the District Swat

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Abstract: Iron is an essential element for the body, its requirements increase during pregnancy. Improper use of iron may lead to anaemia in the mother. Anaemia may lead to complications such as abortion, stillbirth, and congenital abnormalities in the fetus of pregnant women. This study is aimed to determine the iron level and problems associated with iron deficiency in pregnant women in Swat District. Samples were collected from eight hundred pregnant women. The iron status was determined by measuring haemoglobin levels using Sahli's Method and the Haematology Analyzer. In total, 54% women were anaemic. The age group 41-45 was highly anaemic (100%) followed by 15-20 (74%) while the 31-35 age group was the least anaemic (42%). The Underweight was the highly anaemic group (83%) and the obese were the least anaemic group (12%). Pregnant women with second trimester gestational age were the least anaemic (34%) while the third trimester was the highly anaemic (67%). It was concluded that iron deficiency is a common issue in pregnant women in District Swat.

Keywords: Pregnancy, Anaemia, Haemoglobin, Swat.

1. INTRODUCTION

Iron is the fourth most plenteous component in the earth's crust [1]. In the world, the most related nutrient insufficiency is iron insufficiency. From iron insufficiency anaemia almost 500 to 600 million people are effected [2]. In pregnancy, iron supplementation is very essential and its supplementation had a defensive effect on adverse pregnancy outcomes [3]. Among pregnant women on an international level iron deficiency is the commonest nutritional insufficiency [4]. The metabolic processes include tissue oxygenation in that iron is a crucial component. An average individual has a total of 3-5 grams of iron. An average diet can supply up to 15 mg of iron per day. In several developed countries iron deficiency is the most common nutritional problem reaching an epidemic level worldwide. In Pregnancy, the risk of iron deficiency rises because pregnancy is related to increased demand for iron. Least iron stores in their newborn baby will increase the risk of iron deficiency. Further risk increases due to initially stopping breastfeeding and prematurity of reduced iron accumulation [5].

In pregnancy, the amount of haemoglobin and other red blood cells reduces because the volume of plasma is more than that of red blood cell mass. When there is a rise in red cell mass then the whole haemoglobin circulating also rises. This in turn depends relatively on the iron status of the individual. For pregnant women, the normal haemoglobin level is 12-16 g/dl and a value less than 12 is well-thought-out as iron deficiency and those less than 10.5 as anaemia. The pregnancies of anaemic women are less in duration than those of non-anaemic women. The absorption of iron can be influenced by several dietary factors [6]. The pregnant women having normal or high haemoglobin levels mean (>10 g/dl) gave birth to normal babies having weight (3.3 kg) while those having lower haemoglobin means (<10 g.dl) gave birth to babies having 2.6 kg weight [7]. In

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iron-deficient women, the haemoglobin level is decreased due to the incapability to increase plasma volume. The requirements for iron are more in pregnancy than in the non-pregnant state [8].

The normal haemoglobin level of healthy non-pregnant women is 12 g/dl. Pregnancy is a biotic situation and usually has no impact on the general health of a pregnant woman. However, pregnancy results in hormonal, hemodynamic and haematological changes. According to World Health Organization (WHO), haemoglobin should be retained at or above 11.0 g/dl and should not be allowed to fall below 10.5 g/dl in the second trimester. The normal iron supply for non-pregnant menstruating mature women is about 1.36 mg per day. The iron requirements are decreased in the 1st trimester because of an absence of menstruation. These rise subsequently as high as ≥10 mg/day. Iron requirements are increased in pregnancy, particularly in the 3rd trimester when there may be several times more than that in the early stages of the- pregnancy. In pregnancy, the total iron requirement is approximately 840 mmg [8].

Anaemia is a serious worldwide health problem. According to the World Health Organisation (WHO), anaemia is characterised to have haemoglobin (Hb) levels below 12.0 g/dl in females and below 13.0 g/dl in males [9]. Depending on the precise kind and degree of anaemia, several clinical signs and possible problems may be present. The expenditure for the treatment of anaemia varies according on the kind and severity of coexisting diseases, ranging from $29,511 for those with congestive heart failure to $7,092 for people with coexisting rheumatoid arthritis [10]. Further, anaemia decreased globally from 40.2% in 1990 to 32.9% in 2010, with a greater frequency of anaemia seen in females than in men and in those under the age of 5 [11]. The present study is aimed to determine the iron level and problems associated with iron deficiency in pregnant women in Swat District.

2. MATERIALS AND METHODS

2.1 Study Area

District Swat is in the north of Khyber Pakhtunkhwa Province and is famous for its pleasant beauty and culture. The district is lying in the lap of the offshoot of the Hindukush Mountainous Ranges, which are the sub-ranges of the world's greater Himalayan Ranges. District Swat includes seven tehsils: Kabal, Matta, Bahrain, Charbagh, Babozai, Barikot and Khwazakhela. The area of Swat is 5,337 km^2. According to the 2017 census population of swat is 2,309,570. Geographically District Swat lies at 35.2227° N, 72.4258° E [12].

2.2 Sample Size Collection and Questionnaire distribution

The data was collected from pregnant women by getting blood samples and by filling out questionnaires including the information: name, education, place, blood pressure, blood group, height, number of children, relation with husband, number of pregnancies, number of abortions, current pregnancy age, current Hb level, diet, any other disease, congenital abnormality and stillbirth. Random sampling was done from the local population.

2.3 Collection of blood

Venous blood was collected from each individual in a test tube containing ethylenediamine tetraacetic acid (EDTA). The blood samples were taken to the central laboratory for determination of haemoglobin by Sahli's method and Haematology Analyzer.

2.4 Sahli's Method

The haemoglobin tube (STD 14.5 gm=100% concentrated) was filled with N/10 hydrochloric acid (HCl) up to 2 gm marking. The graduated tube was placed in Sahli's hemoglobinometer. The Blood samples collected from capillary or venous blood were drawn in Sahli's pipette up to 20 micro litre mark and added to a haemoglobin tube containing N/10 HCl. Through a glass stirrer, the blood and acid were mixed and then allowed to stand for 5 minutes for the formation of acid hematin. In the comparator colour plates are present and for dilution of acid, hematin distilled water was added drop by drop till it matched with the standard colour plates of a comparator. The result was read as gms/dl present on the haemoglobin tube [13].

2.5 Haematology Analyzer

This is a very simple method used in laboratories. Blood samples collected from pregnant women

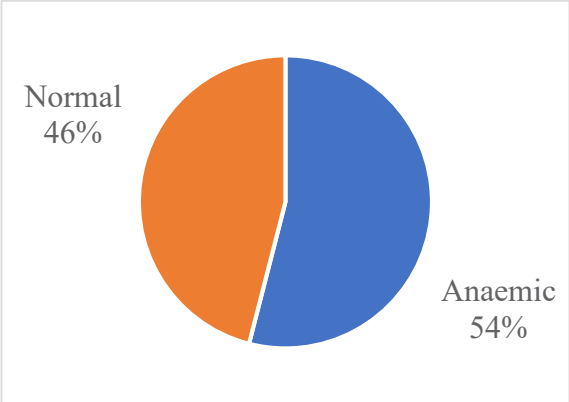

Fig. 1. Status of haemoglobin level in pregnant women.

in EDTA tubes were processed by an automated haematology analyzer. An automated haematology analyzer with an impedance method for complete blood count (CBC) measurement was used to measure Hb, mean corpuscular volume (MCV), and red blood count (RBC) [14].

2.6 Statistical Analysis

The data analysis was done by using SPSS software.

2.7 Ethical Approval

The oral and written consents was taken from the pregnant women from whom data and blood samples were taken.

3. RESULTS

A total of 800 samples were collected from pregnant women at District Swat among which 432 (54%) were anaemic as shown in Figure 1.

The highest number of samples were collected from the age group 26-30 (328) followed by the age group 21-25 (236) in which 160 and 132 cases were anaemic respectively. Samples collected from age group 41-45 were 8 which all were anaemic (Table 1). In our study, abortion was observed in 568 cases, and stillbirth in 92 cases as shown in Figure 2. The correlation between pregnancy age with HB status was also determined. Different observers belonged to different trimesters according to their pregnancy age. The pregnancy age of 124 observers was the first trimester, in which 54 were anaemic and 70

Table 1. Status of haemoglobin level in the different age groups of pregnant women.

Age groups	Normal (11.5 mg/dl)	Anaemic (<10.5 mg/dl)	Total number of observers
15-20	24	68	92
21-25	104	132	236
26-30	168	160	328
31-35	60	44	104
36-40	12	20	32
41-45	0	8	8
Total	368	432	800

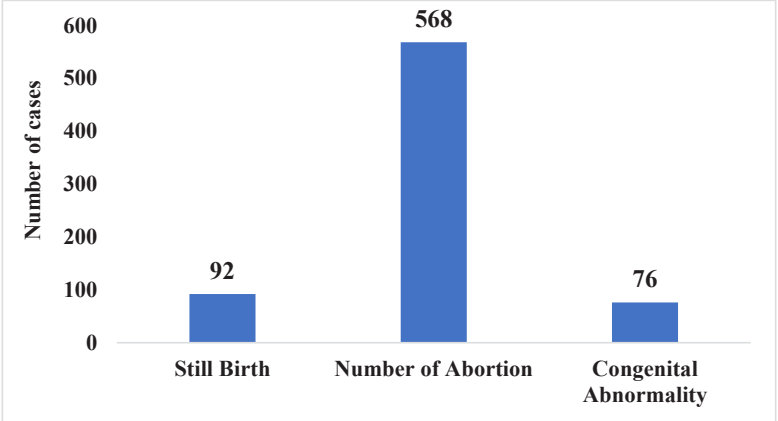

Fig. 2. Haemoglobin deficiency-related issues in pregnant women.

Table 2. Haemoglobin level in different pregnancy age groups.

Pregnancy age	Anaemic (<10.5 mg/dl)	Normal (11.5 mg/dl)	Total number of observers
First trimester	54	70	124
Second trimester	80	152	232
Third trimester	298	146	444
Total	432	368	800

Table 3. Relationship of body mass index with anaemia in Pregnant women.

Body mass index (BMI)	Anaemic (<10.5 mg/dl)	Normal (11.5 mg/dl)	Total number of observers
Normal Weight	34	58	92
Underweight	340	68	408
Overweight	34	58	92
Obesity	24	184	208
Total	432	368	800

were normal. The second-trimester pregnancy age group was comprised of 232 observers of which 80 were anaemic and 152 were normal. The third trimester age group consisted of a total of 444 individuals of which 298 were anaemic while 146 were normal (Table 2). The Body Mass Index was also determined. Total cases were divided into four groups, which were normal weight, underweight, overweight and obesity. The underweight group was more anaemic (83%) followed by normal weight and overweight (37%) (Table 3).

4. DISCUSSION

In the present study, data were collected from 800 pregnant women from a different region of Swat in which 54% of women had low HB levels because they were anaemic. The findings of the present study are similar to the one conducted in Nigeria [15]. Palupi *et al.* [6] carried a research work in developed countries and Raza *et al.* [8] in Mansehra and Abbottabad was nearly similar to our research work. The reports of Shobeiri *et al.* [16] were different from recent results as the cases in Mysore City were <4%. The reasons for the high anaemic ratio in the study area of the current research were the lack of knowledge about anaemia during pregnancy and the poor bioavailability of iron because the ratio of illiteracy is very high in women of District Swat. The work of Susanti *et al.* [14] in Jatinangor and West Java, showed that there were more anaemic observers (86.7%) as compared to the recent work because the said regions lie in the thalassemia belt area.

In the present study, the findings showed that age group that ranges from 40-45 was 100% anaemic followed by 15-20 (74%). Both groups show highly anaemic cases because they need more iron, calcium and other components which are necessary for their growth and development and when they become pregnant at this time then the need for the said components become increase. Due to which low iron stored in their body and cause iron deficiency and that's why more women were anaemic. Loy *et al.* [17] also reported the group-wise cases of Hb-deficient pregnant women in Singapore and their result was different because in their study age group range 25-34 was highly anaemic while the age group ≥35 was least anaemic. The reason was the routine measurement of plasma ferritin and nutritional issues due to which the absorption of iron may be affected.

The present study showed that underweight individuals were more anaemic (83%) while the obese were least anaemic (12%), the similar results were shown by Qin *et al*. [18] in Jiangsu Province, China. The second-trimester pregnancy age group was the least anaemic (34%) and the third-trimester pregnancy age group was the highly anaemic (67%) in our study while the work done by Ejeta *et al.* [19] also showed similar results in Western Ethiopia.

5. CONCLUSIONS

It is concluded from this study that deficiency of iron constitutes a serious and alarming health hazard to the mother and new-born. Both young and aged women are at risk of this problem. The underweight women are more affected due to this problem. Significant dangers to the health of both mothers and newborns are associated with this deficit. To address this issue and safeguard the health and safety of expectant mothers and their unborn children in the area, further efforts and interventions are required.

6. LIMITATIONS

Because of the study's limited sample size, the findings could not be a precise estimate of the district's overall population of pregnant women. Cultural barriers appeared between researchers and individuals, which may have an influence on the precision and thoroughness of the data obtained.

7. ACKNOWLEDGEMENT

The Authors acknowledge the Centre for Animal Sciences and Fisheries to provide the laboratory facilities.

8. ETHICAL STATEMENT

The study was conducted under the Declaration of PM & DC Professional Ethics and Code of Conduct, and the protocol was approved by the Ethics Committee of the University of Sawat.

9. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Impact of Yeast Diet on the Number of Eggs and Larvae Produced in Honey Bee Colonies (*Apis Mellifera* L.) Apidae: Hymenoptera

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Abstract: Honey bees (*Apis mellifera* L.) are important social insects because of the honey production and pollination services they provide. Diet quality affects bee progression through different life stages, adult longevity, fecundity and foraging activity, among other likely phenotypes. This study was conducted to determine the probable effect of colony food availability on the number of eggs and resulting larvae produced by honey bee colonies. Sixteen honey bee hives were used in the study. The hives were split into groups of four, with each group receiving one of the following four treatment diets: (1) T1 – sugar water (1 l water + 250 g sugar), (2) T2 - yeast water (1 l water and 50 g Brewer's yeast–non-floral protein diet in dry form), (3) T3 – water (1 l water), and (4) T4 – no diet. The impact of the colony diet (sugar syrup and yeast with treatments mentioned above) on the number of eggs and larvae produced was determined using a one-way ANOVA conducted using the statistical program "R" version 2.15.3. Where appropriate, means were compared using the least significant difference (LSD). Numerically, the average number of eggs and larvae on sugar solution were 24.20 ± 1.72 and 26.8 ± 1.808 respectively, while on the yeast diet were 33.66 ± 2.92 and 31.55 ± 2.324, respectively. Significantly, the number of eggs (P-value 4.74E-10, F value 21.50528 and F-tabulated value as 2.731807) and larvae (P-value 5.31E-05, F-value 8.70 and F-tabulated value 2.73) produced was significant when colonies were fed with yeast and sugar solution.

Keywords: Larval Duration, Eggs, Honey Bees, Artificial Diet.

1. INTRODUCTION

Proteins, lipids, carbohydrates, vitamins, other minerals, and water are present in plant nectar [1]. Western honey bees (*Apis mellifera* L.) collect nectar and convert it to honey. Honey serves as the bees' carbohydrate source but also provides other essential elements and compounds. Honey bees use these elements in the scarcity of flora and fauna [2]. Honey bees also feed on bee bread; a food resource produced from pollen [3]. Adult honey bees feed directly on these foodstuffs. However, immature honey bees feed on the glandular secretions provided to them by their adult nurse worker sisters, though some nectar and pollen may be added to the larval diet [4]. The quality of the food supplies available in the nest impacts nurse production of the larval diet [5]. The lack of quality food resources causes the nurse honey bees to use the protein and lipid reserved in their own bodies to produce larval food and also for their survival during periods of dearth [2, 6].

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However, the brood growth on alternate pollen food and or supplemental diet in the bee hives is observed as less comparative to the fresh natural pollen [7]. Although the supplemental diet which contains yeast was consumed in surplus comparatively because of their constant behavior and cravings [8] but the fecundity was not in significant difference with the natural and fresh pollen. In the spring season, an increase in the honey bee brood development was observed at the supplemental pollen mixed diet [9]. The provision of a supplemental protein diet mixed with brood pheromones showed an increase in brood and adult bees' strength compared to the colonies with only a protein diet [10].

In the late spring or early winter, the beekeepers supply food to the colonies to encourage brood rearing and or nutritional strain in the dearth periods to cope with nutritious feeding [11]. The physiology and behavior of adult bees can be affected by the quality of the diet they receive [6]. Worker bees provide 25 - 37.5 mg of protein in the diet of each larva [12], some of this deriving from bee bread [13, 14]. In the first three days of larval growth, the sugar content of the larval diet (fructose and sucrose) is about 18%, but it increases to 45% in the final two days of larval development. The honey bees which efficiently consume the supplemental diet in a surplus of 23% of protein could have better potential of rearing larvae [15].

Honey bees that consumed diet with more than 23% protein could rear honey bee larvae. A larval diet encompassing the sugars and dry yeast in the bee hives [16] increases longevity and further can be boosted by raising the amount of glucose, yeast, and fructose in their diet [17]. During the larval developmental period, a larva is regularly observed and little by little nourished about 135−143 times on the food preserved by the worker bees [16, 18]. The supplemental diet enriched with carbohydrates and proteins is consumed more by the bee larvae, so as per a rough appraisal, the entire proteins and carbohydrates for the growth and development consumed by one larva are 25−37.5 mg and 59.4 mg respectively [12].

For proper honey bee colonies management, beekeepers should have the knowledge of different apiculture management practices in depth [19]. Beekeepers often supplement the honey bee diet by feeding colonies a carbohydrate source (usually sugar or corn syrup) and a protein source (typically a pollen patty or supplement). However, the success of these diets often is determined as a colony's consumption of the diet rather than the colony's effective utilization of it. A high-quality food substitute should contain ingredients that supply the essential colony growth nutrients and nutrients for individual bee development, longevity, and productivity.

It is experiential that even if the principles of migratory beekeeping are followed, around 40% of bee hives decomposed through the dearth (scarcity of flora and food resources) time of the year. The stipulation of the supplementary artificial diet to the colonies has unanimously been considered and administered for the brood rearing egg laying, as well as for the foraging activities that possibly can uphold all the colony parameters. A numerous supplemental diet formulations have systematically been developed through combination of various components and observed by a diverse population of workers around the globe regarding commercial beekeeping point of view [20−25].

The primary components of yeast are polysaccharides such mannans, chitins, and glucans. The benefits from yeasts appear to include the production of vitamins that can enhance bee food [26]. Additionally, yeasts were found in large quantities in newly emerging bees and nurse bees, and it has been suggested that they aid in the digestion of pollen and the production of royal jelly [27]. Herein, we determined the impact of feeding colonies with sugar water fortified with yeast on the number of eggs and larvae produced in treated colonies. We expected the increased number of eggs in the cells and larvae in the colony.

2. MATERIALS AND METHODS

This experiment was conducted in the apiary of the College of Agriculture, University of Sargodha located at 32.08° N, 72.67° E and 193 m above sea level. The temperature in the study area was 22.8 °C to a warm 31.9 °C under the sub-tropical, semi-arid and clear climatic conditions. Honey bee colonies which were Langstroth equipped and single deep manufactured containing ten frames per colony were established.

The colonies were equalized regarding the larval strength, empty egg cells, and honey stores before the experiment and exposed to comparable food resources (daily foraging activities of bees and food available in the hive) as well and further placed on iron stands ~0.3 m high to reduce the other contaminants' effects. There was a 200 meters distance among each experimental colony. The colonies were checked for *varroa destructor*. Since the adult mite infested the honey bees, so a close inspection of the drone brood strength was done to check its infestation, however chemical treatment was applied. The experimental colonies were critically checked for diseases (discoloring of sealed brood, brood combs, scattering of sealed brood, punctured cell capping, sunken cells) in order to avoid any sort of error in the experiment.

