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Research Article

Efficacy of Seed Priming through Plant Hormones on the Germination of Bitter Melon (*Momordica charantia* L.)

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Abstract: Bitter melon (*Momordica charantia* L.) is a fast-growing vine and seeds germinated quickly in warm soil even if covered with shaded by crops. Due to its high medicinal benefits, it is the most essential vegetable crop. The findings of this study indicated that the germination percentage of the bitter melon variety i.e. Bitter gourd-1 (BG-1) was highly affected by the application of different concentrations of plant hormone. Such an effect was even prominent in comparison without the application of plant hormones. The seed germination was significantly highest (96.4 %) @ NAA 0.9 (900 mg/1000 ml). The germination time (days) was significantly lower 5.10 days @ NAA 0.3 (300 mg/1000 ml) and consistently increased at higher concentration of PGR (7.46 days at NAA 0.6 (600 mg/1000 ml) and 8.53 days at 1.5 (1500 mg/1000 ml), respectively. The germination index was 1.91 recorded higher at the control treatment. The results further indicated that the germination index of bitter gourd increased as the concentration of PGR increased. Overall, these findings indicated that the impact of PGR on the germination index was obvious, and the index enhanced as PGR concentration increased. The seedling vigour index was significantly maximum 1835 recorded at NAA 0.3 (300 mg/1000 ml) and followed by NAA 1.5 (1500 mg/1000 ml) with 1292, respectively. The results further showed that the seedling vigour index of bitter gourd decreased between 709 and 1024 at the control and distilled water treatments. We concluded that seed priming is a tool for sustainable agriculture.

Keywords: Bitter melon, Seed priming, plant hormones, germination

1. INTRODUCTION

Bitter gourd, *Momordica charantia* L. is one of the significant important vegetables belonging to the Cucurbitaceae family. It is well known as a tropical and subtropical vegetable vine with tendrils cultivated in Asia and Africa. Fruits and seeds both are soft, and vegetables are often harvested at an immature stage. Bitter gourd is more famous for its bitter taste and in fact, it is bitter than all other cucurbits but considered as an essential vegetable due to its highly nutritious values and medicinal purpose. Vegetable fruits contain ascorbic acid and iron which are highly worthless and necessarily required for the human body [1]. Though, it is annually cultivated but can also be grown as a perennial in warm areas having frost-free winters. It is reported that the ideal temperature range is 25-28 ºC for better germination of bitter gourd seeds [2]. However, the widespread practice of sowing bitter gourd is a direct seed, but direct seedlings can also be transplanted to the field. Due to the hard seed coat, delayed emergence is often caused because of slow water absorption. Thus, poor germination, delay in emergence after seedling, slow growth rate and survival of plants are the key issues for limiting the yield of bitter gourd. To notify the issue of seed dormancy, the imbibition process could perform a vital role, thus in hormonal activity, seeds of vegetables or fruits are pre-soaked with an ideal phytohormone concentration which increases the physiological activities of plants and result in better germination, progressive growth of seedlings, better root penetration or growth and which all come in better yield [3]. Apart from this, it also plays a vital role in signalling pathways of plants [4].

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Seed priming is a treatment performed before the sowing of seeds which produces a physiological change that leads the seed to germination more efficiently. Seed priming with phytohormones could alter the molecular and biochemical means which assist the plants to tolerate the stress of abiotic factors. Nowadays, such procedures are very likely [5]. Plant Growth Regulators (PGRs) are biochemical compounds that modulate the development and growth of plants. It takes place through changing the action of hormones. Through PGR, the yield of M. charantia could be increased by enhancing the fruit setting percentage. Globally, the awareness and realization regarding the usage of PGRs have been well documented for their imperative role to maximize crop yield and in this connection, Gibberellic acid (a PGRs) is a well-known growth regulator contributing to the physiological characteristics of plants particularly plant growth [6]. The gibberellic acid indirectly incites the cell extension of plant roots and shoots; meanwhile, Ethrel (another plant growth regulator) improvises the number of plant branches, fruit length, number of fruiting bodies, uniform ripening fruits and fruit colouration [7]. Keeping in view the significant effect of plant growth hormones on the germination and seedling growth of bitter gourd seeds, the present study was carried out to observe the effect of plant growth hormones on the seed germination and seedling growth of bitter gourd.

2. MATERIALS AND METHODS

Bitter gourd-1 (BG-1) is a commonly recommended variety of bitter melon grown in pots in a standardized soil medium at Misan city, South Iraq. Different quantities of synthetic plant hormone such as 1-Naphthaleneacetic acid (NAA) was mainly used throughout the study. Overall, five treatments for experiment were chosen including T1 = Control, T2 = Distilled water, T3 = NAA 0.3(g) (300 mg/1000 ml), T4 = NAA 0.9(g) (900 mg/1000 ml) and T5 = NAA 1.5(g) (1500 mg/1000 ml), respectively. In the experiment, these treatments were replicated thrice, and each replication contained three pots in a Completely randomized design (CRD). The observations to be recorded were Seed germination (%), Germination time (Days), Germination index (GI), and Seedling vigour index (SVI). The seed germination was observed up to one week of plantation and germination (%) was calculated by using the following formula given by Larsen and Andreasen (2004) [8].

$$GP = \sum n/Nx100$$

Where n is number of germinated seeds at each count and N is the total seeds in each treatment. Meanwhile, the mean germination time (d) was calculated by using an equation. $MGT = \sum (nxd)/\sum N$; Where n=number of seeds germinated on each day, d= number of days from the beginning of the test, and N= total number of seeds germinated. The Germination rate index (GRI) was calculated by the formula;

Germination rate index (GRI) = G1/1+G2/2+.... +Gi/i;

where G1 is the germination percentage on day 1, G2 is the germination parentage on day 2; and so on. In last, the seedling vigour index (SVI) was calculated by the following formula,

(SVI) = Seedling length (cm) x germination %

Statistical Analysis

The recorded data were statistically analysed using Statistics computer software (SAS). The mean was separated using Least Significant Difference (LSD) at a probability p-value of 0.05.

3. RESULTS

3.1 Seed Germination (%)

The results for seed germination of BG1 were affected ($P < 0.05$) with the application of different concentrations of plant hormone as well as without the application of hormones as mentioned in (Figure 1). The seed germination was significantly highest (96. 4 %) @ NAA 0.9 (900 mg/1000 ml) followed by NAA 1.5 (1500 mg/1000 ml). The additional application of NAA (@1.5) decreased the germination percentage thus it shows an exact or balanced application of hormone is ideal to increase the germination percentage of bitter gourd. However, the low concentration of PGR 0.3 (300 mg/1000 ml)) also did not improve the seed germination percentage (71.4 %) but it was even better than the seed germination observed in

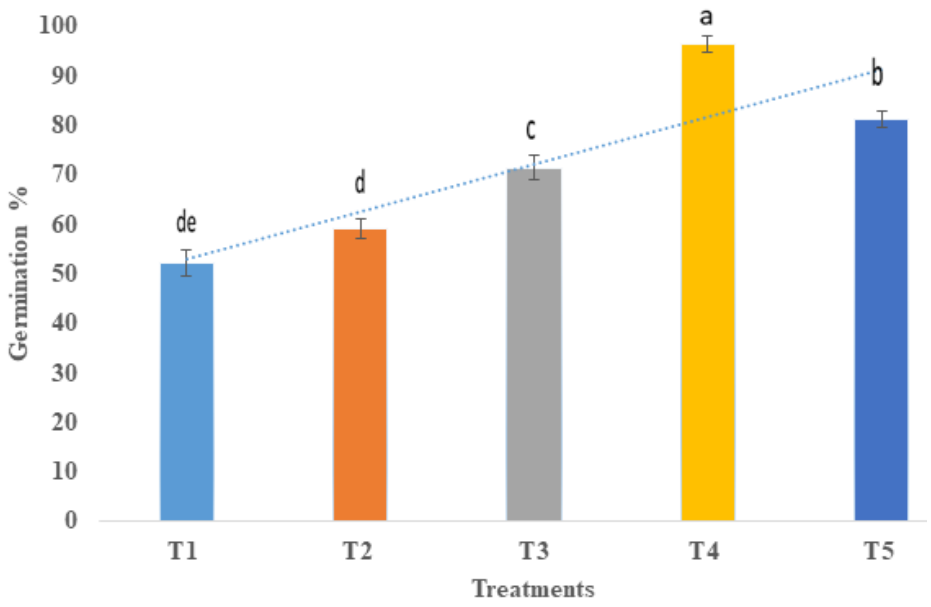

Fig 1. Seed germination percentage of bitter gourd at different hormones concentration

the control treatment (52.2 %) and with distilled water (59.1). The LSD test demonstrated that no significant difference in seed germination ($P > 0.05$) was observed at control treatment and distilled water. The overall results showed that there was a simultaneous improvement in the seed germination with increasing plant hormone concentrations but at a certain level of application.

3.2 Germination Time (Days)

The results for germination time of bitter gourd (BG1) showed a significant difference ($P < 0.05$) with and without plant hormones (at variable concentration) application (Figure 2). The germination time was significantly lower 5.10 days @ NAA 0.3 (300 mg/1000 ml) and consistently increased at higher concentration of PGR (7.46 days at NAA 0.6 (600 mg/1000 ml) and 8.53 days at 1.5 (1500 mg/1000 ml), respectively. However, the germination time was higher in distilled water and in the control treatment with a non-significant difference ($P > 0.05$) than the rest of the other treatments treated with variable concentrations of plant hormones. The highest time for germination of bitter gourd was 10.1 days observed in distilled water. These results clearly illustrated that germination time prominently influenced plant hormones. It suggests that we can get faster germination with the application of suitable plant hormones at exact concentrations.

3.3 Germination Index (GI)

The results regarding the germination index of the bitter gourd by different concentrations of plant hormones and without plant hormone is significantly different ($P < 0.05$) as presented in (Figure 3). The germination index was 1.91 recorded higher at control treatment (T1) followed by 1.89 at N.A.A 1.5 (1500 mg/1000 ml). The results further indicated that the germination index of bitter gourd was increased as the concentration of PGR increased. However, the minimum germination index (G.I) was recorded in distilled water. Meanwhile, there was no significant difference ($P > 0.05$) between T1 and T4 and similar, observations were recorded between T4 and T5. Overall, these findings indicated that the impact of PGR on the germination index was obvious, and the index enhanced as PGR concentration was increased.

3.4 Seedling Vigour Index (SVI)

The results for seedling vigour index (SVI) of BG1 bitter gourd for different concentrations of plant hormones and without hormones showed significantly different ($P < 0.05$) as presented in Figure 4. The seedling vigour index was significantly maximum 1835 recorded at NAA 0.3 (300 mg/1000 ml) and followed by N.A.A 1.5 (1500 mg/1000 ml) with 1292, respectively. The results further showed that the seedling vigour

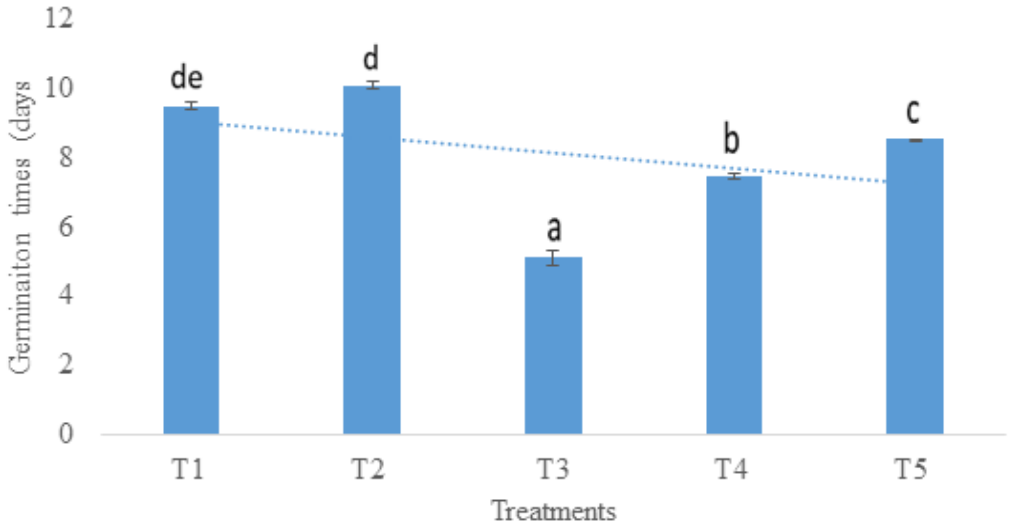

Fig. 2. Seed germination time (days) of bitter gourd at different hormone concentrations

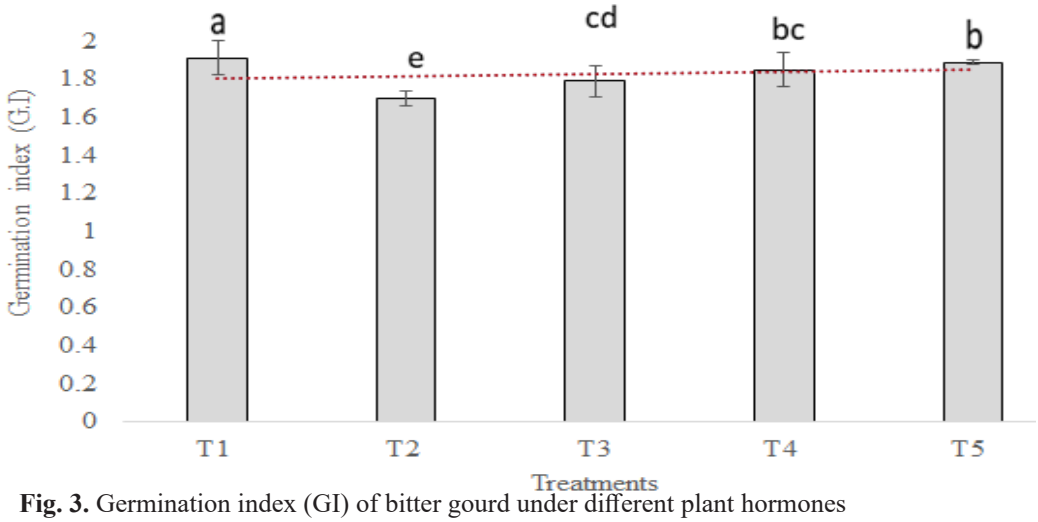

Fig. 3. Germination index (GI) of bitter gourd under different plant hormones

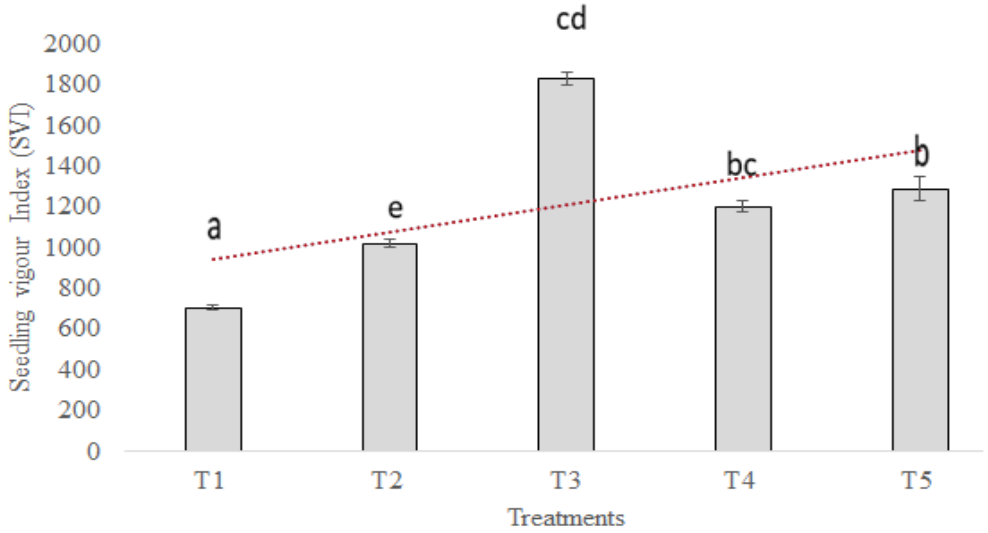

Fig. 4. Seedling vigour index (SVI) of sponge gourd varieties under different plant hormones

index of bitter gourd decreased 709 and 1024 at the control and distilled water treatments.

4. DISCUSSION

Bitter melon (*Momordica charantia* L.) is a fast-growing vine and seeds germinated quickly in warm soil even if covered with shaded by crops [9]. In most countries, these plants are cultivated in small plots in kitchen gardens for personal consumption with commercial cultivation being of secondary importance. However, it is established to appear in the international marketplace [10-11]. Generally, seed priming is a common practice used for improving the crop vegetables' physiology or morphological traits including crop status, germination and yield. A number of seed priming techniques have been introduced nowadays to promote plant growth including hormonal priming, halo-priming, solid matrix priming, Hydro-priming, Bio-priming and osmo-priming. Besides normal plant growth, seed priming is also useful to improve nutrient production and stress tolerance [12]. Keeping in mind the significant importance of seed priming, this study was proposed to know the effect of seed priming on the germination of bitter melon. The findings of this study indicated that the germination percentage of taken bitter melon variety i.e. BG1 was highly affected by the application of different concentrations of plant hormone. Such an effect was even prominent in comparison without the application of plant hormones. The seed germination was significantly highest (96.4 %) @ NAA 0.9 (900 mg/1000 ml) followed by NAA 1.5 (1500 mg/1000 ml). The additional application of NAA (@1.5) decreased the germination percentage thus it shows an exact or balanced application of hormone is ideal to increase the germination percentage of bitter gourd. However, the low concentration of PGR 0.3 (300 mg/1000 ml)) also did not improve the seed germination percentage (71.4 %) but it was even better than the seed germination observed in the control treatment (52.2 %) and with distilled water (59.1). As it is previously studied and reported that the thick coat of embryo confinement and germination is impaired by the application of a mechanical constraint on embryo development [13]. The rate of seed germination is not 100 per cent due to the presence of hard seed coats [13].

Crop priming is an effective method for enhancing seed germination and vigour. Priming increases, the rate of germination, speed of germination, time of germination and seed priming is one of the most critical aspects of improving the yield and consistency of bitter gourd [14]. Similarly, in our study germination time (days) for bitter gourd (BG1) was significantly lower at 5.10 days @ NAA 0.3 (300 mg/1000 ml) and consistently increased at higher concentration of PGR (7.46 days at NAA 0.6 (600 mg/1000 ml) and 8.53 days at 1.5 (1500 mg/1000 ml), respectively. However, the germination time was higher in distilled water and the control treatment than in the rest of the other treatments treated with variable concentrations of plant hormones. The highest time for germination of bitter gourd was 10.1 days observed in distilled water. These results clearly illustrated that germination time prominently influenced plant hormones. It suggests that we can get faster germination with the application of suitable plant hormones at exact concentrations. The germination index was 1.91 recorded higher at the control treatment. The results further indicated that the germination index of bitter gourd was increased as the concentration of PGR increased. However, the minimum germination index (G.I) was recorded in distilled water with no significant difference between T1 and T4 and similar observations were recorded between T4 and T5. Overall, these findings indicated that the impact of PGR on the germination index was obvious, and the index enhanced as PGR concentration was increased. Therefore, in a similar connection seedling vigour index (SVI) of BG1 bitter gourd for different concentrations of plant hormones and without hormones showed significantly different findings. The seedling vigour index was significantly maximum 1835 recorded at NAA 0.3 (300 mg/1000 ml) and followed by NAA 1.5 (1500 mg/1000 ml) with 1292, respectively. The results further showed that the seedling vigour index of bitter gourd decreased 709 and 1024 at the control and distilled water treatments.

Similarly, our results are further supported by many research workers such as Hidayatullah *et al.* [6] applied different concentrations of Maleic hydrazide (MH 200 to 800 µmol L^{-1}), Gibberellic Acid (GA 15 to 45 at 3, -1 -1 & 60 µmol L^{-1}) and -1 ethrel (from 500 to 2000 µmol L^{-1}) on Lagenaria siceraria Molina) plants and

achieved significant effects of these PGRs on the reproductive performance of bitter melon. Cong *et al*. [15] concluded the efficacy of salicylic acid (SA) on sponge gourd when stored for 2 days @ 20 °C for checking its shelf life at the concentration of 1.5 mmol L^{-1}. They found CI postharvest was effective with a significant reduction. Therefore, all these reviews highly support our findings and are in accordance to obtained results of this study. Choudhary and Prakash [16] stated that the practice of seed priming is crop hydration controlled to a certain level which allows metabolic activity to continue at pre germinative. It is a physiological process where seeds of crops are pre-soaked prior to planting, which allow imbibition partially [17]. Saleem *et al.* experienced that to increase the growth and germination of cultivars of seedlings and seeds, seed soaking for 12 hrs are ideal [18]. Similarly, Lin and Sung reported that seed soaking is a prerequisite because it triggers the embryo of the seed which affects germination and results in seedling establishment [19]. Crop soaking is thus considered a fundamental step for successful seedling establishment and even it works better under sub-optimal temperature conditions. Thirusenduraselvi and Jerlin stated that for better seedling development and 100 % pre-germination of bitter gourd seeds, treatment of PGR like panchakavya at 3 % solution for 9 hrs was ideal and similarly for soaking in KNO_3 @ 2 percent [20]. Sher *et al*. also reported various PGRs including NAA, Kinetin, GA3 and ascorbate admiring their role in priming seeds [21]. Ullah et al. experienced that pre-soaking of seeds with gibberellic acid for 24 hrs ppm for the period of the seeds treated with gibberellic acid @ 100 ppm resulted in increased germination of certain hrs improved the genotype of bitter melon water particularly for germination [22]. Besides, it also targeted digestive enzymes of the plant and increased its permeability.

5. CONCLUSION

It has been concluded that seed priming effectively enhanced and improved the seed germination of *M. charantia* based on the selected parameters. The highest seed germination of 96.4 % @ NAA 0.9 (900 mg/1000 ml) was found. The germination time (days) for bitter gourd (BG1) was lower 5.10 days @ NAA 0.3 (300 mg/1000 ml). However, the germination time was higher in distilled water and the control treatment than the rest of the other treatments treated with variable concentrations of plant hormones. The germination index of bitter gourd was increased as the concentration of PGR increased. The seedling vigour index was significantly maximum 1835 recorded at NAA 0.3 (300 mg/1000 ml). Thus, seed priming is the best tool for sustainable agriculture for a number of crops, vegetables and fruits and should be encouraged further for local farmers' usage.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Research Article

Distribution and Damage Potential of Pulse Beetles, *Callosobruchus* spp. (Coleoptera: Bruchidae) in Sindh, Pakistan

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Abstract: *Callosobruchus* spp. are global serious pests of pulses. Therefore, two studies were carried out to determine their distribution in three ecological zones of Sindh, Pakistan along with their damage potential to major pulses. A survey was conducted in Zone one (Ghotki, Sukkur, and Larkana districts), Zone two (Hyderabad, Mirpur Khas, and Shaheed Benazirabad districts), and Zone three (Karachi district) from March – April 2020. Grain samples of main pulses i.e., chickpea, field pea, cowpea, green lentil, and yellow lentil were collected from three locations in each district and brought to Stored Grain Research Laboratory, Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University, Tandojam for further examination. Three pulse beetle species i.e., *C. maculatus, C. chinensis* and *C. analis* were identified from the collected pulse samples as the highest populations of adults, eggs, grubs, and pupae were recorded from green lentil, followed by cowpea and chickpea. No population of pulse beetle was recorded from yellow lentil and field pea. *Callosobruchus maculatus* was the most dominant species in all sampling as higher beetle populations were recorded from Mirpur Khas and Sukkur districts. Therefore, both these strains were further evaluated for their feeding preference and weight loss assessment on various pulses i.e., chickpea, cowpea, green lentil, and yellow lentil in no-choice under laboratory conditions. *C. maculatus* showed a significant feeding preference among pulses with cowpea and chickpea being the most preferred, whereas yellow lentil was the least preferred, hence suffering the highest and lowest weight losses, respectively. Therefore, proper storage and quarantine measures should be taken in the transportation and storage of pulses to restrict the spread and damage of pulse beetles.

Keywords: Beetles, *Callosobruchus*, Damage, Distribution, Population, Stored grains

1. INTRODUCTION

The cultivation of pulses i.e., peas, beans, and lentils can be traced back to ancient civilizations as back as 8,000-10,000 BC [1]. The world's largest producer and consumer of pulses is India, whereas Pakistan, Canada, Myanmar, Australia, and United States are the other major exporters of pulses [2, 3]. The major pulses grown in the world are dry beans, dry peas, chickpeas, and lentils [3, 4]. Pulses are excellent sources of proteins (20-40 %), carbohydrates (50-60 %), minerals, and vitamins i.e., thiamin, niacin, calcium, and iron [4].

During 2018-19, the total sowing area of pulses in Pakistan was 1,174 thousand hectares with a production of 167.4 thousand tons. Chickpea accounts for about 71 % of the total pulse production, whereas green lentil, red lentil, black lentil, and field pea are the other pulses grown in the country [5]. In recent years, a decline has been observed in the area and production of pulses in Pakistan as pulses cover only 7 % of the total cropping land of Pakistan [6]. Moreover, the grain losses due to storage pests ranged from about 5-15 % of the total grain production and may increase up to 50 % in case of severe infestation [7, 8].

More than 150 insect pests attack pulses during storage. Among them, the most important pests are

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bruchids (*Callosobruchus* spp.) i.e., *Callosobruchus maculatus* (Fabricius), *C. chinensis* (Linnaeus), *C. analis* (Fabricius), and *C. phaseoli* (Gyllenhal) [9, 10]. *Callosobruchus* spp. are considered major pests of legumes that originated from tropical and subtropical regions of the world [9, 11]. Mainly, larvae cause damage by feeding inside the grains and in case of severe infestation, can consume entire contents of grains [1]. Moreover, the presence of larvae and pupae inside the pulse seeds deteriorates the quality and marketability of the pulse [12]. Pest attacking grain storage products has become a major concern for all the stakeholders worldwide, thus, needs effective management practices to reduce storage losses by insect pests. Therefore, effective management necessitates assessing the pest status effectively along with the level of losses that might have occurred or likely to occur during the storage [13]. As the infestation and damage of *C. maculatus* is continuously increasing in warehouses in Pakistan and particularly in Sindh province, no considerable research has been done in this regard. Therefore, the study aimed to determine the distribution of *Callosobruchus* spp. at various areas of Sindh along with their damage status to major pulses, so adequate strategies should be devised to manage their spread and losses.

2. MATERIALS AND METHODS

2.1 Distribution of *Callosobruchus* spp.

2.1.1 Study locations

Sampling surveys were carried out in three ecological zones of Sindh i.e., Zone one (Ghotki, Sukkur, and Larkana districts), zone two (Hyderabad, Mirpur Khas, and Shaheed Benazirabad districts), and zone three (Karachi district) (Table 1; Figure 1). The selection of the districts and the particular locations was done on the geography of the Sindh province along with the availability of pulse processing units, and a large warehouse of the pulses. The sampling at various locations was carried out only once during March and April 2020.

2.1.2 Data collection and experimental design

Grain samples of major pulses i.e., chickpea, field pea, cowpea, green lentil, and green lentil were collected from various storehouses. Three sampling points (warehouses), treated as replications, were selected from each zone. A 500 g sample of each pulse was collected from the individual storehouse based on their availability in the particular sampling location. Samples were collected from different points of warehouses and placed in plastic jars immediately with the lids of the jars kept tight. The collected samples were then brought to the Stored Grain Research Laboratory (temperature 30±2 °C and relative humidity 60±5 %) Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University, Tandojam to examine and count both adults as well as immature stages of beetles. The data on the population of larvae and pupae were taken using destructive sampling by breaking the grains with a sharp-edged needle. Species identification was based on the available guidelines from scientific literature [14-16] and taxonomists of the Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University, Tandojam. A sampling of various pulses was done using a completely randomized design as samples were collected randomly on their availability, whereas three samples of each pulse were used as replications.

2.2 Damage Potential of *Callosobruchus maculatus* on Major Pulses

Two population strains of *C. maculatus* i.e., Sukkur and Mirpur Khas were selected for the damage potential studies because of their large-scale presence in all survey locations (results of section 2.1). Fresh 100 g grains of chickpea, cowpea, green lentil, and green lentil were obtained from the local supermarket and placed in separate plastic jars. Five virgins (0-24 h old) pairs (male plus female) of adult *C. maculatus* obtained from the laboratory-reared culture were released in each replicated jar on June 1, 2020. Observations on the population fluctuation of *C. maculatus* were taken fortnightly for three months to record the number of alive and dead beetles. At the end of the experiment, the percent weight loss of pulse grains was calculated using the formula:

% weight loss =
(Final weight of grains/ Initial weight of grains) * 100

Experiments were conducted in the Stored Grain Research Laboratory, Department of Entomology, Sindh Agriculture University, Tandojam, Pakistan (temperature 30±2 °C and relative humidity

Table 1. GPS coordinates of sampling locations

City	Sampling point	Address	GPS	
			Latitude	Longitude
Sukkur	1	Al-Noor Daal Mill, Golimar Sukkur	N 27°42'42.866"	E 68°51'15.466"
	2	Jamal Sons (Ajmeri Daal waala) Liaqat Bazar Sukkur	N 27°41'34.300"	E 68°51'55.911"
	3	Shaikh whole seller, Khudadaad Khan road old Sukkur	N 27°42'14.832"	E 68°52'53.257"
Larkana	4	Super Matni Anaaj Dealer, Jelaus Bazar, Old Anaaj Mandi Larkana	N 27°33'30.255"	E 68°13'12.139"
	5	New Dero road, near Dr. Khalid Mahmood Madarsa, Ayoub Colony Larkana	N 27°33'48.166"	E 68°13'21.617"
	6	Warehouse, near Khosa goth, Rato dero road Larkana	N 27°35'34.184"	E 68°13'24.880"
Ghotki	7	Warehouse, near new bus stand Ghotki	N 27°58'50.464"	E 69°17'16.774"
	8	Laal Dealer, New market Ghotki	N 28°00'31.560"	E 69°18'52.132"
	9	Chander Bhaan Seed store, near noor masjid, new market, Ghotki	N 28°00'31.670"	E 69°18'52.314"
Shaheed Benazira bad	10	Hamdard pansaar and daal centre, chakra bazar Nawabshah	N 26°14'37.676"	E 68°24'37.105"
	11	Haji gareeb shah jadoon daal and rice marchant, markeet road 1. Nawabshah	N 26°14'36.716"	E 68°24'37.347"
	12	Ab Majeed daal, rice and garam masala merchant, market road 1. Nawabshah	N 26°14'36.513"	E 68°24'37.564"
Hyderabad	13	Sagar pulse and rise industry, site area, Hyderabad	N 25°21'23.059"	E 68°23'57.736"
	14	Sun shine Daal Mills, E-29/A site area, Hyderabad	N 25°21'24.119"	E 68°23'52.243"
	15	Sooraj Daal mill, site area, Hyderabad	N 25°21'37.525"	E 68°23'46.161"
Mirpur Khas	16	Zafar Nayab, daal and rice centre, Anaaj market, market chowk Mirpur Khas	N 25°31'40.762"	E 69°00'50.156"
	17	Farooq Nayab, daal centre, Anaaj market Mirpur Khas	N 25°31'40.466"	E 69°00'50.615"
	18	Qalandari daal dealer, near Mehran Cotton factory, Paak coloney Mirpur Khas	N 25°31'41.184"	E 69°00'52.516"
Karachi	19	Haji Kareem (Importer and Exporter) M.R 1/57, Joria Bazaar Karachi	N 24°51'17.668"	E 67°00'14.387"
	20	Zafar Traders and Co. (Importer and Exporter), Joria Bazaar Karachi	N 24°51'18.846"	E 67°00'11.366"
	21	Tariq hole sale, G-13, Batulhina, Gulistan e johar, block-18, Karachi	N 24°54'31.237"	E 67°07'49.160"

60±5 %) in a completely randomized design as each treatment was replicated five times. The humidity of various grains used was standardized at 12 % before the release of insects by treating them in a hot air oven.