Sixteen honey bee colonies were split into four groups of four colonies/group. The groups were assigned one of the following diet regimens: (1) T1 – sugar water (1:1 water + 250 g sugar), (2) T2 – yeast water (1:1 water and 50 g Brewers' yeast; non-floral protein diet for honey bees), (3) T3 – water (1:1 water), and (4) T4 – no diet. The varying diets were provided to the colonies in polythene bags (measuring approximately 2 × 10 ×15 cm), placing them beside the frames.

Twenty randomly chosen, empty cells in the brood nest were monitored on each of six frames for all 16 colonies (120 cells per colony) twice daily (09:00 and 16:00) once the diets were provided to the colonies. The cells were physically marked on each frame with the help of permanent marker. During the monitoring period, we recorded the day that the queen laid an egg in each cell, the day the cells were capped, and the day the resulting adult bees emerged from the cells. Mostly, the larvae were counted through physical observation especially when they were 0-24 hours old. These data allowed us to calculate the length of each developmental period. The impact of the colony diet on the number of eggs, larvae, and adults was determined using a one-way ANOVA conducted using the statistical program "R" version 2.15.3. Where appropriate, means were compared using LSD.

3. RESULTS

3.1 Effect of Diets on Eggs

The effect of different diets on several eggs is presented in Figure 1. The sugar solution diet increased the number of eggs at a rapid rate in the first 10 days, then the increase in number of eggs started to decrease. With a maximum average of 47.55 ± 1.55 and a minimum average of 15.8 ± 1.58, the average number of eggs in the sugar solution was 24.20 ± 1.72. Results showed that a sugar diet has a significant effect on the number of eggs in the colony. There was an excellent increase in the number of eggs in the first 10 days on the yeast diet then the population gradually started decreasing

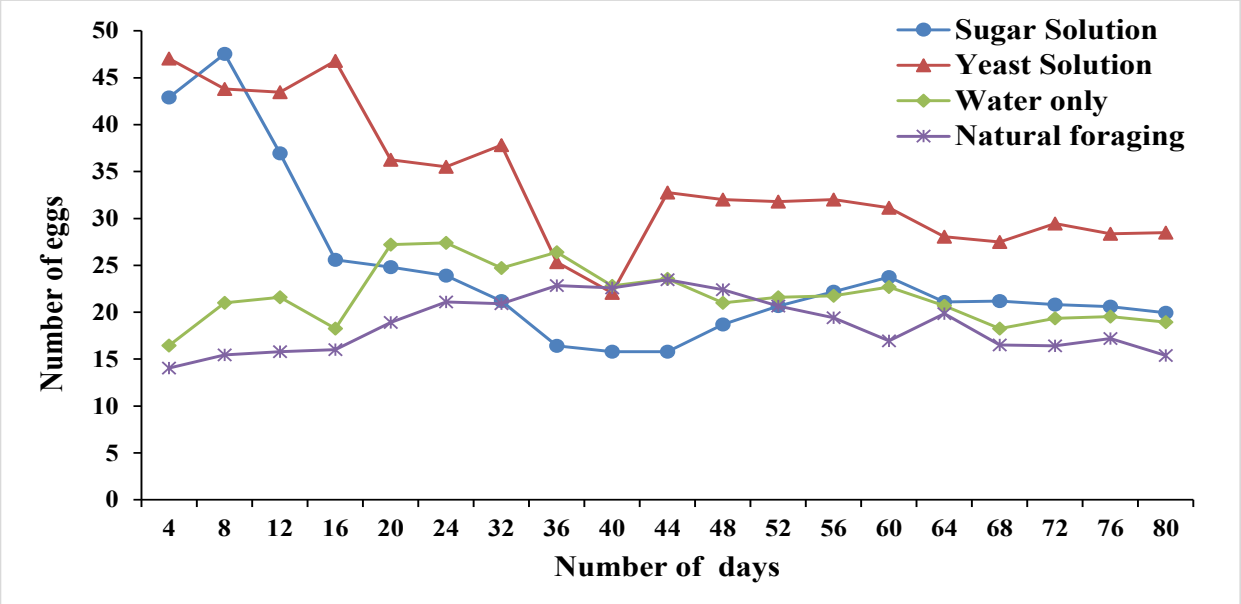

Fig. 1. Effect of varying artificial diets on the number of eggs of honey bees *Apis mellifera.*

throughout. The average number of eggs consumed on this diet was 33.66 ± 2.92, with a maximum average of 47.05 ± 7.94 and a minimum average of 22.05 ± 1.44. The yeast diet has a significant impact on the number of eggs produced in the colony.

The average number of eggs on the water diet was 21.74 ± 1.74 with the highest average of 27.4 ± 2.67 and a minimum value of 16.45 ± 1.29. The results showed that water treatment had a positive effect, but it was modest compared to the other three nutritional parameters and helped increase egg numbers. By natural flora and fauna, the number of eggs increased gradually as well as continuously. This is because the only natural source of protein is pollen for bees. The mean number of eggs in natural foraging was 18.72 ± 1.12, with a highest value of 23.45 ± 1.23 and a minimum of 14.05 ± 0.76. The results showed that natural foraging gave strength to the colony and contained a large number of eggs compared to other parameters.

3.2 Analysis of Variance

ANOVA was piloted to assess the influence of the different diets on the number of eggs in the treated colonies. The ANOVA result showed the significant effect of diet with the F crit value as 2.731807, P-value 4.74E-10, and F value 21.50528 (Table 1). The maximum egg population was 47.55 and the minimum population was 15.4, on 15.03.2014.

3.3 LSD Test for Means Comparison

After finding significant results from the ANOVA, a Post hoc test LSD was used to compare the difference between all diet parameters (Table 2).

The model's findings demonstrated that yeast solution has a greater impact on egg development than any other diet, with an average mean of 33.66 ± 7.22 value for St. error (significant letter "a"). With an average mean of 21.75 and a St. error of 3.08 (significant letter "bc"), the water diet was then found to be effective. The sugar solution with an average mean of 24.20 ± 8.75 (significant letter "b") was discovered to be the best. The average mean and standard error for natural foraging are 18.73 and 2.97, respectively (significant later "c").

3.4 Effect of Diets on Larval Population

On the first day of data collection, due to low sugar content, such as fructose and sucrose in the brood food, which is only about 18% during the first three days of larval development before rising to 45% during the final days of development, the average number of larvae was "0.8" (standard error 0.2). Due to continuous feeding, population growth began after three days and continued for the next 20 days. Following that, the strength of the larvae decreased slightly for the first 14 days before steadily increasing for the following 16 days until the end.

In our experiments, the yeast solution contributed significantly to the development of larval strength compared to other nutritional parameters. The mean number of larval bees on yeast was 20.04, with a maximum mean of 26.8 and a minimum mean of 0.8. A sugar solution

Table 1. Analysis of variance for the number of eggs.

Source of variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between groups	2373.188553	3	791.0628509	21.50527808	4.74E-10	2.731807
Within groups	2648.490526	72	36.78459064			
Total	5021.679079	75				

Table 2. Comparative effects of varying artificial honey bee diet levels on the number of eggs of *Apis mellifera.*

Treatments	Means	St. Error	Significant letters
Yeast solution	33.66	7.22	a
Water only	21.75	3.08	b
Sugar solution	24.20	8.75	bc
Natural foraging	18.73	2.97	c

diet showed a significant difference in larval development as well. On the yeast diet, there was population fluctuation since the increase in larval population was normal in the first 10 days and then it increased at a higher rate from the 11th to14th day and then decreased slowly in the next 11 days. The mean number of sucrose-fed larvae was 23.93, along with a maximum mean of 31.55 and a minimum mean of 1.8.

When watered, populations were low for the first 14 days, then increased for up to 26 days, then decreased for 5–7 days, and then increased again, resulting in increased larval numbers. The average number of larvae at watering was 22.48, with a maximum mean of 32.65 (standard error ± 3.43) and a minimum mean of 0.45 (standard error ± 0.11). Results revealed that water is of great importance for the survival of the larvae. Larval numbers slowly increased as worker bees visited and accessed natural flora and fauna while naturally searching for food (pollen/nectar). The mean larval number that was recorded on this diet was 18.13, with a maximum mean of 26.05 (standard error ± 3.68) and a minimum mean of 0.95 (standard error ± 0.19). The results showed that natural foraging by worker bees to feed larvae is crucial for the development of larval numbers within the colony.

When fed the sucrose solution, the maximum mean number of larvae in worker bees was noted on day 55 of feeding and was 26.8 (standard error ± 1.80), with a minimum mean value of 0.8 (standard error ± 0.2) on the first day of data collection. On the 14th day of data collection for the yeast solution, the maximum mean worker bee larvae were recorded, coming in at 31.55 (standard error ± 2.32), and the minimum mean was recorded, coming in at 1.8 (standard error ± 0.33) (Figure 2).

3.5 Analysis of Variance

An ANOVA was performed to assess the influence of different honey bee diets on honey bee larval numbers. ANOVA showed significant results of diets with a P value of 5.31E-05, an F value of 8.70, and an aggregated F value of 2.73. The highest population was 32.65 and lowest was 0.8 (Table 3).

3.6 LSD Test for Means Comparison

After finding significant results from the ANOVA, a post-hoc LSD test was used to compare differences between all dietary parameters in honey bee larval numbers (Table 4). Dietary differences were compared using the LSD post-hoc test. The LSD results showed the superior effect of yeast solution on the larval population, with a mean of 23.93 and a standard error of 6.34 (significant letter "a") compared to the other three diets. The water-only then followed the yeast solution diet and gave the best results, with a mean of 22.48 and a standard

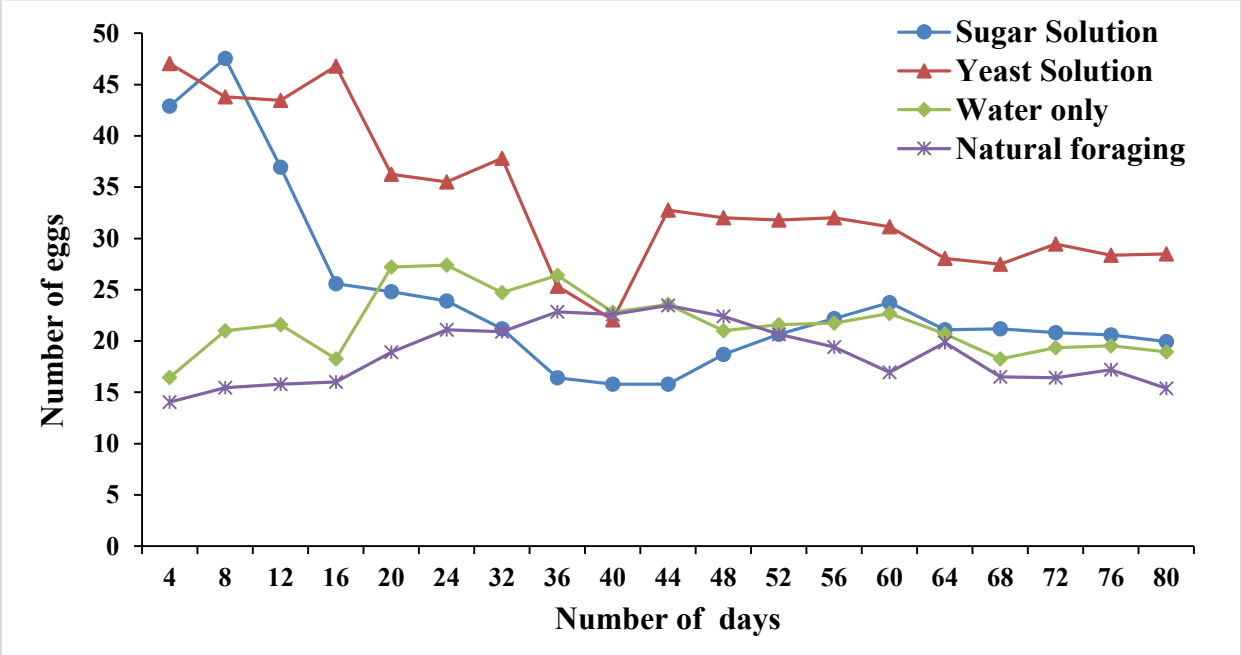

Fig. 2. Effect of varying artificial diets on the number of larvae of honey bees *Apis mellifera.*

Table 3. Analysis of variance for the number of larvae of honey bees.

Source of variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between groups	14.37800817	3	4.79266939	8.70295	<0.000	2.731807037
Within groups	39.65002058	72	0.55069473			
Total	54.02802875	75				

Table 4. Comparative effects of varying artificial honey bee diet levels on the number of larvae of *Apis mellifera.*

Treatments	Means	St. Error	Significant letters
Yeast solution	23.934	6.343	a
Water only	22.489	7.859	a
Sugar solution	20.047	5.503	ab
Natural foraging	18.134	6.036	b

error of 7.85 (significant letter 'a'). The sugar solution then contained a mean of 20.04 with a standard error of 5.50 (significant letters 'ab'), making it a suitable diet for larval development. Ultimately, natural foraging showed lowest effect on larval development. The mean was 18.13 with a standard error of 6.03 for significant 'b'.

4. DISCUSSION

Studies on various dietary parameters associated with worker bee longevity have shown differences in honey bee larval longevity. Four artificial diets (sugar solution, yeast solution, water only, and natural foraging) were used, and these diets showed a significant increase in worker bee lifespan, which had the greatest effect on growth, development, and longevity, and was also favored by larval bees. The maximum average for larvae was 31.55. Many different authors have reported that artificial feeding has positive effects on larval stage dispersal, development, and longevity of honey bees [14]. Steen [28] has explained that the pollen substitutes had a remarkable increase in the life span of honey bees. By frequent or prolonged use of protein-supplemental diets, the bees and castes had negative effects in foraging and other colony activities [29]. However, a lot of scientists reported that supplemental feeding to honey bees can be effective for their growth and development. Yeasts are more attractive to bees for their growth and development because the composition of protein levels is around 50% and normally more fair set of amino acids are also provided by the yeast. Most appropriately yeast is composed of fat 1.0%, 51.8% peptones, proteins, as well as amides, etc., 29.5% Gum along with other carbohydrates, Mineral matter 11%, cellulose as well as the other components 6.7% by difference. Yeast has a great contribution to the survival and growth of honey bees especially at the larval stage and increased the larvae survival up to 80% and 30% [30]. Vandenberg and Shimanuki [31] described in their study that the larval and adult body weight of honey bees can also be increased by the application of yeast, and yeast-mixed diet.

For optimum growth and development of bees, a defined quality of proteins is required. If nurse bees are not capable of acquiring pollen or other proper protein source, their brood food gland secretions are not sufficient for the normal growth and development of larvae and also egg production of the queen. So, as the yeast is composed of 51.8% protein, it strengthens the colony and increases the longevity of larvae. The results showed that *A. mellifera* larvae could be helpful in developing a mixture of supplements, yeast extract, and water without added carbohydrates. However, they cannot pupate and become adults if their diet does not contain enough carbohydrates [32].

5. CONCLUSIONS

It is concluded that the yeast is important for the bees' development. The Langstroth single deep, equalized, and uncontaminated colonies with comparable food resources should be located in a shady place preferably at good floral accessibility for the bees for natural foraging. The colonies should be placed possibly very far from the chemical-treated/sprayed fields. Brewers' yeast, non-floral protein diet for honey bees, sugar mixed diet should be applied in the colonies with proper

mentioned concentration. For better eggs and larvae development, the diet should be placed in the colony beside the frames in polythene bags of proper size and changed every other day with fresh ones. It should be in view that the colony is free of diseases and pests and extra cells in the colonies must be removed.

6. RECOMMENDATIONS

Based on the findings of the current study, it is recommended that the yeast enhance the development of honey bee brood and may be used as a supplemental diet particularly in the dearth period when there is no pollen available in the fields.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

The Role of Hematological Parameters in Atrial Fibrillation Risk Assessment

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Abstract: Atrial fibrillation (AF) is an irregular and rapid heartbeat in the heart's atrial chambers. Conversely, haematological parameters are commonly utilized in clinical settings to evaluate overall health and disease. Our research explored the potential role of haematological parameters in atrial fibrillation within the Pakistani population. In this case-control a total of 400 participants were enrolled from the Punjab Institute of Cardiology, Lahore, Pakistan. The participants were divided into two groups: a control group comprising 200 healthy individuals, and an AF group consisting of 200 individuals diagnosed with atrial fibrillation. Haematological parameters were assessed using an automated hematology analyzer. The AF group had higher levels of white blood cells, red blood cells, and mean corpuscular volume as compared to control group. Conversely, lower levels of haemoglobin, hematocrit, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and platelets were observed in AF group compared to control group. In conclusion, our research established a significant relationship between haematological parameters and atrial fibrillation in the Pakistani population.

Keywords: Atrial Fibrillation, Haematological Parameters, Relationship, Blood Count, Risk Assessment, Pakistani Population.

1. INTRODUCTION

The common heart arrhythmia in the general population known atrial fibrillation (AF) which is linked to an higher danger of several health problems. These complications encompass thromboembolism, stroke, neurological damage, major and minor organ dysfunction or failure, as well as hospital readmissions, leading to significantly increased medical expenses [1-3]. In 2021, Lippi *et al*. [4] reported 3.046 million additional cases of AF were reported globally by the record. With 403 instances per million people, the predicted incidence rate for that year was 31% higher than the incidence rate for 1997. Atrial fibrillation is present in 37.574 million people worldwide, or 0.51% of the world's population. Over the past two decades, the incidence of AF has risen by 33%. High socio-demographic index countries bear the greatest burden of atrial fibrillation, although there has been a significant increase in middle socio-demographic index countries as well. By 2050, atrial fibrillation may cause an absolute rise in cases of nearly 60%, according to future predictions [4].

In clinical practice, reference ranges for haematological and immunological parameters are commonly employed to assess the health status and disease conditions of individuals. These reference ranges play a vital role as biomarkers in evaluating how patients respond to treatment or the progression of their disease. Notably, these reference ranges can change based on racial, gender, age, genetic, and environmental variables [5, 6].

The predictive role of haematological factors in the onset and recurrence of atrial fibrillation has been established. One of the most frequently advised blood tests by physicians is the complete blood count (CBC) which counts different kinds of blood components including platelets, red blood cells (RBCs), and white blood cells (WBCs). The

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complete blood count includes the following tests: The three main tests are the (1) WBC total and differential count; (2) erythrogram (RBC count, hemoglobin (Hb) and hematocrit determination; and (3) platelet count indices calculation, which includes mean platelet volume (MPV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW). The diagnostic process for cardiovascular disease usually includes the CBC, one of the most significant blood tests in clinical practice. Given the possible predictive significance of haematological markers for both new-onset and recurrent AF, a CBC test has been recommended as a component of the diagnostic procedure for AF in clinical practice [7]. Previous investigations have explored the association between haematological factors and AF, but the results have been inconclusive. Despite the extensive research on AF diagnosis and treatment in recent years, the precise mechanism of this complex condition remains incompletely understood [2]. The ongoing controversy and discussions surrounding AF diagnosis and treatment are justified due to the involvement of numerous intricate mechanisms in its development [2, 3]. In the current study, we designed to examine the relationship between haematological parameters and atrial fibrillation in Pakistani population.