2.3 Data Analysis

Collected data for both the studies were analyzed using Analysis of Variance (ANOVA) with the Least Square Difference (LSD) used to separate means with significant differences. The Statistix 8.1 computer software was used for the analysis.

3. RESULTS

3.1 Identification of Collected Pulse Beetles

In seven surveyed districts of Sindh province, three species of pulse beetle i.e., *C. maculatus*,

Fig. 1. GPS co-ordinates (landmarks/legends) of various sampling districts (locations) of Sindh, Pakistan

C. chinensis, and *C. analis* were identified based on their morphological characteristics as suggested by [14-16]. *Callosobruchus maculatus* can easily be identified by its general appearance like coloration on the plate covering the end of the abdomen, *C. chinensis* have a distinguished inner tooth on the hind femur having sides parallel, converging near the apex, whereas the pronotum of *C. analis* has uniformed cuticle (reddish-brown) along with sparse golden setae.

Among the identified species, relatively higher populations of *C. maculatus* were recorded in all the survey districts. Moreover, the population of *Callosobruchus* spp. was observed only on chickpea, cowpea, and green lentil, whereas no population was recorded from green lentil and field peas (Table 2).

3.2 Population of *Callosobruchus* spp. Eggs at Various Survey Locations

The highest mean population of pulse beetle eggs at Sukkur were recorded on green lentil (659.33±168.34 eggs), followed by cowpea (409.33±215.64 eggs) and chickpea (8.67±4.91 eggs), whereas eggs on cowpea (376.33±215.94 eggs), chickpea (17.67±17.67 eggs) and green lentil (1.00±1.00 eggs) were recorded at Larkana. At Ghotki, egg population was recorded only on chickpea (43.67±37.83 eggs), whereas chickpea (89.33±80.02 eggs) and green lentil (6.00±6.00 eggs) showed the population of eggs at Shaheed Benazirabad. The number of *Callosobruchus* spp. eggs at Hyderabad were recorded from green lentil (764±233.89 eggs), chickpea (275.67±151.88 eggs), and cowpea (29.00±29.00 eggs), whereas the population of eggs on the same pulses at Mirpur Khas was 692.67±281.29, 193.67±170.23 and 22.00±11.53 eggs, respectively. Furthermore, in Karachi, commodities that suffered from the population of eggs were chickpea (10.00±6.08 eggs), green lentil (12.00±12.00 eggs), and cowpea (12.00±12.00 eggs). Among sampling districts, Hyderabad and Mirpur Khas showed a relatively higher population of eggs on green lentil (Table 2).

3.3 Population of *Callosobruchus* spp. Grubs at Various Survey Locations

The population of *Callosobruchus* grubs was recorded only in green lentil (56.33±17.07 grubs) and cowpea (43.33±33.12 grubs) at Sukkur, whereas chickpea (56.33±17.07 grubs) and cowpea

(20.00±16.33 grub) showed the presence of grubs in Larkana. In Ghotki district, 6.67±6.67 grubs, and in Shaheed Benazirabad district, 24.33±23.84 grubs were recorded from chickpea only. In Hyderabad, the population of grubs was recorded on chickpea (64.67±32.46 grubs), green lentil (57.00±24.85 grubs), and cowpea (2.00±2.00 grubs), whereas the population of grubs on same pulses in Karachi was 4.67±2.6, 44.00±29.1 and 1.67±1.67 grubs, respectively. At Mirpur Khas, grubs were observed only in chickpea (17.00±11.53 grubs) and green lentil (141.67±95.83 grubs). Among sampling districts, a relatively higher population of grubs was observed at Mirpur Khas in green lentil commodity (Table 2).

3.4 Population of *Callosobruchus* spp. Pupae at Various Survey Locations

Results indicated that the population of pulse beetle pupae was recorded only in cowpea (26.67±14.77 pupae) and green lentil (18.00±5.51 pupae) at Sukkur, whereas cowpea (32.00±16.17 pupae) and chickpea (11.00±11.00 pupae) were the only commodities showing pupae population at Larkana. Chickpea was the only commodity where pupae were recorded at Shaheed Benazirabad (6.67±5.7 pupae) and Ghotki (2.33±2.33 pupae) districts. In Hyderabad, the population of pupae was recorded in chickpea (32.00±23.58 pupae), green lentil (31.33±9.61 pupae), and cowpea (6.00±6.00 pupae), respectively, whereas the population of pupae on same pulses at Mirpur Khas was 6.00±3.46, 86.33±52.7 and 6.00±4.58 pupae, respectively. Furthermore, in Karachi, a population of pupae was observed in green lentil (41.33±32.75 pupae), chickpea (6.00±3.46 pupae), and cowpea (0.67±0.67 pupae). Among sampling districts, a relatively higher population of pupae was observed at Mirpur Khas followed by Karachi (Table 2).

3.5 Population of Adult *C. maculatus* at Various Survey Locations

A great variation was recorded in the population of *C. maculatus* adults from various pulses at different sampling locations (Table 2). Thus, a significant difference (F =6.82, P =0.0045) was recorded in the population of adult *C. maculatus* on various commodities, whereas overall populations at sampling districts were not significantly different (F =1.50, P =0.2425) from each other. In the Sukkur district, the population of *C. maculatus* was recorded only from green lentil (51.33±8.57 beetles) and cowpea (21±10.12 beetles), whereas cowpea (34.33±18.62 beetles) and chickpea (12.00±11.50 beetles) suffered from *C. maculatus* infestation in Larkana. In Ghotki, the *C. maculatus* population was only recorded from chickpea (2.00±1.15 beetles); in Shaheed Benazirabad, chickpea (0.33±0.33 beetles) and green lentil (0.67±0.67 beetles) suffered the beetle attack. The green lentil (18.33±11.67 beetles) and chickpea (6.00±4.58 beetles) showed the population of *C. maculatus* in Karachi. Among sampling districts, Mirpur Khas and Hyderabad showed a relatively higher population of *C. maculatus* on three pulses i.e., chickpea, cowpea, and green lentil. At Hyderabad, the overall population of *C. maculatus* recorded on chickpea, cowpea and green lentil were 22.00±11.06 beetles, 3.00±3.00 beetles, and 43.00±13.01 beetles, respectively, whereas the population of adult beetles on same pulses in Mirpur Khas was 4.00±4.00 beetles, 2.00±2.00 beetles, and 93.00±46.51 beetles, respectively.

3.6 Population of Adult *C. chinensis* at Various Survey Locations

Results also indicated that at Sukkur and Shaheed Benazirabad, population of *C. chinensis* (0.33±0.33 beetles and 16.67±16.67 adults, respectively) was only recorded from chickpea. However, at Mirpur Khas, adult C. chinensis population was recorded from cowpea (2.67±2.67 beetles) and green lentil (4.00±4.00 beetles), only. Furthermore, at Karachi, C. chinensis adults (5.00±5.00 beetles) were only recorded from green lentil (Table 2).

3.7 Population of Adult *C. analis* at Various Survey Locations

It has been also observed that population of adult *C. analis* was recorded only on cowpea (1.67±1.67 beetles) and chickpea (0.67±0.67 beetles) at Sukkur. However, C. analis population in Larkana (1.67±1.67), Ghotki (0.67±0.67), Shaheed Benazirabad (1.67±1.67) and Mirpur Khas (5.00±5.00) was recorded on chickpea, only. Furthermore, at Karachi, population of *C. analis* adults was recorded only on cowpea (2.00±2.00 beetles) (Table 2).

Table 2. Overall mean population of *Callosobruchus* spp. in different pulses at various sampling districts of Sindh, Pakistan.

City	Commodity	Eggs	Grubs	Pupa	*C. maculatus*	*C. chinensis*	*C. analis*
Sukkur	Chickpea	8.67±4.91e	0.00	0.00	0.00	0.33±0.33b	0.67±0.67b
	Cowpea	409.33±215.64a-d	43.33±33.12b	26.67±14.77b	21±10.12b-d	0.00	1.67±1.67ab
	Green lentil	659.33±168.34ab	56.33±17.07b	18.00±5.51b	51.33±8.57b	0.00	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00
Larkana	Chickpea	17.67±17.67de	56.33±17.07b	11.00±11.00b	12.00±11.5cd	0.00	1.67±1.67ab
	Cowpea	376.33±215.94a-e	20.00±16.33b	32.00±16.17b	34.33±18.62b-d	0.00	0.00
	Green lentil	1.00±1.00e	0.00	0.00	0.00	0.00	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00
Ghotki	Chickpea	43.67±37.83c-e	6.67±6.67b	2.33±2.33b	2.00±1.15d	0.00	0.67±0.67b
	Cowpea	0.00	0.00	0.00	0.00	0.00	0.00
	Green lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00
Shaheed Benazirabad	Chickpea	89.33±80.02cde	24.33±23.84b	6.67±5.7b	0.33±0.33d	16.67±16.67a	1.67±1.67ab
	Cowpea	0.00	0.00	0.00	0.00	0.00	0.00
	Green lentil	6.00±6.00e	0.00	0.00	0.67±0.67d	0.00	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00
Hyderabad	Chickpea	275.67±151.88b-e	64.67±32.46ab	32.00±23.58b	22.00±11.06bcd	0.00	0.00
	Cowpea	29.00±29.00de	2.00±2.00b	6.00±6.00b	3.00±3.00d	0.00	0.00
	Green lentil	764±233.89a	57.00±24.85b	31.33±9.61b	43.00±13.01bc	0.00	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Matar	0.00	0.00	0.00	0.00	0.00	0.00
Mirpurkhas	Chickpea	193.67±170.23c-e	17.00±11.53b	6.00±3.46b	4.00±4.00d	0.00	5.00±5.00a
	Cowpea	22.00±11.53de	0.00	6.00±4.58b	2.00±2.00d	2.67±2.67b	0.00
	Green lentil	692.67±281.29a	141.67±95.83a	86.33±52.7a	93.00±46.51a	4.00±4.00b	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00
Karachi	Chickpea	10.00±6.08e	4.67±2.6b	6.00±3.46b	6.00±4.58cd	0.00	0.00
	Cowpea	12.00±12.00de	1.67±1.67b	0.67±0.67b	0.00	0.00	2.00±2.00ab
	Green lentil	439.33±300.83a-c	44.00±29.1b	41.33±32.75ab	18.33±11.67b-d	5.00±5.00b	0.00
	Yellow lentil	0.00	0.00	0.00	0.00	0.00	0.00
	Field pea	0.00	0.00	0.00	0.00	0.00	0.00

3.8 Overall Population of *Callosobruchus* spp. at Various Locations and Pulses

It has been also observed that overall, the highest population of *Callosobruchus* spp. adults (110.67) was recorded in Mirpur Khas district, whereas, the highest population of eggs (1077.33), grubs (158.67), and pupae (98.33) were recorded in Sukkur and Mirpur Khas districts, respectively. Moreover, among pulses, green lentils suffered the highest population of various stages of *Callosobruchus* spp. followed by cowpea (adults and eggs and pupae) and chickpea (grubs) (Table 3).

3.9 Population Fluctuation of Alive *C. maculatus* (Sukkur and Mirpur Khas strains) on Various Pulses

Population fluctuation of both *C. maculatus* strains on four major pulses over the entire study duration exhibited a highly significant variation ($F= 14.15$, $P < 0.001$). According to Table 4, during the first week after the release of *C. maculatus,* the maximum population of alive beetles was observed in cowpea (38.00±3.78 beetles) for the Mirpur Khas strain, followed by green lentil (31.00±3.61 beetles) for the same strain. Afterward, a great variation was recorded in a number of alive and dead beetles in various pulses for both Sukkur and Mirpur Khas strains because of their short adult longevity. Thus, fortnightly the highest mean *C. maculatus* population was recorded in cowpea (800.40±57.55 beetles) for the Mirpur Khas strain, followed by 784.40±36.50 beetles recorded in green lentil for the same strain. The maximum fortnightly mean population for the Sukkur strain i.e., 736.80±57.51 beetles was recorded in green lentil, followed by 676.20±120.94 beetles in cowpea, both observed during the fifth fortnight of the study.

Overall mean population results indicated that among pulses, cowpea suffered significantly ($F = 6.00$, $P = 0.0007$) the highest population of both Sukkur (268±67.04 beetles) and Mirpur Khas (327.64±74.50 beetles) strains of alive *C. maculatus,* followed by the population of Mirpur Khas strain observed in chickpea (263.08±59.29 beetles) and green lentil (252.72±62.33 beetles) (Figure 2). Overall, the lowest alive *C. maculatus* population was recorded in green lentils (139.96±32.32 beetles) for the Mirpur Khas strain, followed by 202.80±60.06 beetles recorded in the same pulse for the Sukkur strain.

3.10 Population Fluctuation of Dead *C. maculatus* (Sukkur and Mirpur Khas strains) on Various Pulses

Like population fluctuation of alive *C. maculatus* in various pulses throughout the study duration

Table 3. Overall mean population of various stages of *Callosobruchus* spp. recorded at various locations (A) and pulses (B)

A.

District	Adults	Eggs	Grubs	Pupae
Sukkur	75	1077.33	99.66	44.67
Larkana	48	395	76.33	43
Ghotki	2.67	43.67	6.67	2.33
Shaheed Benazirabad	19.34	95.33	24.33	6.67
Hyderabad	68	1068.67	123.67	69.33
Mirpur Khas	110.67	908.34	158.67	98.33
Karachi	31.33	461.33	50.34	48

B.

Pulse	Adults	Eggs	Grubs	Pupae
Chickpea	73.01	638.68	173.67	64
Cowpea	66.67	848.66	67	71.34
Green lentil	215.33	2562.33	299	176.99

Table 4. Population fluctuation of alive *C. maculatus* strains (Sukkur & Mirpur Khas) on different pulses under lab conditions

Observation interval (Fortnight)	Chickpea		Cowpea		Green lentil		Yellow lentil	
	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas
One	8.00±1.34i	10.60±1.21i	8.00±1.84i	38.00±3.78i	9.60±3.09i	31.00±3.61i	9.60±4.30i	7.00±2.43i
Two	18.20±6.48i	28.80±4.27i	21.40±10.45i	22.60±3.96i	8.40±6.16i	18.80±3.41i	38.40±15.18i	25.00±4.28i
Three	486.40±53.15ef	622.00±47.68c	605.40±63.83cd	731.00±50.64ab	246.80±72.08h	393.40±27.72fg	297.00±48.83gh	234.20±22.64h
Four	28.40±4.21i	48.80±6.30i	33.40±7.06i	46.20±2.99i	12.40±6.07i	26.00±2.92i	53.20±20.23i	29.60±3.50i
Five	513.20±75.13de	605.20±22.48cd	676.20±120.94bc	800.40±57.55a	736.80±57.51ab	784.40±36.50a	611.00±66.99cd	404.00±19.32f

*Means followed by the same letters are not significantly different from each other (LSD = 103.87, P < 0.05)

Table 5. Population fluctuation of dead *C. maculatus* strains (Sukkur & Mirpur Khas) on different pulses under lab conditions

Observation interval (Fortnight)	Chickpea		Cowpea		Green lentil		Yellow lentil	
	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas	Sukkur	Mirpur Khas
One	0.00±0.00n	0.80±0.37n	0.00±0.00n	1.20±0.49n	9.20±2.73mn	20.80±4.04l-n	8.20±3.58mn	8.00±1.14mn
Two	36.40±11.95j-n	46.40±7.50j-n	33.40±12.97j-n	46.20±3.90	21.80±7.72l-n	31.00±3.45j-n	12.80±5.61	10.60±1.36
Three	32.20±3.97j-n	62.20±5.91i-m	33.60±8.31j-n	112.20±5.94hi	34.40±7.68j-n	48.00±4.74j-n	32.80±6.74j-n	22.80±1.91k-n
Four	600.40±33.02c	703.20±21.92ab	653.40±38.47bc	749.00±16.58a	346.40±57.99e	424.00±38.63d	399.20±71.18de	215.40±20.97f
Five	77.20±9.20ijk	192.80±11.78fg	80.20±13.09ij	165.00±12.47f-h	82.40±14.54ij	145.00±15.39gh	70.20±8.95i-l	28.40±3.08

*Means followed by the same letters are not significantly different from each other (LSD = 55.130, P < 0.05)

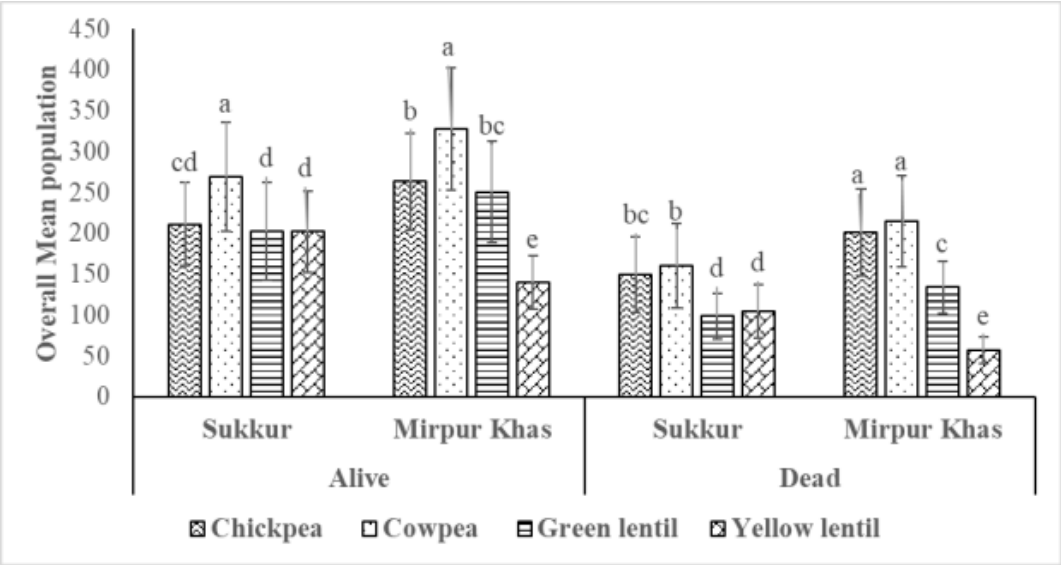

Fig. 2. Overall mean population of *C. maculatus* (alive and dead) on various pulses for Sukkur and Mirpur Khas strains

*Means followed by same letters individual for Alive (LSD = 46.451) and Dead (LSD = 24.655) are not significantly different from each other

(Table 5), a great variation was also observed in the number of dead beetles in different pulses with a highly significant difference (F= 4.39, P < 0.001). Accordingly, the highest number of dead *C. maculatus* (749.00±16.58 beetles) was observed from cowpea for the Mirpur Khas strain, followed by chickpea (703.20±21.92 beetles) and cowpea (653.40±38.47 beetles) for Mirpur Khas and Sukkur strains, respectively.

Overall, a highly significant difference (F = 14.88, P < 0.001) for the *C. maculatus* population was recorded in various pulses for both strains. According to the results, the highest overall mean population of beetles (214.72±55.85 beetles) was observed from cowpea for Mirpur Khas strain, followed by chickpea (201.08±53.10 beetles) and cowpea (160.12±51.23 beetles) for Mirpur Khas and Sukkur strains, respectively. Overall, the lowest population of dead *C. maculatus* (57.04±16.70 beetles) was recorded in green lentils for the Mirpur Khas strain and 98.84±28.06 beetles in the same pulse for the Sukkur strain.

3.11 Cumulative Mean population of *C. maculatus* Strains on Different Pulses

Figure 3 described the cumulative overall mean population of alive and dead *C. maculatus* of both strains on various pulses. According to the results, a significantly higher population of alive (F = 4.26, P = 0.0407) and dead (F = 14.10, P = 0.0002), *C. maculatus* on different pulses was recorded for Mirpur Khas strain i.e., 245.35±29.90 and 151.65±21.87 beetles, respectively than Sukkur strain having an overall cumulative mean population of alive (221.09±28.36) and dead (128.21±20.33) beetles.

3.12 Percentage Damage of Various Pulse Grains due to Feeding of *C. maculatus*

The percentage damage in the shape of weight loss of various pulses due to the feeding of

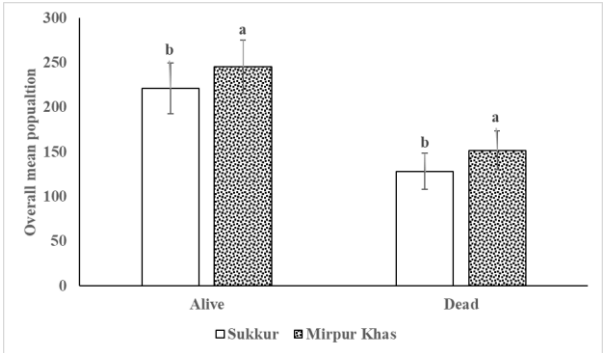

Fig. 3. Collective overall mean population of *C. maculatus* strains (alive and dead) on various pulses

*Means followed by the same letters individually for Alive (LSD =23.225) and Dead (LSD = 12.327) are not significantly different from each other

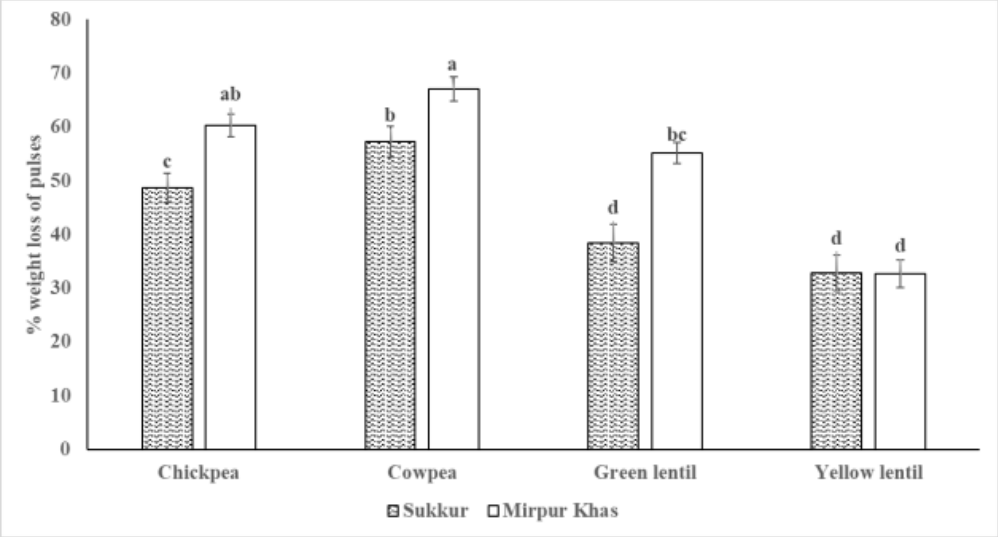

Fig. 4. Percentage damage of various pulse grains due to feeding of *Callosobruchus maculatus*
*Means followed by same letters are not significantly different from each other (LSD = 7.8296, P < 0.05)

C. maculatus is given in Figure 4 which confirmed a significant difference among various pulses (F = 3.39, P = 0.0298). Mirpur Khas strain of *C. maculatus* significantly damage more grains of cowpea (67.07±2.29 %), followed by chickpea (60.24±2.14 %), whereas the highest damage of Sukkur strain was also recorded in cowpea (57.20±2.87%), followed by chickpea (48.64±2.71%). The lowest percentage damage of both the strains was recorded in green lentils i.e., 32.82±3.35 and 32.60±2.56 % for Sukkur and Mirpur Khas strains, respectively.

4. DISCUSSION

Three pulse beetle species i.e., *C. maculatus*, *C. chinensis*, and *C. analis* were recorded from seven sampling locations in Sindh, Pakistan. Moreover, populations of these species were recorded only from chickpea, cowpea, and green lentil, whereas no beetle population was observed from green lentils and field peas. Among insect pests, *Callosobruchus* spp. have been reported as the primary pests of stored grains that cause damage by feeding inside the grains [17, 18]. Moreover, among *Callosobruchus* spp., *C. maculatus* is a more devastating and widely distributed pest of pulses in the tropical and subtropical areas of the world [19]. The results of this study support the findings of our study as at all the sampling locations, *C. maculatus* was the most abundant and widely distributed species on various stored pulses. Many other research studies also suggested that *C. maculatus* is a comparatively more abundant and destructive pest of pulses, where grubs are the most destructive stage that can consume entire contents of the grains in case of severe infestation [18]. The relatively higher and continuous infestation of *C. maculatus* to a wide variety of pulses has also been reported in various geographical regions of the world [10, 20, 21]. Similarly, the infestation of pulse beetles especially *C. maculatus* has not only been found in warehouses but their population is also recorded from the pulses in field conditions [22, 23].

Thus, results of survey and damage potential studies conducted also confirmed significant differences in the presence and damage percentage of *Callosobruchus* spp., especially *C. maculatus* on various pulses. Relatively more population and damage of *C. maculatus* was observed in the cowpea, followed by chickpea and green lentils than green lentils in this study. Such differences in population fluctuations and damage of *C. maculatus* on different pulses may be due to the physical texture, seed coat, and size along with nutrient contents of the seeds. It has been mentioned in previous studies that pulses with smooth seed coats and greater seed weight along with surface area are more preferred by the Callosobruchus spp. for feeding and oviposition [24, 25]. Tripathi et al. (2020) also found a significant impact of various

physical features i.e., colour, shape, testa texture, length, width, and seed hardness of 103 cowpea accessions on oviposition, damage, and percentage weight loss caused by *C. maculatus* [26]. The comparative preference study of *C. chinensis* on lentil, black gram, mungbean, and chickpea showed that it prefers to lay a relatively higher number of eggs on chickpea, whereas black grams were least preferred for oviposition. Accordingly, chickpea and black gram suffered the highest and lowest weight loss due to feeding of the beetle, whereas mungbean and lentil were moderately affected pulses [27, 28]. Due to variable preference of *C. maculatus* on different pulses, they also affect its growth and reproductive parameters as the latest studies by Bidar et al. (2021) found that among chickpea, cowpea, lentil, and green gram, lentil supported the lowest reproduction parameters and higher developmental time, whereas chickpea was the more preferred pulse to support growth and reproductive parameters of *C. maculatus* [29].

In our study, *C. maculatus* showed more feeding preference for cowpea, followed by chickpea, whereas green lentil was the least preferred, hence most of the above-mentioned studies partially supported our findings as most of them found chickpea as the most susceptible pulse against *C. maculatus*.

5. CONCLUSION

Three species of pulse beetles i.e., *Callosobruchus C. maculatus*, C. *chinensis*, and C. *analis* were collected and identified from all sampling locations of Sindh, Pakistan with *C. maculatus* being more abundant and widely distributed. Among sampled pulses, the population of *Callosobruchus* spp. was recorded from green lentil, cowpea, and chickpea, whereas no population was recorded from green lentil and field pea. Overall, the maximum pulse beetle population was recorded on green lentils, with Mirpur Khas and Sukkur districts being the most affected. The feeding preference of Mirpur Khas and Sukkur strains of *C. maculatus* also exhibited significant variation in their feeding towards various pulses with cowpea being the most preferred, followed by chickpea, whereas green lentil was the least preferred, accordingly, suffered the highest and lowest damage and weight loss. Therefore, it is suggested that proper storage and quarantine measures should be taken in the transportation and storage of pulses to restrict the spread and damage of pulse beetles from more susceptible areas and pulses to new areas and relatively resistant pulses.

6. CONFLICT OF INTEREST

The authors declare no conflict of Interest.

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Research Article

Optimization of Organic Mulch Sheet Compositions in Chili (*Capsicum annum* L.) Cultivation: Effect on the Growth and Yield

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Abstract: Climate change causes low production of most vegetables throughout the world by reducing the quality and quantity of yield. Mitigation of the negative impact of climate change on plant productivity must be emphasized in the development of production systems to improve the efficiency of water use, including mulch. Organic mulch sheet (OMS) is an alternative biodegradable mulch made from abundant renewable materials. This study aimed to understand the influence of OMS application on the growth and yield of chili compared to plastic mulch and to optimize the compositions of OMS made from water hyacinth, coconut fiber, and manure for improving the growth and yield of chili. This study was carried out using a simple randomized complete block design with two controls (without mulch (P0) and plastic mulch (P0M)) and five treatments of OMS compositions (the percentage of water hyacinth, coconut coir, and manure) labeled as PO1 (70:20:10), PO2 (60:30:10), PO3 (50:40:10), PO4 (40:50:10) and PO5 (30:60:10). All tested OMS compositions in this study showed insignificantly growth and yield variables of chili compared to the plastic mulch. Optimum OMS composition for yield variables obtained at 59.39% water hyacinth and 0% coconut coir based on response optimizer using response surface analysis. Insignificant different growth and yield produced from OMS and plastic mulch application indicated that all tested OMS compositions, with biodegradable and eco-friendly properties, can be used to substitute for plastic mulch in chili cultivation.

Keywords: Biodegradable mulch, Climate Change, Natural Fiber, Response Surface Methodology

1. INTRODUCTION

Strategies in dealing with climate change and its impacts become one of the objectives of the Sustainable Development Goals (SDGs) with the target of strengthening resilience and adaptive capacity toward climate-related hazards and improving capacity in mitigating, adapting, and reducing the impacts of climate change. Climate change directly impacts the productivity of agricultural products through changes in temperature, climate variability, and extreme weather [1]. Mulch is one of the mitigation strategies to reduce the negative impacts of climate change [2].