2. MATERIALS AND METHODS

This study, which was a case-control investigation, was conducted at the Punjab Institute of Cardiology (PIC), Lahore and the Department of Zoology at Lahore College for Women University. The selection of participants involved a non-probability purposive sampling approach during July 2021 to December 2022. Individuals suffering from atrial fibrillation were identified by physicians at the PIC on the basis of electrocardiogram (ECG). The ECG showed aberrant impulse conduction to the ventricles, uneven R-R intervals, and a lack of P waves. The subjects were not suffering from any other illness.

The Rao program was used to calculate the sample size for this investigation, accounting for a 5% margin of error and the disease's prevalence. A total of 400 participants were recruited, with 200 individuals assigned to the control group, who were in good health and had no family history of atrial fibrillation, diabetes, or hypertension. The remaining 200 participants were included in AF group. Prior to enrollment, each participant gave their permission and answered a series of questions about age, gender, job history, marital status, educational attainment, and renal disease history. The haematological parameters were analyzed using an automated Hematology Analyzer (DC 2400 PLUS, Pakistan) at the research laboratory of Lahore College for Women University. The examined haematological parameters consisted of white blood cell count, total red blood cell count, haemoglobin level, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and platelet count. Reference ranges were applied as follows: WBC count (4-11 x 10^9/L), total RBC count (4.5 - 6.5 million/cm), Hb level (130-180 g/L), HCT (38-58%), MCV (76-96 fl), MCH (27-33 pg), MCHC (30-37%), and platelet count (150-400 x 10^9/L).

Utilizing the Statistical Package for the Social Sciences (SPSS, IBM statistics, version 22.0, NY) software, the statistical analysis was performed. The standard error of the mean (SEM) and mean were used to portray the continuous data, while percentages and frequencies were used to present the categorical data. The T-test is utilized to ascertain whether there is a significant difference between the means of AF sufferers and the control group. A P-value of less than 0.001 was considered very significant, while a P-value of less than 0.05 was considered statistically significant.

2.1 Ethical Approval Statement

The institutional review of PIC (Ref. no: RTPGME-Research-179) and ethical review committee of Zoology Department, LCWU (REF/NO/LCWU/ZOO/690; Dated: 01-01-2021) approved the study. Enrolled subjects also gave their approval to take part in the research.

3. RESULTS AND DISCUSSION

The case-control study involved a total of 400 participants, with males accounting for 51.5% and females comprising 48.5% in a control group, whereas in AF group, males were 60% and females were 40%. The electrophysiologic characteristics change according to gender. In comparison to men, women often have a QT interval that is 10–20 ms

longer [8]. During adolescence, this differential in ventricular repolarization manifests itself as a persistent shortening of the QT interval in males. The alteration is thought to be connected to androgen hormones, while the underlying mechanism for this difference is not fully known. Sex hormones may have an impact on the density of potassium channels and the transmural dispersion of calcium channels in the ventricular myocardium, according to animal studies [9]. The electrophysiologic characteristics of the atria differ throughout genders, but these differences have not been as well investigated as those in ventricular repolarization. There is a dearth of published research on the sex variations in atrial electrical remodelling between men and women [10]. The baseline demographic characteristics of the participants are shown in Table 1.

Table 2 shows the mean values for the following parameters: age, total red blood cells (RBCs), white blood cell count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count. Age, total RBCs, and MCV of the research groups did not differ statistically, according to the analysis. WBC, Hb, HCT, MCH, MCHC, and platelet counts, however, showed statistically significant variations across the groups. While the mean values for hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count were lower in the atrial fibrillation group when compared to the control group, the AF group showed greater mean values for white blood cell count, red blood cell count, and MCV.

This study's goal was to examine the possible significance of haematological markers in atrial fibrillation patients, which was carried out at the PIC in Lahore, Pakistan. The study's findings showed a significant association between AF and a number of haematological measures, such as platelet count, mean corpuscular hemoglobin concentration, hematocrit, white blood cell count, and hemoglobin level. Regarding mean corpuscular volume and total red blood cell count, however, no meaningful correlations were found.

Our investigation revealed that the AF group had a higher white blood cell (WBC) count than the control group; moreover, very significant differences were discovered, as shown in Table 2. Inflammation markers have been consistently associated with atrial fibrillation in various studies. WBC count is a commonly used and easily accessible indicator of systemic inflammation. Evidence suggests that a greater WBC count was linked with occurrence of atrial fibrillation during a 5-year follow-up [11]. WBCs may contribute to atrial remodelling

Table 1. Baseline demographic characteristics of studied groups.

Variables [n (%)]	Control Group (n=200)	AF Group (n=200)
Gender		
Male	103 (51.5)	120(60)
Female	97 (48.5)	80(40)
Education status		
No	14 (7)	155 (77.5)
Yes	186 (93)	29 (14.5)
Marital status		
No	08 (4)	22 (11)
Yes	192 (96)	133 (66.5)
Employment status		
No	06 (3)	139 (69.5)
Yes	194 (97)	16 (08)

Table 2. Biochemical analysis of control and AF groups.

Variables	Control group (n=200)	AF group (n=200)	P-value
Age (years)	52.07 ± 15.91	54.31 ± 15.82	0.158
White blood cell count ($\times 10^9$/L)	8.27 ± 2.06	12.28 ± 10.10	0.001
Total red blood cells (Million/cm)	5.04 ± 0.75	6.04 ± 7.91	0.074
Haemoglobin (g/L)	138.43 ± 20.84	127.41 ± 21.47	0.001
Hematocrit (PCV) (%)	54.12 ± 43.18	38.90 ± 7.08	0.001
Mean corpuscular volume	79.06 ± 8.83	80.46 ± 8.81	0.112
Mean corpuscular haemoglobin (pg)	32.29 ± 8.64	26.35 ± 3.26	0.001
Mean corpuscular haemoglobin concentration (%)	34.18 ± 1.89	32.76 ± 1.71	0.001
Platelets ($\times 10^9$/L)	306.54 ± 80.15	230.55 ± 122.91	0.001

at multiple levels, including electrical and structural changes. Numerous cytokines released by activated WBCs cause intrinsic inflammatory cascades in fibroblasts that cardiomyocytes, and even leukocytes. Consequently, biopsies taken from AF patients have demonstrated a connection between inflammation and the development of atrial fibrosis [12-14]. Atrial fibrillation is growing in both prevalence and frequency. One indicator of systemic inflammation and a danger cause for cardiovascular disease is the white blood cell count. Across the Japanese population as a whole, higher WBC counts were significantly linked to a higher prevalence of AF, particularly in smoker women [15].

While the overall red blood cell count did not differ significantly across the groups under investigation in our study, Table 2 shows that the atrial fibrillation group had more mean RBCs as compare to the healthy group. However, the heterogeneity of red blood cell size and volume was reflected by the red blood cell distribution width (RDW). RDW is an cheap and simply measurable index that has been associated with various cardiovascular diseases. Growing evidence recommends that RDW can serve as a prognostic indicator for atrial fibrillation in different therapeutic contexts [16]. A distinct risk factor for stroke and mortality in patients with atrial fibrillation is left ventricular hypertrophy (LVH). In AF patients, Yao *et al.* [17] show a connection between LVH and RDW. Those with AF and LVH showed considerably higher RDW than those in the non-LVH group. RDW has also been connected to a number of other illnesses. A number of cardiovascular disorders have been related to the relationship between RBC distribution width, a measurement of erythrocyte volume change and AF. RBC distribution width was related with the occurrence of AF which included middle-aged individuals from the general public, independent of other nutritional, medical, and cardiovascular factors. Although several hypotheses have been put up, the processes underlying these correlations are still unknown. Elevated RDW was linked to a considerable reduction in heart rate variability in a current study of individuals suffering from systolic left heart failure [18].

The atrial fibrillation group in this research had lower mean corpuscular hemoglobin and hemoglobin levels than the control group. Table 2 shows that there were noteworthy alterations in both hemoglobin and mean corpuscular hemoglobin between the analyzed groups. The red blood cells that contain the protein called hemoglobin are responsible for transporting oxygen from the lungs to the body's tissues and releasing carbon dioxide when exhaled. Chronic or acute blood loss can lead to reduced haemoglobin levels. In the context of AF, individuals might be at an higher danger of bleeding, especially if they were taking anticoagulant medications to prevent blood clots. A reduction in the mean corpuscular haemoglobin levels in individuals with atrial fibrillation may be indicative of anaemia or other underlying health issues. An indicator of the usual quantity of Hb in each RBC is the MCH. This discovery was even with the findings of a research study by Lim *et al*. [19], which showed that, after controlling for other cardiovascular and demographic risk variables, there was a U-shaped relationship between hemoglobin concentrations and the chance of atrial fibrillation. The study showed that having high or low Hb levels presented the largest risk of AF, while keeping levels within the normal range was linked to the lowest risk. Additionally, Katayama *et al.* [20] observed that in individuals with normal left ventricular (LV) systolic function, there was an independent relationship between left atrial (LA) enlargement and both a lower Hb concentration and a higher LV mass index. This shows that hemodynamic abnormalities associated with Hb levels may have a role in LA remodelling and the start of AF prior to the manifestation of other abnormalities such systolic dysfunction or LV hypertrophy .

In comparison to the control group, the atrial fibrillation group exhibited lower hematocrit levels, according to our study's findings. Additionally, a very significant difference was found between the groups that were investigated, as revealed in Table 2. The percentage of cellular blood in total blood volume is known as the hematocrit, and it is a measure of this proportion. It is commonly used to diagnose and monitor various medical conditions. Chronic blood loss, whether due to gastrointestinal bleeding, genitourinary bleeding, or other sources, can lead to a decrease in hematocrit levels. This may be relevant in AF patients if they have an underlying condition causing bleeding. It was found that the rate of hematocrit change from sinus rhythm to atrial fibrillation varied among paroxysms in patients with multiple episodes. A 5-point increase in hematocrit leads to hemoconcentration,

which results in an approximately 10% increase in the plasma concentration of macromolecular compounds, including hemostatic agents. This hemoconcentration typically occurs within hours or less after the onset of the paroxysm. Given the observation of localized blood stasis in the left atrial appendage during AF, the abrupt development of specific hemoconcentration may promote the formation of an intracardiac thrombus. According to this, when AF first develops, platelet initiation and coagulation happen in a time-dependent way [21-23].

In the present study, we found that the AF group had higher mean corpuscular volume levels and lower mean corpuscular hemoglobin concentrations as compared to a control group. According to Table 2, there were significant variations in the study groups' mean corpuscular haemoglobin concentration but not in their mean corpuscular volume. Similarly, Takahashi *et al*. [24] previously reported that atrial fibrillation patients exhibited higher mean corpuscular volumes. Another study found that patients with lower mean corpuscular volumes had more dilated left ventricles, which has been linked to iron deficiency. It was noted that women typically had smaller mean corpuscular volumes compared to men, and the authors suggested that iron deficiency may contribute to the association between the female gender and a higher risk of thromboembolism in AF, along with other hormonal factors [25, 26].

As demonstrated in Table 2, platelets in the investigated groups likewise revealed extremely significant differences. The mean value of platelets in the AF group was found to be lower than in the control group, according to research results. Similarly, thrombocytopenia, characterized by a platelet count below 100×10^9/L, was estimated to affect approximately 6% to 24% of patients with AF [27, 28]. Although there were no obvious abnormalities in platelet aggregation among atrial fibrillation patients, fluctuations in plasma indicators of platelet function were observed. However, despite the decreased thrombogenesis associated with warfarin use, treatment with either aspirin or warfarin did not demonstrate important benefits in terms of platelet activation (fibrin D-dimer). This suggests that platelet initiation may not significantly influence the aetiology of thromboembolism in AF [29].

Furthermore, Liu *et al*. [30] revealed that active platelets release significant amounts of Transforming growth factor β 1 (TGF-β1) into the bloodstream after stimulation by angiotensin II (Ang II). Concurrently, initiated platelets enter the atria and discharge TGF-β1 at the local level. The presence of TGF-β1, in conjunction with angiotensin II infusion, promotes atrial fibrosis and increases atrial fibrillation inducibility by enhancing the activity of atrial fibroblasts both locally and systemically. It is worth noting that platelet-fibroblast interaction, along with other factors released by platelets, also contribute to atrial fibrosis. Since platelets play a role in atrial thrombosis, antiplatelet treatment may be beneficial in preventing both thrombosis and fibrosis . Haematological parameters can serve as valuable indicators for assessing the danger of problems and associated comorbidities in atrial fibrillation patients. An increased danger of stroke, thromboembolism, and other cardiovascular events might be linked to abnormalities in haematological parameters. The evolution of atrial fibrillation and the problems that accompany it can be understood by looking at changes in haematological markers over time. Frequent monitoring of these measures can be used to track the effectiveness of therapeutic interventions and detect any deterioration in the patient's health. Patients with atrial fibrillation may use haematological measures as biomarkers to assess how well their medication is working. It is possible to assess if the treatment strategy is working or whether changes need to be made by keeping an eye on changes in these parameters. It might be useful in identifying possible links between haematological anomalies and the onset or development of atrial fibrillation.

4. CONCLUSIONS

Overall, this research indicated that there was a significant alteration in platelet levels, MCH, MCHC, Hb, HCT, WBC count, and HCT among the control and atrial fibrillation groups. Nevertheless, among the populace we analyzed, there were no discernible variations in total RBCs or MCV. However, this study did not clarify the exact mechanism by which these haematological characteristics lead to the development of AF. In common, examining haematological markers in individuals with atrial fibrillation can help with risk assessment, diagnosis, monitoring, and therapy evaluation. It can also shed light on the underlying causes of the disorder.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Research Article

Comparative Effect of Honey and Antibiotics against Multi Drug Resistant Bacteria Isolated from Surgical Site Infection

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Abstract: Patients undergoing surgery are predominantly exposed to surgical site infections (SSI) resulting in serious consequences. A cross-sectional study comprising 100 samples, was collected from various surgical sites of admitted and non-admitted patients in Lady Reading Hospital, Peshawar. 87 samples were tested positive for bacterial growth. After isolation and identification, the highest prevalence was recorded among isolates of S*taphylococcus aureus* 38 (43.6%*)* followed by *Pseudomonas sp*. 25 (28.7%), *Streptococcus pyogenes* 13 (14.9%) and *E. coli* 11 (12.6%). Antibiogram pattern was determined which showed high sensitivity of all bacterial isolates toward Clindamycin, Clarithromycin, and Piperacillin, followed by Erythromycin, Doxycycline, Co-amoxiclav, while maximum number of isolates showed resistance against Vancomycin, Ciprofloxacin, Amikacin. According to quantitative analysis, *Ziziphus* and *Acacia* honey inhibited *E. coli* at different Minimum Inhibitory Concentration (MIC). The results revealed that *Ziziphus* honey inhibited *E.coli*, at 100 µl concentration on the lowest MIC at 0.25 µl while *Acacia* honey inhibited *E.coli* at 75 µl concentration on lowest MIC at 0.5 µl. *Ziziphus* honey inhibited P*seudomonas sp.* at 100 µl concentration on lowest MIC at 0.25 µl while *Acacia* honey inhibited *Pseudomonas* at 100 and 75 µl concentration on lowest MIC at 1 µl. *Ziziphus* honey also inhibited *Staphylococcus aureus* at 100 and 75 µl concentration on lowest MIC at 0.25 µl, while *Acacia* honey inhibited *S. aureus* at 100µl concentration on lowest MIC at 0.5 µl. This study concludes that *Ziziphus* honey was more effective in curing surgical wounds compared with *Acacia* honey. However, further Studies needed to be done for *Ziziphus* honey to utilize it as an efficient treatment approach.

Keywords: Surgical Site Infection, Antibiotics, *Acacia, Ziziphus*, Bacterial Isolates.

1. INTRODUCTION

Skin is a natural barrier against infection. The most important function of skin is to inhibit microbes that reside in the skin's superficial layer and minimize the proliferation of pathogens from invading underlying tissues. Although there are many protocols and precautions which are responsible for preventing infection but exposure of subcutaneous tissues to certain bacteria easily destroys skin's structure, thus provides a nutritious, warm, and moist environment that is favorable to microbial proliferation and colonization. Surgical Site Infections (SSIs) are the most important cause of the accumulation of flora at or near the surgical site. In contaminated surgery, urinary, intestinal genital, and respiratory flora also infect the site. Physicians name these infections as SSIs because they occur in body parts that have undergone surgery. As described by Centers for Disease Control and Prevention (CDC) three different kinds of SSIs occur which include superficial incisional SSI, deep incisional SSI and organ or space SSI. SSIs reveal signs and symptoms such as fever, redness, swelling accompanied by tenderness, pain warmth and pus [1].

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SSIs are one of the most common complications for patients undergoing surgical procedures and the second most frequently occurring healthcare-associated infection (HAI) [2, 3]. Due to SSIs, most of the patients are readmitted to hospital after surgery [4]. Initial infections, develop within one week of surgery, are frequently more serious [5]. Infections after surgery are caused by microorganisms. Microorganisms may invade an operational wound by different means of contact, such as touching a contaminated caregiver or surgical tools, aerosols, or microbes that are already on or in your body and then spread into the wound. Several bacterial species were isolated from wounds which include *Streptococcus*, *Staphylococcus* and *Pseudomonas;* however, *Staphylococcus aureus* was the most abundant species among wound isolates [6]. In severe wounds and burned cases, *Pseudomonas aeruginosa* is mostly isolated [7]. *Methicillin-resistant Staphylococcus aureus* causes 37% of SSI infections in community hospitals [8].

After surgery, SSIs can still occur within 30 days and may have a chance to occur within 1 year in patients who experienced implantation during surgery. Majority of SSIs, almost 12.84% are first diagnosed after the discharge of patient from the hospital. The risk of SSI is decreased by the use of short doses of antimicrobial drugs. Selection of antimicrobial agents depend on the pathogens, usually related to protocols being performed. Broad spectrum β lactam antibiotics are frequently used during surgical site preparation, and in order to kill infection causing anaerobes Metronidazole is used if needed and Vancomycin is not suggested for routine prophylaxis. The primary dose should be given at appropriate time to confirm that bactericidal values are present in tissue and serum at the incision time and necessary to maintain bacterial amounts for few hours after stitching wound in the operation theatre [9].

Antibiotics inhibit bacterial infections, but unfortunately, the efficacy of drugs is reduced with the passage of time because of increasing use of drugs especially different generations of antibiotics. Manufacturing novel antibiotics is difficult as huge financial expenses are required to test them by keeping in view the side effects which may occur after drug use. During recent years, health care professionals investigated high number of infections due to strains resistant to some antibiotics mostly because of drugs abuse. Natural resources have regained their preference as the primary alternatives for treating SSIs. Between these are the bee hive products such as honey, traditionally regarded as a very effective non-toxic material with antimicrobial properties and wide range of health benefits. Honey was considered to be used for medical and health purposes since 2000 BC. Currently, the use of honey to treat wounds is widely considered [10].

Honey is the natural sweet substance extracted from parts of plants which has been collected by honey bees *(Apis mellifera*) and then stored by them in the hives for future use [11]. However, in traditional medicine, honey is widely used, whereas in modern medicine it is inadequate [12]. Honey is a treatment option for many illnesses and frequently used for dressing surgical wounds, burns, and skin ulcers. It activates the growth of new tissues and heals wounds; it is also a pain reliever and eliminates odor [13]. The enzymes present in honey are the major factors due to which it is useful to human health. Honey is composed of three key enzymes, i.e., diastase (amylase), invertase (saccharase) and glucose oxidase [14]. In raw honey, glucose oxidase is not activated instead shows activity upon dilution with wound lesions. The enzymatic activity in honey results in production of hydrogen peroxide. Honey's inherent antiseptic properties render it exceptionally valuable for various applications. Its optimal antibacterial action is achieved when used within the range of 30−50%, surpassing the efficacy of conventional drugs typically employed to treat urinary tract infections [15]. Honey has healing properties, it heals the wound by making it moist and rapidly treats infection, prevents and decrease exudation, edema and inflammation. It causes abrupt activation of angiogenesis, granulation and epithelialization, thus making the healing process more rapid [16]. Honey exhibits antimicrobial action because of its pH, osmolarity and production of hydrogen peroxide and due to presence of phytochemical components, e.g., methylglyoxal [17].