Mulch is frequently used in crop cultivation practices, such as chili (*Capsicum annum* L.), to increase water use efficiency, manipulate the microclimate, and improve yields [3-4]. Indonesian chili export is included in the top 20 chili and peppers exporting countries with a quantity reaching 2093 tons in 2017 [5]. Furthermore, the national chili production of Indonesia reached 1045 million tons with 123 thousand ha harvested in 2016 [6], thus the mulch demand is needed in large quantities. The conventional and commercially available mulch used in cultivation is plastic mulch which has limitations associated with its waste handling and sustainability, leading to a potential environmental risk after long-term application [7-8]. Various degradable mulch alternatives were studied because they could reduce global warming potential by 5 % and non-renewable energy use by 14 % compared to the use of polyethylene mulch [9]. The biodegradable film is also widely developed as an alternative mulch material, but it is more expensive and not very durable compared

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with plastic mulch. Paper mulch (organic mulch sheet) is a promising alternative related to abundant material sources and relatively low manufacturing costs [10].

Organic mulch sheets (OMS) are made from natural fibers that can be derived from agricultural waste such as coconut coir and overabundant aquatic weeds such as water hyacinth [11-13]. Biodegradable mulch derived from natural fibers is widely studied, but the optimum composition that satisfies desirable yield as an appropriate substitute for polyethylene mulch still needs further research and improvement. This research aimed to understand the effect of OMS application on the growth and yield of chili compared to plastic mulch and to optimize the composition of OMS made from coconut fiber, water hyacinth, and manure for improving the growth and yield of chili.

2. MATERIALS AND METHODS

The study was conducted in East Java, Indonesia with an altitude of 500 meters asl and rainfall ranging from 1750-2500 mm per year. Materials needed in this study were raw materials for making OMS (water hyacinth petiole, coconut coir, and manure), water, chili seeds, goat manure, inorganic fertilizer, and pesticides. The procedures of making OMS following the method of Iriany *et al*. [14] were conducted.

2.1 Experimental Design

This study was carried out using a simple randomized complete block design (RCBD) with two controls (bare soil(P0) and plastic mulch (P0M)) and five treatments i.e OMS compositions), with three replications. The treatments were various OMS compositions (the percentage of water hyacinth and coconut coir combined with 10 % manure) namely PO1 (70:20:10), PO2 (60:30:10), PO3 (50:40:10), PO4 (40:50:10) and PO5 (30:60:10).

2.2 Measured Variables and Data Analysis

Measured variables were plant growth and plant yield. Plant growth variables were plant height, stem diameter, number of leaves and number of flowers. Plant yield variables were a number of fruits per plant, fruit fresh weight per plant (g), fruit fresh weight per fruit (g), fruits dry weight per plant (g), fruit diameter (mm), and fruit length (cm).

The analysis of variance was performed to understand the effect of the treatment, then HSD (Tukey test) α 5 % was complete to determine the best treatment. Surface response method analysis was also carried out to analyze the optimum OMS compositions based on growth variables and a number of fruits data using Minitab 19.

3. RESULTS AND DISCUSSION

3.1 Effect of OMS Compositions on the Growth of Chili

OMS application produced a similar plant height of chili with plastic mulch (P0M) and higher than produced by bare soil (P0) (Figure 1). Plant height produce from chili grown with various OMS compositions showed an insignificant difference compared to the plastic mulch (P0M) but did significantly higher than without mulch (P0) at the end of observation (7 weeks after planting (WAP)) (Table 1). The PO1 treatment (70 % water hyacinth, 20 % coconut coir, and 10 % manure) showed higher plant height than other OMS compositions although not significantly different.

Various OMS compositions and plastic mulch (P0M) treatments showed a similar number of leaves (Figure 2) yet higher than without mulch (P0). Treatments did not significantly influence the number of leaves at the end of observation (7 weeks after planting) (Table 1). However, OMS made from 50 % water hyacinth, 40 % coconut coir, and 10 % manure (PO3) showed more number of leaves than other OMS compositions even though it was not significantly different.

OMS produced insignificantly different stem diameters compared with control (plastic mulch and without mulch) as shown in Figure 3. The PO2 composition (60 % water hyacinth, 30 % coconut coir, and 10 % manure) showed a higher stem diameter than other OMS compositions although it was not significantly different (Table 1).

OMS composition treatment did not produce more number of flowers compared to the plastic mulch but it was significantly different from

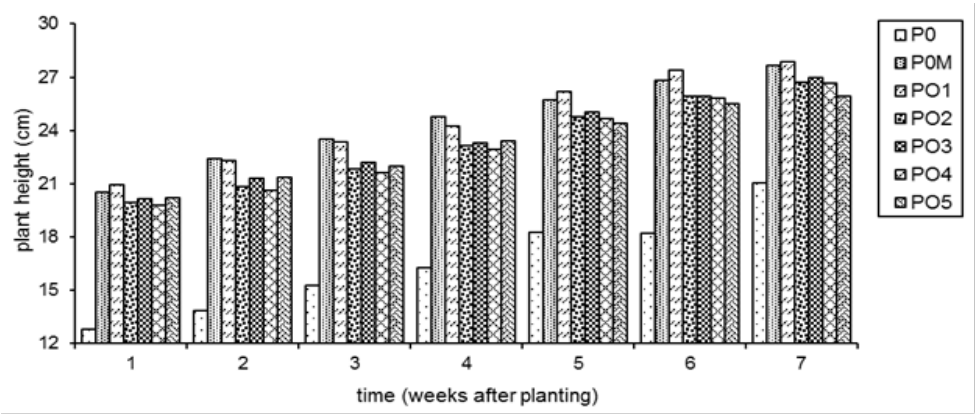

Fig. 1. Plant height of chili grown in various OMS compositions from 1st until 7th WAP. P0; P0M; and PO1, PO2, PO3, PO4, and PO5 represent without mulch, plastic mulch, and various OMS compositions, respectively.

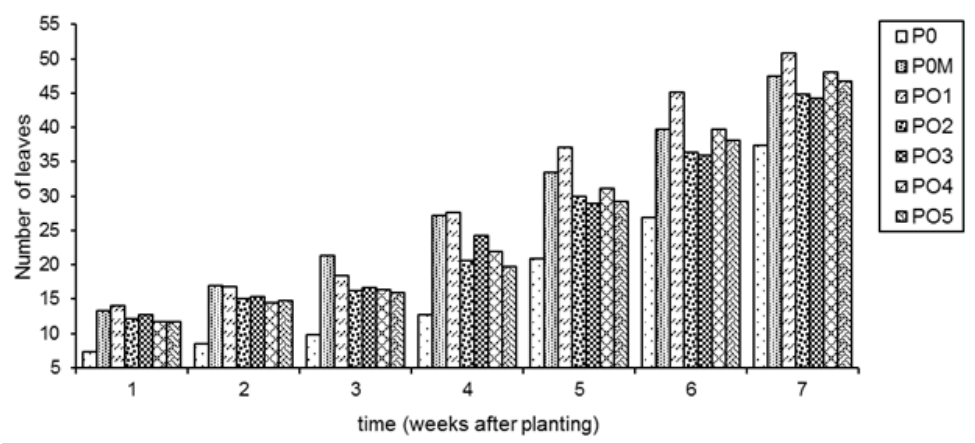

Fig. 2. Number of chili leaves grown in various OMS compositions from 1st until 7th WAP. P0; P0M; and PO1, PO2, PO3, PO4, and PO5 represent without mulch, plastic mulch, and various OMS compositions, respectively.

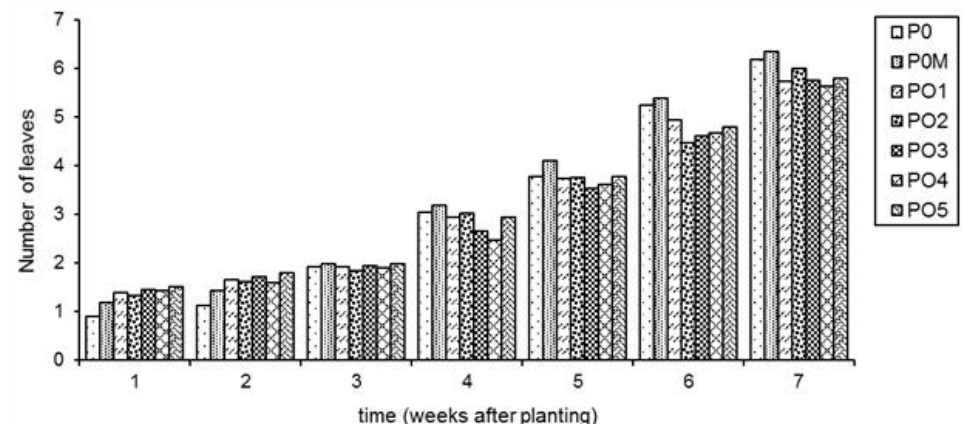

Fig. 3. Stem diameter of chili grown in various OMS compositions from 1st until 7th WAP. P0; P0M; and PO1, PO2, PO3, PO4, and PO5 represent without mulch, plastic mulch, and various OMS compositions, respectively.

without mulch as shown in Figure 4. There was no significant different number of flowers between treatments and control at the end of observation (Table 1). Even though it was not significantly different, PO3 composition (50 % water hyacinth, 40 % coconut coir, and 10 % manure) showed more number of flowers than other OMS compositions.

The influence of OMS composition on the chili growth in this study was consistent with previous studies. The composition ratio of OMS materials did not influence plant growth. Sekara *et al*. [15] stated that tomatoes grown in biodegradable mulch (made from biopolymer of starch) showed an insignificant difference in total biomass, residual biomass, and LAI compared to mulch film (LDPE). Iriany *et al*. [12, 16] assert that the OMS compositions ratio showed an insignificant difference in number of leaves and plant height of shallot and cauliflower compare to the plastic mulch. It can be indicated by the similar microclimatic condition with the application of OMS and plastic mulch. Furthermore, there is an insignificant difference in microclimate factors due to variations in OMS compositions [12].

3.2 Effect of OMS Compositions on the Yield of Chili

Generally, plastic mulch produced higher fruit fresh weight, more number of fruits, and higher fruit dry weight per plant compared to OMS treatments even though it was not significantly different from various OMS compositions (Table 2). Furthermore, plastic mulch and OMS application showed insignificantly different fruit lengths also mulch application did not influence fruit fresh weight and fruit diameter. This finding emphasized that OMS can be applied for the substitution of plastic mulch in chili cultivation which produced similar (not significantly different) crop yields. OMS application increased number of fruit per plant, fruit weight per fruit, and dry fruit weight per plant up to 25 %, 10 %, and 59 %, respectively, compared to the bare soil. OMS compositions influenced the number of fruits per plant significantly with PO5 (30 % water hyacinth, 60 % coconut coir, and 10 % manure) as the highest value. OMS compositions did not affect the fresh fruit weight per plant, fresh fruit weight per fruit, fruit diameter, and fruit length variables significantly, although PO4 (40 % water hyacinth, 50 coconut coir, and 10 % manure) showed higher fresh fruit weight per plant than other OMS compositions.

The earlier research on the application of degradable mulch from various raw materials showed an insignificant effect between the ratio composition of mulch raw material with the marketable yield of several crop species. This present study also has a similar result that crop yield produced from biodegradable mulch (OMS) and plastic mulch was insignificantly different. The difference in OMS compositions (paddy straw, water hyacinth, and tannery waste) shows an insignificant difference compared to the mulch film on the weight and diameter of cauliflower curd and the number of the tuber of shallot variables [12, 16]. Coolong [7] asserts that the difference in paper mulch types did not affect the marketable yield of summer squash significantly compared to plastic mulch. Sekara *et al*. [15] stated that biodegradable mulch and mulch film produced no significant difference in the marketable yield variable and harvest index of tomatoes. Furthermore, there was an insignificant difference in number of fruit, marketable yield, and the total weight of tomatoes grown in biodegradable mulch (made from cornstarch or and cellulose) and mulch film [17, 18].

Application of different commercial degradable mulch product types showed insignificantly yield and an average head weight of lettuce; average curd of broccoli; yield, average fruit weight, and number of fruits of bell pepper; and average fruit weight of watermelon. Mulch type (degradable and non-degradable) also does not influence the marketable yield of cucumber [19-20]. Furthermore, Haapala *et al*. [21] stated that the influence of biodegradable mulch on cucumber yields began to be seen in the second cycle of cultivation. It could be due to the minor effect of weathered buried mulch (starch-based, cellulose-based, and polylactic acid) on the soil quality index (based on the five indicators, namely microbial biomass, and β-glucosidase activity, TOC, EC, and pH) which plays an important role in crop production [22].

3.3 Optimum Compositions of OMS on the Growth and Yield of Chili using RSM

Response surface methodology (RSM) is a multivariate statistic technique for optimizing the

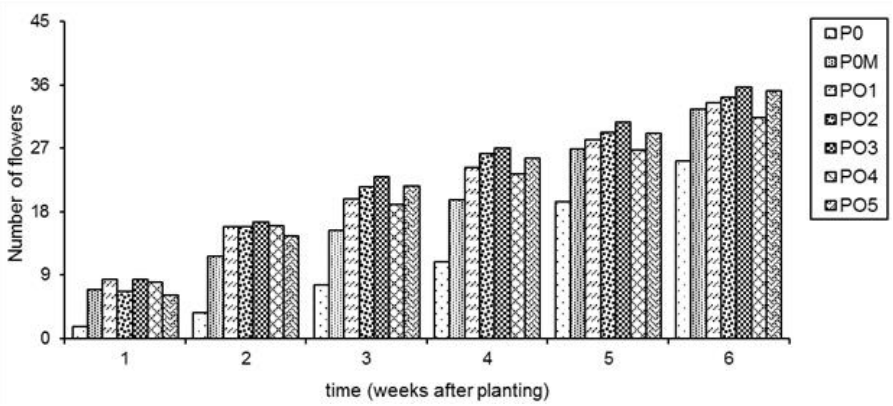

Fig. 4. Number of chili flowers grown in various OMS compositions from 1st until 7th WAP. P0; P0M; and PO1, PO2, PO3, PO4, and PO5 represent without mulch, plastic mulch, and various OMS compositions, respectively.

Table 1. Growth observation variables of chili grown in various OMS compositions on 7 WAP

Treatment	Plant height (cm)	Stem diameter (cm)	Number of leaves	Number of flowers
P0	21.0±3.1 b	6.2±0.3 a	37.3±0.7 a	25.2±1.6 a
P0M	27.7±1.2 a	6.4±0.6 a	50.9±4.3 a	32.6±0.6 a
PO1	27.8±0.9 a	5.7±0.3 a	44.8±4.7 a	33.4±1.7 a
PO2	26.7±1.3 ab	6.0±0.5 a	44.2±3.3 a	34.2±2.4 a
PO3	27.0±1.7 a	5.8±0.9 a	48.1±3.0 a	35.7±1.3 a
PO4	26.6±1.8 ab	5.6±0.2 a	46.8±1.1 a	31.3±1.0 a
PO5	25.9±2.1 ab	5.8±0.3 a	47.4±2.2 a	35.2±5.6 a
HSD 5 %	7.20	2.52	14.18	12.70

Notes: Values were expressed as means ± standard error.
The means followed by the same letter within the same column is insignificantly different based on the HSD test with α 5 %.
Treatments: P0 control (bare soil), P0M (Plastic mulch), PO (OMS compositions i.e the percentage of water hyacinth, coconut coir, and manure namely PO1 (70:20:10), PO2 (60:30:10), PO3 (50:40:10), PO4 (40:50:10) and PO5 (30:60:10).

Table 2. Yield observation variables of chili on various OMS compositions

Treatment	Number of fruits per plant	Fresh fruit weight per plant (g)	Fruit weight per fruit (g)
P0	37.8±0.4 c	326.6±31.2 ab	5.2±1.9 a
P0M	47.4±2.7 a	395.6±1.3 a	5.7±1.7 a
PO1	39.7±1.5 bc	297.0±0.8 b	5.5±1.8 a
PO2	36.8±3.1 c	311.7±3.8 b	5.4±1.8 a
PO3	46.1±1.4 ab	341.5±4.4 ab	5.7±1.7 a
PO4	36.3±1.2 c	347.0±2.7 ab	5.4±1.8 a
PO5	47.1±0.1 a	319.9±20.1 b	5.7±1.7 a
HSD 5 %	7.4	74.4	1.1
	Dry fruit weight per plant (g)	**Fruit diameter (mm)**	**Fruit length (cm)**
P0	27.3±0.8 c	10.1±0.3 a	10.6±0.4 a
P0M	54.8±4.0 a	10.9±0.9 a	9.5±0.4 ab
PO1	38.8±0.7 bc	10.8±1.5 a	8.9±0.4 b
PO2	34.7±0.4 bc	9.9±0.7 a	9.3±0.4 ab
PO3	43.3±5.7 ab	11.3±1.2 a	9.3±0.4 b
PO4	35.6±1.1 bc	10.7±1.3 a	10.2±0.7 ab
PO5	30.9±0.6 bc	11.9±1.2 a	9.4±0.7 ab
HSD 5 %	14.2	2. 26	1.3

Notes: Values were expressed as means ± standard error.
The mean followed by the same letter in the same variable was not significant based on Tukey's test with α 5%.
The means followed by the same letter within the same variable is not significantly different based on the HSD test with α 5%.
Treatments: P0 control (bare soil), P0M (Plastic mulch), PO (OMS compositions i.e the percentage of water hyacinth, coconut coir, and manure namely PO1 (70:20:10), PO2 (60:30:10), PO3 (50:40:10), PO4 (40:50:10) and PO5 (30:60:10)).

levels of independent variables to gain the desired response [23]. RSM can be used to optimize composition and process in biopolymer, bioplastic, biodegraded composite, and biodegradable aliphatic-aromatic polyester film production [24]. In this study, the optimum composition of OMS that supports growth and produces desirable yield was determined using RSM.

Contour and surface plot for the effect of OMS compositions on the growth of chili are shown in Figure 5. The optimum plant height was obtained with OMS made from 70 % water hyacinth and 60% coconut coir as shown in Figure 5a. The optimum composition of OMS to produce a maximum number of leaves was 70 % water hyacinth and 60 % coconut coir (Figure 5b) while the maximum stem diameter was 30 % water hyacinth and 10 % coconut coir (Figure 5c). Related to yield variables, both fruits' weight per plant and fruit length were produced optimally with an OMS composition of about 30 % water hyacinth and 15 % coconut coir (Figure 6a and Figure 6c). Besides, the maximum number of fruits was seen at the OMS composition of 5 % water hyacinth and 60 % of coconut coir (Figure 6b). Based on the response optimizer, the optimum OMS composition for yield variables (fruit length, number of fruits, and fruits weight) was 0 % coconut coir and 59.39 % water hyacinth (Table 3). the composite desirability value (D) that close to one indicated that the composition appeared to achieve favorable outcomes for all responses overall [25].

Table 3. Optimization of OMS compositions for yield variables (fruit length, number of fruits, and fruits weight)

Desirability	**Optimum OMS composition (%)** Water hyacinth: coconut coir 59.39:0
Composite desirability	0.86200
Fruit length	0.82069
Number of fruits	0.94593
Fruit weight	0.82517

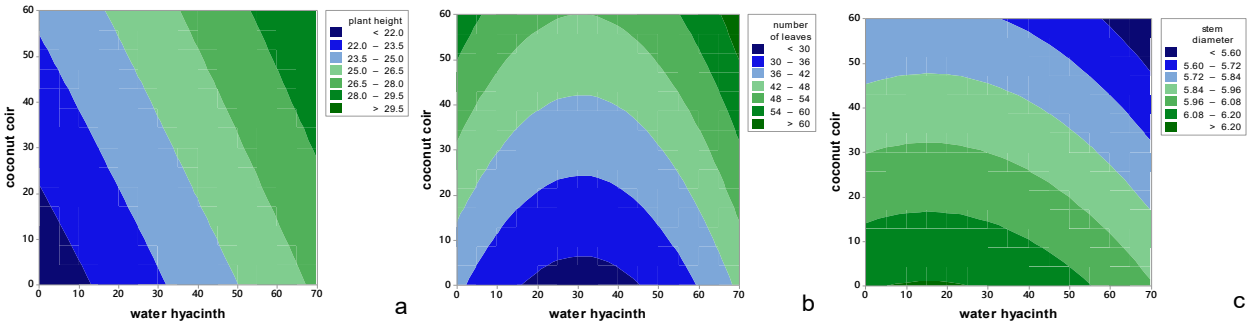

Fig. 5. Contour plot for the effect of OMS composition (the percentage of water hyacinth and coconut coir) on the growth of chili (a. Plant height; b. Number of leaves; and c. Stem diameter).

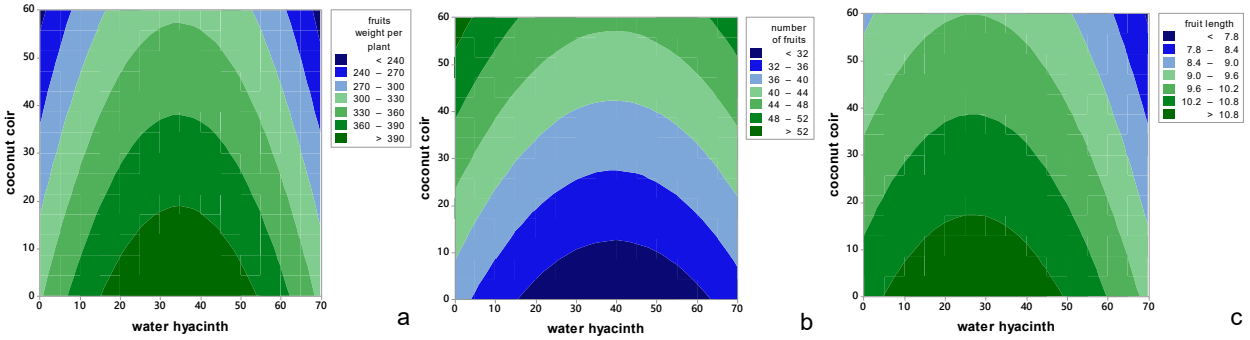

Fig. 6. Contour plot for the effect of OMS composition (the percentage of water hyacinth and coconut coir) on the growth of chili (a. Fruits weight per plant; b. Number of fruits; and c. Fruit length).

Generally, contour and surface plots showed that the optimum composition of OMS for supporting plant growth needs more water hyacinth than coconut coir, but producing a desirable yield needs less water hyacinth than coconut coir. This result was in accordance with the mean value of the observed variables. Although optimum OMS composition has obtained using RSM, all tested OMS compositions showed an insignificant difference in plant growth and crop yield variables based on the HSD test. This study revealed that the use of OMS produced similar growth and yield of chili with plastic mulch. Therefore, OMS composition can be adjusted with the availability of raw materials and made based on the formulation in this research. The significance of this finding is the use of OMS as biodegradable mulch for crop production that supports sustainable agriculture practices so that sustainable development will be achieved.

4. CONCLUSION

All tested OMS compositions in this study showed insignificantly different growth and yield variables of chili compared to the plastic mulch. Optimum OMS composition for yield variables was obtained at 59.39 % water hyacinth and 0 % coconut coir based on RSM analysis. Insignificant different growth and yield produced from OMS and plastic mulch application indicated that all tested OMS compositions, with biodegradable and eco-friendly properties, can be used as a substitute for plastic mulch in chili cultivation.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors have no conflict of interest.

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Research Article

Hepatic and Renal Histopathological Effects of Local Fruit Juices containing Sodium Benzoate as Preservative

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Abstract: Packed fruit juices are commonly consumed on daily basis around the globe which usually contain sodium benzoate as a food preservative to enhance their shelf life and its amount may vary from defined food standards among brands. This mammalian model-based research was designed to investigate the impact of locally packed fruit juices' regular intake on consumers. For this purpose, albino mice (Mus musculus) were acclimatized in the laboratory environment for ten days and then categorized into control and two experimental groups. The selected local brands were Murree brewery lemon malt and Shezan mango juice and on their bottles and tetrapacks, it was already mentioned that they contain sodium benzoate as a preservative. For further confirmation, the titration method was used to detect the presence of sodium benzoate in selected locally packed juice samples. After dose preparation and optimization, 0.1 ml of the prepared dose was given to each experimental group I (treated with lemon malt) and II (treated with mango juice), whereas the control group was treated with the equivalent amount of distilled water. The obtained histopathological results emphasized that regular intake of sodium benzoate having fruit juices may cause severe damage to hepatic and renal tissues, usually in the form of necrosis, vascular congestion sometimes dilation and other cellular alterations i.e., in glomeruli and bile duct. Moreover, it may result in the onset of tumorogenesis. The conclusion of this study is local food authorities should ensure the addition of a defined amount of sodium benzoate as a juice preservative in locally packed juices to provide healthy products to consumers.

Keywords: Fruit juices, food preservative, sodium benzoate, necrosis, tumorogenesis.

1. INTRODUCTION

Fruit juices are the products of daily consumption. They may be prepared either through fresh extraction or supplied to markets in tetra packs which contain different types of food preservatives to avoid microbial growth and to improve their shelf life. The amount of these food preservatives varies from brand to brand and it is also an important parameter to compare the quality of locally packed fruit juices with international food standards. In this regard, reported data also highlight that the appliance of diverse synthetic compounds as food preservatives may cause detrimental health effects on consumers [1].

The commonly used juice preservatives are benzoate (SB), sodium metabisulphite (SM), potassium sorbate (PS), and their compositional mixtures but none of them is side effect free [11]. Among them, sodium benzoate is considered less harmful or sometimes safe, if added according to internationally defined standards by food and health organization [12]. But in general practice, especially in the case of local less renowned brands, such food standards are not uniformly followed which often alters the attributes of juices and beverages and indirectly results in adverse health effects.

Reported data also supports that if a higher concentration of sodium benzoate enters in living body, which is often used as a food preservative in fruit juices too, has been reported to affect renal function and as result may elevate the blood plasma level and disturbs cellular carbohydrate metabolism which can be observed in histological sections of

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kidneys [2]. This compound has also been found to induce immunosuppressive effects and disrupts the process of blood filtration and detoxification in the liver and kidney [3]. Recent mammalian model-based studies have also supported these above-mentioned side effects of sodium benzoate present in different food items as a preservative [4]. That is why; this research was designed to estimate the possible side effects of sodium benzoate as a preservative of locally packed fruit juices.

2. MATERIALS AND METHODS

2.1 Animal Collection and Habituation

Thirty female albino mice (Mus musculus) of 6 weeks of age were purchased from the University of Veterinary and Animal Sciences, Lahore. The mice were placed in an animal house, School of Zoology, Minhaj University, Lahore, at room temperature (25 °C ± 2 °C) and after habituation of 10 days, they were divided into 3 groups: control group, experimental group I (treated with Murree Brewery lemon malt) and experimental group II (treated with Shezan mango juice).

2.2 Detection of Sodium Benzoate

For detection and confirmation of sodium benzoate in selected juice samples, the acidimetry-alkalimetry titration method was used and the percentage of sodium benzoate was calculated [13].

2.3 Dose Optimization

For dose optimization, 0.5 ml of each selected packed juice was diluted in 9.5 ml of distilled water and 0.1 ml of this prepared dilution was given to both experimental groups whereas control group mice were given 0.1 ml of distilled water orally during 4 weeks of experimentation.

2.4 Histopathology & Microscopy

The mice were dissected weekly and kidney and liver tissues were collected and preserved in 10 % formalin solution for further tissue processing through an automatic tissue processor. Their histological sections were prepared by microtomy and the prepared slides were stained by using Hematoxylin and Eosin staining. The microscopic observations and micrometry of prepared slides were done at 40X and their photomicrographs were recorded at 10X with the help of PixelPro software.

2.5 Statistical Analysis

Single-factor ANOVA was applied for statistical data analysis [5].

3. RESULTS AND DISCUSSION

First of all, the detected percentage of sodium benzoate in the experimental group I sample was 0.096 % and in the experimental group II sample was 0.11 % which was higher than the defined amount of sodium benzoate 0.1% used as food preservative according to the food authorities and especially the formulation of lemon malt should be revised [14]. Moreover, the weekly observations of body weight (Table 1) indicated considerable variations in both experimental groups after i.e. at the end weight gain was observed at 37.33 g of 1st week group II but at the end of 2nd week weight reduction was noticed while in group I body weight increased up to 34.33 g. Similarly, as compared to the previous week though body weight was reduced in group II (30 g) it was still significantly higher than group I (27 g) on completion of 3rd week. Whereas, 24.67 g and 36.67 g body weights were noted in groups I and II, respectively, at the end of the experimentation [15]. Dermal tumors were also observed in mice of experimental groups. These side effects occurred in presence of sodium benzoate which may induce toxicological and adverse changes in the biochemical markers and physiological processes of consumers [6].

For histopathological analyses, first of all, the variations in blood vessel diameter were observed. The diameter of renal arteries was significantly changed during 2nd week, in group I vasoconstriction (50.70 µm) and group II vasodilation (84.53 µm) were noted (Table 2). The reduction in arterial diameter was noticed in both experimental groups I and II, 48.65 µm and 75.02 µm, respectively and same pattern continued till the end of 4th week (Figure 1). Whereas in the case of renal veins diameter, at the end of the 4th week, vasoconstriction was observed in group I (61.29 µm) while vasodilation was noted in group II (3.48 µm). The histopathological observations regarding glomerular diameter

(Table 3) indicated the considerable shrinkage of glomeruli at the end of 2nd week in both groups I and II, 46.49 µm and 35.93 µm, respectively, in comparison to the control group because exposure to sodium benzoate as preservative damage structure of kidney and disturbs its functions [15]. Moreover, the results related to the diameter of necrotic renal tissue presented maximum damage in groups I and II of 83.48 µm and 81.36 µm, respectively, at the end of 3rd week. It indicates that regular use of sodium benzoate-containing food items may lead to serious vascular complications like calcification and congestion of renal blood vessels [7].

In addition to this, histopathological changes related to the diameter of liver blood vessels were examined and among them, hepatic arteries showed prominent vasoconstriction in groups I and II of 46.49 µm and 85.92 µm, respectively, at the end of 2nd week but later on the trend was found opposite till the end of experimentation (Table 4). Similarly, vasodilation was also noticed in diameter of hepatic veins, 117.23 µm (group II) and 54.96 µm (group I) after 2nd week. Then till the end of the experimentation, a gradual reduction in the diameter of the vein was noticed in both experimental groups (Figure 2) due to biochemical alterations [8].

For the measurements of the diameter of bile duct, highlighted the gradual increase, from 1st to 4th week (Table 5). But the striking size of the damaged and necrotic area of hepatic tissue was observed at 86.65 µm and 110.95 µm, in experimental groups I and II respectively, at the end of 3rd week [9]. Thus, locally available juice products are not reliable and their components may induce broad spectrum health side effects [10].

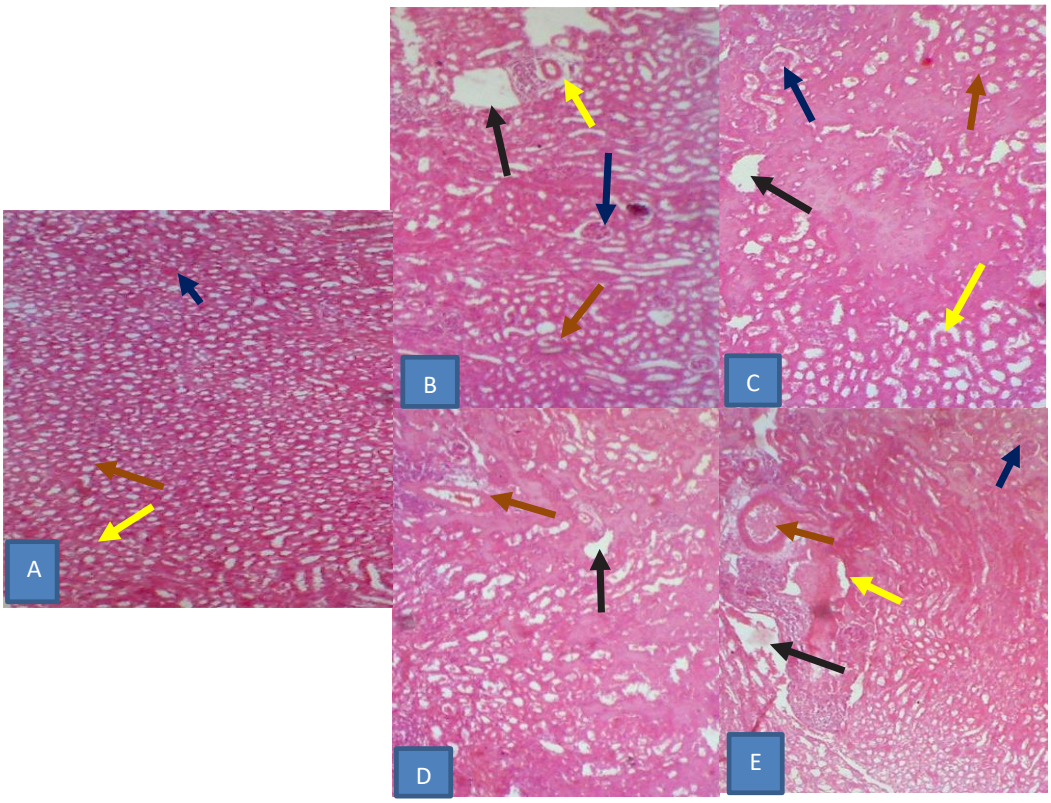

Fig. 1. Comparative histopathological renal observations of (A) control group, (B) 1st week of experimental group I, (C) 1st week of experimental group II, (D) 4th week of experimental group I, (E) 4th week of experimental group II. Black arrows show damaged area diameter (µm), blue arrows show glomerulus diameter (µm), yellow arrows show artery (µm) and red arrow show vein diameter (µm). All microphotographs are of 100X magnification.

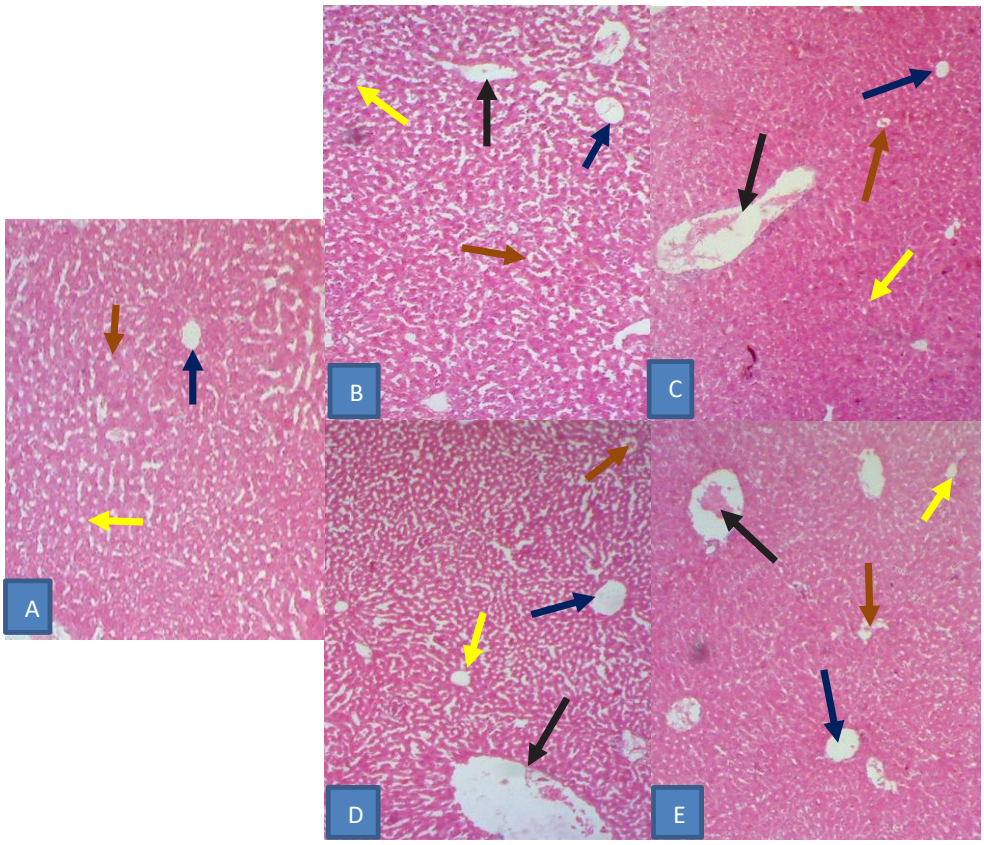

Fig. 2. Comparative histological hepatic observations of (A) control group (B) 1st week of experimntal group I (C) 1st week of experimental group II, (D) 4th week of experimntal group I, (E) 4th week of experimntal group II. Black arrows show the damaged area diameter (µm), blue arrows show the diameter of bile duct (µm), yellow arrows show the artery (µm) and red arrow show vein diameter (µm). All microphotographs are of 100X magnification.

Table 1. Effect of sodium benzoate as juice preservative on body weight (g)

Week	Control group	Experimental Group I	Experimental Group II
1	35±2.52 (3)	31.33±1.33 (3)	*37.33±2.33 (3)
2		*34.33±4.81 (3)	31.67±3.53 (3)
3		27±2.65 (3)	*30±2.31 (3)
4		24.67±1.45 (3)	*36.67±4.41 (3)

*Values represent Mean ± S.E.M. (n). Data was compared by employing single factor analysis of variance and results were found significant at a 5% level (*).

Table 2. Effect of sodium benzoate as juice preservative on renal artery and vein diameter (µm)

Week	For renal artery			For renal vein		
	Control group	Experimental Group I	Experimental Group II	Control group	Experimental Group I	Experimental Group II
1	85.60± 4.84 (3)	57.06 ± 12.81 (3)	49.66 ± 10.08 (3)	102.50 ± 2.80 (3)	72.91 ± 3.17 (3)	70.80 ± 2.80 (3)
2		50.70 ± 10.21 (3)	**84.53 ± 7.40 (3)		53.89 ± 5.50 (3)	59.17 ± 2.80 (3)
3		48.61 ± 9.40 (3)	** 75.02 ± 9.212 (3)		71.85 ± 2.11 (3)	47.56 ± 5.48 (3)
4		*57.06 ± 5.50 (3)	53.89 ± 11.13 (3)		61.29 ± 8.25 (3)	83.48 ± 8.65 (3)

*Values represent Mean ± S.E.M.(n). Data was compared by employing single factor analysis of variance and results were found significant at 5% (*) and 1% (**) levels.

Table 3. Effect of sodium benzoate as juice preservative on diameter of glomerulus and renal necrotic area (µm)

Week	Glomerular diameter			Diameter of renal necrotic area	
	Control group	Experimental Group I	Experimental Group II	Experimental Group I	Experimental Group II
1	86.65 ± 9.21 (3)	102.50 ± 32.35(3)	45.44 ± 7.40(3)	68.68 ± 15.35 (3)	67.63 ± 20.08 (3)
2		**46.49 ± 5.60 (3)	35.93 ± 2.80 (3)	54.95 ± 6.93 (3)	116.23 ± 8.65 (3)
3		**67.63 ± 2.11 (3)	62.34 ± 11.20 (3)	83.48 ± 4.61 (3)	81.36 ± 17.01 (3)
4		66.57 ± 12.001 (3)	67.63 ± 4.6 (3)	62.34 ± 62.34 (3)	63.40 ± 9.69 (3)

*Values represent Mean ± S.E.M.(n). Data was compared by employing single factor analysis of variance and results were found significant at 1 % level (*).

Table 4. Effect of sodium benzoate juice as juice preservative on diameter of liver artery and vein (µm)

Week	For hepatic artery			For hepatic vein		
	Control group	Experimental Group I	Experimental Group II	Control group	Experimental Group I	Experimental Group II
1	73.97 ± 9.21 (3)	102.50 ± 32.35 (3)	45.44 ± 7.40 (3)	126.8± 30 (3)	68.68 ±15.35 (3)	67.63±20.08 (3)
2		**46.49 ± 5.60 (3)	35.93 ± 2.80 (3)		54.95 ± 6.93 (3)	*116.23±8.65 (3)
3		67.63 ± 2.11 (3)	62.3433 ± 11.2 (3)		83.48 ± 4.61 (3)	81.36±17.01 (3)
4		66.57 ± 12.001 (3)	67.63 ± 4.61 (3)		62.34 ± 3.81 (3)	63.4±9.69 (3)

*Values represent Mean ± S.E.M.(n). Data was compared by employing single factor analysis of variance and results were found significant at 5% (*) and 1% (**) levels.

Table 5. Effect of sodium benzoate as juice preservative on bile duct and necrotic area diameter of liver (µm)

Week	For diameter of bile duct			For diameter of hepatic necrotic area	
	Control group	Experimental Group I	Experimental Group II	Experimental Group I	Experimental Group II
1		48.61 ±14.80 (3)	42.27±2.11 (3)	69.74 ± 12.81 (3)	83.48 ± 10.41 (3)
2		62.34 ± 13.74 (3)	60.23±3.66 (3)	77.14 ± 19.74 (3)	85.59±12.68 (3)
3	65.51±9.21 (3)	58.12 ±10.72 (3)	68.68 ±14.68 (3)	86.65 ± 8.6492 (3)	110.95±8.39 (3)
4		78.19 ± 13.86 (3)	57.06±6.34 (3)	71.85 ± 3.81 (3)	100.4 ± 16.51(3)

*Values represent Mean ± S.E.M. (n). Data was compared by employing single factor analysis of variance and no significant result was found.

4. CONCLUSION

It can be concluded that sodium benzoate is not a suitable juice preservative and the consumption of products having this food preservative may cause hepatic and renal physiological disturbances and their long term consumption may lead to adverse health complications. The current study showed that it affected the functioning of the liver and kidney. In this regard, local food authorities should take appropriate action to secure the health of consumers.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

7. ETHICS APPROVAL

The experimental protocols and procedures used in this study were approved by the Ethical Committee of the Directorate of Academics, Minhaj University Lahore, Pakistan with reference number: MUL/DA/11356.

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Research Article

Curative Potentials of Garlic (*Allium sativum*) Extract against Di-(2-Ethylhexyl) Phthalate Induced Reproductive Toxicity in Female Mice

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Abstract: The present study was intended to find out the curative potentials of Garlic (*Allium sativum*) against di-(2-Ethylhexyl) phthalate (DEHP) induced toxicity in the reproductive system of female mice. Forty female mice were divided into four groups (n=10) as the (a) control group was given normal feed and drinking water, (b) aqueous garlic extract treated group (500 mg/kg), (c) DEHP group received 500 mg/kg in corn oil, and (d) DEHP + aqueous garlic extract each at a dosage of 500 mg/kg body weight. All treatments were given daily through oral gavage for 28 days. After completion of the experiment, all the animals were dissected through cervical dislocation to get reproductive organs. Collected organs were weighed and processed through the conventional histology technique of staining with eosin and hematoxylin. This study indicated that DEHP exposure caused a significant decrease ($P<0.001$) in body weight and weight of the complete female reproductive tract as compared to the control group, while the garlic co-administered group showed prominent improvement in body and organ weight when compared to only DEHP treatment. The adverse effect of DEHP on the histology of the ovary such as a decreased mean number of developing follicles, thin and irregular corona radiata, disruption of cumulus oophorus, and reduction in the size of the antral cavity of the tertiary follicle was observed. However, a significant recovery in the development of follicles was seen in DEHP plus garlic-treated group. DEHP plus garlic extract treatment showed protective effects on the uterus, such as a significant increase in the diameter of the uterus ($P<0.001$), muscularis, mean number of endometrial glands ($P<0.001$), and endometrial epithelial heights as compared to only DEHP exposed group. Hence, garlic extract showed significant ameliorative potential against DEHP-induced reproductive anomalies in female mice.

Keywords: DEHP; reproduction; toxicity; garlic extract

1. INTRODUCTION

Di-(2-Ethylhexyl) phthalate (DEHP) is extensively utilized in the plastic industry for enhancing the durability, transparency, and flexibility of polyvinyl chloride (PVC) based polymers [1, 2]. DEHP is widely used in non-polymeric substances such as solvents in cosmetics, perfumes, insect repellents, inks, paints, adhesives, additives in hair sprays, building materials, and lubricating oil in the industry [3]. As DEHP is weakly associated with plastics and consequently easily releases and pollutes the environment by leaching, abrasion, and evaporation from the products [4]. The mode of introduction of phthalates in people can be oral, dermal or inhalation [1]. The presence of DEHP metabolites in several human urine samples has raised concerns about its harmful effects on human populations [5-7]. As previous studies have reported DEHP metabolites in reproductive fluids like amniotic and follicular [8-10] fluids, suggesting their ability to assimilate from circulating plasma.

Meanwhile, limited shreds of evidence suggest that exposure to DEHP may have adverse female reproductive outcomes including, decrease ovulation [11], reduced size of follicular granulosa cells [12], inhibition of synthesis of estradiol [13],

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accelerated apoptosis [14, 15], delayed follicular development [16], extended estrous cyclicity [17], reduced folliculogenesis [12], endometriosis [18], and delayed puberty onset [19]. Moreover, DEHP had also been associated with failure of implantation and fetal loss [10].

Several studies have shown that mono (2-Ethylhexyl) phthalate (MEHP), a major metabolite of DEHP, reduces the meiotic ability of oocytes and affects the blastocyst phase. In vitro and in vivo studies suggest that DEHP or MEHP can induce metaphase II spindle anomalies, negatively modulate the transition of prophase I to metaphase II, and thereby affect meiotic development [14, 15, 20]. Furthermore, these metabolites can also cause prolonged cumulus cell-oocyte aggregates development to the blastula stage [21,22]. Previous studies suggested that DEHP levels detected in 'everyday' environments (200 $\mu gKg^{-1}day^{-1}$) and work-related environments (2000 $\mu gK^{-1}day^{-1}$) cause zygote division and arrest zygote growth from the second-cell stage [23].

Modern scientific studies highlighted the wide usage of plants as a source of herbal medicine. Conventionally Garlic (*Allium sativum*) is utilized against protozoans, toxins, viral, fungal, and bacterial agents [24].

As garlic has different composites, due to multivitamins, but sulfur component mainly represents the biological and medicinal properties of this plant [25]. It has several therapeutic effects on different vital organs and tissues [26-35]. Garlic oil thickens the zygotic wall, which demonstrates that garlic oil has a positive effect on ovarian function and increases the secretion of estrogen [36]. Garlic extract triggers the emission of gonadotropic hormones and ovarian hormones via stimulation of the pituitary gland, increasing the Golgi complex with drawl rate, cell cycle, and accelerating binding to estrogen receptors [37]. Many plastic industry female workers across the world are vulnerable to heavy amounts of phthalates in daily life. The use of utensils, food wrappers and packing made of phthalates made the general population prone to exposure to phthalates. Microwave application is responsible for the leeching and mixing of phthalates in food. Female reproductive disorders are increasing day by day, so this research was premeditated to investigate the adverse effects of DEHP exposure on the female reproductive system and to check the ameliorative effects of commonly used cheapest spice i.e garlic extract against DEHP-induced reproductive deformities in adult female albino mice as an experimental model.

2. MATERIALS AND METHODS

2.1 Animals

Laboratory-raised *Mus musculus* (female albino mice) were utilized as experimental animals in the current research. Animal House of the Department of Zoology, University of Sargodha, Sargodha was used to take care of the animals under standard environmental conditions such as 24-hour day and night cycles, temperature 25±2 °C, and 45 % humidity. Steel cages bedded with wood shavings were used to keep animals. Feed and water were provided ad-libitum.

2.2 Treatment

Forty female albino mice aged 10-11 weeks and weight 28-30 gm were used in this research and divided into the following four groups=10).

1. Control Group received only standard feed and water.
2. Garlic Group received 500 mg/kg aqueous garlic extract in 0.2 mL distilled water.
3. DEHP Group was given 500 mg/kg body weight DEHP dissolved in 0.2 mL corn oil. 4-DEHP+Garlic Group was administered with 500 mg/kg DEHP in corn oil (0.2 mL) followed by (gap of 2 hours) 500 mg/kg body weight garlic extract. Doses were given by oral gavage once daily for 4 weeks [1, 40,41].

2.3 Organ's Recovery

After the completion of the experimental time, all the animals were dissected after a cervical dislocation [38] to obtain the ovary, oviduct (fallopian tube), and uterus. Organs were placed in saline solution and the fats that cover the reproductive organs were removed. Fat-free organs were weighed on a digital weight balance (HI-500) and fixed in Conroy's fixative for further processing [1].

2.4 Histological Preparation

After gradual alcoholic dehydration (50 %, 70 %, 90 %, and 100 %), and clearance in xylene, the organs were embedded in paraffin wax to obtain serial sections of 5µ thickness through rotary microtome (ERMA TOKYO 42). Sections on frosted slides were deparaffinized in xylene, hydrated in descending grades of alcohol, stained by dipping in hematoxylin, washed by tap water, counter stained with 1 % Eosin, dehydrated by ascending grades of alcohol, cleared in xylene and mounted with Canada balsam. Microphotographs of Hematoxylin and Eosin-stained sections were taken by using a digital camera of Samsung company (Model no. SM-J260F/DS-8.00 megapixel) attached to the trinocular microscope (Labomid CXR2) at 100× and 400× magnification [39]. The photomicrographs of the concerned histological portion were processed in Coral DRAW 7 for sharpness, shading, and editing

2.5 Micrometry

The readings were taken from five arbitrarily chosen areas on the computer screen utilizing a pre-aligned advanced scale in Coral DRAW 7 from digital photographs. The calibrations were made from computerized photograph shots of the stage micrometer on a similar magnification. The mean number of mucosal folds of the ampulla, and the number of primary, secondary, and tertiary follicles of the ovary were taken at 100× objective of a microscope. Diameter of the primary and secondary follicles, the average cross-sectional area of tertiary follicles, the epithelial height of the mucosal fold, the muscular thickness of the ampulla, number of uterine glands, diameter of round uterine glands, the diameter of the uterus, endometrium and myometrium thickness, and epithelial height of uterus were measured at 400 × magnifications.

2.6 Statistical Analysis

Results were analyzed using One-Way Analysis of Variance (ANOVA) and post hoc Tukey's tests in the IBM SPSS version 23.

2.7 Ethical Statement

This study was approved by the Biosafety and Ethical Review Committee of the University of Sargodha, 40100 Pakistan (Ref: SU/Acad/1167/2, Dated: November 06, 2019).

3. RESULTS

3.1 Body Weight

IStatistically, no difference was observed in the mean initial body weight of animals belonging to different groups. While the mean final body weight of the DEHP-treated group was significantly ($P<0.001$) reduced as compared to the control, garlic extract, and DEHP+ Garlic ($P<0.01$) given group (Fig. 1).

3.2 Mean Weight of Female Reproductive Tract

Highly significant decrease was found by statistical analysis in mean weight of complete female reproductive tract of the DEHP treated group as compared to control ($P<0.001$), garlic ($P<0.01$) treated and DEHP+Garlic ($P<0.01$) administered group (Figure 2). Effect of garlic in attempt to reduce DEHP toxicity was obvious in co-administered group.

3.3 Histological Observations

Histological study of ovary of the control group showed developing primary, secondary, tertiary follicles and corpus luteum in the cortex of ovary, blood vessels, and normal stroma in the center (Figure 3A). Ovary of garlic extract-treated group (Figure 3B) indicated proper ovarian follicles with normal blood vessels and stromal cells resembling that of the control. Histological examination of ovaries of mice given DEHP (Figure 3C) showed a significantly reduced number of ovarian follicles and that of corpus luteum, blood vessels appear congested and dilated. In follicles, oocytes (O) became distorted and granulosa cells were irregular. Whereas DEHP+Garlic treated group (Figure 3D) indicated an improvement in the ovarian structure with decreased number of atretic follicles.

Histological analysis of primary follicles of the control group and garlic extract-treated group at higher magnification (Figure 4) showed normal structure having a central oocyte surrounded by granulosa cells encapsulated by theca cell. DEHP

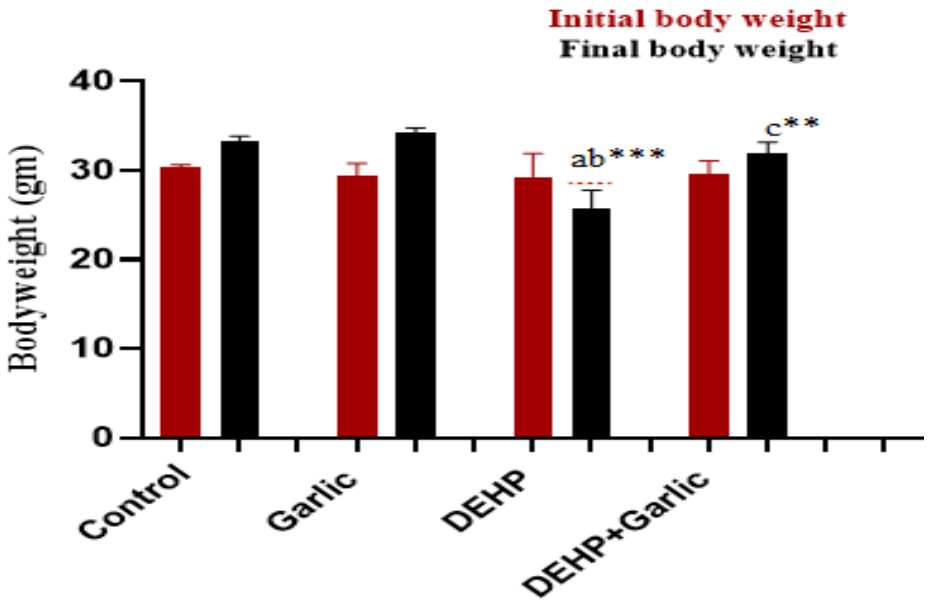

Fig. 1. Effect of 28 days oral exposure of 500 mg/kg b.wt. garlic extract, DEHP and DEHP+garlic extract (500 mg/kg each) on mean body weight of female albino mice. Data is represented as Mean±SEM, **P<0.01, ***P<0.001

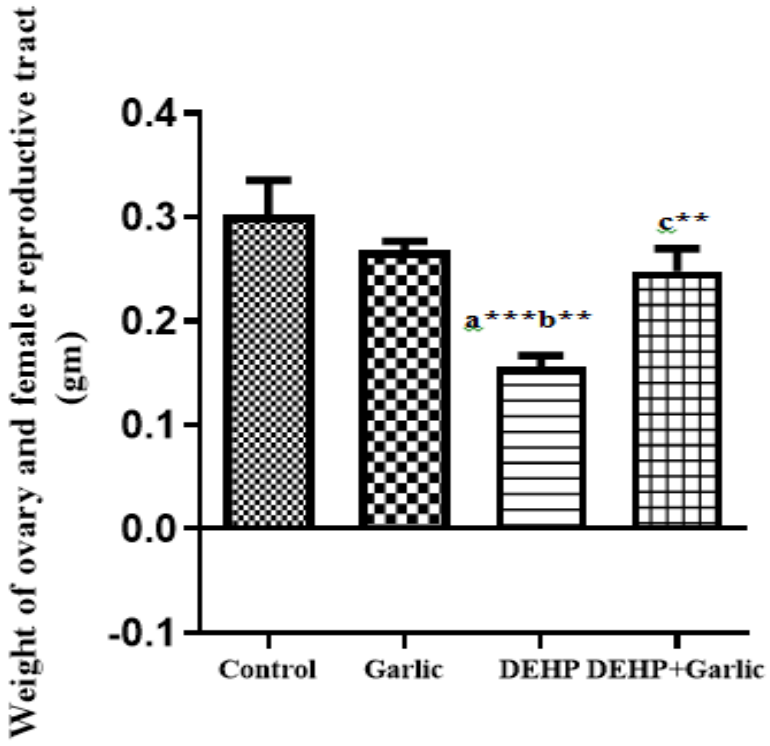

Fig. 2. Effect of oral intake (28 days) of garlic extract, DEHP and DEHP+garlic extract (500 mg/kg b. wt. each) on the mean weight of the complete reproductive system of female albino mice. Data is represented as Mean±SEM, a = control vs treated groups, b=Garlic extract group vs other treatments and c=DEHP treated group vs DEHP+garlic group. **P<0.01, **P<0.001

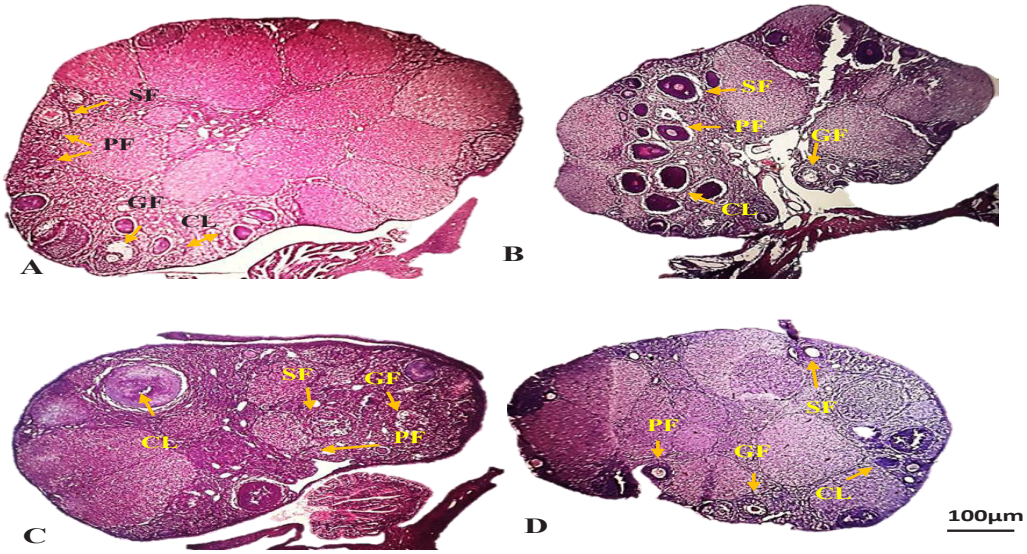

Fig. 3. Hematoxylin and Eosin-stained sections of adult female mice ovary exposed to garlic extract (500 mg/kg), DEHP (500 mg/kg), and DEHP+ garlic extract (500 mg/kg each) through gavage for 28 days (10X). Control (A) and garlic extract (B) groups showing normal ovary with well follicular development such as primary follicle (PF), secondary follicle (SF), Graafian follicle (GF) and corpus luteum (CL). DEHP group (C), ovary has reduced number of all stages of the follicle. While ovaries of animals treated with DEHP+garlic extract (D) showed improvement in the number of ovarian follicles.

exposed group represented primary follicles with a lesser number of granulosa cell and distorted theca cells. However, the DEHP + Garlic extract group indicated a normal-sized primary follicle with compact granulosa and theca covering.

In the control and garlic extract groups, secondary follicles presented normal structures having central oocyte surrounded by compact zona granulosa. Between the zona granulosa and oocyte, a thick glycoprotein coat of zona pellucida was present (Figure 4). Whereas in DEHP exposed group, the secondary follicles were filled with autophagic granulosa cells with a thin thecal layer. Toxic effect was evident in the combined treatment group as the secondary follicles were surrounded by vacuolated granulosa layers (Figure 4).

While the tertiary follicle of control group showed a normal mature oocyte surrounded by granulosa layers with normal corona radiata and cumulus oophorus. Animals treated with garlic extract represented the normal structure of the preovulatory follicle. The most evident histological changes in DEHP exposed group include vacuolation in granulosa cells of preovulatory follicles. Moreover, other changes indicated thin and irregular corona radiata, disruption of cumulus oophorus, and reduction in the size of the antral cavity of the Graafian follicle. Animals given DEHP+Garlic showed the ameliorative effect of garlic against toxicant on the mature follicle as shown in Figure 4.