The current study was designed to diagnose the root cause of surgical site infection and drug resistance patterns of different isolates for facilitating the medical experts to select empirical antimicrobial therapy. The recent nosocomial infection scenario revealed the emergence of multi-

drug resistant bacteria, so potential therapeutic agents are needed to control, eradicate and investigate these resilient pathogens. An initial in vitro evaluation of honey originating from *Acacia modesta* and *Zizyphus jujube* was also described in previous studies [18].

The aim of the proposed study was to determine antibiogram of honey from *acacia* and *ziziphus* compared with commonly used antibiotics against multi drug resistant (MDR) bacteria isolated from surgical site infections using broth dilution and spectroscopic technique.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Collection and processing of hundred surgical wound samples were carried out aseptically using cotton swabs which were carefully sterilized. Samples were collected from patients of both genders aged >24 years, visiting Lady Reading Hospital (LRH), Peshawar. After collection, samples were transported to the Microbiology Research Laboratory (MRL), Abasyn University Peshawar.

2.2 Sample Inclusion and Exclusion Criteria

Collection of samples was carried out from the wounds of surgical sites, from pus discharge in surgical wounds along with serous or sero-purulent discharge, and untreated sepsis patients. A complete history of sex, age, type of illness, diagnosis, the associated co-morbid diseases and type and duration of surgery performed, were obtained from the patients except those patients already using antibiotics, were included in the history.

2.3 Bacteriological Assessment of Microorganisms Present in the Wound Samples Colony Morphology

The sample was cultured on MacConkey's agar, nutrient agar, and blood agar plates and incubated for 24 hours at 37 ºC. After incubation, pure culture study (cultural characteristics, Gram staining, and different biochemical) using standard operating procedures was performed to identify bacterial isolates.

2.4 Microscopy/Gram Staining

Standard Gram staining procedure was performed for microscopy, smear of bacterial isolates was prepared and heat stabilized on clean slides. Two drops of crystal violet were applied on smear and washed with distilled water after 2 min. The second stage involved staining Smear and Gram's iodine for 45 seconds before washing it with distilled water. The smear was exposed to decolorized 95% ethyl alcohol for 15 seconds in the following stage. Safranin was then used to cover the smear before being rinsed. Afterwards, all the slides were examined under the oil emulsion objective lens (100X).

Table 1. Colony features, gram stain reaction, and biochemical tests for identification of bacteria.

S. No.	Colony Features	Gram Reaction	Coagulase	Oxidase	H_2S	Indole Test	Citrate Test	Urease Test	Catalase Test	TSI Test	Identified Organisms
1.	Large, opaque, flat colonies with irregular margins showing greenish coloration	-ve Rods	-	+	-	-	+	-	+	A/A	P. aeruginosa
2.	Thick, greyish white, moist, smooth, opaque	-ve Rods	-	-	-	-	-	-	+	A/ AG	E. coli
3.	Round, smooth, raised, gray to deep golden yellow	+ve Cocci	+	-	-	-	-	+	+	A/A	S. aureus
4.	Round, raised, shiny, gray, and have complete edges	+ve Cocci	-	-	-	-	+	+	+	A/A	S. pyogenes

Key: 'A'= Acidic., 'Alk' = Alkaline., AG' = Acid and Gas., '-' = Negative '+' = Positive '+/-' = May show negative or positive

2.5 Biochemical Assessment

Gram +ive and Gram -ive isolates were recognized by using different biochemical tests, i.e., urease test, Coagulase, Triple Sugar Iron (TSI) test, catalase, Citrate test, Oxidase test and Indole test (Table 1).

2.6 Antibiotic's Susceptibility Profile against Isolated Bacteria

Kirby Bauer disc diffusion method was implemented to detect antibiotic susceptibility patterns. The isolates were subculture on Muller Hinton Agar (MHA) plates. Results of susceptibility pattern were determined according to guidelines of Clinical Laboratory Standards Institute, 2019 [19].

2.7 Antimicrobial Sensitivity Testing

The entire 4 isolates were tested against 10 antibiotics, i.e., Co-amoxiclav (10 µg), Vancomycin (30 µg), Erythromycin (15 µg), Cephradine (30 µg), Clindamycin (30 µg), Clarithromycin (30 µg), Ciprofloxacin (5 µg), Cefotaxime (30 µg), Doxycycline (30 µg), Gentamicin (10 µg), Amikacin (30 µg) and Piperacillin/Sulbactam (30 µg) to detect MDR strains by following Agar disc diffusion sensitivity method as mentioned in the guidelines of the National Committee for Laboratory Standards (NCLS) [20].

2.8 Determination of Minimum Inhibitory Concentration (MIC)

To determine MIC of different brands of honey against isolated bacteria broth dilution method was used. For this purpose, bacterial cultures containing broth media were transferred to sterile tube. To these culture tubes, different concentrations of honey were added. One test tube was left containing media and test organism as control. In shaking incubator, all the tubes were incubated at 37 °C and 120 rpm for 12 hours. Following that, MIC was calculated by using a spectrophotometer to measure optical density (OD) values at 610 nm [21].

2.9 Antibacterial Activity of Honey against Isolated Bacteria

A screening well diffusion test was conducted with some alterations inoculating nutrient agar plates (Oxford, U.K.) by rubbing sterile cotton swabs that were dipped into bacterial suspensions (on nutrient agar cultures grown at 37 °C and adjusted to 0.5 McFarland in sterile saline for 12 hours) above the whole surface of the plate. After incubation using a sterile cork borer 8.2 mm diameter wells were bored into the agar surface. Test honey of about 100 µl was poured into every well. Then these plates were incubated at 37 °C for 24 h and plates in which P. aeruginosa were present were incubated at 30 °C. Methylene blue was used for diffusion control with the help of Vernier caliper (Draper). The zones of inhibition were found out with a scale. The diameter of the well and diameter of the zone was measured. Each assay was carried out in triplicate.

3. RESULTS

In the current research study, a total of 100 samples were collected from different patients having surgical site infections, visiting Leady Reading Hospital, Peshawar. Samples were collected from both male and female patients (Table 2). Out of 100 collected samples, 87 samples were found positive for bacterial growth, whereas 13 were found negative among positive samples, bacteria were identified based on Gram staining, colony morphology, its general characteristics and biochemical tests. The frequency of identified isolates is given in Table 3.

Table 2. Gender-wise distribution of surgical site infection.

Gender	Total number of samples of SSI	Positive
Male	43	39
Female	57	48

Table 3. Frequency of bacterial species isolated from surgical sites.

S. No.	Isolates	Frequency	Percentage
1.	*Staphylococcus aureus*	38	43.7%
2.	*Pseudomonas aeruginosa*	25	28.7%
3.	*Streptococcus pyogenes*	13	15.0%
4.	*Escherichia coli*	11	12.6%

3.1 Antibiogram Analysis of Bacterial Isolates

Antibiotic susceptibility was performed on Muller Hinton Agar media using 12 different antibiotics. The results showed high sensitivity of all bacterial isolates toward Clindamycin, Clarithromycin, Piperacillin, Erythromycin, Doxycycline, and Co-amoxiclav, while maximum number of isolates showed resistance against Vancomycin, Ciprofloxacin, and Amikacin as shown in Table 4. The isolated bacterial species of *Staphylococcus aureus* were tested for antibiotics sensitivity profile, Clarithromycin, Vancomycin and Cephradine showed highly sensitivity against *S. aureus* and Ciprofloxacin, Amikacin and Co-amoxiclav showed highly resistant. The isolated bacterial species of *Pseudomonas aurgenosa* were tested for antibiotics sensitivity profile. Clindamycin, Clarithromycin, and Cephradine showed high sensitivity and Ciprofloaxin, Vancomycin, and Amikacin are highly resistant. The isolated bacterial species of *Streptococcus pyogens* were tested for antibiotics sensitivity profile. Piperacillin, Erythromycin, and Clindamycin showed high sensitivity, and Vancomycin, Ciprofloxacin, and Amikacin are highly resistant. The isolated bacterial species of *Escherichia coli* were tested for antibiotics sensitivity profile. Amikacin, Clindamycin, and Co-amoxiclav showed high sensitivity and Vancomycin Ciprofloxacin, and Cephradin are highly resistant.

3.2 Antimicrobial Activity of Honey against Isolated Bacterial Species

The present study has been conducted to evaluate the antibacterial activity of honey collected from *Acacia* and *Ziziphus*. The honey was dissolved in DMSO. The antibacterial activity was performed against *S. aureus, P. aeruginosa, S. pyogenes,* and *E. coli.*

3.3 Antibacterial Activity

Acacia and *Ziziphus* honey screened in the study showed significant Antibacterial activity against various Gram +ve and Gram –ve bacteria. *Acacia* honey and *Ziziphus* honey were tested for their Antibacterial activity against bacterial isolates (*S. aureus, P. aeruginosa, S. pyogenes* and *E. coli*) and their potency was quantitatively assessed by the presence or absence of zone of inhibitions. The *Ziziphus* honey has good antibacterial activity followed by *Acacia* honey *Ziziphus* honey had greater activity against *E. coli, P. aeruginosa,* and *S. pyogenes* having the highest zone of inhibition (35 mm) respectively, followed by *S. aureus* having zone of inhibition (30 mm). *Ziziphus* honey and *Acacia* honey all had lesser activity at 50 µl as compare to 100 µl (Figure 1).

Table 4. Antibiotic susceptibility pattern of bacterial species.

Antibiotics	*S. aureus*		*P. aeruginosa*		*S. pyogens*		*E. coli*	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
Gentamicin	22	16	12	13	6	7	06	05
Doxycycline	23	15	14	11	9	4	07	04
Co-amoxiclav	16	22	15	10	8	5	08	03
Amikacin	12	26	09	16	4	9	10	01
Vancomycin	29	09	07	18	3	10	02	09
Erythromycin	25	13	17	8	11	2	05	06
Cephradine	26	12	19	6	8	5	03	08
Clindamycin	27	11	21	4	10	3	09	02
Clarithromycin	29	09	20	5	9	4	06	05
Ciprofloxacin	5	33	4	21	4	9	02	09
Piperacillin	31	7	13	12	11	2	06	05
Cefotaxime	25	13	11	14	7	6	08	03

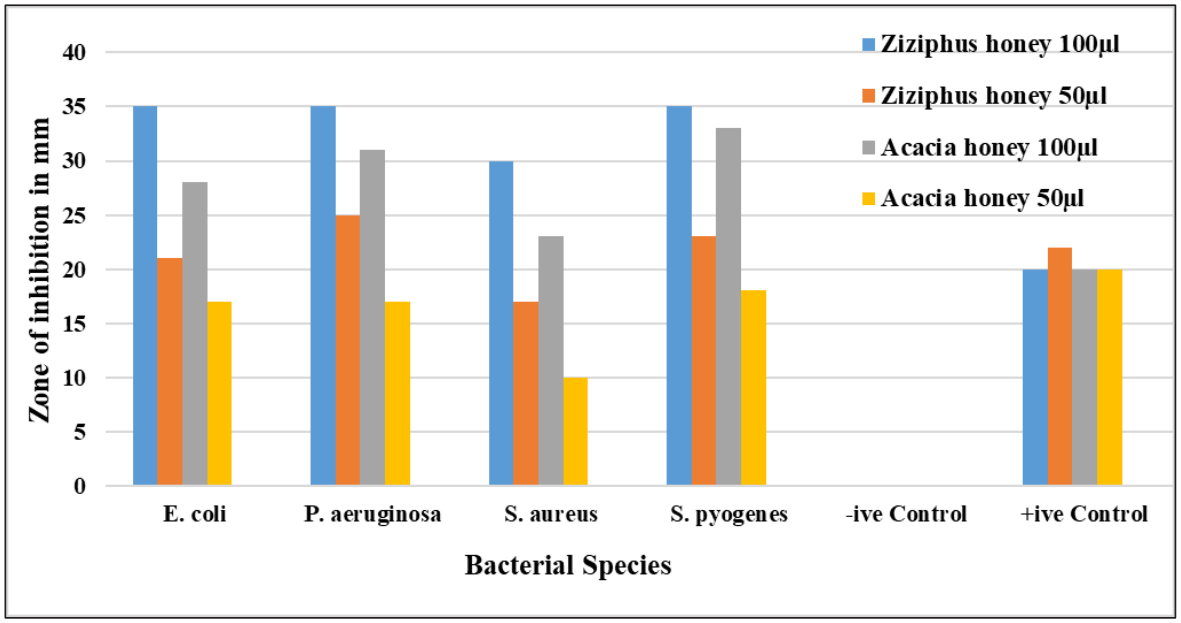

Fig. 1. Antibacterial activity of *Ziziphus honey and Acacia honey.*

3.4 Minimum Inhibitory Concentrations (MIC) Assays of Honey against Bacterial Isolates

The quantitative analysis of *Ziziphus* and *Acacia* honey (Figure 2(a)) showed that *Ziziphus* and *Acacia* inhibited *E. coli* at different concentrations. The results showed that *Ziziphus* honey inhibited *E. coli*, at 100 mg/ml concentration and showed lowest

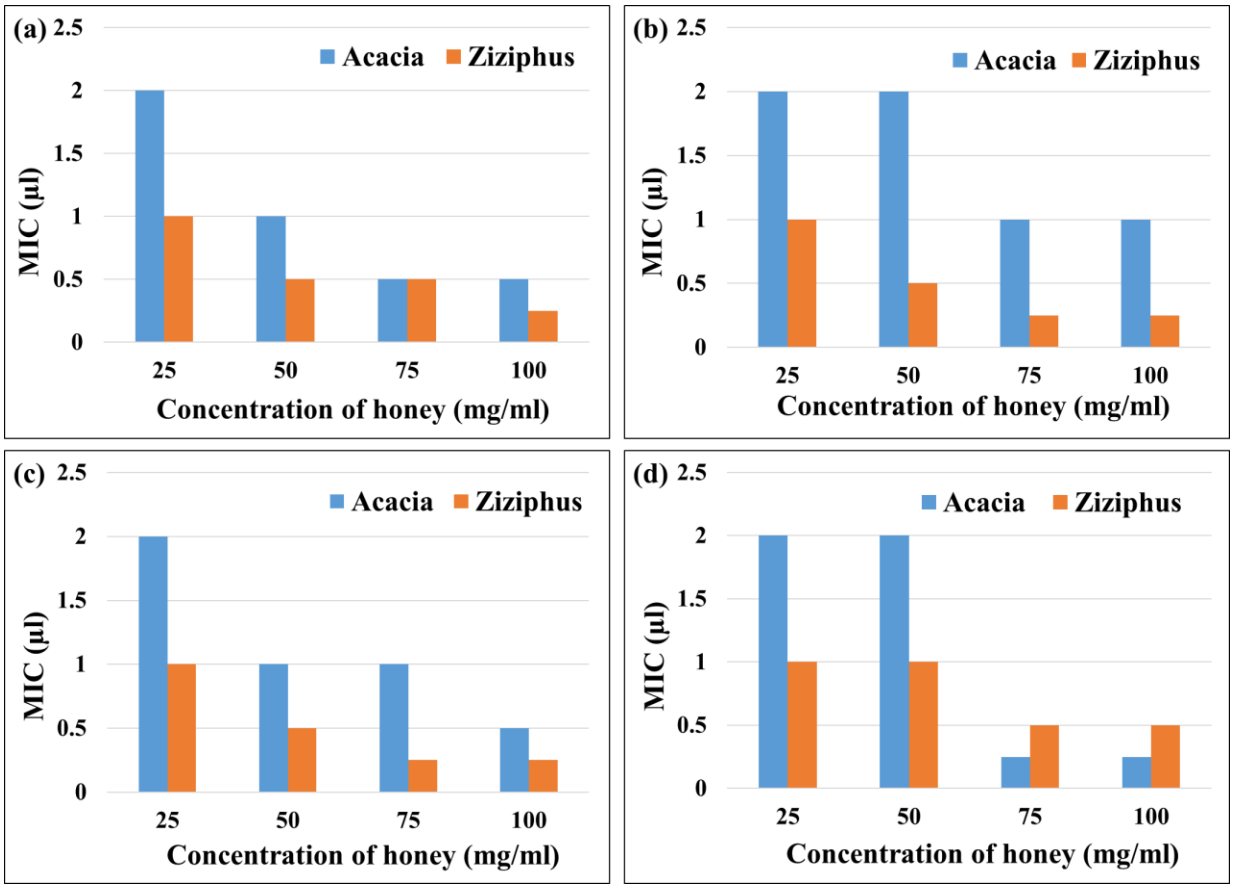

Fig. 2. MIC of *Acacia* and *Ziziphus* honey against (a) *E. coli*, (b) *P. aeruginosa*, (c) *S. aureus*, and (d) *S. pyogenes.*

MIC result of 0.25 µl. *Acacia* honey inhibited *E. coli* at 75 mg/ml concentration low MIC at 0.5 µl.The results revealed that *Ziziphus* honey inhibited *Pseudomonas* at 100 mg/ml, 75 mg/ml concentration on the lowest MIC result is 0.25 µl (Figure 2(b)). *Acacia* honey inhibited *Pseudomonas* at 100 mg/ml and 75 mg/ml concentration on low MIC is 1 µl. It is revealed from Figure 2(c) that *Ziziphus* honey inhibited *S. aureus*, at 100 mg/ml and 75 mg/ml concentration on lowest MIC at 0.25 µl. *Acacia* honey inhibited *S. aureus* at 100 mg/ml concentration on the lowest MIC at 0.5 µl. The results from Figure 2(d) show that *Acacia* honey inhibited *S. pyogenes*, at 100 mg/ml and 75 mg/ml concentration on the lowest MIC at 0.25 µl. *Ziziphus* honey inhibited *S. pyogenes* at 100 mg/ml and 75 mg/ml concentration on lowest MIC at 0.5 µl, respectively.

4. DISCUSSION

Surgical Site Infections are one of the most common complications for patients undergoing surgical procedures and also the second most frequently occurring healthcare-associated infection (HAI) [22, 23]. SSIs led to increased readmission cases in hospitals, increased morbidity, and mortality, reoperation, and prolonged hospital stays which exceeded the health care expenses and may result in significant production of drug-resistant bacterial species [24-26]. Initial infections are frequently more serious, and develop within one week of surgery [5], infections after surgery are caused by microorganisms. Microorganisms may invade an operational wound by different means of contact, such as touching, contaminated care providers or surgical tools, aerosols, or through microbes that are already on or in your body and then spread into the wound [6].

In the present study, a total of 100 samples collected from Surgical Site Infection (SSI) were processed for bacteriological analysis. Out of 100 collected samples, 87 (87%) were positive and showed presence of *S. aureus, P. aeruginosa, S. pyogenes, and E. coli,* while 13 (13%) were negative. The bacterial isolates were identified with *Staphylococcus aureus,* 38 (43%), *Pseudomonas aeruginosa* 25 (28.7%) followed by *Streptococcus pyogenes* 13 (15%), and *E. coli* 11 (12.6%), respectively. According to Bhattacharya *et al.* [27] total of 3004 cases of SSI were considered in which bacterial isolates frequency was as followed: *S. aureus* (34.93%), *E. col*i (20.34%), *Klebsiella spp* (18.08%) *Pseudomonas* (7.99%), respectively. The difference in the identified flora and their respective frequencies of Bhattacharya *et al.* [27] finding in comparison with our research findings might be due to the predominance of those particular microorganisms in that particular locality.