Histological analysis of oviduct section of control group presented normal anatomical structure as branched and highly invaginated mucosa surrounding the oviduct lumen. A compact and organized epithelium of the oviduct was observed with normal-sized ciliated principal cells and richly populated basal cells (Figure 5A&B). Oviduct of garlic extract-treated group indicated normal organization of mucosal folds with deep grooves and prominent epithelium with fine ciliated principal cells and round basal cells near lamina propria (Figure 5C&D). Histological deformities were seen in mucosal folds and epithelial layer of the DEHP exposed group as compared to the control. Branching invaginations of mucosal folds were decreased and a wider lumen was prominent. DEHP also showed disorganized loosely packed epithelium where basal cells were more abundant as compared to columnar cells and exfoliation of principle cell was the prominent feature in epithelium (Figure 5E&F). The DEHP+Garlic treated group showed tall mucosal folds with a

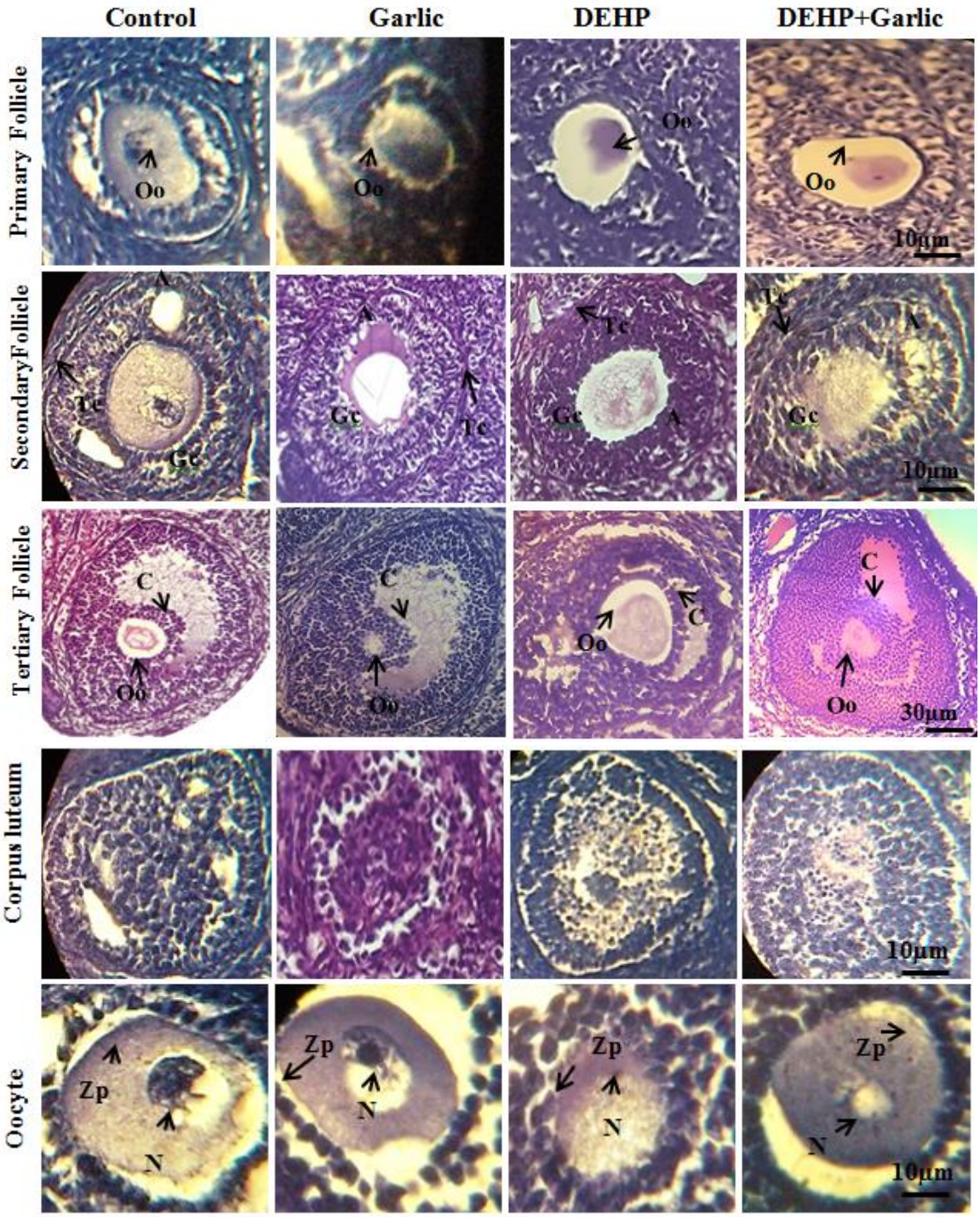

Fig. 4. Photomicrographs of all stages of follicles, secondary oocyte and corpus luteum of control and treated groups at higher magnification. Effect of Phthalate is prominent on all follicles as hypertrophy in granulosa (Gc) and theca cells (Tc), congested antrum (A), reduced corona radiata (C), disrupted zona pellucida (Zp) and regressed oocyte as compared to control and other treatments. Garlic coadministration can be seen as a rescuing treatment against DEHP intoxication. H&E staining.

slight portion of lumen. Epithelium clearly showed rehabilitative potential of garlic as size and shape of principle and basal cells resemble to that of control but still, exfoliation can be seen (Figure 5G&H).

In control and garlic-treated groups, uterus had a very dense external muscular layer, the endometrium, and myometrium. Deep invaginations were observed within the endometrial stroma (Figure 6 A&B). Histomorphological alterations were observed in uteri of DEHP-given animals as compared to the garlic and control groups. The size of uterus was decreased having less invaginated epithelium, and endometrium was thicker with marked proliferation and hypertrophy in the stromal cell (Figure 6C). While the DEHP+Garlic extract treated group showed normal anatomical structure of uterine layers and lumen as garlic seems to attenuate the effect of toxin in this group (Figure 6D).

In control and Garlic treated sections of uterus, densely populated and well-organized (circular and elongated) endometrial glands were present in the uterine endometrium (Figure 7B&E). However, comparatively small-sized, undeveloped and congested endometrial glands were observed in the DEHP-treated group (Figure 7H). While small circular and glands were prominent in the DEHP+Garlic extract treated group where the effect of toxin was prominent (Figure 7K).

In the control and garlic extract-treated uterine sections, the epithelium was well-defined, and thick having compact tall columnar ciliated cells with a prominent basement membrane on which basal nuclei were lying (Figure 7C and F). However, in the DEHP group, the organization and shape of epithelium, and basement membrane were so distorted that stromal tissues and endometrium epithelium were lacking a clear demarcation (Figure 7I) Epithelial sloughed cells were present in lumen. In the in DEHP+Garlic treated group, cuboidal shaped principal cells were present with hypertrophy in round basal cells. Detaching epithelial cells can be seen toward lumen (Figure 7L).

3.3 Micrometric Analysis of Ovary

3.3.1 Mean Number Ovarian Follicles and Corpus Luteum

Statistically, no significant difference was noted in mean number of primary follicles in ovaries of treated females as compared to control group. Whereas, mean number of secondary and tertiary follicles was reduced ($P<0.05$) by DEHP treatment as compared to control and garlic extract given group. DEHP treatment also caused a reduction in mean number of secondary follicles when compared to the co-exposed ($P<0.01$) group as given in Table 1.

Table 1. Mean number of primary, secondary, tertiary follicles, and corpus luteum in control and treated groups after 28 days oral exposure to DEHP and garlic extract alone and in combination.

Groups	Mean number of Follicles			
	Primary	**Secondary**	**Tertiary**	**Corpus luteum**
Control	4.33±0.33	3.50±0.23	1.00±0.00	3.50±0.28
Garlic	5.00±0.44	3.50±0.23	1.00±0.00	3.83±0.40
DEHP	3.83±0.47	2.83±0.16 ^{ab*}	0.66±0.14^{ab*}	2.00±0.24^{a**b***}
DEHP+Garlic	4.83±0.30	3.55±0.12^{c**}	0.83±0.11	3.66±0.22^{c**}

Values are expressed as Mean ± SEM and comparison is made by Tukey's test. a = Control vs treated groups, b = DEHP group vs Garlic and DEHP+Garlic group and c = Garlic vs DEHP+Garlic group. *P<0.05, **P<0.001, ***P<0.001

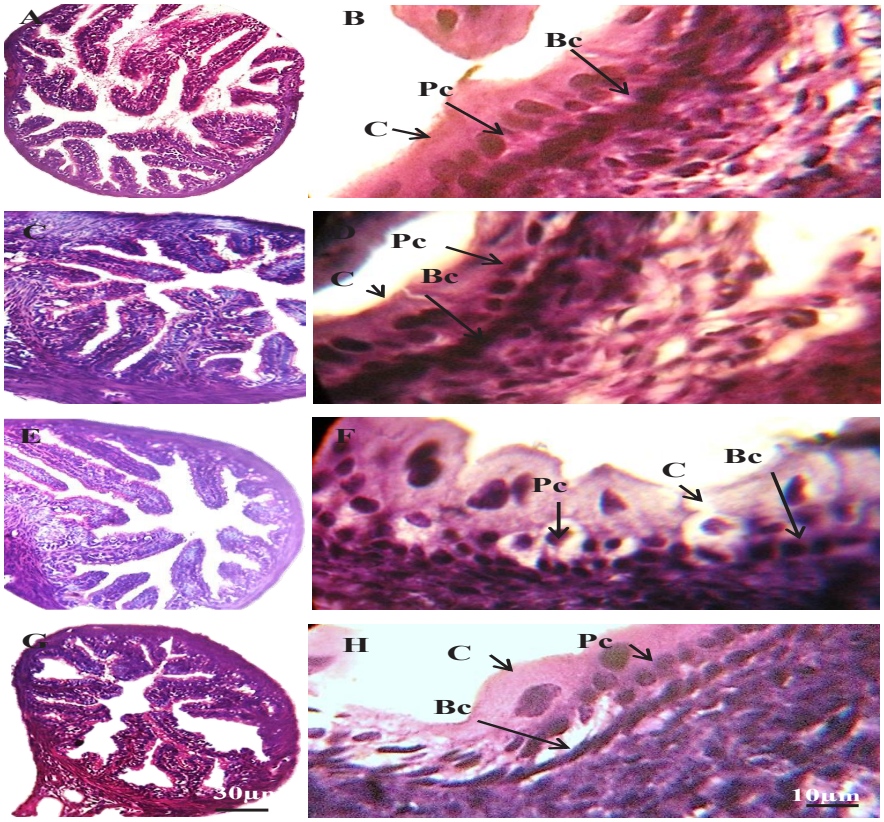

Fig. 5. Hematoxylin and Eosin-stained sections of Ampulla from the oviduct of control and treated mice at low and high magnification. Control group (A) shows branched, tall mucosal folds with deep grooves, muscle layer, and epithelium (B) containing ciliated columnar principal cell lying on round basal cells. Garlic extract-treated group (C) shows many mucosal folds, thick muscle layer, and epithelium (D) with an organized adluminal layer of ciliated principal cells and a dense layer of basal cells. While DEHP exposure (E) resulted in reduced muscle layer thickness, less branches and disorganized epithelium (F) with loose flaking principal cells and hypertrophy in basal cells. Combined group (G) showing increased mucosal folds, and epithelium (H) presented much improved signs as compared to only toxin-treated epithelium as regular-sized principal cells and basal cells was prominent.

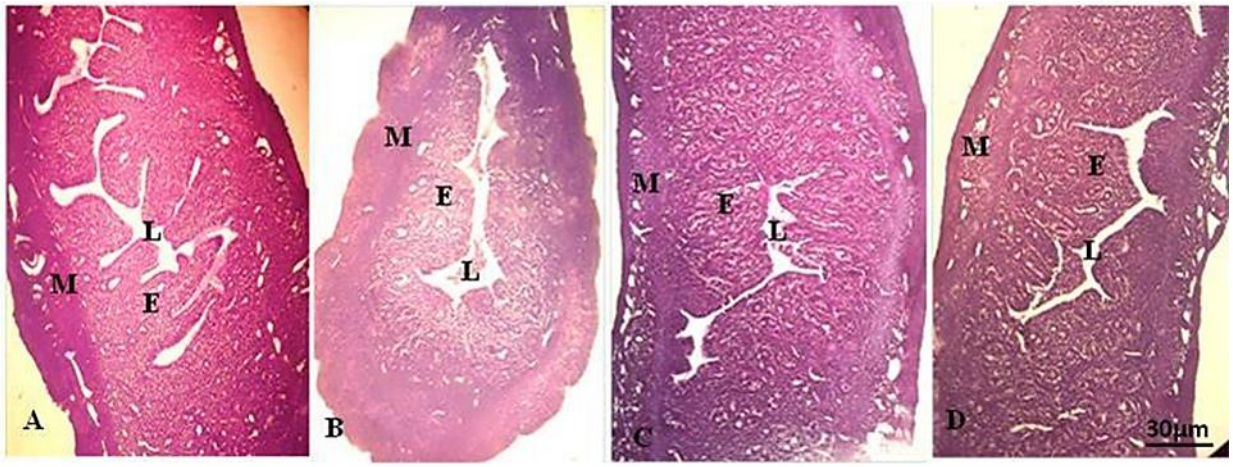

Fig. 6. Hematoxylin and Eosin-stained uterine longitudinal sections of adult female control and treated mice. Control (A) and garlic extract group (B) sections exhibited prominent invagination and branching in endometrium (E), well-organized myometrium (M), and clear lumen (L). While the DEHP group (C) showed congestion in all layers of uterus with a significantly reduced lumen. DEHP+Garlic extract group (D) showed improvement signs in uterine tissue as compared to only DEHP given group.

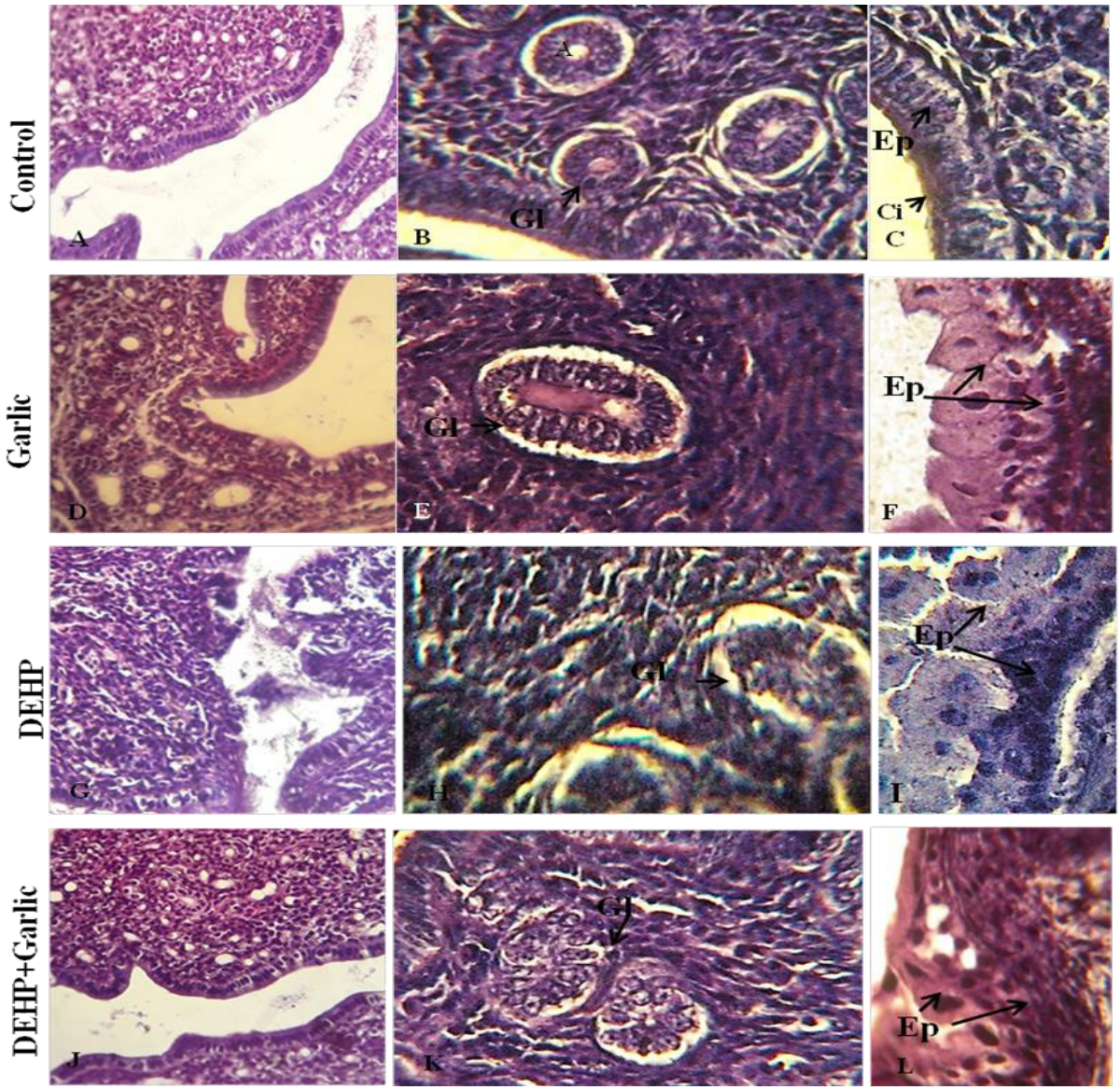

Fig. 7. Photomicrographs taken from sections of endometrium and uterine epithelium (Ep) of adult female mice of control and treated groups at different magnifications. Control (A, B, C) and garlic extract (D, E, F) group show regular-sized uterine glands (Gl) embedded in rich stromal tissue, while in epithelium columnar cells with prominent basal nuclei on a clear basement membrane were obvious. While DEHP exposed group (G,H,I) present distorted glands in the disorganized stroma, epithelium with shorter principle cells and irregular-shaped cells sloughed in lumen. DEHP+Garlic treated group (J,K,L) showed improvement in columnar epithelium with normal basement membrane and seems to lessen the effect of DEHP but exfoliating cells are also prominent. A,D,G,I at 10X and all other at 100X.

3.3.2 Mean Diameter of Ovarian Follicles

Statistical analysis indicated that combined treatment of DEHP+Garlic caused a significant ($P<0.05$) increase in mean diameter of primary follicles as compared to control, garlic and DEHP alone administered ($P<0.01$) females. Mean diameter of secondary follicles of mice given different treatments was not statistically different from that of control. Significant ($P<0.05$) decrease in the mean diameter of tertiary follicles was noticed in DEHP-exposed mice as compared to control and garlic-treated mice (Table 2).

3.4 Micrometric Analysis of Oviduct

Statistical analysis (ANOVA) showed that treatment of DEHP caused a highly significant ($P<0.001$) decrease in the mean number of mucosal folds as compared to the control and other treatment

Table 2. Mean diameter of primary, secondary and tertiary follicles in control and treated groups after 28 days of oral exposure of DEHP and garlic extract alone and in combination.

Groups	Diameter of Follicles (µm)		
	Primary	**Secondary**	**Tertiary**
Control	1.34±0.11	3.58±0.18	8.62±0.51
Garlic	1.36±0.07	3.89±0.30	8.63±0.57
DEHP	1.20±0.05	3.05±0.14	7.17±0.20^{ab*}
DEHP+Garlic	1.74±0.14^{ab*c**}	3.77±0.28	8.49±0.20

Values are expressed as Mean ± SEM and comparison is made by Tukey's test. a = Control vs treated groups, b = DEHP group vs Garlic and DEHP+Garlic group and c = Garlic vs DEHP+Garlic group. *P<0.05, **P<0.01.

groups. While mean number of mucosal folds was significantly (P<0.001) increased in DEHP+Garlic treated ampulla as compared to DEHP treated oviduct which showed the protective ability of garlic extract. Mean epithelial height was observed to be significantly (P<0.001) increased in garlic and DEHP+Garlic exposed group as compared to control. Whereas, the DEHP-given group displayed a considerable (P<0.001) reduction in mean thickness of ampullary epithelium as compared to control and other treatment groups. Toxic effect of Phthalate was prominent on epithelium of ampulla. Epithelial height was also reduced (P<0.05) in DEHP+ Garlic treatment as compared to only garlic extract given group. Muscle layer thickness of the oviduct was not significantly affected by any of the treatments (Table 3) as compared to control.

3.5 Micrometric Analysis of Uterus

3.5.1 Uterine Diameter and Epithelial Height

According to one-way ANOVA, a highly significant increase was found in the mean diameter of uterus of female mice exposed to DEHP+Garlic when compared to the control while other treatments caused no noticeable difference when related to control. Among treated groups, significant (P<0.001) decrease was observed in the mean diameter of the uterus of DEHP exposed group as compared to the garlic and DEHP+Garlic administered group. Noteworthy (P<0.001) increase was seen in mean epithelial height of uterus in garlic extract-given group as compared to control and all other treatment groups. Whereas, DEHP and combined treatment caused a prominent (P<0.01) decrease in mean epithelial height as compared to control (Table 4).

3.5.2. Number of Uterine Glands

Data after statistical analysis revealed a significant (P<0.001) decrease in the mean number of uterine glands in Garlic and DEHP alone treatments as compared to the control group while co-administration of both increased (P<0.001) in mean number of glands in uterine stroma as compared to control. Among treated groups, the mean number of uterine glands was significantly (P<0.001) decreased in DEHP exposed group as compared to garlic and DEHP+Garlic extract given groups. However, combined exposure of DEHP+Garlic extract resulted in highest (P<0.001) number of uterine glands as compared to other treatments (Table 4).

3.5.3 Muscular Thickness: Endometrium and Myometrium

One-way ANOVA showed no significant effect on the mean thickness of endometrium by any treatment as compared to control. While a significant (P<0.05) reduction was observed in the mean thickness of

Table 3. Mean number of mucosal folds, muscular thickness and epithelial height (µm) in ampulla region of oviduct in control and treated groups after 28 days of oral exposure to DEHP and/or garlic extract.

Groups	Ampulla		
	Mucosal folds	Epithelial height (µm)	Muscularis (µm)
Control	28.33±0.81	0.446±0.01	1.00±0.10
Garlic	28.16±0.70	0.457±0.01^{a***}	1.36±0.15
DEHP	17.33±0.71^{ab***}	0.361±0.01^{ab***}	1.06±0.11
DEHP+Garlic	26.75±0.70^{c***}	0.455±0.02^{ac***b*}	1.16±0.13

Values are expressed as Mean ± SEM and comparison is made by Tukey's test. a = Control vs treated groups, b = DEHP group vs Garlic and DEHP+Garlic group and c = Garlic vs DEHP+Garlic group. *P<0.05, ***P<0.001

Table 4. Mean diameter of uterus, epithelial height, mean number of uterine glands and thickness of endometrium and myometrium in control and treated groups after 28 days of oral exposure to DEHP and/or garlic extract.

Groups	Uterus (µm)			Thickness (µm)	
	Diameter	Epithelial height	Uterine glands	Endometrium	Myometrium
Control	4.82±0.28	0.47±0.02	171.83±5.67	1.24±0.11	0.70±0.07
Garlic	5.65±0.29	0.58±0.02^{a***}	143.00±3.91^{a***}	1.20±0.08	0.64±0.06
DEHP	3.98±0.23^{b***}	0.38±0.01^{a**b***}	106.66±10.47^{ab***}	0.98±0.11	0.50±0.03^{a*}
DEHP+Garlic	6.01±0.24^{a**c***}	0.38±0.01^{a**b***}	192.83±12.93^{abc***}	1.20±0.09	0.65±0.02

Values are expressed as Mean ± SEM and data was compared by Tukey's test. a = Control vs treated groups, b = DEHP treated vs Garlic and DEHP+Garlic treated groups and c = Garlic vs DEHP+Garlic group. ***P<0.001, **P<0.01, *P<0.05

myometrium in female mice treated with DEHP as compared to the control group (Table 4).

4. DISCUSSION

DEHP, a potent toxicant, is widely used in various products and found everywhere in the environment. The initial studies suggest that the reproductive system is more vulnerable to phthalates as compared to other organs [42]. On the other hand, oxidative stress caused by environmental contaminants can be prohibited or reduced by dietary natural antioxidants through their capacity by scavenging these products [43]. This study was intended to investigate the lesser-known protective effects of aqueous extract against DEHP-induced reproductive toxicity in adult female mice.

Our study indicated that the number of developing primary, secondary, and tertiary follicles and corpus luteum were adversely affected by DEHP exposure in mice. DEHP treatment also caused histomorphological alteration such as degeneration of ovarian follicles, oocytes degradation, antral reduction and granulosa cells hypertrophy. It has been reported by Wang et al. (2012) [29] that antral follicle growth of mice was reduced by using all doses (0.1, 1, 10, 100 µg/mL) of MEHP and DEHP. Furthermore, DEHP can alter the antioxidant system causing the accumulation of superoxide ($O2^{-}$) that in turn damage the antral follicle [44]. In the current study, DEHP-induced superoxide addition might be the cause of degenerative features in ovarian tissue.

Our result clearly showed that the

histomorphology of the ovary was improved by garlic extract supplementation. Diameter and number of developing primary, secondary, antral follicles and corpus luteum were ameliorated upon garlic extract supplementation in DEHP-intoxicated mice. A previous study reported that garlic oil supplementation played a direct and indirect role in enhancing the activity of ovaries by stimulating the release of sex hormones together with estrogen [36]. Another research found that garlic extract induces secretion of ovarian hormones and gonadotropins through the stimulation of the pituitary gland, cell cycle, and accelerates estrogen receptors binding [37]. These reported effects of garlic on female reproduction are also established in this study in an improved form of ovarian histological architecture in garlic-exposed groups that might be due to enhanced gonadotropins input and boosted hormone receptor binding. All these reports point toward the importance of garlic intake as an important nutraceutical to nullify effect of environmental toxicants.

In the mammalian oviduct, the ampulla is a major anatomical region in which fertilization takes place and it is the main site of early embryo development [45, 46]. Results of our research indicated that DEHP exposure adversely affected the oviduct of mice by a reduction in the number of mucosal folds along with histological alteration in the ampullary region in comparison to the control and garlic extract-treated group. According to another research oviduct with a reduced number of ciliated cells [47] and low circulating estrogen [48] level would lead to ectopic pregnancies in comparison to normal intrauterine gestation. The structural changes in the ampulla of oviduct in this study might be attributed to DEHP interference in hypothalamic gonadal axis.

Our results demonstrated that histomorphology of oviduct was improved upon garlic extract supplementation in the co-administered group. It has been reported in previous studies that garlic has antioxidant properties and often prevents cell damage, aging and cancer [36]. These properties of garlic seem to be playing role in attenuating the toxic effects of DEHP in co-exposed ampullary tissue in this study.

In the uterus, a significant decrease overall diameter of uterus, muscularis, mean number of uterine glands, and epithelium heights were observed in DEHP exposed group as compared to control in the current study. Another similar research also emphasized on the toxic effects of DEHP on uterus which disturbed the morphology and physiology of the uterus by causing a change in stromal cell patterns comparable to changes observed in females on progestin contraceptives [49]. Altered patterns of cell proliferation were observed in the DEHP-exposed uterus leading to de-regulation of the normal oestradiol-progesterone responses in the uterus which in turn causing to an anti-estrogenic effect [50]. DEHP-imposed anti-estrogenic effects might be the reason behind histopathological alterations in uterine tissue in this study as uterus ia an estrogen-dependent organ. While our result dictated the ameliorative abilities of aqueous garlic extract on uterine histology and morphometry due to its antioxidant and gonadal axis stimulatory abilities.

5. CONCLUSION

Various histo-protective effects in reproductive organs were noticed in the combined group (DEHP+Garlic) as compared to only DEHP exposed group that reflected the antioxidant abilities of garlic extract. These findings indicated that garlic is a common, cheapest, and most effective remedy against reproductive toxicity induced by environmental pollutants. Many female workers of plastic industries and other industries where phthalates are used in abundance are exposed to heavy amounts of a mixture of phthalates and are at much higher risk of developing reproductive abnormalities. However, simple natural products such as garlic can be useful to reduce the damage caused by these environmental pollutants. As we know that exposure to toxicants generates reproductive abnormalities so daily intake of diet-based cheap and easily available remedies like garlic are suggested by this study.

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7. CONFLICT OF INTEREST

All authors unanimously declare no competing conflict of interest in any regard for this research work.

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Research Article

Response of Rangeland Vegetation to Recent Trends in Seasonal Climate in Mansehra, Pakistan

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Abstract: The deterioration of rangeland resources as a result of environmental changes is a serious concern in the Himalayan mountainous region of Pakistan. The present study is aimed to evaluate the response of vegetation cover of rangeland to recent trends in climate parameters, such as the seasonal temperature and rainfall in the Mansehra district of Khyber Pakhtunkhwa province, Pakistan. Correlation analysis was performed between the MODIS data products, i.e., NDVI (Normalized difference vegetation index) and LST (Land surface temperature), and TRMM rainfall datasets of the 2000-2018 period. NDVI indicated a negative correlation with LST of winter (R= -0.56), spring (R= -0.7), summer (R= -0.24), and autumn (R= -0.23) significant ($p<0.05$) for winter and spring seasons only. In contrast, the correlation of NDVI was observed positive with seasonal rainfall exhibiting coefficient of correlation values of 0.41, 0.79, 0.64, 0.7 for winter, spring, summer, and autumn significant ($p<0.05$) for the last two seasons only. The low correlation observed between NDVI and LST of summer and autumn seasons is likely because of the prevailing stress condition of chlorophyll contents of the vegetation cover under warming conditions. However, this situation appears to be compensated by the rainfall as indicative of the moderate to strong correlation between the NDVI and rainfall of these two seasons. The least NDVI values observed during the winter season indicate limited vegetation cover for grazing opportunities in the lower valleys. However, an in-depth investigation of production patterns would further facilitate analyzing the grazing potential to support decision-making for long-term grazing management.

Keywords: Climate change, Rangeland, Vegetation, Remote sensing, NDVI

1. INTRODUCTION

The vegetation cover of Himalayan rangelands forms a major source of feed for livestock and is closely linked to the socio-economic systems of the region [1]. Assessment of climate change impacts on vegetation cover is an important subject to livestock management. Vegetation cover is a commonly used indicator to assess terrestrial environmental and rangeland conditions and any change in the cover pattern will alter the structures and functions of the environment. According to FAOSTAT [2], the world's total grassland has been reduced nearly by 1% during the 1994-2012 period. Climate change is believed to be one of the reasons impacting rangeland ecosystem processes [3, 4]. However, evaluating rangeland's capacity and productivity in a spatially and temporally dynamic way concerning climate change effects is still a great challenge. There are many techniques and or/ indices that can be used to monitor and assess vegetation cover using the remote sensing data (e.g., Normalized Difference Vegetation Index (NDVI), Leaf Area Index (LAI), and Fractional of Photosynthetically Active Radiation (FPAR) [5-9]. The normalized difference vegetation index (NDVI) driven by remote-sensing tools is used as a substitution for field-based vegetation monitoring studies which are usually time and cost-consuming. Moderate-Resolution Imaging Spectro-radiometer (MODIS) land products like LST (Land Surface Temperature) and NDVI are freely available and have been widely

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applied for monitoring rangeland production [7, 10-12]. Similarly, the microwave instruments onboard the Tropical Rainfall Measuring Mission (TRMM) satellite have contributed significantly to various environmental applications worldwide [13, 14].

In Pakistan, rangelands constitute nearly 65 % area up to 4000 m elevation and over 60 % of small ruminants' food and 5 % supply of large ruminants' food are reliant on this resource [15]. The sustainability of rangelands is one of the fundamental problems in the perspective of environmental challenges in the Mansehra district of Khyber Pakhtunkhwa province of the country. Currently, no study exists about the impacts of recent trends of seasonal climate on the vegetation cover of mountain rangeland in the Mansehra district. It was hypothesized that long-term changes in seasonal climate would have a great impact on the vegetation cover of the rangelands in this mountainous region. The primary focus of this study is to assess the impact of climate parameters, such as seasonal temperature and rainfall on the vegetation cover of Mansehra district, Khyber Pakhtunkhwa province of Pakistan using MODIS data products such as NDVI, LST, and TRMM rainfall data of 2000-2018 period. Time-series analysis of the Normalized Difference Vegetation Index was performed to understand the projected status of the rangeland vegetation and its relationship with the influential climatic parameters (i.e., temperature and rainfall). This study can help in understanding the effects of changes in temperature and rainfall on the vegetation cover and evaluating the season in which these effects will become critical in this Himalayan region.