In our study out of total 100 samples, 43 were taken from male and 57 were from female patients. Among 43 samples from male patients, 39 (44.8%) were positive, while out of 57 female patients, 48(65.5%) were positive while in a similar study conducted by Bhattacharya *et al.* [27], the findings of this parameter revealed 62.54% male and 37.45% female were positive. This difference in gender wise positivity of their comparative studies might be due to a large population size of the later researcher.

In the current study, out of 87% positive samples, the site wise distribution was as follow: Cholecystectomy (90%), Appendectomy (93.7%), Diabetic Toe Amputation (80%), Diabetic Foot Amputation (89.2%), and Herniotomy (84.6%). These findings are in contradiction with the results of Bhattacharya *et al.* [27] who collected samples from different surgical wards including; Surgery (12.49%), Orthopedics (11.85%), Urology (3.67%) & Pediatrics Surgery (2.25%) instead of particular surgical sites.

Commercially available antibiotics were evaluated in current study for their antimicrobial activity against the identified bacterial isolates. The results of the experiments revealed that most potent antibiotics found against all the tested bacterial isolates were: Clindamycin Clarithromycin, Piperacillin, and Cefotaxime, Co-amoxiclav, Vancomycin, and Cephradine, respectively, while maximum number of bacteria displaying resistance against Gentamicin, Ciprofloxacin and Amikacin. Our findings regarding antibiotics profiling against Co-amoxiclav are similar to those of Abubakar [28], who also found Co-amoxiclav as the most sensitive of all tested antibiotics in his study. The findings of Bhatt *et al.* [29] are in agreement with our studies in terms of evaluating the potency of commercially available antibiotic against isolated specimen. The tested bacteria displayed highest resistance to Gentamycin and Amikacin and displayed sensitivity

to Piperacillin antibiotic in their study. The most possible reason for the difference in sensitivity profile might be the improper use of antibiotics, over dosage or self-medication. Therefore, proper and prescribed dosage of medicine was recommended along with the maintenance of good hygienic conditions within hospitals as well as in routine life. In addition, theantibiotics must be used after performing susceptibility tests. Following these protocols might reduce the phenomenon of antibiotic resistance to a greater extent.

In the current study, *Acacia* honey and *Ziziphus* honey were tested for their antibacterial activity against *S. aureus, S. pyogenes,* and *E. coli,* at different concentrations of 50 µl and 100 µl for both honey brands. The *Ziziphus* honey showed highest zone of inhibition for*, S. pyogenes, P. aeruginosa,* and *E. coli* the zone of inhibition is (35 mm) while *S. aureus* showed (30 mm) zone of inhibition. At 50 µl the highest zone of inhibition was (25 mm) for *P. aeruginosa. Acacia* honey *S. pyogenes* showed highest zone of inhibition for both concentrations 50 µl and 100 µl which are (19 mm) and (32 mm), respectively. In contrast to our study Rajeswari and Mandal [30] studied two brands of honey: Manuka honey and Nilgiris honey were tested against pathogenic bacterial isolates. The zone of inhibition were determined against *E. coli, S. typhi, S. aurous* and *P. aeruginosa*, the zone of inhibition were (13 mm – 14 mm), and (17 mm – 19 mm), followed (20 mm – 21 mm) and (25 mm – 27 mm), respectively. A difference in results was due to variation in samples size, geographical difference and location from where honey was collected. Hussain *et al.* [31] conducted study on Maunka honey and local honey brand *E. coli, S. typhi, S. aureus* and *P. aeruginosa* zone of inhibition ranging from 13 mm, 14 mm and 16 mm, respectively. It was thus concluded that honey is an alternative to treat bacterial infection and helped to reduce the chance of emergent drugs resistance to a great extent.

MIC values are used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents. In current study four different concentration of honey were used, i.e., 100 mg/ml, 75 mg/ml, 50 mg/ml, and 25 mg/ml for isolated *Staphylococcus aureus, Pseudomonas, Streptococcus pyogenes,* and *E. coli.* The MIC was determined by measuring optical density (OD) values at 610 mn using spectrophotometer [21]. Yilmaz [32] reported three different concentrations, i.e., 100 µl/mg, 50 µl/mg, 10 µl/mg in his findings. In *ziziphus* honey *S. aureus* growth was inhibited at 100 mg/ml and 75 mg/ml concentration and the MIC was 0.25 µl. In *Acacia* honey the growth of *S. aureus* was inhibited at 100 mg/ml concentration, the MIC was 0.5 µl. Similarly, for *Acacia* honey the growth of *P. aeruginosa* was inhibited at 100 mg/ml and 75 mg/ml, the lowest MIC is 1 µl. *P. aeruginosa* growth was inhibited in *Ziziphus* honey at 100 mg/ml, 75 mg/ml and the lowest MIC is 0.25 µl for both concentrations. *S. pyogenes* growth was inhibited at 100 mg/ml, 75 mg/ml concentrations and the lowest MIC is 0.25 µl for *Acacia* honey, whereas *Ziziphus* honey inhibited growth at 100 mg/ml, 75 mg/ml concentration on the lowest MIC at 0.5 µl, respectively. In *Ziziphus* honey *E. coli* growth was inhibited at 100 mg/ml concentration and the lowest MIC is 0.25 µl; however, *Acacia* honey inhibited the growth of *E. coli* at 100 mg/ml, 75 mg/ml concentration and the lowest MIC was 0.5 µl. Mandal *et al.* [33] determined the Antibacterial mechanism of honey toward pathogen *E. coli* (n = 5), *P. aeruginosa* (n = 5), *S. enterica* serovars *typhimurium* (n = 8). Also, MIC and PIC in their findings ranged from 1.75-3.0 and 3-3.5, respectively. The differences observed were due to the variation in sample size, geographical location and quality and type of honey used.

5. CONCLUSIONS

It was concluded from the present study that Surgical Site Infections (SSI) was among the highest prevalent diseases, affecting millions of people throughout the world. This can be achieved by optimal preoperative, intra-operative and post-operative patient care. This would be supported with proper infection control measures and balanced antibiotic policy. Infection by multidrug-resistant bacteria enhances the need for antibiotic policy guidelines in hospitals. Furthermore, it can be concluded that honey has effective antimicrobial properties against bacterial isolates. The honey showed excellent antibacterial activity against *Staphylococcus aureus*, *Pseudomonas spp, Streptococcus pyogenes, E. coli* at the Surgical Site Infections. From bioassay, it was revealed that the most potent antibiotics found against all the tested bacterial isolates showed maximum resistance against Gentamicin, Ciprofloxacin, and Amikacin.

Moreover, it is also established that honey would be effective against bacterial isolates responsible for SSI and must be used as alternative of antibiotics because of resistance found in commercially available antibiotics. Furthermore, due to high antimicrobial activity of honey, further investigation is suggested in this regard as an alternative therapy for wound healing.

6. ETHICAL STATEMENT

The study was conducted in accordance with the declaration of Helsinki, and the protocol was approved by the Institutional Ethics Review Committee, Abasyn University Peshawar.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Decentralization of OPDs of Basic and Rural Health Care Units of Punjab, Pakistan

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Abstract: Decentralization is associated with political administration and historical development and it varies according to region and implementation factors. In the 1980s, the International Monetary Fund (IMF) and World Bank demonstrated that corruption and bad administration in society are due to centralization policies which concentrated authority and power in the hands of the elite upper class. This criticism was an initial point to start the period of structural decentralization adjustment. Decentralization is a substantial process for equal distribution of healthcare service delivery in the community. The outpatient department (OPD) is one of the most important parts of any health care unit to diagnose and treat patients who do not require overnight care or stay in hospitals. The main purpose of this research was to analyze the effects of decentralization and improvements in health services for OPD by analyzing the number of patients in Primary Health Care Centers (Basic Health Unit (BHU) and Rural Health Centers (RHCs) in Punjab before and after decentralization. Non-probability convenient and simple random sampling technique was employed and patients visiting PHCs (primary healthcare centers) OPD, were included in the study population. OPD patients of rural and basic health care units were categorized into three pairs OPD 1, 2 and 3, and the means and other statistical parameters were calculated by using SPSS. The average mean of all groups of OPD patients of RHCs were 119441.1111, 192536.5185 and 153487.1358, respectively. The average mean of all groups of BHU was 94818.5062, 109331.7160, and 124231.0123, respectively. These results showed that the number of patients in the outdoor patient department increased after decentralization due to more health facilities.

Keywords: Decentralization, Rural Health Centers, Basic Health Unit, OPD.

1. INTRODUCTION

Primary healthcare centers are responsible for the essential health care of the entire community. Primary care refers to population-oriented care, including health promotion and screening. According to Alma Ata Declaration, 85% to 90% of health problems can be resolved at primary health care centers or by enhancing the ability of clinical testing, and it is possible to maintain 96% of patients at primary healthcare facilities [1, 2]. Primary health care guarantees that individuals receive high-quality, comprehensive services, including several promotions, prevention and therapies, rehabilitative services, and pain management, as close to their homes as possible. Decentralization can be characterized in terms of 'who' gets decision-making authority. According to public administration "who" can differentiate between deconcentration and devolution. In deconcentration local agencies have more power to make decisions, while in devolution, local government with associated responsibility of several other sectors gets a choice to make decisions [3]. Public health has a layer structure system in which Basic Health Unit (BHU) and Rural Health Centers (RHCs) constitute a primary level of health services at the Union Councils level. Tehsil Headquarter

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Hospitals provide secondary healthcare at tehsil and District Headquarter Hospitals in every district [4, 5]. The government of Pakistan delivers health facilities throughout Pakistan through primary (PHCs), secondary, and tertiary health care units. PHCs are primary level health facilities such as emergency services, hospitalization amenities, surgical procedures, and specialized clinics. It is composed of a rural health center and basic health units. Basic Health Unit (BHU) is the main service, from where many patients are referred to the RHCs (Rural Health Centers) [6]. These primary health care services are observed and administered by district-level health officers. All services of PHC, including basic and rural health centers, are controlled by qualified physicians with excellent exposure in the therapeutic and medicinal field. RHCs and BHU are intended to enhance access to primary health care services; they can be public or private health facilities [7, 8]. They have required a team approach of doctors and non-physician staff like nurses, assistants, etc. Outpatient departments of rural and basic health care centers are part of the clinical facility designed to treat outpatients who do not require overnight care or bed at that time. Modern-time OPDs have various services, surgical processes, and diagnostic tests [9, 10]. OPD is an important health facility component and integrated with in-patient services and consultant physicians. All the collected data of research was analyzed using SPSS version 26. Mean ± S.D used for quantitative data, paired t-test was also applied where applicable to compare the means in the treatment and control groups and test of normality (Kolmogorov–Smirnov test and the Shapiro–Wilk test) was done to check the normal distribution of data. The main purpose of this research was to analyze the effect of decentralization and improvements in health services for OPD by analyzing the number of patients in BHU and RHCs of Punjab before and after decentralization. Whether this is an effective way to enhance the quality and provision of health services to every citizen of Pakistan or not.

2. MATERIALS AND METHODS

2.1 Study Design

This study was a cross-sectional survey based, on a random stratified sample of Rural and Basic Health Care facilities. This study was carried out at BHU and RHCs OPD patients of nine highly burdened selected districts of Punjab. A total of 350 healthcare facilities were selected for data collection. Data was collected during a field survey from 2009 to 2010, 2012 to 2014, and 2017 to 2018, after ethical standards were approved by the IRB (Institutional Review Board) of the University of Lahore.

2.2 Sampling Technique

Non-probability convenient and simple random sampling technique was employed and patients visiting PHCs OPD, were included in the study population.

2.3. Data Analysis Procedure

Collected data was analyzed using SPSS version 26. Mean ± S.D. was used for quantitative data and to check the normal distribution of data, test of normality including Kolmogorov-Smirnov and Shapiro-Wilk was also calculated.

3. RESULTS

OPD patients of rural and basic health care unit were categorized in three pairs OPD 1 (2009 to 2010), 2 (2012-13) and 3 (2017-2018). The average mean of all groups of OPD patients of RHC were 119441.1111, 192536.5185 and 153487.1358, respectively. The average mean of all groups of BHU was 94818.5062, 109331.7160, and 124231.0123, respectively. Statistical factors of all groups such as 95% Confidence Interval for Mean Lower and Upper bound, 5% Trimmed Mean, Median, Variance, Standard Deviation (to measure the amount of variability or dispersion), Range, Interquartile range, Skewness (to measure the asymmetry of the distribution) and Kurtosis (to measure the tailedness of distribution) of RHCs and BHU were also calculated, and are given in Table 1 and 2. To test the normal distribution of data, the test of normality, including Kolmogorov-Smirnov and Shapiro-Wilk was done. In the present data, the significant value of the Shapiro-Wilk test was smaller than 0.05 in all groups, which showed that data significantly deviated from a normal distribution. Kolmogorov-Smirnov p-value was also significantly deviated from the significant level (0.05) in the present research data, as shown in Tables 3 and 4. The same data from the same

Table 1. Descriptive stats of OPD patients in rural health centers.

Statistical factors	2009 to 2010	2012 to 2013	2017 to 2018
Mean	119441.1111	192536.5185	153487.1358
95% Confidence Interval for Mean			
Lower bound	61586.3002	76933.3790	78246.3246
Upper bound	177295.9221	308139.6581	228727.9470
5% Trimmed Mean	86892.5679	106816.0316	107873.1509
Median	20739.0000	26684.0000	37540.0000
Variance	68458935541.050	273331976704.403	115786552332.419
Std. Deviation	261646.58519	522811.60728	340274.23107
Minimum	.00	.00	.00
Maximum	824756.00	2.89E+6	1.13E+6
Range	824756.00	2891558.00	1128026.00
Interquartile Range	58623.00	59227.00	102189.00
Skewness	2.234	3.857	2.478
Kurtosis	3.235	16.617	4.431

Table 2. Descriptive stats of OPD patients in Basic health unit.

Statistical factors	2009 to 2010	2012 to 2013	2017 to 2018
Mean	94818.5062	109331.7160	124231.0123
95% Confidence Interval for Mean			
Lower bound	24061.7966	25933.1926	25600.6753
Upper bound	165575.2157	192730.2395	222861.3494
5% Trimmed Mean	34046.7243	37209.1118	42552.1619
Median	16731.0000	16714.0000	14518.0000
Variance	102396813318.253	142255120524.831	198962953625.937
Std. Deviation	319995.02077	377167.23151	446052.63549
Minimum	0.00	0.00	0.00
Maximum	1.89E+6	2.06E+6	2.75E+6
Range	1888901.00	2056156.00	2747230.00
Interquartile Range	102252.00	110062.50	109622.50
Skewness	4.898	4.860	5.420
Kurtosis	23.314	22.661	29.711

pairs were also analyzed to produce a Normal Q-Q Plot and detrended Normal Q-Q Plot as shown in Figures 1 to 4 (Supplementary Data).

3.1 Paired T-test

For paired T-test, three pairs of all study duration eras were formed; pair 1 included 2009-10 and 2012-13, pair 2; included 2009-10 and 2017-18 and pair 3 included 2017-18 and 2012-13. Paired sample T-test of all the groups was also calculated to compare the mean of the two pairs. The means and standard deviation of all the groups are given in Table 5. Paired sample correlation of all three pairs of BHU were 0.998, 0.925, and 0.947 and RHCs were 0.816, 0.933 and 0.562 ($p < 0.001$) respectively showing a significantly positive correlation among pairs as shown in Table 6. T values of paired T-test

Table 3. Test of normality (Kolmogorov-Smirnov[a]).

Pairs	OPD in RHCs			OPD in BHU		
	Statistic	df	Sig.	Statistic	df	Sig.
2009-10	0.444	81	0.000	0.446	81	0.000
2012-13	0.452	81	0.000	0.445	81	0.000
2017-18	0.424	81	0.000	0.413	81	0.000

a. Lilliefors Significance Correction.

Table 4. Test of normality (Shapiro-Wilk).

Pairs	OPD in RHCs			OPD in BHU		
	Statistic	df	Sig.	Statistic	df	Sig.
2009-10	0.475	81	0.000	0.282	81	0.000
2012-13	0.403	81	0.000	0.275	81	0.000
2017-18	0.461	81	0.000	0.270	81	0.000

Table 5. Paired sample T-test **.**

Pairs	OPD in RHCs			OPD in BHU		
	Mean	Std. deviation	Std. error mean	Mean	Std. deviation	Std. error mean
2009-10	119441.111	261646.58519	29071.84280	94818.5062	319995.0207	35555.00231
2012-13	192536.518	522811.60728	58090.17859	109331.716	377167.2315	41907.47017
2009-10	119441.111	261646.58519	29071.84280	94818.5062	319995.0207	35555.00231
2017-18	153487.135	340274.23107	37808.24790	124231.012	446052.6354	49561.40394
2012-13	192536.518	522811.60728	58090.17859	109331.716	377167.2315	41907.47017
2017-18	153487.135	340274.23107	37808.24790	124231.012	446052.6354	49561.40394

Table 6. Paired samples correlations.

Pairs	Correlation (RHCs)	Correlation (BHU)	Sig.
2009-10 & 2012-13	0.816	0.998	0.000
2009-10 & 2017-18	0.933	0.925	0.000
2012-13 & 2017-18	0.562	0.947	0.000

Table 7. Paired sample T-test of rural health centers.

Pairs	Paired differences					T	df	Sig. (2-tailed)
	Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
1	-73095.40	344372.7	38263.63	-149242.47	3051.66	-1.910	80	0.060
2	-34046.02	134426.6	14936.29	-63770.20	-4321.84	-2.279	80	0.025
3	39049.382	435085.5	48342.83	-57155.92	135254.6	0.808	80	0.422

Table 8. Paired sample T-test of basic health unit.

Sr. no.	Mean	Std. deviation	Std. error means	95% confidence interval of the difference		T	df.	Sig. (2- tailed)
				Lower	Upper			
1	-14513.20	61468.09	6829.7	-28104.92	-921.49	-2.125	80	.037
2	-29412.50	21485.85	21485.8	-72170.71	13345.70	-1.369	80	.175
3	-14899.29	150018.4	16668.7	-48071.08	18272.49	-.894	80	.374

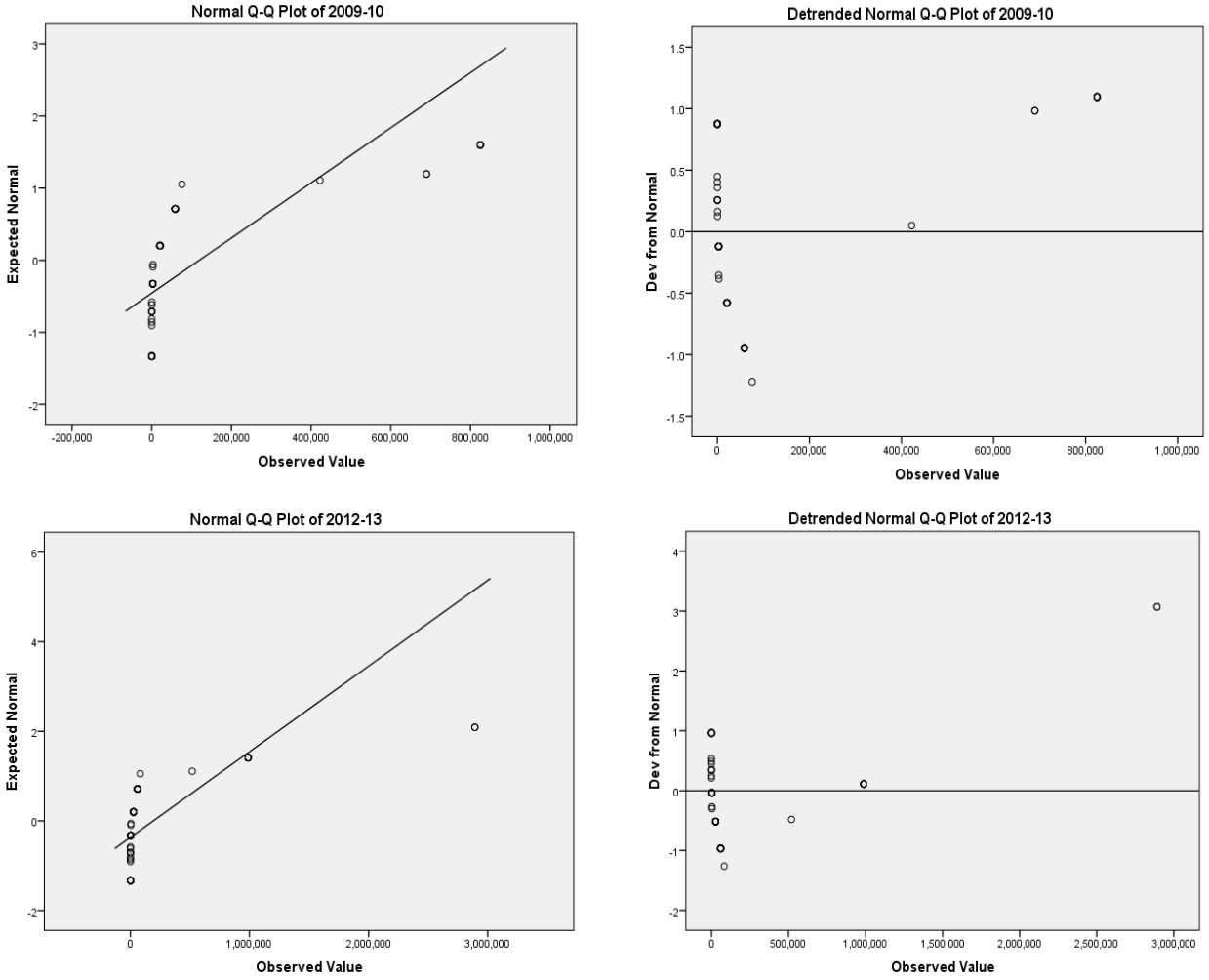

Fig. 1. Normal and detrended Q-Q plot showing correlation among OPD patient's data of RHC:2009-10 and 2012-13.

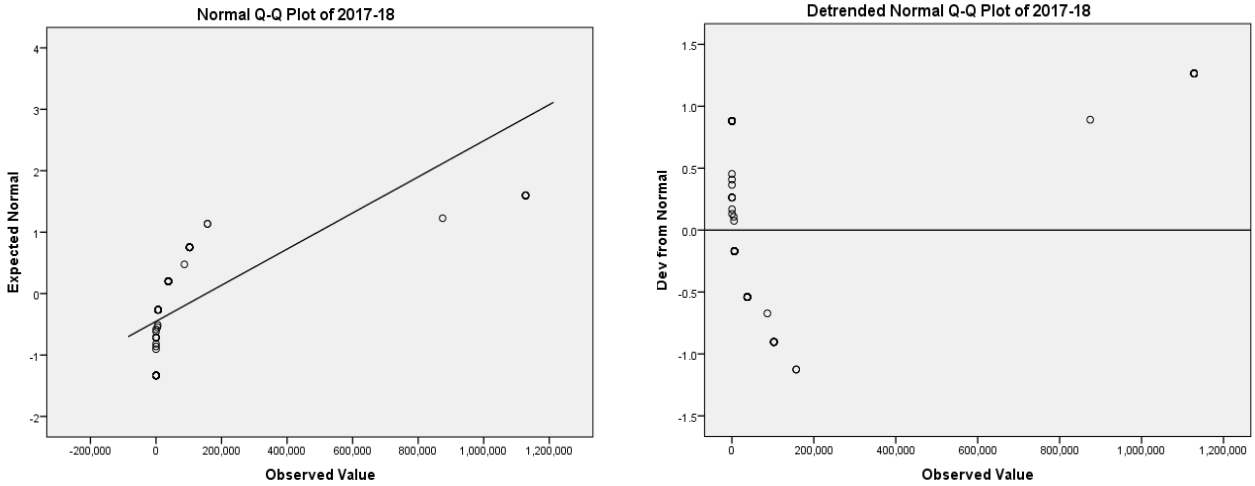

Fig. 2. Normal and detrended Q-Q plot showing correlation among OPD patient's data of RHC: 2017-18.

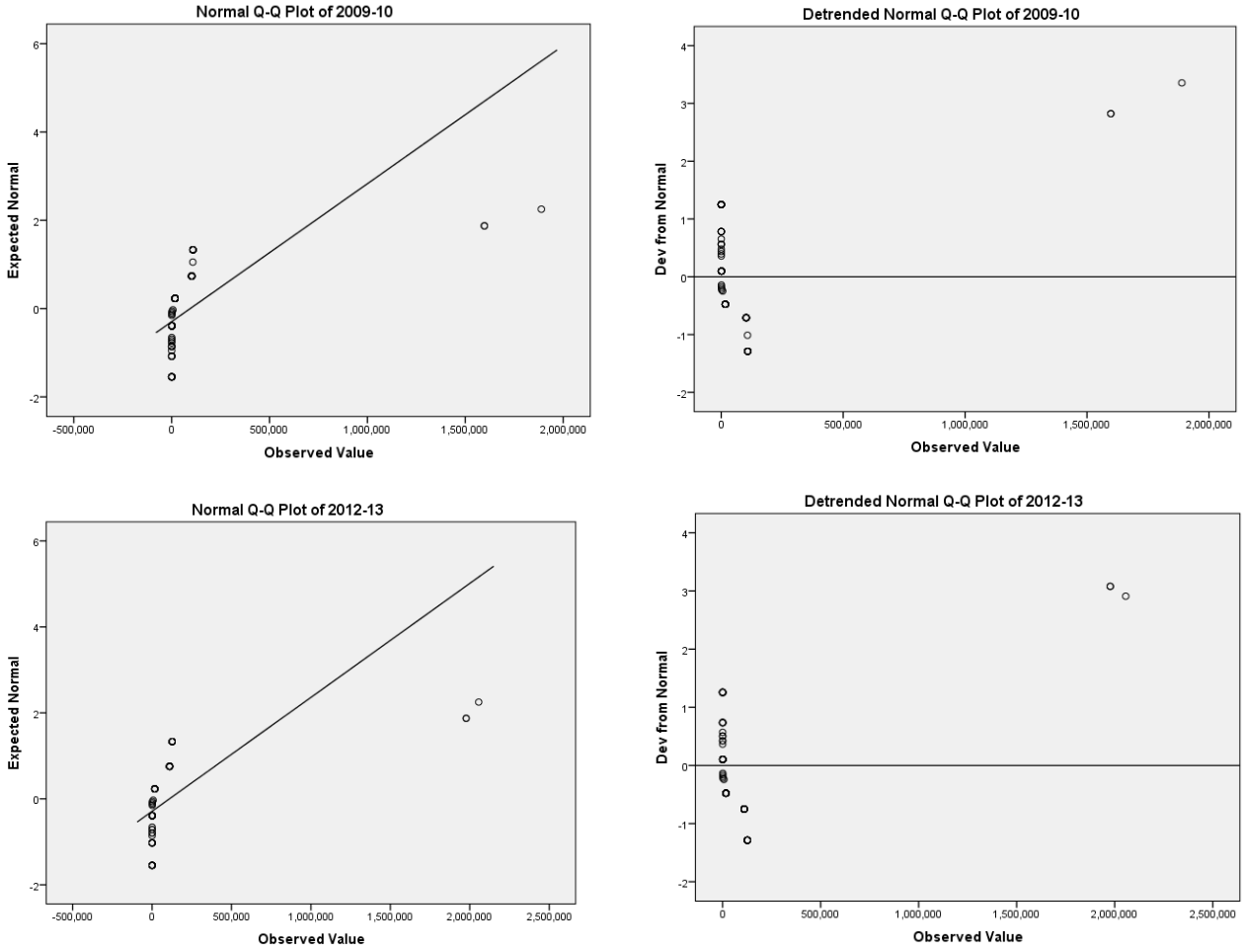

Fig. 3. Normal and detrended Q-Q plot showing correlation among OPD patient's data of BHU: 2009-10 and 2012-13.

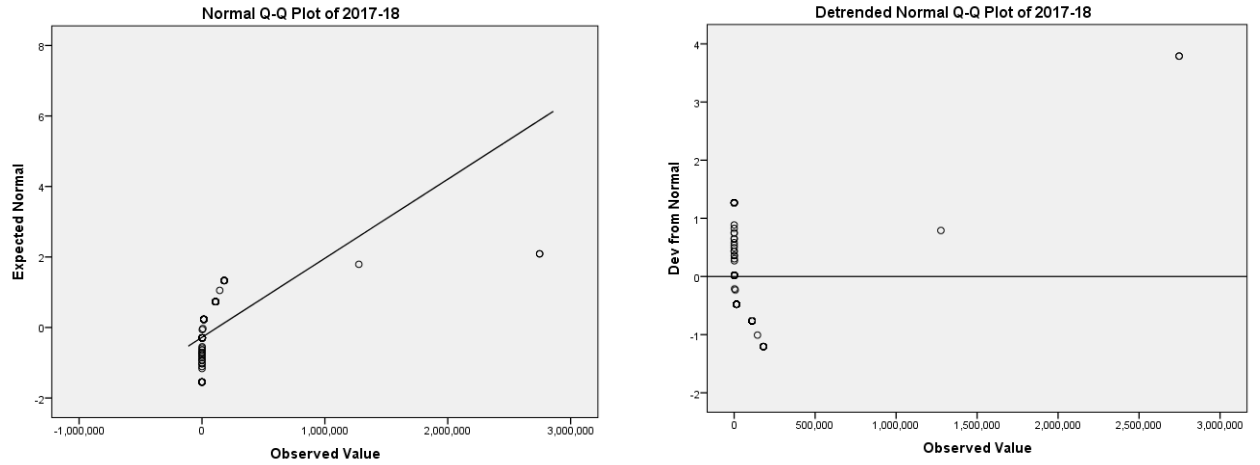

Fig. 4. Normal and detrended Q-Q plot showing correlation among OPD patient's data of BHU; 2017-18.

of all the pairs of RHCs were -1.910, -2.279, 0.808 and Sig. (2 tailed values) was 0.06, 0.02 and 0.42 with degree of freedom 80 and T value of paired T-test of all the pairs of BHU was -2.125, -1.369 and -0.894 respectively, and Sig. values were 0.37, 0.175 and 0.374 with degree of freedom 80 as shown in Tables 7 and 8.

4. DISCUSSION

Decentralization is the process in which authority transfers responsibilities and power from the federal government to the subordinate government. Decentralization has been applied and promoted to enhance public goods betterment, responsiveness,

government accountability, service delivery, popular participation in decision making, stability of state and contribution to better governance [11, 12]. Health quality is a global issue and the main goal of health services is to improve community. Health care service, delivery and its management are significant concerns for Pakistan's government and public strategic administrative approaches. Health is a global communal good so, better management is associated with better delivery of primary and secondary health services to patients.

The satisfaction level of patient is one of the important factors of health care units to measure health quality and it is directly associated with the use of health care facilities. Patient's satisfaction can be defined as an attitude derivate by the receiver of health services whether the patient's expectations for services like availability of doctors, nurses, proper diagnosis and treatment have been considered or not [10]. Healthy communities characterized by a significant decrease in mortality, morbidity and disability became the main aim in Punjab. This sort of purpose can be attained by well-organized and efficient health services provided to patients [13]. Out-patient care is important for strengthening the primary health care services and structure of health networks in basic and rural health care centers [14]. Our results confirmed that better services after decentralization provided at OPD of BHU and RHCs health facilities have improved the number of patients in OPD. Previous research carried out in Norway [15], Iran [16] and Pakistan [17] revealed that good services are a substantial determinant of the delivery of health services for outdoor patients.

5. CONCLUSIONS

The present research concludes that improving healthcare services and management in OPD can be an effective strategy to attract patients to the eradication of disease and increase in survival rate. This study can be useful to analyze the beneficial effects of a decentralized health system in different districts of Punjab and how decentralized system can improve the quality-of-service delivery by some interventions at larger cohort.

6. DECLARATION AND ETHICAL STATEMENT

The results of this manuscript are original. The same materials are neither published nor under consideration elsewhere. The approval of all authors has been obtained before publication. The ethical standards were approved by the IRB (Institutional Review Board) of University of Lahore.

7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Understanding Farmers' Knowledge, Attitude, and Practices in Managing Water Quality for Effective Insecticide Performance: A Case Study in Agriculture

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Abstract: Pesticides are frequently used in agriculture to manage the pest populations below threshold levels; however, most of the time, their applications do not get the desired outcome. There are many factors for the low performance of pesticides, the quality of water being one of them. Local farmers of Sindh, Pakistan have little or no knowledge of the role of water quality in the performance of pesticides. Therefore, a survey study was conducted to determine the farmer's knowledge, attitude, and practice regarding it. Ten villages were selected from each Hyderabad and Tando Allahyar districts as the information was obtained from five farmers from each village. Descriptive analysis results indicated that most of the respondents prefer to grow cotton (average 24.07 acres) with the majority of them being illiterate (54 %) averaging 39.74 years of age and averaging 18.80 years of farming experience. Most farmers (92 %) used groundwater for spray solution, whereas only 3% of farmers tested the water in their fields. The majority (74.51 %) of farmers considered lower-quality pesticides for their poor performance. Therefore, farmers normally apply up to sixteen sprays to control pests. It was also observed that although farmers have knowledge about the role of water quality in the performance of pesticides to get pest control, they lack the attitude and practice it in their fields. Therefore, it is suggested that awareness should be created among the local farmers regarding water quality to be used in spray solutions to get desired pest control.

Keywords: Water Quality Management, Pesticides, Insecticides, Pest Control.

1. INTRODUCTION

The application of pesticides on crops is although discouraged at large, nevertheless the pesticide is supposed to be an inevitable component by the farming communities to keep their crops safe from pest infestations and to maximize crop production [1]. Likewise, in Pakistan, the habit of applying insecticides to various crops is referred as a common practice as only during 2020-2021, 37,441 tons chemical insecticides of worth Rs. 30,083 million were imported to manage crop pests [2]. The phenomenon of 'injudicious use of pesticides' is normally correlated with inefficacy of pesticides [3]. While the related literature concludes that the number of factors involved in failure or lessening of the pesticides' efficacy in controlling pest problems. In this regard, insect resistance is also considered a huge issue these days [4, 5]. Yet some of the researchers are of the opinion that the mishandling, improper calibration, and selection of incorrect pesticides could also contribute as significant causes behind pesticides ineffectiveness [6]. Therefore, the general opinion of farmers, researchers and institutional representatives related to insecticide incompetency are quite diverse and somehow complicated.

All the contributors of this study believe that before applying pesticides to the crops, the farmers

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must be aware and should adopt all the significant measures and steps that may not disturb the effectiveness of a pesticide. In this regard, the water quality has always been ignored by most of the farmers. However, improper quality of water may potentially bring adverse impact on the performance of pesticides, as water functions as a solvent for mixing pesticides before applying. Hence, recommended water quality plays an imperative role in maintaining pesticide's effectiveness [7]. The available literature is correspondingly evident that the water hardness, pH, turbidity, and temperature display negative effect on the performance of various synthetic pesticides [8-15]. The pH of the spray solution can impact significantly on insecticide efficacy. The variation in pH of solution may lead to hydrolysis which cleaves the chemical molecules into smaller compounds. Pesticides may undergo alkaline hydrolysis, in which a pH greater than seven causes chemical degradation of certain pesticides in the presence of ions. The rate of alkaline hydrolysis is enhanced as the pH increases [16]. For example, organophosphate (acephate and chlorpyrifos), carbamate (methiocarb), and pyrethroid (bifenthrin, cyfluthrin, and fluvalinate) undergo alkaline hydrolysis in the presence of alkaline water. Moreover, the half-life of many insecticides can also be affected with the variation in pH of solvent or spray mixture [15]. Therefore, it is strongly recommended by various researchers that for most of the insecticides favor to use water with pH only ranging from 4 to 7 for preparation of spray mixture [16, 17]. As it was earlier discussed that 80 percent of the total pesticides are applied merely on cotton crop for the purpose of plant protection [18].

In Pakistan, cotton is not only a cash crop which contributes 1.0% in Gross Domestic Product (GDP) but also covers a share of 5.5% in value added goods of agriculture. Furthermore, the cultivation area has increased at 6.5% in 20119-20 as compared to 2018-19, however, there was a decline in the production from 1,676,000 metric tons to 1,560,000 metric tons. Moreover, during the same period of time, cotton backed up to US$ 10.22 billion to foreign exchange of Pakistan [19]. Logically, increasing in cotton yield may directly proportion to the pesticides' application, which may disturb the foreign exchange reserves at one end, on the other hand it may also augment environmental degradation and health hazards to the local people. Hence, the sustainable development requires certain level of awareness among farming communities to find out real issue related to 'ineffectiveness of pesticides. Once the matter of water quality prerequisite to pesticide spray mixture is carefully managed then one can go beyond it and think about pest resistance [1].

It is the foremost important to have considerable knowledge regarding basic or suggested parameters to make a mixture of pesticides, but unfortunately the literacy rate of rural people is quite unsatisfactory where only 53.3% can have ability to read and understand simple text in local languages [20]. On the contrary, the labels of pesticides especially from international companies are in English and have technical terms which are not possible for the local farmers to understand. Therefore, current situation of farmers motivated the researchers to conduct a study in which the level of farmers' knowledge, their attitude and practice regarding water quality for preparing a pesticide mixture may be investigated. The outcome of the study may help to identify the real issue regarding pesticide ineffectiveness and could propose some implications to resolve targeted issues.

2. MATERIALS AND METHODS

2.1 Data Collection from Farmers

A comprehensive single-time survey was conducted using multi-stage cluster sampling method, in which two major cotton growing districts of Sindh, Pakistan i.e., Hyderabad and Tando Allahyar were selected. Both districts are rich with agricultural land and variety of crop cultivation. In next stage, ten villages were selected from each district, and five farmers were interviewed from each village (Table 1; Figure 1). Keeping in mind the specific objective (gathering perceptions of farmers), a descriptive research design was selected, and a cross-sectional data were gathered on a reliable and valid scale [21–25]. Information was collected via primary sources through interviewing method, where close ended questionnaire using 10-point Likert scale was used supposing the pronounced variation in farmers' perceptions [26]. Descriptive research was used for obtaining people's perceptions of social issues and social facts concerning with the status of phenomena, in which central tendencies and cross tabulation are calculated [22].

Table 1. GPS coordinates various villages of Hyderabad and Tando Allahyar Sindh, Pakistan.