2. MATERIALS AND METHODS

2.1 Study Area

Mansehra district stretches over an area of about 4579 km^2 within longitudes 72.81^{o}E–74.13^{o}E and latitudes 34.18^{o}N–35.18^{o}N in the Khyber Pakhtunkhwa province of Pakistan (Figure 1). The elevation ranges from 200 m in the south to over 4500 m above sea level towards the north. The climate is humid with annual rainfall exceeding 1200 mm. Heavy snowfall occurs during the winter season. The mean temperature at the nearest meteorological station (Balakot) is about 16^o C in the winter and 32^o C in the summer season. The district is significant for its biological resources. The rangeland of the district belongs mainly to the Subtropical Lower foothills; Subtropical Chirpine

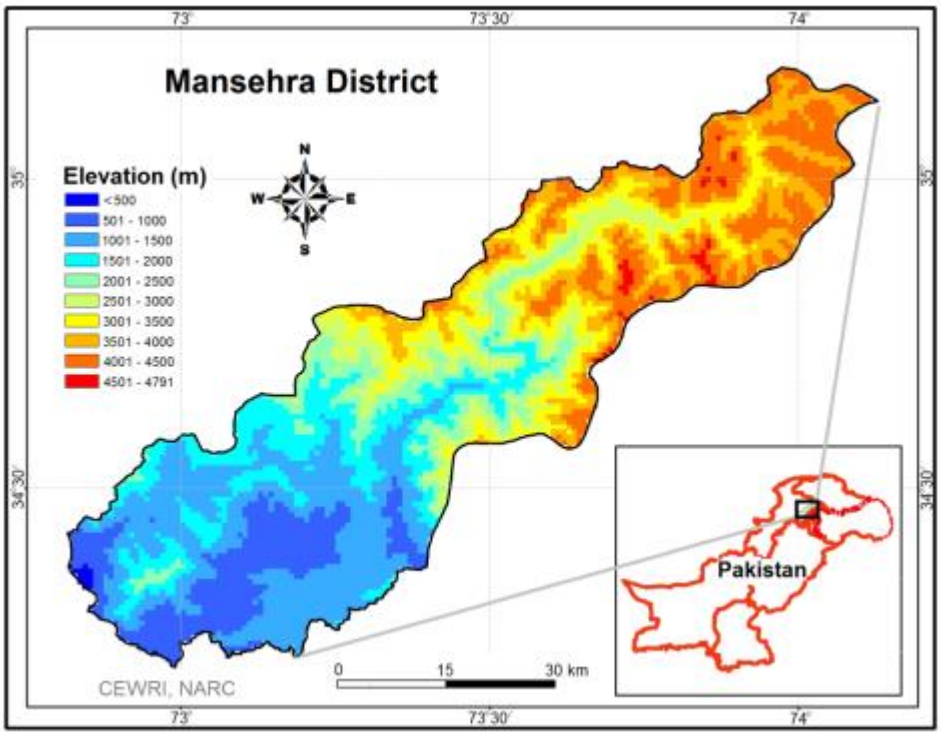

Fig.1. Location of the study area in Pakistan

zone; Moist temperate zone, and Sub-alpine and alpine area. The subtropical Lower foothill zone below 1000 m elevations is usually utilized by nomads during the winter season when the higher reaches are normally snow-covered [16]. They utilized grasses and forbs along with green leaves of species like *Olea ferrugenia, Grewia optiva, Acacia modesta*, and *Prosopis sp*. for livestock rearing. In the subtropical Chirpine zone above 1000 m, the understory of the Pinus is managed by the community for producing grasses and the moist temperate zone above 1700 m elevation is used for livestock grazing during autumn. Above 3000 m, the sub-alpine zone consists of vegetation species like Abies pindrow, Juniperus sp. accompanied by Artemisia as understory at lower reaches and in the alpine zone, the dominant species are *Kobersia sp*. turf. *Sibbaldia cuneata, Trifolium* sp. and *Poa pratensis* [17].

2.2 Data and Methodology

The climate data is mostly available from the valley-based meteorological stations in this region which do not represent the climatic conditions of higher altitudes, so we used RS-based products for monitoring the response of vegetation cover to climatic factors in this study. The processed data products of MODIS, i.e., NDVI and LST were acquired from the United States Geological Survey (USGS) Center for Earth Resources Observation and Science (EROS) for the 2000-2018 period. The NDVI product (MOD13Q1, Level 3) with a spatial resolution of 250 m received at 16 days interval [18] was used in this study. NDVI is considered a qualitative and quantitative measure of vegetation cover and is measured by deducting the red band value from the near-infrared (NIR) value and then divided by the sum of the values of NIR and red bands.

$$NDVI = \frac{NIR-R}{NIR+R} \quad (1)$$

The NDVI values range from –1 to +1, with high values (closer to one) being associated with a greater level of photosynthetic activities. Healthy vegetation reflects more near-infrared energy than stress vegetation. The methods involved in preprocessing of time series of MODIS vegetation indices are described in detail in several previous studies [19, 20]. The MODIS 8-day LST products (MOD11A2/MYD11A2) are the averaged LSTs of the daily MOD11A1/MYD11A1 products over 8 days [21]. Therefore, the MODIS/Terra 8-day LST product (MOD11A2) Level-3 with a spatial resolution of 1 km and temporal resolution of 8 days was used in this study. The LST or the emissivity-corrected land surface temperature Ts is computed as follows [22]:

$$T_s = \frac{\mathrm{BT}}{\{1+[(\lambda BT/\rho)]\mathrm{In}\ \varepsilon_\lambda]\}} \quad (2)$$

where Ts is the LST in Celsius (oC), BT is at-sensor BT (°C), λ is the wavelength of emitted radiance (for which the peak response and the average of the limiting wavelength (λ =10.895), $\varepsilon\lambda$ is the emissivity as calculated in Barsi et al. [23].

$$\rho = h\frac{c}{\sigma} = 1.438 \times 10^{-2}\ m\ K \quad (3)$$

where σ is the Boltzmann constant (1.38 × 10−23 J/K), h is Planck's constant (6.626 × 10−34 J s), and c is the velocity of light (2.998 × 108 m/s) [24]. The LST data was validated using the mean temperature data of the nearby station and found a mean drift of 2oC in the seasonal LST data from the observed data. According to Srivastava et al. [25], the accuracy of LST at places may indicate a difference of ±2°C with actual ground temperature measurements. This difference is obvious as LST represents the ground surface temperature while the latter exhibits air temperature above the ground surface.

The TRMM Multi-satellite Precipitation Analysis (TMPA) product Ver.7 consists of three products at different temporal resolutions: 3-hourly (3B42), daily (3B42 derived), and monthly (3B43). We used monthly 3B43 products with a spatial resolution of 25 km to evaluate the rainfall in the study area. Many studies on this region have proven the effectiveness of TRMM monthly precipitation products when compared with meteorological station data [26, 27]. According to these studies, a strong correlation exists between 12 hourly TRMM and field data in this region as the correlation coefficient comes out to be 0.9 for monthly, seasonal, and annual TRMM data values. We relied on this correlation analysis as validation of the seasonal TRMM data in this study.

The natural vegetation of rangeland serving as grazing land for the livestock was selected to study the influence of seasonal temperature and rainfall. Global Landcover dataset [GlobeLand30, 2010] downloaded from http://www.globallandcover.com/ was used to analyze land cover (Figure 2) and delineate rangeland boundaries which served as a sample area for vegetation cover analysis in our study. The sample boundary of rangeland was used to extract mean values from the three time-series remote sensing products (2000-2018 period) using the zonal statistics tool of the spatial analyst function of ArcMap GIS software. This tool helped in determining the mean values of raster datasets of variant-scale parameters (e.g. NDVI data of 250 m, LST of 1 km, TRMM data of 25 km) within the defined zone or boundary.

We performed trend analysis using time-series data of seasonal NDVI, LST, and TRMM of the study area. Four seasons were defined, i.e. winter (Jan-Mar), spring (Apr-Jun), summer (Jul-Sep) and Autumn (Oct-Dec) to execute statistical analysis in this study. The time-series data of each parameter was plotted using line graph type and a linear trend-line was added to exhibit the direction of the parametric trend in Microsoft Excel software. The relationship of NDVI was studied with LST as well as with rainfall over 19 years. Among several types of the correlation coefficient, the most popular one – Pearson's correlation (also called Pearson's R) was used to measure the relationship between the two variables in the Excel software. This correlation coefficient is commonly used in linear regression to obtain the strength and direction of the relationship between two variables. The correlation value varies between -1 and 1, where, 1 indicates a strong positive relationship, -1 is a strong negative relationship, and zero no relationship at all.

3. RESULTS

3.1 Seasonal NDVI, LST and Rainfall Analysis

INDVI values were observed within the range of 0.18–0.32 during autumn and within 0.15–0.27 during the summer season of the 2000-2018 period in the rangeland of the Mansehra district. During autumn, maximum NDVI was observed in the year 2011 and minimum in the year 2000, while during summer, maximum NDVI was found in the year 2015 and minimum in the year 2002 (Figure 3). The NDVI values of winter and spring seasons were observed within the range of 0.15–0.26. The

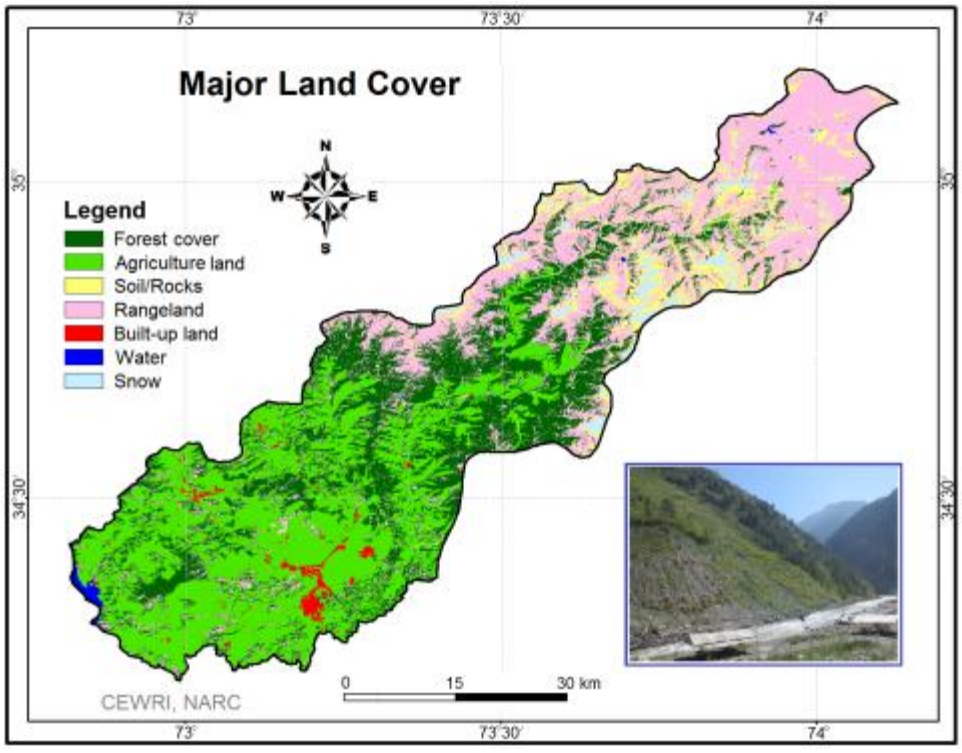

Fig. 2. Major land cover distribution in the study area

maximum NDVI during winter was in the year 2016 and the minimum in the year 2000, while the maximum NDVI during the spring season was in the year 2014 and the minimum in the year 2001. High LST values were observed during summer (i.e., within the range of 31° C–37.1° C), followed by the spring season (within the range of 30.9° C–36.8° C) during the 2000-2018 period. During summer, maximum LST was observed in the year 2009 and minimum in 2015, while during the spring season, maximum LST was found in the year 2010 and minimum in the year 2014 (Figure 4). The LST of winter was found least, i.e., ranging from 17oC to 24.4° C, highest in the year 2000 and lowest in the year 2005. The LST of autumn ranged between 29° C and 33.4° C, lowest in the year 2010 and highest in year 2013. The LSTs of winter, summer, and autumn appear to be more or less stable, while the LST of spring seems to be on the lower side across the 19 years. Variable patterns of seasonal rainfall were observed during the 2000-2018 period in the study area (Figure 5). Higher rainfall was observed during the summer (i.e., within the range of 111–339 mm), followed by the spring season (within the range of 7–265 mm). The rainfall of winter ranged between 22 mm and 127 mm, and of autumn between 22 mm and 167 mm. Overall, the seasonal rainfall indicated rising trends across the 2000-2018 period (Figure 5).

3.2 Correlation of NDVI with LST & Rainfall

We compared seasonal NDVI with corresponding LST and rainfall patterns of the 2000-2018 period in the study area. The upland areas in the northern parts of Mansehra showed low NDVI values likely due to a decline in temperature and rainfall in the upper reaches, whereas the lowlands exhibited increased NDVI values because of higher temperature and rainfall conditions (Figure 6). NDVI values were observed within ranges of -0.16 to 0.82, 0.15 to 0.78, -0.15 – 0.8, and -0.13 – 0.86 during the years 2000, 2006, 2012, and 2018 respectively. LST had shown positive change during each season mostly in the lower parts of the study area during the 2000-2018 period. However, it exhibited a negative change in the uplands of the study area during the spring and autumn seasons.

NDVI indicated a moderately negative correlation with the LST of winter (R= -0.56) and spring (R= -0.7) significant at $p<0.05$ (Figure 7). The correlation of NDVI was low and negative with the LST of summer (R= -0.24) and autumn seasons (R= -0.23). In contrast, the correlation of NDVI with rainfall was observed positive for all seasons,

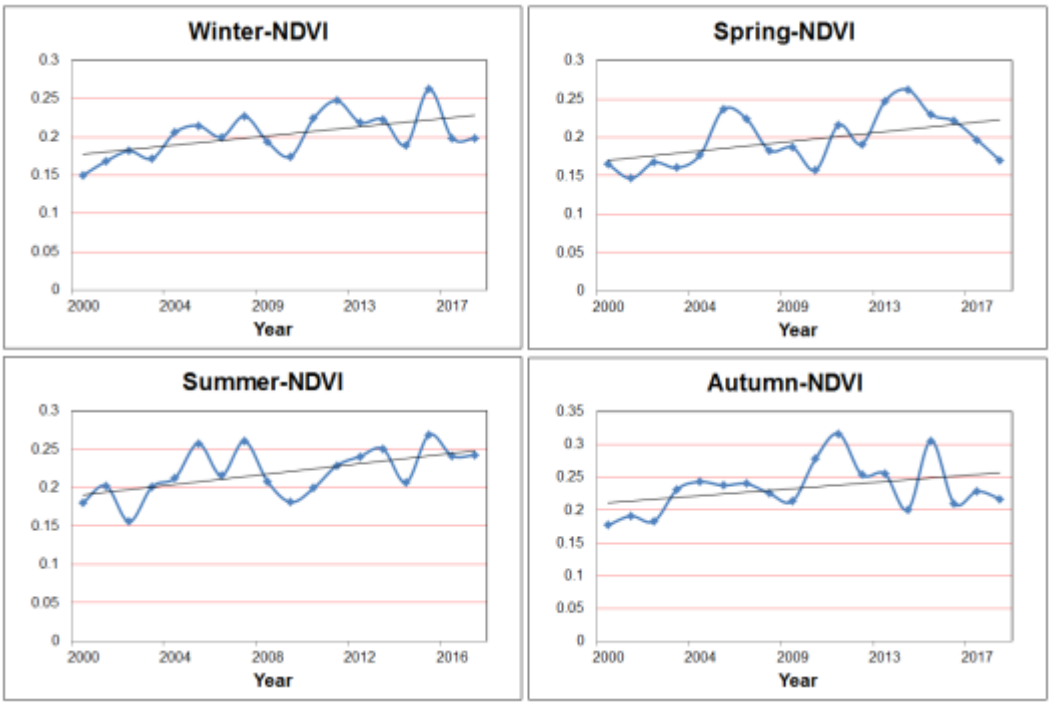

Fig. 3. Seasonal trends in NDVI during 2000-2018 period

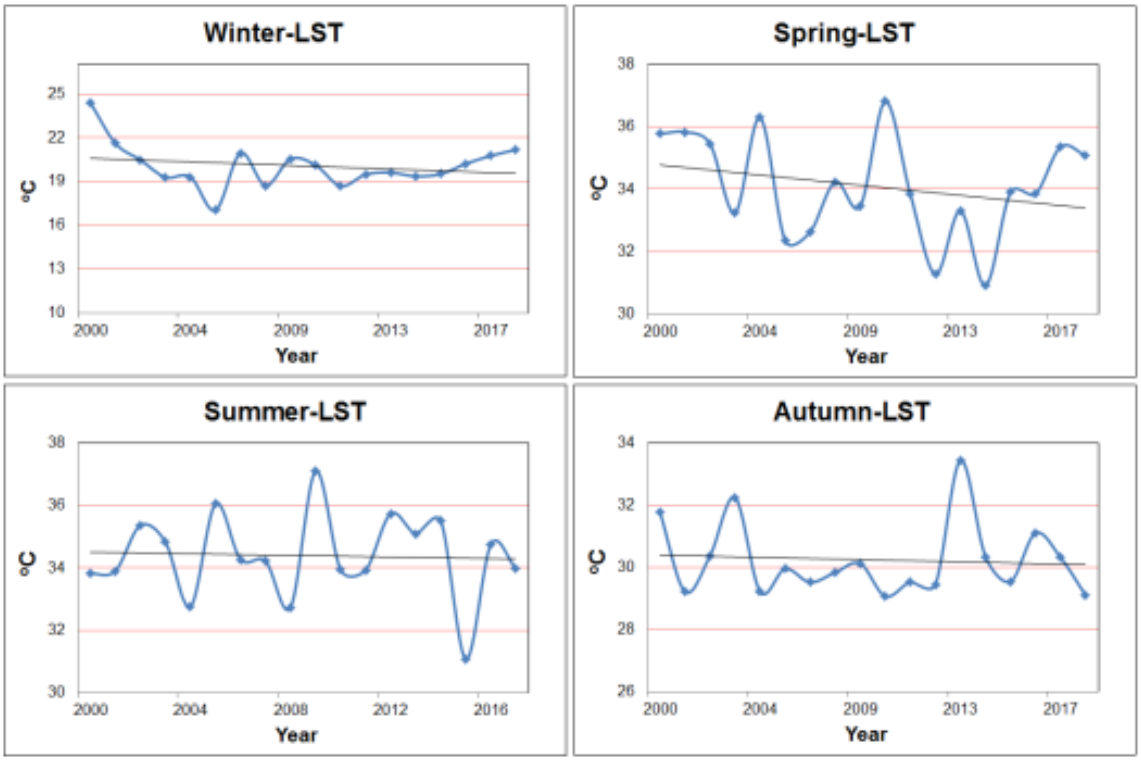

Fig. 4. Seasonal trends in LST during 2000-2018 period

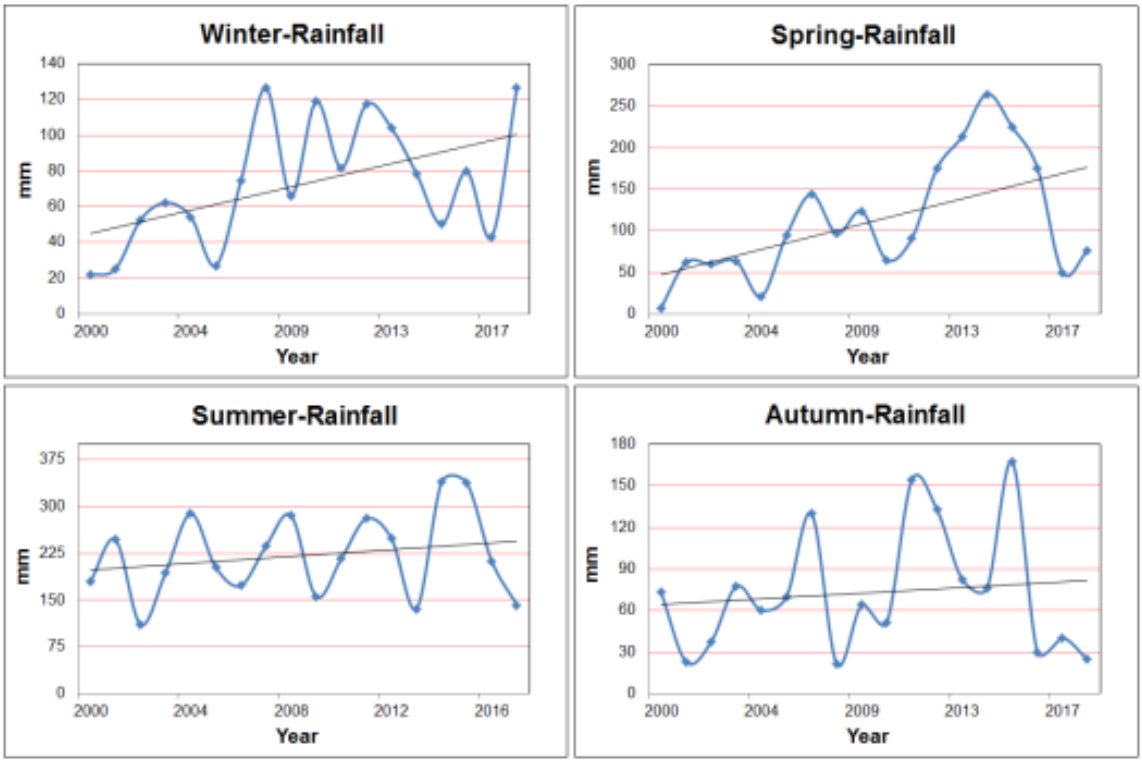

Fig. 5. Seasonal rainfall trends in the study area during 2000-2018 period

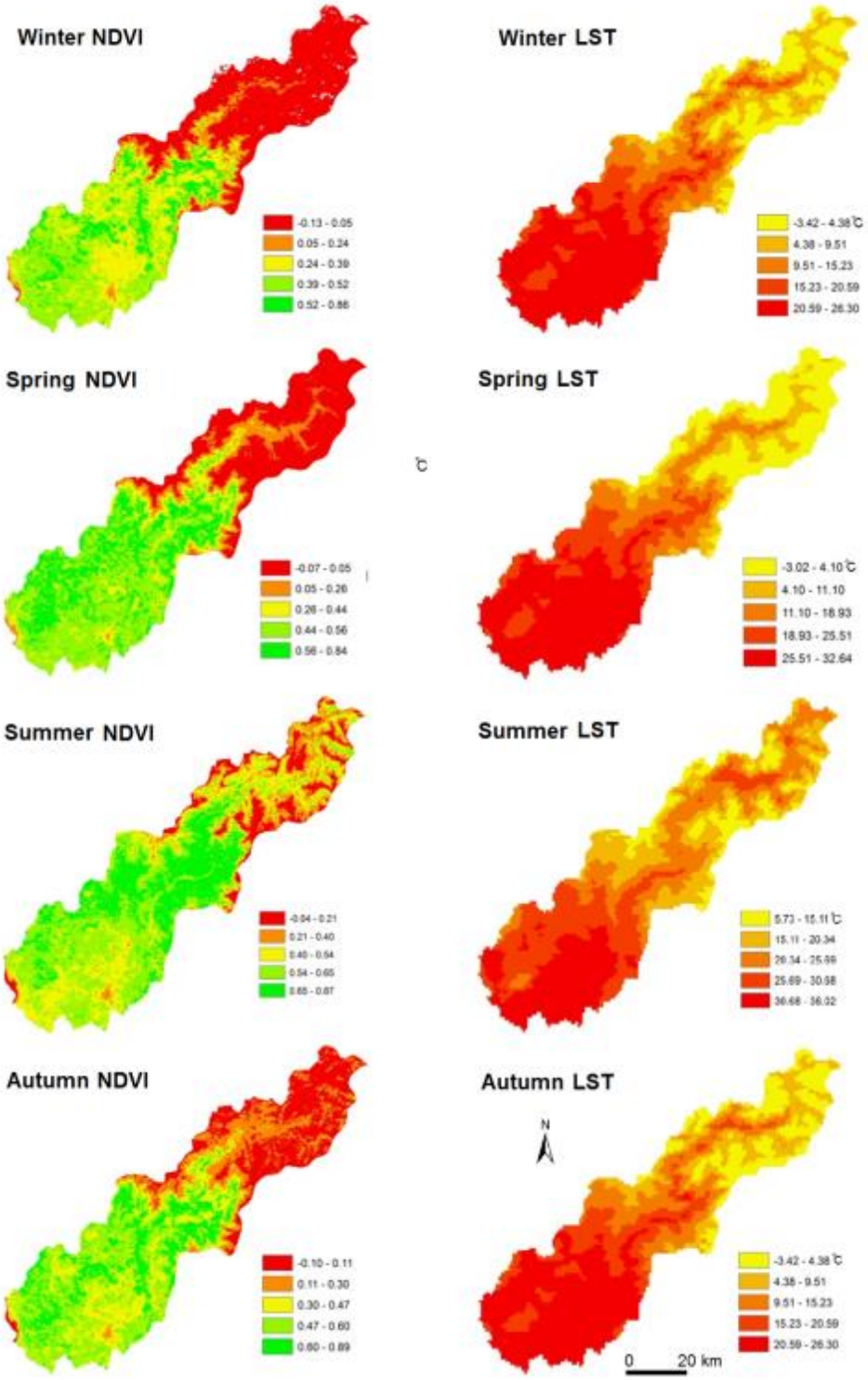

Fig. 6. Seasonal NDVI and LST in the study area (2018)

moderate for summer (R= 0.64) and autumn season (R= 0.7) significant at p<0.05 (Figure 7). The correlation of NDVI was strong with rainfall of spring (R= 0.79) and low with rainfall of winter season (R= 0.41).

4. DISCUSSION

The rising trend observed in seasonal rainfall in the study area during 2000-2018 (Figure 5) is also depicted in the findings of Hasson *et al.* [28] and Latif *et al.* [29] according to which precipitation had shown an increasing trend in the Upper Indus Basin (UIB) during 1995–2012 period. Chaudhry [30] reported a 25 % increase in average annual rainfall and 18 % – 32 % in summer rainfall over the monsoon region of Pakistan during the last century. The situation of higher rainfall trends appears to be favorable for rangeland vegetation which depends mainly on moisture availability

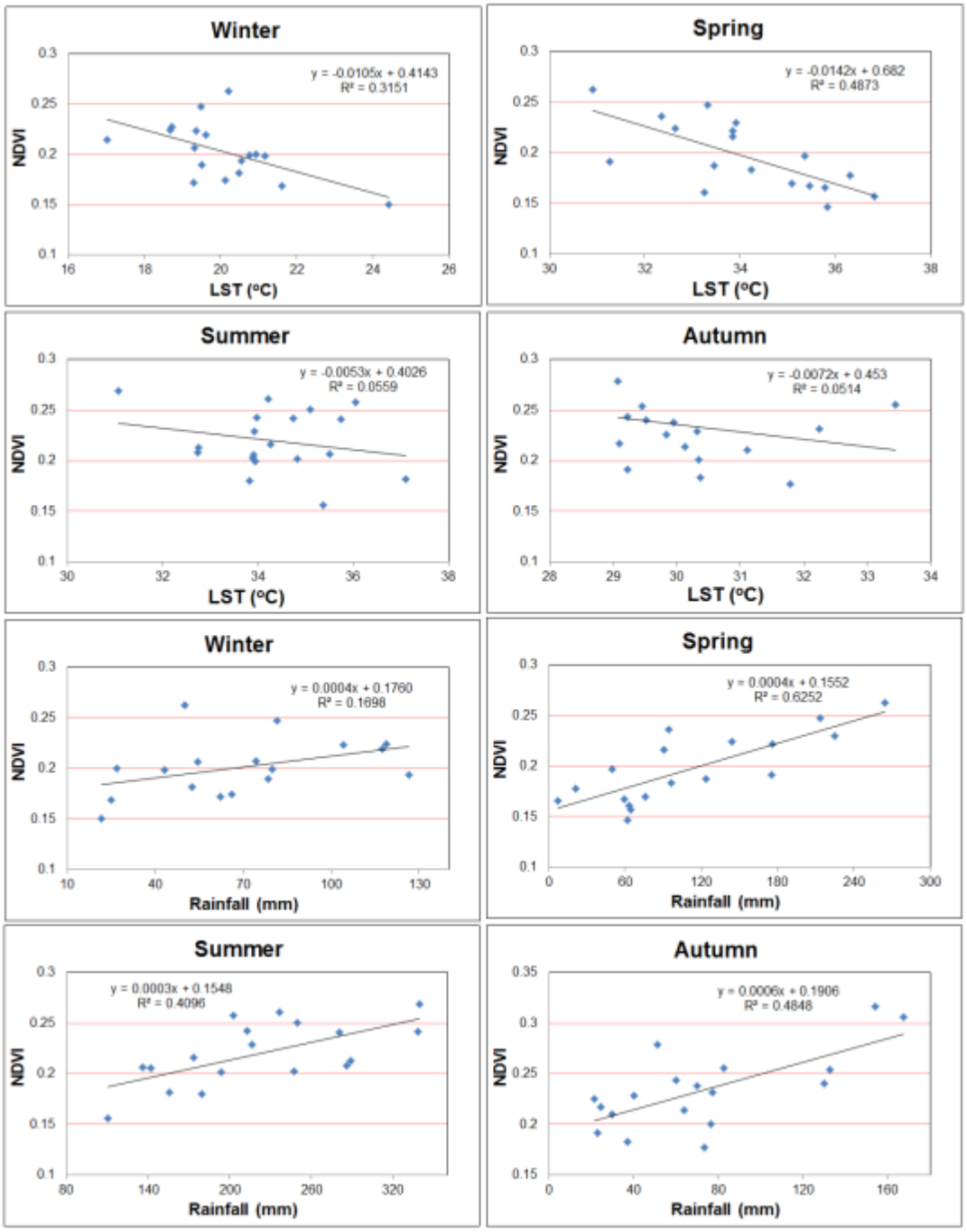

Fig. 7. Relationship of NDVI with seasonal LST and rainfall (2000-2018)

and so indicated a positive correlation with rainfall in different seasons (Figure 7). The stable and in some cases declining trends in LST observed in different seasons indicate the influential effect of the vegetative cover of various types in the study area, as Chaudhry et al. [31] observed 1.5°C rises in temperature during the last 40 years in the northern mountainous region of Pakistan. The NDVI had shown relatively higher variability during spring, autumn, and summer seasons than in winter (Figure 6), however, positive trends in NDVI were observed during all four seasons. According to a study by Liu and Lei [32], the autumn and spring seasons indicated high variations in NDVI, while the summer NDVI showed lesser variability in a part of China. The lowest NDVI values were observed during the winter months from December to March, which may be attributed to heavy snowfall occurrence during this season. Generally increasing trends in seasonal NDVI were observed in the lowlands, however, the trends were on the negative side in the uplands, especially during the spring and autumn seasons. Besides the decrease in warm conditions, high grazing pressure during pre- and post-snowfall conditions might contribute to reducing NDVI in the upper reaches of the district. The increased NDVI values observed during autumn followed by the summer season during the 2000-2018 period are likely due to increasing trends in rainfall in the area. The least NDVI values noticed in different seasons of 2000-2002 were likely due to prevailing drought conditions during that period [33], which influenced the vegetation resource of this region. However, not only drought conditions, but the overuse of rangeland coupled with intense monsoon rainfall leads to massive erosion and landslides [34] which may cause a lowering of NDVI values. The study by Rehman *et al.* [35] indicated that the NDVI in Ketibunder Sindh Pakistan had a moderately negative correlation with LST between 2000 and 2010 and a strongly negative correlation with LST during 2014.