District	Union Council (UC)	Village	GPS		Respondents
			Latitude	Longitude	
Hyderabad	Moosa Khatyan	Imam Bux Pusio @ Pasha farm	N 25°27'33.453"	E 68°32'05.882"	5
		Muhammad Bachal Gopang	N 25°26'49.312"	E 68°32'10.381"	5
		Abu Talib Sipio	N 25°27'55.433"	E 68°31'05.432"	5
		Moosa Khatyan	N 25°28'11.455"	E 68°31'19.362"	5
		Mureed Sipio	N 25°28'56.294"	E 68°30'18.239"	5
	Shahpur @ Arif Khatyan	Arif Khatyan	N 25°28'46.567"	E 68°33'27.262"	5
		Suleman Khan Khatyan	N 25°28'16.607"	E 68°32'18.905"	5
		Allah Dino Sandh	N 25°29'54.390"	E 68°31'45.355"	5
	Tandojam	Noor Khan Lashari	N 25°26'23.959"	E 68°32'03.637"	5
		Piyaro Khan Behan	N 25°26'48.725"	E 68°32'47.318"	5
Tando Allahyar	Shah Inayat	Tando Soomro @ Qazi A. Majeed	N 25°30'46.627"	E 68°40'09.261"	5
	Dasori	Jhando Mari @ Piyaro Lund	N 25°35'32.647"	E 68°41'43.081"	5
	Missan Wadi	Shadiyoon Walhar	N 25°23'33.739"	E 68°52'55.562"	5
	Purani Mirabad	Mirabad	N 25°38'54.459"	E 68°40'52.635"	5
	Tajpur	Jam Samo, Kisana Mori	N 25°27'53.643"	E 68°34'16.469"	5
	Nasarpur	Abdul Rouf Kakepoto	N 25°30'07.748"	E 68°37'31.507"	5
	Shahpur Rizvi	Shaikh Moosa Khabar Chachar	N 25°32'20.974"	E 68°43'30.441"	5
	Sawan Khan Gopang	Darya Khan Nahyoon	N 25°23'33.918"	E 68°43'55.038"	5
	Khokhar	Bukera Sharif, Darya Khan Masak	N 25°21'53.801"	E 68°39'17.908"	5
	Tando Allahyar	Dhingano Bozdar	N 25°26'17.022"	E 68°39'12.390"	5
2 Districts	13 UCs	20 Villages	-	-	100

2.2 Data Analysis

The collected data were subjected to statistical analysis software SPSS-21 for descriptive analysis to access the knowledge, approach, and practical nature of farmers in preparing the insecticide solution.

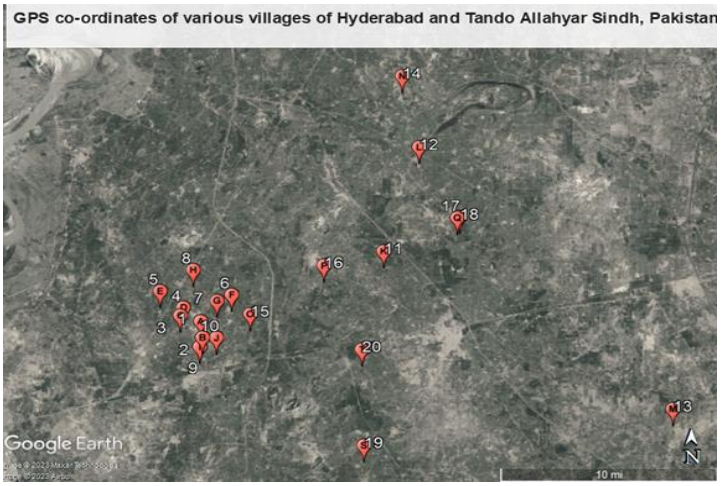

Fig. 1. GPS co-ordinates (landmarks) of various villages of Hyderabad and Tando Allahyar Sindh, Pakistan.

The present research focused on the district Hyderabad and Tando Allahyar because both districts are also well-known in cotton production, where cotton is grown over 72,894 acres. Likewise, local farmers consume a big proportion of pesticides to save their cotton crop from various insect pests. Hence, using purposive sampling method [25], one hundred cotton growers were approached, and multi-stage cluster sampling method was used to reach at respondents. For this purpose, total fifteen union councils from both districts were selected, followed by twenty villages were visited to collect data from one hundred farmers/respondents. In this regard, the researchers employed KAP (Knowledge, Attitude and Practice) method to know the levels [26, 27], yet the procedure (KAP Assessment) is quite common for perception-based studies in public health discipline [27, 28] but rarely considered for agricultural studies. The data collected using KAP scale is not only beneficial to identify the respondents' knowledge, but their behavior could easily be segregated for future implications [29].

To the best of researchers' knowledge, the issue (water quality used for insecticide mixture) has not been attempted by any other researcher, however, few related articles [30, 26] were reviewed that were on herbicide spray mixture. In addition, some companies' brochures also supported and guided to develop and finalize questionnaire for this research. Ten-point Likert scale (1 = Nil to 10 = Excellent) was decided to perceive the perceptions of the respondents. After questionnaire was developed, it was translated into Sindhi language in order to make the data collection process easier and comprehensive. The respondents for this study were confined to the farmers those were making pesticide mixture and applying to their crops.

Validity and reliability of data are the most important steps in scientific research. Without considering these steps, generalization and prediction cannot be achieved which is the rudimentary target of scientific research. As far as the content and validity of the questionnaire (scale) is concerned, the validity was ensured through Confirmatory Factor Analysis (CFA), because CFA is a satisfactory tool to establish validity of a scale [31], where factor loadings of items ≥ 0.40 are acceptable if items are in lesser quantity [32].

The AMOS software was used to know confirmatory Factor Analysis (CFA) through Average Variance Extraction (AVE) and found that Knowledge (AVE = 0.59), Attitude (AVE = 0.59) and Practice (AVE = 0.54) were valid to a greater extent [33], where four items in each variable were recorded valid (Figures 2, 3 and 4) through quantified analysis.

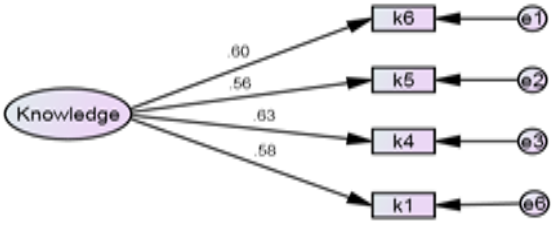

Fig. 2. Knowledge level of farmers regarding the use of water in insecticide application.

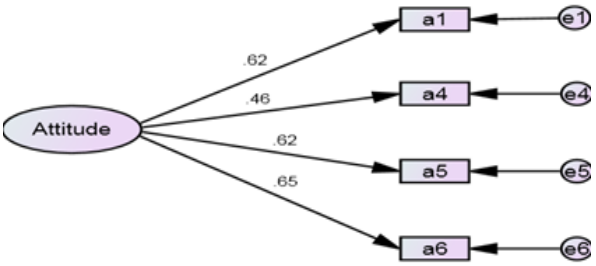

Fig. 3. Attitude of farmers regarding the use of water in insecticide application.

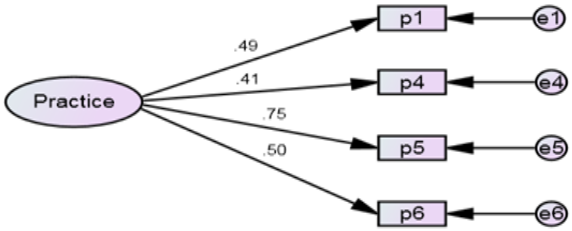

Fig. 4. Practice of farmers regarding the use of water in insecticide application.

The four items which were found valid includes: water and pesticide ration; temperature of water and timing of the spray; nozzle size or type with pesticides sprayed; and related pesticides used for the spray. However, two main questions concerning to water quality to prepare spray solution were identified as invalid by the software because of extreme variations in responses, therefore eliminated for final analysis. Cronbach's alpha technique was applied to confirm the reliability of the instruments by inserting initially twenty-five questionnaires [33] into SPSS [4]. The acceptable Cronbach's Alpha value varies from 0.70 to 0.99, while less than 0.7 is viewed as questionable. The concepts/variables' reliability was estimated, and results found accordingly as described in (Table 2).

3. RESULTS AND DISCUSSION

Table 3 shares the demographic information of the respondents, which shows that the majority (mode) of the respondents were of the age of 46 years,

Table 2. Reliability of the constructs.

Appendix	Concept/ Variable	No. of Items	Rank	Cronbach's *Alpha*
I	Knowledge	4	Good	.811
II	Attitude	4	Excellent	.915
III	Practice	4	Good	.853

Table 3. Demographic information.

	Mean	Maximum	SD	Mode
Age of the farmers (years)	39.74	58	11.108	46
Farming experience (Years)	18.80	43	10.345	15
Status of respondents	Farmers (*Haari*)		74%	
Education	Illiterate		54%	

where average age of the respondents was almost 40 years, however one among them was aged 58 years. The result also indicated that the majority of the respondents were well-matured and averagely they spent 19 years with agricultural profession. Moreover, less than three fourth (74%) respondents were farmers (haari) and had direct relationship with crop management and one could easily rely upon the perceived related (crop management) practical information. Yet, more than simple majority (54%) of the respondents were illiterate, therefore the researchers were not expecting any progression from them, where they were commonly using their previous experience and traditional methods for crop management.

Table 4 indicates about the crop cultivation trend of the respondents, which shows the worth of cotton crops by the local farmers in the study area. According to survey results, average 24.07 acres out of 29.51 acres of land was used during the year 2019 for cotton cultivation which refers the priority or common practice to grow cotton crop in summer season. However, only an average 1.28 acres agricultural land remained fallow due to variety of reasons, which included water logging and salinity area of 1.07 acres, conflicts, etc. Some farmers also

Table 4. Cotton crop cultivation trend among the selected respondents.

Area (In acres)	Mean	SE	SD	Mode
Total land	29.51	4.865	46.457	10
Cotton cultivated area	24.07	4.590	45.898	8
Fallow land	1.28	0.193	1.928	1
Water logging and salinity	1.07	0.026	0.256	1
Area for other crops	1.19	0.176	1.764	1

responded that they have domestic animals to feed therefore at least 1.19 acres normally allocate for fodder crop.

The information given in Table 5 revealed that an overwhelming majority (92%) of the respondents preferably use underground water to make insecticide mixture for spraying, whereas only 3% farmers confirmed that they tested their irrigation water used in their fields. Moreover, an informal discussion with the farmers during survey also disclosed, normally water quality is fixed by its taste. A significant majority of the respondents generally prefer underground water for spray because underground water is crystal clear, which does not interrupt/block the nozzle, resultantly the process saves their time and also avoid bothersome, and more importantly, the underground water is easily available round the year. It was also shared by the simple majority (51%) of the respondents/ cotton growers that usually they could not achieve desired results, and out of them almost three fourth majority (38 farmers) of the respondents blamed the companies in response to failure of any insecticide. While the related literature concludes that a number of factors are involved in failure or lessening the pesticides' efficacy in controlling pest problems. In this regard, insect resistance is also considered a huge issue these days by the many research studies [34 – 37], yet some of the researchers are of the opinion that the mishandling, improper calibration, and selection of incorrect pesticides could also contribute as significant causes behind pesticides ineffectiveness. Therefore, the general opinion of farmers, researchers and institutional representatives related to insecticide incompetency are quite diverse and somehow complicated.

Table 5. Water quality and performance of pesticides.

	Total farmers	**Frequency**	**%**
Using underground water for pesticide mixture	100	92	92
Water was tested	100	3	3
Often fail to achieve desired results of pesticides	100	51	51
Blaming pesticides for inefficiency	51	38	74.51
Spray frequency for cotton crop	M=5.34; SD=1.32	Mode=6	Max.=16

It was also disclosed that local farmers normally do 5 to 6 sprays of various pesticides on cotton crop to achieve a reasonable pest control to maximize its production [35]. Moreover, studies also suggested that in Pakistan around 80% of total pesticides used in the country are sprayed on cotton [37], showing an upward trend by 11.69 % in last two decades, where the number of sprays has reached to fifteen with an average of nine [37]. Therefore, in continuity of the same one farmer in the study area also claimed that he sprayed sixteen times to his cotton crop to achieve a maximum output, however, the researchers agree to eliminate that figure (16 sprays) before final analysis because it could disturb the whole mean value.

Table 6 explains the results regarding farmers' knowledge, attitude and practice related to the impact of different water features on the performance of pesticides to spray. The results show a logical description in which 'knowledge' remained bit higher as compared to 'attitude' and 'practice.' However, respondents' knowledge in relation to importance of water for making pesticide mixture was quite rudimentary (M = 3.44, SD = 0.68), as some of the farmers responded amazingly against the water quality questions that "is there any effect of water quality on a pesticide performance?". In addition, the majority of the respondents' educational level (illiterate) was also evident that the selected farmers could have quite a little knowledge on the issue. Neither they receive any directions from their landlords about the caring of water quality nor any governmental or non-governmental organization guided them about the acceptable features of water to make a pesticide mixture for achieving desired results. However, farmers must be aware about optimum quality of water while making a pesticide mixture because substandard water may potentially bring adverse impact on the performance of pesticides [7]. In this regard, any variation in water hardness, pH, turbidity, and temperature may contribute negatively on the performance of synthetic pesticides [9, 13, 14, 38]. For example, water having pH greater than 7 may cause chemical degradation of certain pesticides in the presence of ions. The rate of alkaline hydrolysis is enhanced as the pH increases on the other hand, for most of the insecticides favor to use water with pH only ranging from 4 to 7 for preparation of spray mixture [15, 38].

Table 6. Cotton farmers' perception about water quality to make a pesticide mixture using KAP scale.

KAP Scale	Items	Mean	St. Deviation
Knowledge	4	3.4417	0.68591
Attitude	4	3.1710	1.2580
Practice	4	3.0683	0.81184

Since, knowledge makes attitude, the lesser the knowledge may be predicted as the lower the attitude to adopt a certain thing. An attitude is a settled way of thinking or feeling about something that may brought from having knowledge. Results described in (Table 5) divulges the same situation where 'attitude' of the respondents representing their knowledge level about the matter and recorded (M = 3.17, SD = 1.25) again at lower level. Followed by the behavior of the respondents in shape of 'practice' were perceived concerning to water features for pesticide mixture and results (M = 3.06, SD = 0.81) indicates that only a few farmers do care about the water features while making a pesticide mixture; however, there is no such practice (caring about water quality for pesticide mixture) being implemented in the study area. Neither from governmental personnel nor from pesticide companies guide them about the matter. Hence, a significant majority of the respondents were of the opinion that it is wastage of time caring about water features. If the water taste is acceptable it is fit for all purposes to use. However, most of the scholars believe that before applying pesticides to the crops, the farmers need to adopt all the significant measures, but water quality has always been ignored by a significant majority of the farmers [15]. The pH of the carrier water or spray solution can impact significantly on insecticide efficacy. The variation in pH of solution may lead to hydrolysis and organophosphate (acephate and chlorpyrifos), carbamate (methiocarb), and pyrethroid (bifenthrin, cyfluthrin, and fluvalinate) undergo alkaline hydrolysis in the presence of alkaline water. Moreover, the half-life of many insecticides can also be affected with the variation in pH of solvent or spray mixture [16].

4. CONCLUSIONS

Most of the farmers in Sindh use underground water for pesticide mixture, however, they seldom test it before the application because, apparently, it looks clear without any impurities. Most farmers were also unaware of water quality standards for the pesticide mixture, hence their effectiveness on their performance. Generally, 5–6 sprays are made in cotton as pesticide companies are mostly blamed if the farmer fails to get desired results. Therefore, it is suggested to take appropriate measures to aware farmers regarding water quality parameters to get optimum pesticide effectiveness, which could help in reducing pesticide hazards to humans and their environment.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Evaluation of Biological Activity of Crude Extracts from Plants used by Indigenous Communities of Pothohar Plateau, Pakistan

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Abstract: The present study aimed to evaluate the potential of eight plant extracts that are used by communities of the Pothohar Plateau. Selected plants were *Brassica campestris*, *Brassica oleracea* var. *italica*, *Allium sativum*, *Piper nigrum*, *Cinnamomum zeylanicum*, *Allium cepa*, *Olea europaea* and *Moringa oleifera*. The antimicrobial assessment was carried out by using the agar diffusion method and antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl free radical assay, phosphomolybdate assay, and reducing power assay, against the selected isolates. Antimicrobial and growth rate studies were carried out by using two Gram-negative, one Gram-positive, and two pathogenic fungal strains. Among all tested extracts *M. oleifera* appeared to have the highest bioactivity with a percentage inhibition of 89% against DPPH free radical, followed by *Allium sativum 81%, Allium cepa 75%, Olea europaea* 67%, *B. campestris 60%, B. oleracea 58%*. During the phosphomolybdate assay similar trends were obtained such as: *M. oleifera* 91% followed by *A. sativum* 85%, *A. cepa* 78%, *Brassica campestris* 72%, *Brassica oleracea* 70% and *Olea europaea* 65% in higher concentration (1000 µg/ml). In the case of antibacterial assay *Moringa oleifera* showed maximum zone of inhibition against *Staphylococcus aureus* (20 mm) followed by *Klebsiella pneumonia* (18 mm) and *Escherichia coli*, (17 mm) whereas, crude extract of *Allium sativum* showed maximum zone of inhibition 14.4 mm against *A. flavus;* whereas *M. oleifera* gave maximum zone of inhibition against *A. alternata*, followed by *A. sativum* and *A. cepa*. All the tested extracts showed bioactivities. This study indicates the antimicrobial and antioxidant potentials of *M. oleifera,* and *A. species*. Hence, it is recommended that the extracts of these plants should be further evaluated for their possible application as antimicrobial and antioxidant agents.

Keywords: Moringa, Pothohar, Plateau, Antimicrobial, Antioxidant.

1. INTRODUCTION

Since ancient times, plants have been used as medicine [1]. There are many different traditional systems of medicine, i.e., Ayurveda, Unani, and Chinese, which are still utilized by native people [2]. After the 17th century, modern synthetic medicine gained more trust of pharmacologist, due to their availability and rapid mode of action [3]. However, with time, due to the development of resistance in microbes, synthetic medicine is facing more challenges [4]. Therefore, these days there has been an increasingly growing interest in investigating plants for the development of new antimicrobial and antioxidant agents as well as an alternative for the utilization of chemically synthetic representatives [5]. Plants have different types of bioactive compounds, i.e., phenolic compounds, carotenoids, tocopherols, etc. These natural antioxidants are obtained from different sources and different plant parts. Sources such as fruits (grapes, pomegranate), vegetables, (broccoli, pumpkin), herbs, and spices decreased lipid oxidation and microbial activity [6]. *M. oleifera* is known as the miracle tree, people

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have used it for centuries due to its health benefits. *Olea europaea and Brassica campestris* are known for their strength to retard microbial growth, maintain the organoleptic characteristics of food and inhibit the pathogen's growth [7].

Pakistan occupies 80,943 km^2 and has a large biodiversity, with 6000 species of higher plants. But regrettably, only 10% of the plant species in Pakistan are reported for their medicinal potential. Pakistan is rich in native herbs and supplies a large scope for ethnobotanical studies. In Pakistan Traditional Unani medicine is largely used among the local communities. Pothohar plateau falls in a rainfed region of Pakistan [8]. It occupies the north of Punjab province of Pakistan with an elevation ranging from 1500-2000 feet.

Even though a great deal of work has been conducted on the ethno-medicinally used flora of this region. However, no significant scientific study was conducted on the bioactivities of important plants in this region. Therefore, the present study was planned to evaluate the bio-activities of traditionally utilized plants of the Pothohar plateau i.e., *Brassica campestris, Brassica oleracea* var. *italica*, *Allium sativum, Piper nigrum, Cinnamomum zeylanicum, Allium cepa, Olea europaea* and *Moringa oleifera.*

2. MATERIALS AND METHODS

2.1 Plant Selection

Species selected were *Brassica campestris*, *Brassica oleracea* var. *italica*, *Allium sativum, Piper nigrum, Cinnamomum zeylanicum, Allium cepa, Olea europaea* and *Moringa oleifera*.