The correlation of NDVI was observed low with LST of the summer season (R= -0.24) likely because of the contribution of warm conditions in reducing chlorophyll contents of the vegetation cover. In a study by Yang et al. [12], a negative correlation between NDVI of grasslands and temperature was found in Mongolia during the autumn and summer seasons of the 1982-2011 period. Several studies conducted in various parts of the world also concluded that NDVI is strongly affected by temperature as compared to precipitation [36-39]. The positive correlation observed between NDVI and rainfall for all seasons (significant for summer and autumn seasons at $p<0.05$) is likely because of the favorable influence of increasing wet conditions on the rangeland vegetation. According to a study by Wang *et al.* [38], NDVI is strongly influenced by the precipitation pattern during the growing and proceeding winter.

5. CONCLUSION

In the present study, time-series data of MODIS products, i.e., NDVI & LST, and TRMM rainfall were used to analyze seasonal trends and examine the relationship between NDVI with climatic parameters in the lesser Himalayas of Pakistan during the 2000-2018 period. Higher NDVI values were observed during the summer and autumn seasons due to the positive effect of wet conditions on the vegetation during the 19 years. The low correlation observed between NDVI and LST during the summer season is likely because of the prevailing stress condition of chlorophyll contents of the vegetation cover under warm conditions. However, this situation appears to be compensated by the rainfall of the summer and autumn seasons as indicative of the moderate to strong correlation between the NDVI and the rainfall of these seasons. Overall least NDVI values were observed during the winter season, which may be attributed to the prevalence of snow cover over higher altitudes, i.e., above 2300 m, and the availability of limited vegetation cover and grazing opportunities in the lower valleys of the district. The dynamics and snow cover change and its impacts on the vegetation of high reaches need in-depth research for better understanding and getting scientific evidence. The large swath and resolution of the satellite images provided rapid observations of bioclimate conditions over a large part of the area in a synoptic view thus facilitating spatial variability and change analysis. NDVI data product was found effective in monitoring the status, distribution, and trends of rangeland vegetation and biomass variability in this mountainous region. In the absence of high-altitude observational data on climate, validation of the gridded climate data is challenging and can be improved through the provision of a network

of high-altitude climate observatories. Regular monitoring of land use, plant phenology, and socioeconomic conditions is needed for effective rangeland management and to develop viable resource conservation strategies for this fragile mountain ecosystem in the future.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

There is no conflict of interest among authors

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Pakistan Academy of Sciences

Research Article

Socioeconomic and Environmental Impacts of Tobacco Farming in Khyber Pakhtunkhwa, Pakistan

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Abstract: Tobacco production and curing cause a threat to the environment through injudicious use of pesticides, imbalanced use of chemical fertilizers, and excessive consumption of local firewood. Keeping all this in retrospect, this study has been designed to assess the impact of tobacco farming on family workers, and witness on spot involvement of children and nursing/ expecting mothers in tobacco-related health-hazardous tasks. General objective of the study is to document both the positive and negative environmental and socioeconomic externalities of tobacco production in the study area. It is based both on primary data and secondary statistics. Primary data has been collected from sixty farmers for the tobacco season 2021; including forty contract and twenty non-contract sample farmers. Farmers conceive that tobacco farming has a bad impact on human health (93 %), and causes degenerative deforestation and resource depletion (68 % each). Use of Green Tobacco Sickness gloves and Personal Precautionary Equipment was reported by eighty and sixty-two percent of the farmers, respectively. Impact of the disease on the workers' health was reported by thirty-five percent of contracted farmers, and 90 percent of non-contracted ones. Thus, the impact of the disease on human health was severe on non-contracted farms, mainly due to little or no use of Personal Precautionary Equipment. Cost of Personal Precautionary Equipment per season at the contracted farms was much higher (US$ 18.0) than at non-contracted ones (US$ 2.0). While the treatment cost of Green Tobacco Sickness or other agrochemical-related diseases of contracted farming households was 2.5 times lower than non-contracted ones. As per the results of the double log Ordinary Least Squares regression model about medical treatment cost, the coefficient of age of household head and use of Personal Precautionary Equipment have negative expected signs and are statistically significant. Laboratory tests of soil and water samples have revealed hazardous levels of Sodium Chloride and Dissolved Oxygen in canal water, which indicate imbalanced use of fertilizers by the growers and leaching of excessive nutrients below the root zone. Tobacco companies' contracted growers were found to have less occupational health hazards. Thus, raising awareness among tobacco growers about the importance of adopting precautionary measures and use of Personal Precautionary Equipment can reduce the negative effects of tobacco farming.

Keywords: Deforestation, Resource degradation, GTS, PPE, Tobacco, Pakistan

1. INTRODUCTION

Tobacco (*Nicotiana Tabacum*) is an important cash crop in the Khyber Pakhtunkhwa and Punjab provinces of Pakistan. Khyber Pakhtunkhwa and Punjab contribute nearly 65 and 32 percent in total tobacco cropped area, and about 79 and 20 percent in total tobacco produce, respectively. Its role in the economy can be judged by the fact that it is a major source of government revenue in the form of excise taxes. Pakistan earns considerable foreign exchange by exporting tobacco and its products. The country exported raw tobacco and its products of worth US$ 77.34 million in the fiscal year 2021 [1]. In Khyber Pakhtunkhwa province tobacco growing districts by production in decreasing order are Swabi, Mardan, Charsadda, Mansehra, Buner, Malakand, and Nowshehra. Swabi district contributes about 30 % in area and 38 % to the provincial production of tobacco due to suitable agronomic and environmental conditions. Total number of tobacco growers and barns in

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the district are 5500 and 6500, respectively. Area under tobacco cultivation in the district is 13179 hectares with a total production of 34.08 million kg, and productivity of 2586 kg per hectare. Tobacco is the main cash crop and source of livelihood for the farmers in the Swabi district of Khyber Pakhtunkhwa province of Pakistan [2].

As already stated, it contributes to the national economy through excise taxes and foreign exchange earnings. According to Pakistan Tobacco Board (PTB), almost 350 thousand people are involved in the tobacco industry in Pakistan, creating an income of Rs. 300 billion per annum. Similarly, tobacco production is a livelihood source for 1.2 million people. There are 75000 tobacco growers in the country, out of these more than 45000 growers (60 %) are in Khyber Pakhtunkhwa, which implies the importance of the tobacco crop in the study area [3]. Farooq *et al* [4] reported that there are 53 tobacco companies registered with PTB. While the cigarette market in Pakistan is highly skewed as only two multinational companies control 98 % of the market. In the country, PTB and United Kingdom-based multinational companies viz. Pakistan Tobacco Company (PTC) and Phillip Morris Pakistan Limited (PMPKL) are actively involved in the research and development of tobacco. These multinational companies distribute certified high-yielding seeds of the crop to their contracted growers. They disseminate knowledge to the farmers about recommended production package of the crop, including fertilizer application, keeping crop protection agents' (CPAs) in lockups, use of personal protective equipment (PPE) including masks, protective clothes, goggles, Green Tobacco Sickness (GTS) gloves, shoes, etc. Moreover, through the corporate social responsibility (CSR) program, these companies work for the social welfare of the farming community with the involvement of community support organizations. These companies operate mobile first aid providing medical units, and arrange summer schools for children through contractual farming to eliminate child labour from hazardous tasks of tobacco production viz, harvesting, post-harvest handling and processing viz. leaf picking, stick tying and barn loading, etc. Women are provided alternate sources of income to discourage their involvement in tobacco-related activities by arranging vocational training centers for them. Thus, women folk is protected from health hazards and empowered to become skillful and productive members of society [3].

It is a fact that tobacco cultivation causes an irrevocable cost to the environment, as tobacco curing requires about one ton of firewood per barn for one week. Sami *et al.* [5] estimated that 14,156 kg of firewood per hectare is used for tobacco curing in the Swabi district, and firewood cost shared 38 percent of the total cost of production. Similarly, Nasrullah *et al.* [6] reported that firewood cost was Rs. 39,957 per hectare, with a share of 25 percent of the total cost of tobacco production in year 2019. Thus, it results in huge pressure on forest resources along with soil erosion & degradation, water pollution, changes in climate and cropping seasons, etc. Furthermore, tobacco cultivation causes losses to farming households in form of sickness/health costs and missing farming days due to the non-adoption of PPEs. Though, contractual farming of tobacco completely has banned child labour, and involvement in hazardous tasks of tobacco leaf handling activities of nursing women as well expecting mothers. However, at non-contractual farms due to unawareness, they are at the risk of GTS disease caused by the entrance of nicotine into the human body through skin pores from the wet tobacco leaves specifically in teenagers after rains [7].

Different chemicals are used on tobacco crops from sowing to harvesting, among them some most commonly used pesticides are *Imidacloprid, Chlorpyrifos, Dichloropropen, Aldicarb,* and *Methyl Bromide* causing chronic infections and soil degradation. According to Khan *et al*. [8], the majority of farmers suffer mild to moderate poisoning as a result of pesticide usage. According to Food and Agriculture Organization, the tobacco-producing regions in the developing countries are wood deficient and are below the proficiency level, specifically in Asia and Africa, resulting in deforestation and biodegradation [9]. It is worth mentioning here that in the 1990s tobacco curing caused 19 % of deforestation in Pakistan as reported by British American Tobacco (BAT), which is a visible footprint for climate change [10]. Moreover, multinational companies are providing PPEs to the contract growers for agrochemical use, harvesting, leaf picking and grading. They provide

extension services and training facilities for use of PPEs, as well as the adoption of protective measures against GTS disease. This is supported by proper promotion of less toxic chemicals having low residues, and the disposal of empty bottles of agrochemicals to protect the environment besides the health of farming households. The companies claim to provide safety training with a special focus on the use of PPEs to around 89 % of the contracted tobacco growers [11]. The use of PPE was quite low in the country about a decade ago, as Khan *et al*. [8] reported that during pesticide spraying only 30 % of tobacco growers were using shoes, 14 % were using masks and 9 % used protective gloves in year 2010. In order to promote the use of PPEs, PMPKL and PTC created mass awareness among the growers. PMPKL also recruited both farm workers to monitor the use of PPEs and all tobacco-related activities performed by male and female workers.

According to Ali *et al*. [12], Pakistan's tobacco yield is 3017 kg per hectare, which is significantly higher than the average yield in developed countries i.e. 1900 kg per hectare. Nasrullah *et al.* [6] estimated that the net revenue of tobacco production in district Mardan was Rs. 405,636 per hectare in the year 2014. Similarly, Aman and Khan [13] reported net revenue of Rs 401,982 per hectare of tobacco crop in the Swabi district in year 2019. They also identified that the problems faced by tobacco growers are low quality of seed, high input prices, child labour and farmer's health issues, and lack of proper health facilities. They recommended the need for advanced research in these areas to solve these issues. According to the World Health Organization-Framework Convention on Tobacco Control (WHO-FCTC), [14] research is needed on social impeding i.e. child labour, deforestation, pesticide exposure and occupational health hazards like GTS disease caused by tobacco crop in developing countries. In a need assessment for WHO-FCTC implementation in Pakistan by European Commission [15], a gap was identified in the lack of proper policy administering the environment and health of tobacco farming households. Likewise, according to another cluster study report by Farooq *et al.* [4] improper disposal of tobacco byproducts and excessive use of chemicals result in health issues and loss of biodiversity. They suggested public sector to take urgent notice of the health and other hazards involved in tobacco curing and emphasized strongly on research to address the persistent issue of health and resource degradation in tobacco farming. Rahman *et al.* [16] considered tobacco farmers' lack of awareness and guidance to tackle the hazardous tasks affecting their health and environment as serious factors that need to be studied behind exquisite tobacco farming. It is worth mentioning here that, threats caused by tobacco cultivation have been identified in Pakistan. Whereas, economic analyses of the crop are limited to cost of production and profitability.

There is a gap in research as per determination of the impact of tobacco farming in terms of health risks for the farmers in general and nursing/ expecting mothers in particular, involvement of child labour, adoption of PPEs, support services provided by multinational companies involved in tobacco farming, issues related to soil degradation and water pollution are concerned. Findings of this study would help stakeholders involved in tobacco farming in the country in finding ways and means for controlling environmental threats, natural resource degradation, and health issues of farming households. Overall objective of the study is to document both the positive and negative environmental and socioeconomic impacts of tobacco production in the study area. While specific objectives of the research endeavor are; to highlight the socioeconomic characteristics of tobacco farmers in the study area; to determine farmers' awareness level regarding the impact of tobacco cultivation on the environment and human health; to gauge the incidence of child and women labour in tobacco farming; to determine the use of GTS gloves and other PPE by tobacco farmers; to document health hazards and estimate the incidence of sickness among the farming households due to pesticide exposure; to determine the impact of the growers socioeconomic, geographic & safety traits on the cost of treatment of the diseases caused by tobacco farming; and to examine the impact of tobacco farming on natural resources in the study area.

2. MATERIALS AND METHODS

The study is simultaneously exploratory and descriptive in nature. It has been carried out based on primary data, secondary sources of information,

and laboratory tests of soil and water samples. The primary data has been collected by designing a comprehensive survey tool. The data is collected through the purposive random sampling technique to give representation in data set to both contracted and non-contracted tobacco growers. Contracted sample farmers are interviewed randomly from the lists of contracted farmers provided by Phillip Morris Pakistan Limited (PMPKL), Mardan. Face-to-face interviews with 60 tobacco growers are conducted, including 40 contract farmers of PMPKL and 20 non-contract farmers. The contracted farmers were from the surrounding villages of Yar Hussain town, which is located at a distance of 25 km from headquarters of the Swabi district, while the non-contracted farmers were residents of Marghuz and adjoining villages. Marghuz is located 12.5 km away from headquarters of the Swabi district. The data for the study was collected at the end of tobacco season of year 2021. Field survey was conducted in August, 2021. The questionnaire covering the tobacco farming households' socioeconomic characteristics, health hazards caused by excessive use of chemicals, incidence of Green Tobacco Sickness (GTS), involvement of child labour, and nursing/ expecting mothers in tobacco farming was prepared, pre-tested, and modified accordingly.

Secondary information about the status of tobacco cultivation in the province has been collected from PTB Peshawar. Similarly, PMPKL, Mardan and PTC, Nowshera offices were visited to get details of services offered to the farmers, and to get technical input on the survey tool used for the study. To estimate the environmental impact of tobacco cultivation the methodology has been adopted from Kutab and Falgunee [17] in which different parameters of soil and water such as pH and DO (Dissolved Oxygen) in the study area were determined for the presence of different toxic chemicals such as Phosphorous and Potassium. The soil samples were taken 5 inches deep from different geographical points in the study area, while water samples from the canal and tube-well channels were taken to observe the presence of different toxic chemicals.

Primary data has been analyzed for descriptive statistics viz. frequency distributions, mean values, percentage, etc. Health cost expressions (equations 1 and 2) used in this study have been adopted from Atreya [18] to determine the cost incurred on farmers' health in monetary terms. As tobacco-related diseases have a negative impact on farmers' welfare as a result of illness, lost wages, medical treatment expenditures, decrease in farm productivity, and loss of income. The monetary loss to the farmers caused by the diseases is calculated by taking into account the wages of leaf pickers along with the missing working days of both patients and their attendants. Thus, health cost is estimated for contracted/ user of preventive measures/equipment and non-contracted farmers/ control group by expressions 1 and 2, respectively.

$$HC_u = SD_u * CI_u + PC_i \quad (1)$$

$$HC_n = SD_n * CI_n \quad (2)$$

Where HCu and HCn are health costs for users and non-users of preventive measures, respectively. Similarly, SDu and SDn are sickness days of users and non-users of preventive measures, respectively. Clu and Cln are the average cost spent on treatment of illness per day, including travel costs, opportunity cost of time spent in traveling to dispensaries/ clinics/ hospitals, doctors' consultation fees, hospitalization charges, medication costs, patients' dietary expenses, and lost work productivity of patient and its attendant, of users and non-users of preventive measures, respectively. PCi is the cost of the PPE (masks, protective clothes, goggles, GTS gloves, shoes, etc.) per cropping season by the individual contracted farmers. Components of health cost were converted in US$ on basis of the prevalent exchange rate at the time of data analysis i.e. January 2022 that was PKR.176.98 equivalent to one US$, to make the findings comparable with the results of the studies conducted in other countries. To study the factors affecting the health cost, regression analysis has been carried out and the health cost function was estimated using a generalized model expressed by equation 3.

$$HC = f(S, IF, DPM) \quad (3)$$

Where HC represents the health cost, S is the socioeconomic characteristics of farmers such as age and education affecting health cost, IF are the institutional factors affecting health cost such as access to Hospitals, Basic Health Units (BHUs) and medical dispensaries, etc. DPM is the dummy variable for use of preventive measures/PPEs during harvesting, leaf picking and stick tying, or

otherwise. The specified double log health cost model used for the study is given by equation 4.

$$LnHC_i = \beta_0 + \beta_1 \Sigma LnAge_i + \beta_2 \Sigma LnDis_i + \beta_3 D_{pm} + \mu_i \quad (4)$$

Where HC is health cost, βo is a constant term, 'Age' is the age of the household head of respondent i, 'Dis' is the distance from the medical facility of respondent i, Dpm is the dummy variable for use of protective measures during tobacco farming by the respondent i, and μi is the usual error term. In reference to health costs, Dasgupta *et al*. [19] pointed out that blood testing is more appropriate to check the cause of the illness as self-reported symptoms are weak indicators of health impacts. But blood sampling in this study was not done due to technical and financial constraints on part of the research team.

3. RESULTS AND DISCUSSION

3.1 Socioeconomic Characteristics of Tobacco Farmers

Socioeconomic characteristics of the sample tobacco farmers are presented in Table 1. Most of the tobacco growers were in the middle age group, i.e. 55 percent of contracted farmers, and 65 percent of non-contracted farmers were in the age bracket of 30 to 50 years. Mean education of sample contracted and control group farmers was 7 and 8 educational years, respectively. Similarly, literacy rates of contracted and non-contracted farmers were 60 and 75 percent, respectively. Thus, high literacy level indicates that tobacco leaf pickers may have awareness regarding the harmful effects of tobacco leaf picking. Since instructions prescribed by tobacco companies regarding Green Tobacco Sickness (GTS) on pesticide containers/ bottles are written in English and Urdu so farming households, especially women with low/ no literacy levels are unable to read instructions or even understand symbols given on poisonous materials and their levels of human health hazards. Knowledge about GTS disease, its precautions and self-protection measures were expected to be low among the tobacco leaf pickers in the study area. The findings of the study are in line with our prior expectations in the case of non-contracted farmers. One-half of the women leaf pickers from non-contracted farming households (50 %) are found to have little knowledge of GTS disease. Tobacco growers in the Swabi district are resource-poor, as the monthly crop income of most of the sample farmers (98 % contracted and 95 % non-contracted) was less than 100 US$ (PKR 17698). Thus, women and children are to participate in tobacco farming activities including stick tying, for which they are paid PKR 2.5 per stick.

3.2 Farmer's Awareness about the Impact of Tobacco Farming on Environment and Human Health

Findings about farming household awareness about the impact of tobacco cultivation on the environment and human health in the study area are ranked in Table 2. The results highlighted that tobacco farming is affecting farmers' and their families' health. Overall 93 percent of the respondents were of the view that tobacco farming/handling activities has a bad impact on human health. As per the data, 68 percent of the sample farmers reported that curing tobacco consumes a large amount of local firewood i.e. 800 kg firewood per barn, which ultimately results in deforestation. Sixty-three percent of the contracted farmers and eighty percent of the non-contracted farmers reported that tobacco farming is causing deforestation in the study area.

The difference in the response of contracted and non-contracted farmers is because tobacco companies prescribe their contracted farmers to use sustainable basis. They bound farmers to obtain approval for the use of fire wood. Similarly, tree plantation campaigns are organized annually by these companies to control deforestation, and this is the reason the contracted farmers perceive that tobacco farming is not an environmental threat inthe study area. This implies that tobacco companies are playing a pivotal role in making their contracted farmers conscious is overcome the environmental impact caused by tobacco cultivation and curing, hence reducing biodiversity loss in the study area. Similarly, 68 percent of tobacco farmers in the research area said to use firewood on additional fertilizers, irrigations and pesticides for tobacco cultivation due to reduced soil productivity and high insect-pest infestation. About one-third of the farmers (35 %) were of the view that tobacco farming pollutes water to some extent due to dissolved oxygen. They reported that crop farming

Table 1. Socioeconomic characteristics of the sample farmers

Characteristics	Contracted (n=40)	Non- Contracted (n=20)	All (n=60)
Age (years)			
18-30	6 (15)	4 (20)	10 (17)**
30-50	22 (55)	13 (65)	35 (58)
50-70	12 (30)	3 (15)	15 (25)
Mean Age	43.0 ± 10.6	41.2 ± 10.2	42.4 ± 10.4
Education			
Illiterate	16 (40)	5 (25)	21 (35)*
Literate	24 (60)	15 (75)	39 (56)
Mean Education	6.5 ± 5.8	7.7 ± 5.7	6.9 ± 5.7
Family Size	7.8 ± 3.9	7.2 ± 4.3	7.6 ± 4.0
Monthly household income from tobacco crop			
Below 100US$ (PKR17,698)	39 (98)	19 (95)	58 (97)**
Above 100US$ (PKR 17,698)	1 (2.5)	1 (5)	2 (3)
Mean monthly household income from tobacco crop US$ (PKR)	75.4 ± 24.4 (13344 ± 4318)	75.6 ± 19.5 (13380 ± 3451)	75.5 ± 22.7 (13362 ± 4017)

** and ** indicate that values are significantly different at 1 and 10 percent levels*

Table 2. Farmer's awareness about the impact of tobacco farming on the environment and human health

	Contracted (n=40)	Non-Contracted (n=20)	All (n=60)	Overall Rank
Bad impact on human health	37 (93)	19 (95)	56 (93)	I
Causes deforestation	25 (63)	16 (80)	41 (68)	II
Increasing use of fertilizers, pesticides and irrigation	24 (60)	17 (85)	41 (68)	II
Causes water pollution	15 (38)	6 (30)	21 (35)	III
Reduces other crops productivity	5 (13)	5 (25)	10 (17)	IV
Deteriorate underground water quality	9 (23)	0 (00)	9 (15)	V
Loss of biodiversity	4 (10)	2 (10)	6 (10)	VI

Note: Figures in parenthesis are percentages

reduces the productivity of other crops (17 %), deteriorates the quality of underground water (15 %), and results in loss of biodiversity (10 %).

All this is due to preventive actions taken by Phillip Morris Pakistan Limited (PMPKL), Mardan to protect the environment, water, soil, health of farming households, and by providing lockups for keeping crop protection agents. PMPKL issues strict guidelines to the contracted farmers to install lockups 6ft above ground level to evade children's access. Farmers are guided not to dispose-off bottles of used toxic chemicals openly in canals, ponds, or in the garbage. They are instructed to either combust or bury these properly or return to PMPKL in exchange for household grocery items. The monitoring teams of PMPKL make regular field visits to the farmers and in case of violation, they raise Prompt Action Issue (PAI) against farmers and cancel their contract license.

3.3 Involvement of the Farm Families in Tobacco Farming

Findings about involvement of the household members in tobacco farming are presented in Table 3. The data shows that 70 percent of adults, including 27 percent of nursing/expecting mothers participate in tobacco farming related activities. Similarly, a little more than one-third of the sample households (35 %) reported children's involvement in tobacco farming. Adults and expecting/ nursing women are reported to perform all types of tobacco farming activities from leaf picking, grading of green leaves, stick tying till barn loading. While, children are being involved in green leaf and

Table 3. Involvement of sample households in tobacco farming

Involvement in tobacco farming	Contracted (n=40)			Non-contracted (n=20)			All (n=60)		
	Adults (M+F)	Nursing/ Expecting mothers	Children	Adults (M+F)	Nursing/ Expecting mothers	Children	Adults (M+F)	Nursing/ Expecting mothers	Children
Overall	25 (63)	13 (33)	11 (28)	17 (85)	9 (45)	10 (50)	42 (70)	22 (27)	21 (35)
Stick tying	25 (63)	7 (18)	11 (28)	17 (85)	4 (20)	10 (50)	42 (70)	11 (18)	21 (35)
Green leaves' grading	25 (63)	0 (0)	2 (5)	17 (85)	2 (10)	7 (35)	42 (70)	2 (3)	9 (15)
Barn loading	25 (63)	6 (15)	0 (0)	17 (85)	3 (15)	0 (0)	42 (70)	9 (15)	0 (0)
Leaf picking	25 (63)	0 (0)	0 (0)	17 (85)	2 (10)	0 (0)	42 (70)	2 (3)	0 (0)
Reasons for nursing/expecting mothers' involvement in tobacco farming									
Reasons	**Contracted (n=40)**			**Non-contracted (n=20)**			**Overall (n=60)**		
Financial reasons	28 (69)			20 (100)			50 (83)		
Non-availability of labour	12 (31)			0 (0)			10 (17)		

Table 4. Precautionary measures

Measures	No. of tobacco farmers		
	Contracted (n=40)	Non-contracted (n=20)	All (n=60)
Use of GTS gloves for leaf handling	38 (95)	10 (50)	48 (80)**
Use of PPEs (protective masks, goggles, long shoes, protective uniform, GTS gloves etc.)	35 (88)	2 (10)	37 (62)*
PPE's provided by tobacco companies	39 (98)	0 (0)	39 (65)**

** and ** indicate that values are significantly different at 1 and 10 percent levels, respectively*

stick tying. Involvement of nursing/ expecting mothers and children in the farming was low at contracted farming as compared to non-contracted ones. Thirty-three and forty-five percent of the tobacco farming households reported involvement of nursing/expected women, respectively. In the same way, the involvement of children in tobacco farming was reported by twenty-eight and fifty percent of the contracted and non-contracted farming households, respectively. Low involvement of nursing/expecting mothers and children at the contracted farms is due to consistent efforts of tobacco companies i.e. PMPKL and PTC. The companies have been launching various campaigns among the tobacco farming households to limit the involvement of vulnerable household members in the farming, to use Personal Protective Equipment (PPE) & Lockups for storage of Crop Protection Agents (CPAs), and regularly monitoring the tobacco farms. Companies play an important role in corporate social responsibility to reduce children (those who are generally on summer vacations) and nursing/expecting mothers' involvement in crop farming. Summer schools for children and embroidery centers for women of contracted households are launched. Attendance records of these institutions are regularly monitored to ensure the attendance of children and women. Irrespective of farming categories i.e. contracted and non-contracted farmers, 83 percent of them reported that children and women are involved in tobacco leaf picking and other leaf handling activities, such as barn loading, stick tying due to limited financial resources and due to non-availability of labour during tobacco season.

3.4 Precautionary measures adopted by tobacco leaf pickers

The use of precautionary measures by the leaf pickers at the farms of contracted growers was higher than that of non-contracted tobacco ones (Table 4). The usedz of precautionary measures includes; installing CPA lockups six feet above the ground level, to avoid kids' access to hazardous chemicals that are used in tobacco farming, wearing

protective masks, goggles, long shoes, protective uniform and gloves by the leaf pickers. Ninety-five percent of the contracted tobacco farmers and fifty percent of the non-contracted farmers reported the use of gloves by the leaf pickers at their farms to prevent Green Tobacco Sickness (GTS) during harvesting the crop. Similarly, the use of Personal Protective Equipment (PPE) was reported by ninety-five of the contracted growers and by just ten percent of the non-contracted growers. Though, the use of PPEs is considered to reduce injurious effects on their health due to exposure to pesticides, and other pollutants during leaf picking. While low use of protective measures puts the lives of tobacco pickers especially the children and nursing/expecting mothers at risk. Generally, tobacco leaf pickers perceive that few health hazards are associated with exposure to pesticides and consider leaf picking and handling as a normal and safe working activity. Therefore, the adoption of preventive measures among non-contracted leaf pickers is minimal, while the opposite is true in the case of contracted farmers. The use of GTS gloves & PPE was statistically different among contracted and non-contracted farmers (Table 4).

As tobacco companies are playing key role in creating awareness about health hazards in tobacco farming families. Out of the total sample of sixty tobacco farmers, 80 and 62 percent of the farmers reported the use of GTS gloves and PPE, respectively. Almost all of the contracted farmers surveyed for the study (98 %) reported that tobacco companies provided them the personal protective equipment. Tobacco companies are playing role in improving the use of PPEs by the farmers' overtime. Khan *et al*. [8] assessed the risk of pesticide exposure on the health of tobacco farmers in the Swabi district of Khyber Pakhtunkhwa province of Pakistan and reported that few farmers use shoes (31 %), masks (14 %), and gloves (9 %) during pesticides spray and tobacco leaf handling activities.