2.2 Collection of Plant

Among selected plants, i.e., *Moringa oleifera* and *Olea europaea* leaves were collected from PMAS Arid Agriculture University, Rawalpindi on 3rd, March 2020. Leaves of *Brassica campestris* and *Brassica oleracea* var. *italica,* the bark of *Cinnamomum zeylanicum*, the seed of *Piper nigrum*, and bulbs of *Allium sativum* and *Allium cepa* were collected from the local market on 15th, April 2020. The specimens were identified with the help of taxonomists.

2.3 Processing and Drying

All the unwanted plant parts were removed, shade dried, at room temperature, ground to powder, and stored in an airtight container with labels, a schematic diagram for crude extract is shown in Figure 1.

2.4 Extract Preparation

Plant extracts were prepared by following the methodology of Qin *et al.* [9]. The ratio between the plant powder material and the solvent was 1:6. The mixture was allowed to stand for seven days and filtered. The filtrates were again treated with the same procedure and filtrates were evaporated. The extract obtained was stored at 4^{o}C. The solvent we used is methanol. Our goal is the isolation of polar natural products such as polyphenols, flavonoids, etc. For the extraction of such polar natural products, a highly polar solvent such as methanol is particularly suited.

2.5 Bioactivities of Crude Methanolic Extracts

2.5.1. Antioxidant activities of crude extracts

For antioxidant activity, the plant samples were mixed in methanol solvent. It was then diluted by making different concentrations (62.5, 125, 250, 500, and 1000 µg/mL).

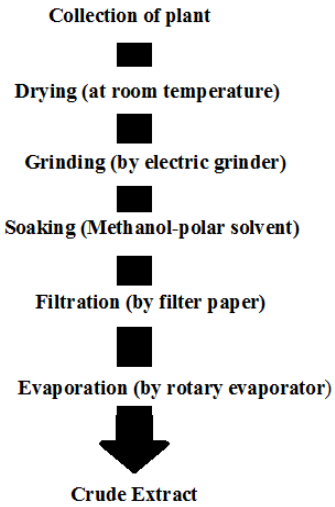

Fig. 1. Scheme for crude extract preparation.

2.5.1.1. DPPH radical scavenging activity assay

Antioxidant potential for crude extracts was evaluated by using the DPPH standard. Free radical scavenging bioassay was carried out by Hara *et al.* [10] with few modifications. To prepare a stock solution, DPPH was dissolved in methanol solvent. The stock solution was then stored in darkness at 20 °C for future use. Then DPPH solution was diluted by methanol, to obtain a particular absorbance at 517 nm. The samples were then mixed with a small quantity of obtained solution. The solution was incubated for 15 minutes in darkness. Positive control was Ascorbic acid while negative control was DMSO.

2.5.1.2. Phosphomolybdate assay

The antioxidant activities were examined by the method phosphomolybdate given by Gupta *et al.* [11]. The samples were mixed with our prepared reagents. These test tubes were then covered using aluminum foil. These covered tubes were incubated in a water bath at 95 °C. The covered tubes were cooled down at room temperature. Absorbance was calculated at 765 nm in comparison with a blank by using a spectrophotometer. Ascorbic acid was used as a positive control. The percentage increase in absorbance was measured.

2.5.1.3. Reducing power assay

The reducing power assay was done by the methodology of Gupta *et al.* [11]. The plant extract solutions, phosphate buffer, potassium ferri-cyanide, and tri-chloroacetic acid were mixed and incubated. These mixtures were then diluted then $FeCl_3$ was mixed. Then absorbance was observed at 700 nm by using a spectrophotometer. The increased absorbance value was the indication of high reducing power activity.

2.5.2. Antibacterial assays

2.5.2.1. Sample preparation

The antibacterial potential of selected plant extracts was tested at different concentrations (62.5 µg/ml – 1000 µg/ml). The extracts of selected species were weighed. Samples were dissolved in DMSO to prepare a stock solution. The stock solution was used to prepare five different concentrations, i.e., 62.5, 125, 250, 500, and 1000 µg/ml. Positive control Ciprofloxacin was prepared with the same concentrations.

2.5.2.2. Test microorganisms

The three different bacterial strains were used in this study, two Gram-negative (*Escherichia coli*, and *Klebsiella pneumonia*) and one Gram-positive (*Staphylococcus aureus).* Muller-Hinton Broth (MHB) was used to maintain microorganisms at 4°C until further use. For the re-culturing of microorganisms, the nutrient broth medium was dissolved in distilled water. Sealed the flask with a cotton plug before allowing for autoclave. The selected bacterial cultures were inoculated under aseptic conditions. These cultures were grown at 37 °C for 24 hours in a shaker incubator at 150 rpm (rotation per minute).

2.5.2.3. Agar well diffusion method

The antibacterial activity was examined by the methodology given by Dilshad *et al.* [12] with few modifications. Nutrient medium was prepared and poured in pre-labeled petri plates up to 1/3 of its volume. The media was inoculated with 10 mL of inoculum. The petri plates were left till solidification. Sterilized cork borer was used to make wells. Wells were made in every plate, for extracts, negative and positive control. Then nutrient media (20 µl) was added in each well. Tested Extracts were poured into respective wells. These plates were incubated at 37 °C for a day and a zone of inhibition was calculated. The experiment was done thrice, and then the mean value was calculated.

Positive control = Diameter of inhibition zone by standard drug (Ciprofloxacin)

2.5.3. Antifungal activity

2.5.3.1. Fungal strains

Aspergillus flavus and *Alternaria alternata* were used in the study. These fungal cultures were preserved on SDA media (Sabouraud dextrose agar) in a slanting position at 27 °C. After 7 days, the fungal spores were ready for determination of antifungal activity.

2.5.3.2. Agar tube dilution method

Antifungal activity was determined by the tube dilution method of Dilshad *et al.* [12]. Sabouraud dextrose agar (SDA, Merck) was dissolved in distilled water (32.5 g in 500 ml). Placed the mixture on a hot plate, a magnetic stirrer was used for proper mixing. Then 5 ml of media were poured and then autoclaved at 120 °C for 15 minutes and allowed to cool. After cooling just before solidification, plant extract was added in several concentrations, i.e., 10, 25, and 50 µg/ml. Then these tubes were shaken well and allowed to solidify. After that, a 4 mm inoculum was placed in each tube and incubated for 7 days at 28 °C.

3. RESULTS AND DISCUSSION

To evaluate the antioxidant properties of plants, it is suggested to use multiple tests with a variety of conditions [13]. In the present study, three different assays were performed, i.e., DPPH free radical, reducing power assay, and phosphomolybdate assay. The eight plant species were examined (Table 1).

Upon evaluation, it was observed that crude extract of *Moringa oleifera* showed maximum activity against DPPH free radical with percentage inhibition of 89% (1000 µg/ml), 81% (500 µg/ml), 77% (250 µg/ml), 65% (125 µg/ml), 56% (62.5 µg/ml). Results of *M. oleifera* were followed by *Allium sativum* 81% (1000 µg/ml) and *Allium cepa* 75% (1000µg/ml). The percentage inhibition was found dependent on concentrations. The minimum antioxidant activity was shown by plants belonging to the Brassicaceae family. *B. campestris* and *B. oleracea* displayed percentage inhibition of 60% and 58% in higher concentration (1000 µg/ml) and the lowest value was 23% and 20% at 62.5 µg/ml, respectively (Figure 2). The percentage inhibition declared by *Olea europaea* is better than the plants belonging to the Brassicaceae family 67% (1000 µg/ml), 56% (500 µg/ml), 40% (250 µg/ml), 35% (125 µg/ml), 26% (62.5 µg/ml). A similar percentage inhibition (40%) was displayed by *B. campestris* and *O. europaea* at 250 µg/ml.

Table 1. Selected plants and their documented uses.

Plants	Phytochemical	Antimicrobial activity	References
Allium sativum	Sulfoxides	Antibacterial	[24]
Piper nigrum	Alkaloid	Antimicrobial	[24]
Olea europaea	Secoiridoids	Anifungal. antibacterial	[25]
Cinnamomum zeylanicum		Antibacterial, antifungal	[26]
Allium cepa		Antibacterial	[27]
Brassica campestris	Flavonoid	Antibacterial	[28]
Brassica oleraceae var. italica		Antibacterial	[29]
Moringa oleifera		Antibacterial	[30]

Minimum inhibition was seen in the lowest concentration (62.5 µg/ml) in all selected species. Against phosphomolybdate ion, the percentage inhibition in the case of *M. oleifera* was 91% followed by *A. sativum* (85%), *A. cepa* (78%) *Brassica campestris* (72%), *Brassica oleracea* (70%) and *Olea europaea* (65%) in higher concentration (1000 µg/ml). *P. nigrum* and *C. zeylanicum* showed the same percentage of inhibition 53% (250 µg/ml). Similar *P. nigrum* and *B. oleracea* showed the same percentage inhibition 70% (1000 µg/ml). Similar findings were obtained during the reducing power assay. The high antioxidant potential of *M. oleifera* has previously been reported [14]. Khunchalee and Surapat [15] also tested different extracts of *M. oleifera* and got a very high activity against DPPH free radical, i.e., leaves extract in ethanol (89.33%), leaves extract in methanol (84.05%), leaves extract in water (74.85%), seeds extract in methanol (89.09%) and pod extract in methanol (86.36%), respectively.

In our study, the other two species that showed antioxidant activity were *A. cepa and A. sativum*. Alliums. Mainly, *A. cepa, A. sativum, A. ursinum,* and *A. ampeloprasum*, are a rich source of bioactive compounds, which have a major role in the pharmacological properties of plants. The extracts of *A. cepa* bulb peel and *A. sativum* bulb have previously been studied and expressed notable antioxidant activity in *in vitro* antioxidant assays, such as DPPH, ABTS, TAC, and FRAP [16]. Allium species have been recognized in the treatment of infective diseases as an effective antimicrobial agent. Many fungi, bacteria, and viruses have been

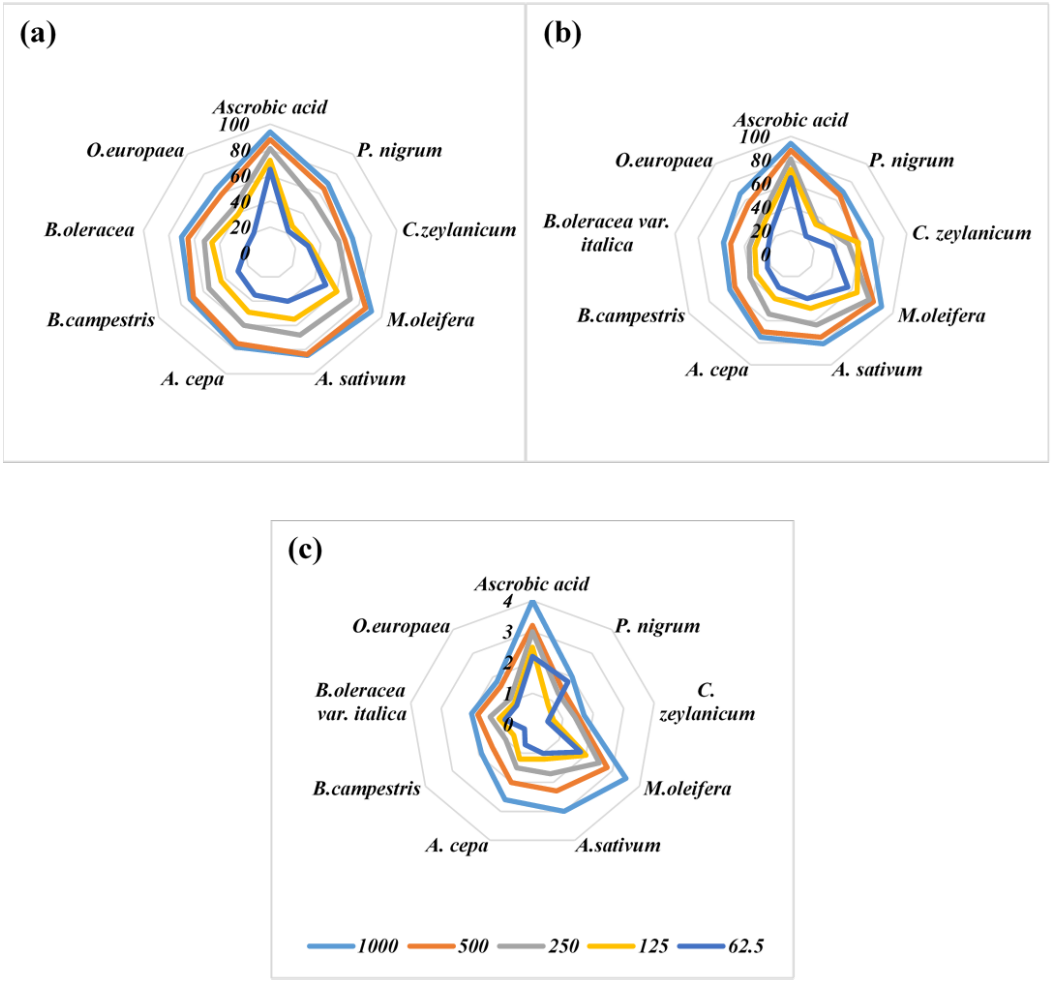

Fig. 2. Antioxidant activity of crude methanolic extract of selected species by (a) Phosphomolybdate assay, (b) DPPH Assay, and (c) Reducing power assay.

found vulnerable to various Allium solvent extracts [17]. They are known to have antioxidant potential due to the presence of high amounts of organosulfur compounds, polyphenols, and flavonoids [18].

The antimicrobial screening of the plants is considered an important parameter for the assessment of any plants to be used as preservatives for food and food products [19]. To study the antibacterial activity of crude methanolic extract of selected species both Gram-positive and Gram-negative bacterial strains were used (Figure 3). The

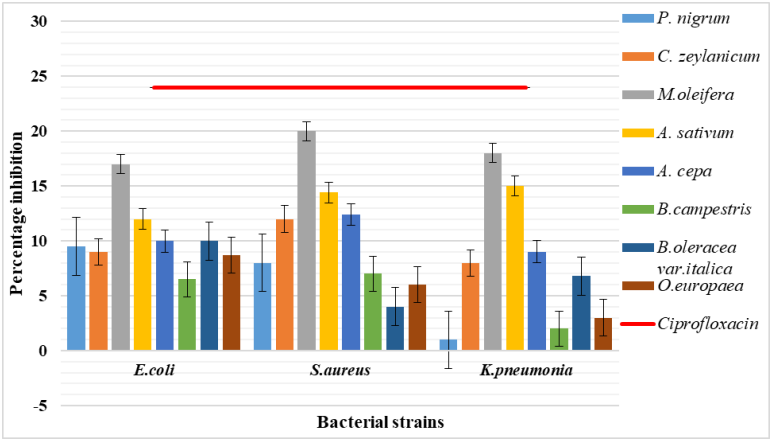

Fig. 3. Antibacterial activity of crude methanolic extract of selected species.

bacteria species were chosen as they are common pathogenic bacteria infecting humans and animals. Similarly, two fungal strains (*Aspergillus flavus* and *Alternaria alternata*) were used in the present study to evaluate the fungal activity of selected species (Figure 4). The crude methanolic extract of selected species represented the antibacterial activity against three bacterial strains. The crude extract of *Moringa oleifera* showed a maximum zone of inhibition against *Staphylococcus aureus* (20 mm) followed by *Klebsiella pneumonia* (18 mm) and *Escherichia coli* (17 mm).

It was noticed that after *Moringa oleifera* the antibacterial activity of crude extract of *Allium sativum* was highest which was further followed by *A. cepa. A. sativum* showed 14.4 mm against *S. aureus*, followed by 12 mm against *E. coli.* The minimum antibacterial activity was shown by *Piper nigrum* (1 mm) against *Klebsiella pneumonia*. Similarly, *Brassica campestris* (2 mm) and *Olea europaea* (3 mm) showed a minimum zone of inhibition against *Klebsiella pneumonia*. *Piper nigrum* and *Cinnamomum zeylanicum* showed the same results against *E. coli.* Among all bacterial strains, *Staphylococcus aureus* showed maximum resistance. *Moringa oleifera* has previously been reported by many studies to have antibacterial activity. Awasthi *et al.* [20] conducted a comparative assessment of ethnobotany and antibacterial activity of *Moringa oleifera* Lam. in Nepal. They concluded that *M. oleifera* is a promising medicinal plant and More research is needed on its ethnomedicinal and biochemical capabilities. Miladiarsi *et al.* [21] prepared an ointment from Moringa leafs and claimed that it has antibacterial activity against *Staphylococcus aureus* with an inhibition zone of 15% (16.1 mm).

Allium species also appear to have high antibacterial activity. According to Oyawoye *et al.* [22] the high antibacterial activity of allium species is due to the large quantity of phenolic compounds in them. Benkeblia [23] assessed the antibacterial activity of different phenolic compounds against *Staphylococcus aureus a*nd *Salmonella enteritidis* and obtained similar results. The crude methanolic extract of selected species was investigated against *Aspergillus flavus* and *Alternaria alternata.* According to the findings, the crude extract of Allium sativum showed a maximum zone of inhibition14.4mm against Aspergillus flavus whereas *Moringa oleifera* gave a maximum zone of inhibition against *Alternaria alternata,* followed by *Allium sativum* and *Allium cepa*. The minimum antioxidant activity was shown by *Olea europeae*. During our evaluation, *M. oleifera* appeared to be the most potent plant followed by *Allium sativum* and *Allium cepa*. Previous studies also showed that extracts obtained from leaves of *M. oleifera* contain bioactive properties such as antioxidant, anti-inflammatory, antilipidemic, antimicrobial, anti-hyperglycemic, among others [12]. The high antioxidant activity of plant extracts is due to the presence of phenolic compounds such as tannins, flavonoids, and steroids. Some of the bioactive activities of plants such as antimicrobial, anti-inflammatory, anticarcinogenic, and antiatherosclerotic are also due to the antioxidant capacity of plants.

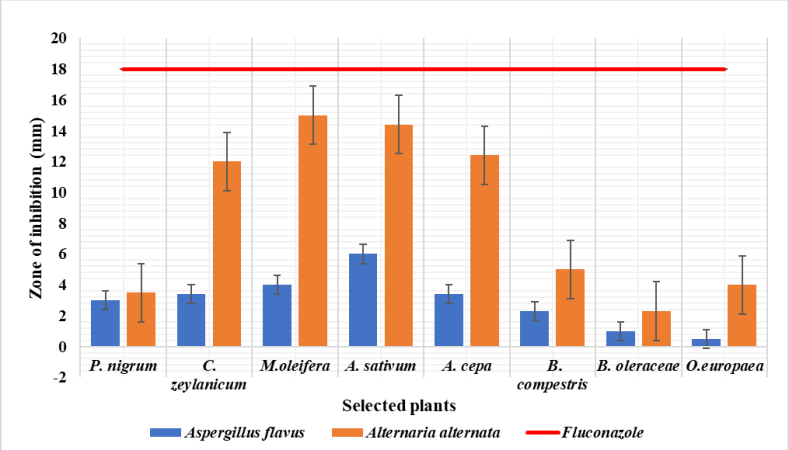

Fig. 4. Antifungal activity of crude methanolic extract of selected species.

4. CONCLUSIONS

Plants used by local communities of Pothohar Plateau, Pakistan appeared to have notable bioactivities. Particularly *M. oleifera* is the most active plant with strong activity against free radicals (89% DPPH, 91% phosphomolybdate) and infectious microorganisms *(Staphylococcus aureus,* 20 mm; *Klebsiella pneumonia,* 18 mm; *Escherichia coli* 17 mm). Other plants that showed activity were *A. sativum* and *A. cepa*. Therefore, it is recommended that these plants should be further explored and characterization of active compounds should be performed with the aim of novel drug development against drug-resistant microbes.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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