3.5 Incidence of Sickness among the Farming Households due to Pesticide Exposure

Khan *et al*. [8] in the Swabi district of Khyber Pakhtunkwa and Silva *et al.* [20] in Brazil reported that exposure to tobacco leaves causes an increase in GTS among tobacco leaf pickers during harvesting season. Most common symptoms of GTS in farm workers due to tobacco farming include headache, weakness, dizziness, and nausea/vomiting. Their clothing becomes saturated from tobacco that is wet from rain or morning dew, or perspiration. Though all the sample households reported GTS in their family workers due to tobacco farming. However, most of the time its impact on human health is temporary. The impact of the disease on the workers' health and their inability to perform farm operations was reported by 35 percent of contracted farming households and 90 percent of non-contracted households (Table 5). Thus, the impact of the disease on farmers' health was more severe on non-contracted farms than on contracted ones, mainly due to little or no use of PPE. Similarly, the frequency and number of cases per household was also higher among non-contracted farmers than their counterparts. Contracted farmers are obligated to use of precautionary measures during tobacco production, harvesting, and curing by the tobacco companies. As already mentioned, these households are provided PPEs by the companies and are frequently monitored for their use. Charges for PPE are deducted by the companies at the end of each season. The cost of PPE at the contracted farms was much higher (US$ 18.0) than at non-contracted ones (US$ 2.0).

All the sample farmers reported having access to health facilities, including Basic Health Units (BHUs)/ dispensaries and hospitals. Mean distance of BHUs/dispensaries from the tobacco farms was less than one kilometer. However, the mean distance of contracted farms from hospitals was more (21.6 km) than non-contracted farms (18.1 km). Half of the sample respondents reported to consult health staff/ doctors for a formal treatment of the diseases caused by tobacco farming in the crop season 2021. As the impact of the disease at the contracted farms was low, and the mean distance of hospitals from the contracted farm was comparatively more, thus they reported consulting health staff/ doctors less (45 %) than non-contracted farmers (60 %). People having less immunity i.e. children, under-nourished women, particularly nursing/expecting mothers, old aged persons are more susceptible than adults to various diseases caused by tobacco farming due to the presence of pesticide fumes, dust and allergens, etc. at farms. Tobacco leaf picking can cause both temporary and chronic impacts on human health. Another, reason for a high incidence

of GTS reported by women respondents is that in spite of their awareness of the disease, they consider stick tying without gloves easy and quick way in comparison to that of wearing gloves. Similarly, in a few farm households where expecting/nursing mothers were experts in stick tying and the ones in which they can't afford to hire labour, these women continue to remain involved in operations and the situation may turn out to be worse.

In international literature, a wide range of GTS incidence has been reported by various researchers, ranging from a few cases to a large number of tobacco workers. As an example, Sujoso and Martiana, [21] reported sufferings from GTS among 11 % of tobacco farmers in Indonesia. While Oliveira *et al*. [22] reported GTC incidence in 82 % of the farmers in Brazil. As review of the literature reveals that the incidence of the disease depends on dewfall, rainfall, air humidity, and the use of PPE. Similarly, a clear description of the criteria for reporting the incidence also matters much, as the symptoms of the disease vary from dizziness, and headache to nausea/vomiting. Guddad *et al*. [23], and Saleeon *et al*. [24] reported a direct relationship between GTS incidence and increased humidity during tobacco crop harvesting in the monsoon (rainy) season. They reported that moisture helps to enhance nicotine absorption through the skin. Although the researchers were unable to quantify this relationship. Similarly, Oliveira *et al*. [22] described that number of patients having GTS symptoms and signs increases during rainy days. Gehlbach *et al*. [25] and Ghosh *et al*. [26] stated that nicotine absorbed in dew drops affects persons who work in the tobacco fields in the morning, particularly those harvesting tobacco leaves. While, on the other hand, farm workers in shed tobacco farms had substantially fewer GTS symptoms [27].

As far as sickness cases from tobacco farming in the study area are concerned, few of the contracted tobacco farmers reported adverse impacts on the health of the farm workers (35 %) due to proper use of PPE in comparison to non-contracted growers (90 %). However, the results should be taken with a bit of caution, as sickness in the farm workers may also be due to factors other than that of tobacco leaf picking. Similarly, healthy workers can be less vulnerable to bad impacts of tobacco leaf picking than those who have already certain health issues or have less immunity i.e. farmers workers under 18 years of age, nursing/expecting mothers and aged persons are more vulnerable to nicotine absorption due to tobacco leaf picking and thus to various diseases. Mean treatment cost of diseases per farm household per season is US$ 8.3 and US$ 29.2 for contracted and non-contracted farmers, respectively. Similarly, Hussain *et al.* [28] reported that medical treatment cost for contracted tobacco farmers in Bangladesh was also low as compared to their counterparts due to the use of personal protective equipment. When farmer workers notice costs associated with exposure to pesticides and green tobacco leaves in the form of illness symptoms and associated costs, they tend to use precautionary measures in true letter and spirit [29].

3.6 Impact of the Growers' Socioeconomic, Geographic & Safety Traits on Cost of the Medical Treatment

Double log model has been estimated to find out the impact of growers' socioeconomic, geographic & safety traits on cost of treatment of the diseases caused by tobacco farming. The results of the model are presented in Table 6. F-value indicates that the test is statistically significant. The value of the R-squared indicates that about 20 percent variability in the dependent variable i.e. treatment cost (US$) of the diseases caused by tobacco farming is explained by the variable considered in the regression model. This means that the treatment cost of tobacco-related diseases depends to a considerable extent on the general health status of the workers, their immunity level, number of children, nursing/expecting mothers, and old aged persons involved in the farming. The coefficient of age of the household head has an expected negative sign and is statistically significant. It means that aged heads of tobacco farming households are experienced in tobacco farming, thus have better know-how to avoid health issues. The coefficient of distance has a negative sign. Understandable reason for this is low treatment costs in contracted farming households than in their counterparts. While the mean distance of contracted farms was higher than non-contracted ones. However, the coefficient of distance from the hospital is statistically insignificant. Dummy variable for the use of PPE has an expected negative sign and is

statistically significant. The value of its coefficient indicates that an increase in the use of precautionary measures/ personal protective equipment results in a decrease in treatment/ health-related costs.

3.7 Impact of Tobacco Farming On Natural Resources

Results of lab tests of soil and water samples to gauge the quality of natural resources, and assess the impact of tobacco cultivation on them are reported in Table 7. Although, the pH level of both tobacco and other crops' soils in the study areas is higher than the ideal range of 6.5 to 7.5; however, soils are alkaline and are ideal for tobacco cultivation. The level of Nitrate (NO_3) is less than the standard range, which indicates the imbalanced use of fertilizers by the growers and the leaching of excessive nutrients down to root zones and water courses. Leaching of Nitrate and Phosphate in water courses can stimulate algal and other water plants' growth. Thus, it lowers the productivity or fertility of such an ecosystem, thus farmers are to apply higher levels of fertilizers to improve soil fertility and crop productivity. While the level of Potassium is found higher than the tolerable range both in soils used for the production of tobacco and other crops. Higher level of Potassium affects the absorption of other critical nutrients by plants. While lowering soil potassium can also prevent excess Phosphorus from running into waterways.

Both canal and hand pump water in the study area are found alkaline in nature, having pH levels of 7.5 and 7.6, respectively. These levels fall almost in the ideal range; thus, the water quality of hand pumps is good for drinking by humans as well as animals, and that of canal water is fair for the production of tobacco and other crops in the study area. Similarly, levels of Sodium Chloride in drinking water are in the ideal range, while in the canal water is much higher than the acceptable limit.

Higher level of Chloride in canal water interferes with nitrogen uptake by plants, reduces crop growth, and stops plant reproduction. Possible reasons for a high level of Chloride in canal water are higher use of fertilizers and disposal of sewerage water and trash in canals. Levels of Dissolved Oxygen are higher than acceptable standards both in hand pump and canal water. This is harmful to animal health and hinders plant root growth as plants are getting everything, they need with a smaller surface area. Total dissolved solids (TDS) in hand pump water (< 300 ppm) indicate

Table 6. Impact of the farmers' characteristics on the medical treatment cost

Variables	Unit	Mean (SD)	Coefficients
Age of Household Head	Years	42.4 ± 10.4	-1.428 (0.090)**
Distance from hospital	Km	20.4 ± 10.8	-0.125 (0.448)ns
Precautionary measures	Dummy (Users: 1, Non-users: 0) Users %	62	-1.160 (0.007)*
	Constant		8.291 (0.010)*
R^2			0.195
	F Value		4.266 *

** and ** indicate that values are significant at 1 and 10 percent levels, and ns stand for non-significant*

Table 7. Soil and Water Quality in the study area

Parameters	Tobacco Crop Land	Other Crop Land	Permissible Limits	Sources
I. Soil Parameters				
pH	7.6	7.9	6.5-7.5	Nabi *et al.* [30]
Nitrate (NO_3)	0.46	1.16	11.0 to 20.0 mg /kg	Pattison *et al.* [31]
Phosphate	5.79	6.14	4.0 to 7.0 mg/kg	Wall and Plunkett [32]
Potassium	138	140	40 to 80 mg/kg	
II. Water Parameters				
Parameters	**Hand Pump**	**Canal**	**Permissible Limits**	**Source**
pH	7.6	7.5	6.5 to 8.5	
Chloride (ppm)	196.8	794.2	< 250	WHO, [33]
Dissolved Oxygen (ppm)	11.00	11.23	6.5 to 8.0 mg/L	
TDS (ppm)	201.9	786.2	1000 ppm	

that it is excellent for drinking. While TDS in the canal water is in a fair range (600 to 900 ppm) and its consumption can be harmful to animal health. Higher levels of TDS in water make it unfit for consumption and may cause several diseases like nausea, lung irritation, rashes, vomiting, dizziness, etc. Few of the farmers perceived that tobacco farming is polluting the water. However, analysis of water samples revealed that the quality of pumped water is good, while canal water is harmful to animal and plant health due to the presence of high levels of dissolved solids.

4. CONCLUSION

Though the tobacco crop is profitable for the farming households in the study area; however, it also has various socioeconomic and environmental implications for them. They are to face Green Tobacco Sickness (GTS) and other agrochemical-related health hazards. Furthermore, the imbalanced use of agrochemicals is resulting in the degradation of land and water resources. Private companies are playing an important role by creating awareness among contracted growers, providing them with Personal Protective Equipment (PPE), and properly monitoring their use. Similarly, summer schools for children and embroidery centers for women of tobacco farming households are organized to limit their involvement in tobacco-related activities. Resultantly, the use of precautionary measures during tobacco leaf picking and handling is relatively fair among contracted farming households. While workers at farms of non-contracted households face bad health impacts due to improper handling of the crop. This can be attributed to a lack of institutional support for the creation of awareness about tobacco-related health hazards, and training them to take proper precautionary measures during crop handling. A voluntary cadre of health workers can also be developed to give first aid to affected farm workers. In this reference, local support organizations, public sector education, agricultural extension, and health departments must come forward to the maximum possible extent. Findings of the study can be used as a benchmark for designing programs for proper handling of GTS and other tobacco-related diseases. Similarly, these can be helpful to develop monitoring and evaluation programs that would help in the generation of national-level data sets to counter environmental degradation and deforestation. Findings of the study are based on a single crop season; thus, these should be taken with a little caution and must be reaffirmed with a multi-seasonal or panel data study. The study is based on health-related data of tobacco leaf pickers. It is suggested that the impact of tobacco farming on health-related issues of whole farming households should be covered in future studies. Furthermore, symptoms of GTS incidence should be based on a clearly defined criterion of headache, nausea, and vomiting separately, instead of taking these as a whole.

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6. CONFLICT OF INTEREST

The authors declare unanimity in the findings of the study and description of the results.

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Research Article

Nested-PCR based Detection of Hepatitis C Virus: Low-cost Strategy in Pakistan

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Abstract: One of the most common blood-borne illnesses is hepatitis C virus (HCV), Hepatitis C is referred to as the inflammation of the liver and caused by the HCV virus, HCV is estimated to cause 53000 fatalities per year over the world. The majority of HCV-infected patients are unaware of their infection. No vaccine is available for HCV although Interferon is used to treat HCV but effective only 20-38 %, but at present, only a minority of infected persons have been tested and are aware of their diagnosis. The expense of testing may play a substantial role in patients' ability to get rid of the hepatitis C virus (HCV). Costs in many low- and middle-income nations, including Pakistan, force the development of novel and economically advantageous testing methods. The major aim of this study is about the effective diagnostic procedure for detecting Hepatitis C in the samples obtained from Balochistan, for this purpose the samples were collected from the health organization BINUQ (Balochistan Institute of Nephrology and Urology Quetta). Twenty (20) HCV antibodies positive patients in the Molecular Laboratory Department of Biotechnology were processed and then subjected to RNA extraction. cDNA was synthesized by reverse transcriptase enzyme. cDNA was used for qualitative analysis of HCV-RNA through nested PCR. According to the study, 09 samples were detected as positive and 7 samples were HCV negative out of 16 patients' samples. The findings of the present study show comparison of the price for HCV- RNA tests per sample from patients with hepatitis C at various labs. When compared to the other five PCR-based tests in the laboratory-conducted anti-HCV, HCV qualitative, quantitative, and genotyping tests, Shoukat Khanum laboratory reported the most expensive costs for HCV -RNA tests. Dow laboratory HCV-RNA test is comparatively lower than Shoukat Khanum laboratory, while reported lowest and most cost-effective test of Molecular Diagnostic laboratory for anti-HCV. So, our molecular tests for HCV- RNA detection and quantitation showed very good diagnostic and clinical performance over all five public health laboratories.

Keywords: Hepatitis C, Comparison Diagnostic Test, Polymerase Chain Reaction

1. INTRODUCTION

Hepa means "liver", Hepatitis abolishes liver cells and also sources of inflammation of the liver, liver cirrhosis and carcinoma originated from various factors, such as drugs, viruses and alcohols. Hepatitis C is a thoughtful lethal challenging all over the world. Hepatitis C virus (HCV) is a blood-borne pathogen mostly affected through skin contact with the unwell individual. Hepatitis C is a considerable public health challenge worldwide [1-4]. Physical conditions of Hepatitis C are yellowing of the skin, Enlargement of the liver and fluid in the abdomen. A, B, C, D, E, and G are the subtypes of the hepatitis C virus. These have an adverse effect on human livers and result in fatal infections. The hepatitis C (HCV) virus, which is the primary cause of (non-A non-B) hepatitis, was first discovered in 1980. It is an RNA virus and its complete genome has been recognized and sequenced [5]. HCV is a member of the flavivirus viral family.

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In Pakistan, the number of HCV-related chronic liver infections is increasing due to many associated risk factors. According to reports, around 60 % of Pakistani patients with liver cancer are anti-HCV positive [6] The incubation period of the HCV virus has ranging from 2 to 52 weeks.

According to HCV reported rate in the US national HCV prevalence from 2013 to 2016 was 0.93 % and varied by jurisdiction between 0.45 % and 2.34 % [7-8] The viral particle is mainly composed of a positive sense strand, single-stranded RNA genome with about 9,500 nucleotides, a nucleocapsid, and an RNA envelope made from host membranes into which virally encoded glycoproteins (E1 and E2) are inserted. Highly conserved untranslated regions (UTR) flank a sizable translational open reading frame that codes a polyprotein with 3,000 amino acids in the genome's 5 and 3 termini. [9 -10]. The burden of hepatitis C in Pakistan is the second-highest in the world. 80% fewer new cases of hepatitis C are expected by 2030, according to a strategy created by WHO to end the harm that it poses to public health [11]. Low- and middle-income countries (LMICs) are responsible for between 50 and 80 percent of the world's hepatitis C burden [12] due to high diagnosis rates of HCV. Hepatitis C can be caused by immune cells in the human body affecting the liver and enhancing autoimmunity, infections from bacteria and viruses like A, B, and C, parasites, liver damage from drugs like an acetaminophen overdose, which can be lethal. Hepatitis C positive patients can be screened by ELISA tests and more investigative approaches e.g. abdominal ultrasound, liver function test (LFT), etc. [13]. My research work emphasizes the collection of samples from the Balochistan Institute of Nephrology and Urology, Quetta (BINUQ) to validate the 'Hepatitis C' can be detected with an efficient and effective diagnostic test among the Balochistan population.

2. MATERIALS AND METHODS

2.1 Collection of Blood Samples

BINUQ Balochistan Institute of Nephrology and Urology Quetta Pakistan collected 20 blood samples from adult patients (18-50 years) for HCV-RNA detection. Blood sample (5 mL) was obtained from each subject in disposable sterile syringes. Then whole blood was shifted in the Molecular Lab, BUITEMS, Quetta, Department of Biotechnology. All patients signed or provided written informed consent forms. The expected time of infection and the patient's previous contacts were all included in the thorough history that was gathered. Exclusion criteria for this study was patients having any signs of infection disease causing other pathogens, such as HBV, HIV, or HDV were omitted from the study.

2.1.1 Confirmatory Tests/Diagnosis

Anti-HCV antibodies were detected in suspected acute hepatitis C patients using the serological assay for entirely patient samples. Further testing was done on the positive samples.

Twenty HCV-positive patients The Molecular Laboratory Department of Biotechnology processed the Ab tests. Nucleospin For extraction of the viral genome (extraction Kit Macherey-Nagel, Germany) with slight modifications was used.

2.1.2 Extraction of Viral RNA from Blood Samples

Following the manufacturer's instructions, 600 ul of lysis buffer with 150 ul of serum sample were used to extract the viral RNA. All of the samples were then incubated at 70 °C for 5–6 minutes in a water bath. After that 600 µL of pure alcohol was added to all samples. Collect the samples in the column tubes and centrifuged at 13000 rpm for one minute. RAW Buffer (500 µL)was added then again centrifuged for 6 minutes.After that, 200 µL RAV3 buffer was added to the samples, again cenntrifuged. After drying the samples,column tubes were incubated for 5 minutes at 70 °C.In the last and final step, 50 µl of elution buffer was added in colume tubes for RNA elutation for further processing.

2.2 Steps for Polymerase Chain Reaction (PCR)

For cDNA from extracted RNA Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies Inc., USA) was used HCV RNA was detected by amplification through PCR. The primers were synthesized from the (5'Untranslated region) of the HCV genome, which was described by Chen and Weck [14]. Primer3 plus computer program was used to design the primers (http://www.bioinformatics.nl/cgi bin/primer3plus/

primer3plus.cgi/).

RT PCR was performed using the following PCR ingredients as follows. The extracted RNA (10 µL) was amplified by using HCV RT mix (5X first strand buffer (It includes 200 U of the MMLV reverse transcriptase enzyme along with 50 mM Tris-HCl (pH 8.3), 7.5 mM KCl, 3 mM $MgCl_2$, 0.1 M DTT, and 10 mM dNTPs) and (1 µL) HCV-antisense primer (10 umol/µl). A final volume of 20 uL was used to conduct the RT-PCR experiments. PCR program was used for cDNA to be synthesized from extracted RNA for 50 min at a temperature of 37 °C with a primer specific for the core region and then for 3 minutes at 95 °C. It was spun down and kept at -20 °C until it was utilized for first-round PCR.

2.2.1 Nested Polymerase Chain Reaction of HCV cDNA

For qualitative investigation, the HCV 5′ UTR cDNA was amplified. In the first step (round) the outer sense and antisense primers were used in the PCR process, followed by the second round of nested PCR with inner primers. To perform the first round of PCR, the following PCR ingredients were added 20 ul of the PCR reaction mixture were inserted in a tube. Primers used during the study is given as exterior sense nucleotides sequence 695-718 (5'-CATGGTCCCAGCCTCCTCGCTGGC-3') and exterior antisense primer nucleotides sequences 873-896 (5'CCGCGAGGAGGTGGAGATGCCATG-3').

The cDNA RNA (2 µL) was amplified by using (16 µL) HCV PCR reaction mixture mix (100 uM of each of the four deoxynucleotides and 2.5 mM $MgCl_2$) (dNTPs), 0.5 µL forward primer (10 pM/ µl), 0.3 µl reverse primer (10 pM/ µl) and (1 µl) Taq DNA polymerase (200U). The overall volume for the PCR reactions was 20 µl.

For amplification, the thermocycler (BIORAD PCR I cycler version 3.021, USA) was programmed. Initial denaturation was done at 93 °C for 4 min, followed by 30 cycles, each of denaturation at 93 °C, 45 sec annealing at 55 °C, for 30 sec and 45 sec extension at 72 °C, with a final extension at 72 °C for 7 min. After completion of the first round, PCR bands were visualized on 2 % agarose gel. In the second round of nested PCR was carried out with the same reaction mix as taking the first round PCR as a template, but using different Inner sense and antisense primer sequences for amplification of PCR as given below (5'-CAACATTCCGAGGG GACCGT-3') and antisense primer (5'-GAAGGAAGGCCCTCGAGAACAAGA-3'). During the PCR, standard precautions were taken to avoid contamination. To check the cross-contamination negative control was also run in each round.

2.3 Agarose Gel Electrophoresis for Amplified Product Conformation

For conformation of PCR amplification, 2 % agarose gel was prepared in TBE buffer. According to the migration pattern of a 100-bp DNA ladder, the sizes of PCR products were determined (Fermentas Life Sciences). After the nested PCR 360 base pair was the size of the PCR products. Four of the twenty samples tested negative for HCV, while twelve tested positive.After qualitative analysis by PCR

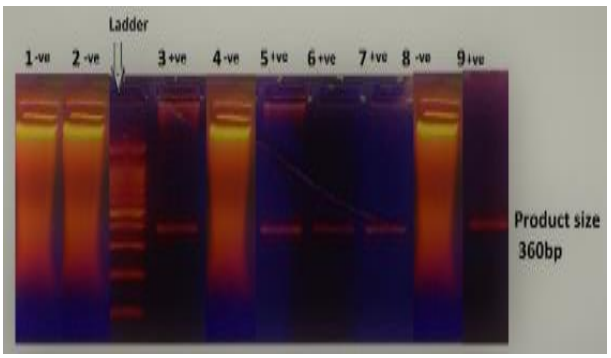

Fig. 1. Showing results of HVC on 2 % agarose gel.

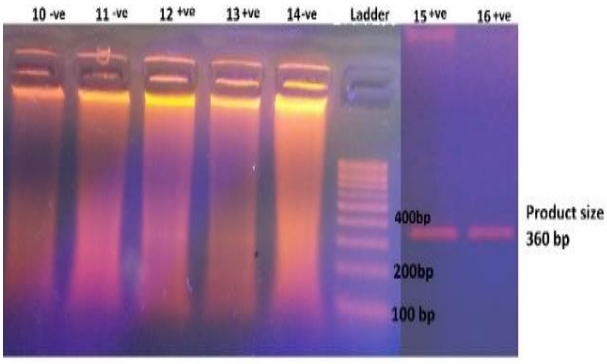

Fig. 2. Showing (HCV) RNA results after PCR amplification.

showing the figures 1 and 2.

3. RESULTS

To detect HCV-RNA, a total of 20 blood samples from adult patients (aged 18 to 50) were collected by the Baluchistan Institute of Neurology and Nephrology (BILNUQ) in Quetta, Pakistan. 5 mL of blood was extracted from each volunteer in disposable sterile needles. Anti-HCV antibodies were identified in all of the samples in patients having acute hepatitis C. HCV-RNA detection results in individuals. Table 1 shows a total 20 number of blood samples amongst 16 patients that tested positive for anti-HCV; 09 samples out of 16 were positive for HCV RNA. Following HCV RNA quantification, 16 samples with viral loads less than 500 IU/mL and 07 samples with viral loads greater than 500 IU/mL were examined.

Table 1. HCV-RNA detection in patients showing positive/negative PCR amplification.

Sample	PCR Results
1	PCR Negative
2	PCR Negative
3	**PCR Positive**
4	PCR Negative
5	**PCR Positive**
6	**PCR Positive**
7	**PCR Positive**
8	PCR Negative
9	**PCR Positive**
10	PCR Negative
11	PCR Negative
12	**PCR Positive**
13	PCR Negative
14	**PCR Positive**
15	**PCR Positive**
16	**PCR Positive**

3.1 Serological Assays

Antibody tests for HCV are used to check for previous exposure and present infection. And for diagnosing a hepatitis C virus (HCV) infection, therapy guidance, or monitoring for the treatment of HCV. It looks for antibodies to the virus, which indicates HCV infection. EIA screening an enzyme immunoassay (EIA) for anti-HCV immunoglobulin G is used to diagnose HCV (IgG). Whole genome of the HCV codes 3,011 to 3,033 amino acids for a polyprotein that is processed into 10 structural and nonstructural (NS) proteins [15]. This test will not tell you if you have an active or prior HCV infection.

There is some confirmation that a slightly positive test could be a false positive. According to the Centers for Disease Control and Prevention (CDC). An entirely positive antibody test should be followed by an HCV RNA test, which detects the presence of viral RNA in the blood to determine whether or not the subject is currently infected, The HCV antibody test can be used to establish which of the most prevalent hepatitis viruses is causing a person's symptoms as part of an acute viral hepatitis panel. The tests listed below can help identify an infection and suggest and monitor treatment:

RNA from HCV Qualitative tests are performed to determine whether an infection is present or past. If any HCV viral RNA is identified, the result is reported as positive or detected; otherwise, the result is reported as negative or not detected.

The quantitative test of Hepatitis C detects the number of international units of HCV RNA virus for one millimeter (mm) of HCV-positive patients' serum or plasma (IU/mL) [16]. This test can be used to check the virus's existence and detect a live infection. Quantitative assays (viral load) are used before and throughout treatments to compare the amount of virus present before and after treatment to measure cure response. HCV viral genotyping is used to define the genotype and kind of HCV present for assistance and monitoring of disease.

4. DISCCUSION

HCV is a single-stranded RNA virus that causes hepatitis C. RNA virus from the Flaviviridae family. HCV was initially recognized as Non-A, Non-B hepatitis virus in 1974 until the cloning of the etiologic agent in 1989. HCV infection affects about 3 % of the world's population (170 million people). 1.8 % of the population in the United States, or 3.9 million persons, are HCV seropositive. It is estimated that 10 million people in Pakistan are living with HCV infection [17]. Hepatitis C (HCV) is primarily transmitted by the use of contaminated needles in combination with a blood carrier after receiving infected blood from

impacted individuals. The majority of people are affected by sexual transmission in person, which can be extremely risky and lead to serious issues. [18-19]. Hepatitis C (HCV) virus is not transmitted by breast feeding, coughing, food, or water.

More than 50 subtypes have been identified, with genotype 1 accounting for roughly 75 % of occurrences in the United States. The medications chosen for treatment are influenced by the genotype of HCV viral infection. For the treatment of chronic viral hepatitis, molecular biology-based diagnostics are useful tools. They can be used to screen blood donations, diagnose active infection, determine prognosis, guide treatment options, and evaluate virological response to medication. The detection and quantification methods that usually are used in other labs are based on PCR and these are used in the management of hepatitis B and C virus infection. 20 serum samples from different individuals were evaluated simultaneously through active Anti- HCV.

The appearance of HCV RNA virus in plasma expresses active infection in patients and HCV-RNA can be detected 1 to 3 weeks post [20]. Different laboratories in Pakistan detect HCV-RNA. Due to the small amount of HCV- RNA in infected individuals, tests can only be performed using commercially available assay kits or in-house, home-made techniques in infected individuals. Reverse transcriptase enzyme (RT-PCR) are target amplification method for converting RNA to cDNA, which is then used as a template for the nested-PCR [21]. Primers whose sequences parallel to the 5′ untranslated regions (5′UTR) are commonly used because this is the most conserved region of the genome HCV-virus [22].

Table 2 shows an analysis to compare the price for HCV- RNA tests per sample in hepatitis C patients in different laboratories. Maximum expensive charges were reported from Shoukat Khanum laboratory for HCV -RNA tests when compared to other five PCR-based tests in laboratory-conducted Anti-HCV, HCV Qualitative, Quantitative and Genotyping tests. While on the other hand, average charges reported in the Alshifa laboratory were conducted for an HCV -RNA test for Anti-HCV (Rs. 2,300/-) Qualitative (Rs. 6,450/-) for Quantitative (Rs. 16,000/-and for Genotyping (Rs. 6,500/-). Dow laboratory HCV -RNA test is comparatively lower than Shoukat Khanum laboratory. The sample per-person test cost is highest for the Shoukat Khanum laboratory that has conducted HCV tests while reported lowest and most cost-effectiveness tests of the Molecular Diagnostic Laboratory for anti-HCV (Rs. 600/-). HCV - RNA for qualitative (Rs. 2000/-) for quantitative (Rs. 5000/-) and for Genotyping (Rs. 6000/-) per sample. So, our molecular tests for HCV- RNA detection and quantitation showed very good diagnostic and clinical performance over all five public health laboratories.

Daniel *et al*. 2005 described the RIA that was proposed for the recognition of anti-HCV antibodies. RIA proved 99.3 % sensitivity and 99.0 % specificity, individually [23]. In 2013, Firdaus *et al*. assessed the clinical effectiveness of RIA samples of HCV patients [24]. Around 15.74

Table 2. Shows analysis to compare the price for HCV RNA tests

Diagnostic Laboratories	Shoukat Khanum cost/sample	Agha Khan cost/ sample	Chughtai cost/ sample	Dow cost/ sample	Alshifa cost/sample	Molecular Diagnostic Lab (cost/sample)
Anti- HCV	2,100/-	2,400/-	2,200/-	800/-	2,300/-	600/-
HCV-RNA Qualitative	7,850/-	7,850/-	7,500/-	2,800/-	6,450/-	2000/-
HCV-RNA Quantitative	17,000/-	17,500/-	16500/-	5,500/-	16,000/-	5000/-
HCV- Genotyping	8500/-	8500/-	7000/-	7,500/-	6,500	6000/-

% of these samples were RNA-positive by nested RT-PCR, and 11.02 % were HCV seropositive by ELISA. Hence, the consequences of their study revealed that the RIA alone could not be relied on as an absolute diagnostic tool for screening HCV and besides this the nested PCR is a good implement for RNA detection [25] So, It is possible to successfully use the molecular diagnostic tests and reliable through different laboratories in Pakistan. In the HCV-RNA situation, our procedures can possesses high sensitivity for detecting infections and good specificity over all other five laboratories. It offers rapid, accurate screening and diagnostic testing that improves human understanding of the disease, provides earlier detection and reduces the time to diagnosis. We have developed a method for the qualitative analysis of hepatitis HCV- RNA virus, at a lower cost as compared to other labs. This will help to control practicing modern molecular tools for disease diagnosis and encourage/contribute in the area of molecular diagnostics. All these insignificant expanses of our tests are all ready to be marketed.

5. CONCLUSION

The finding of our study identified a useful method for the rapid qualitative detection of HCV infection by comparing it with the price for HCV- RNA tests per sample in hepatitis C patients in different laboratories. Although our procedures provide accurate, reliable screening and diagnostic testing for the detection of diseases hepatitis C virus at low rates as compared to other laboratories for prevention of the further spread of HCV. This study also suggested that a one-step ELISA-based HCV-RNA screening method as well as a two-step PCR-based test technique would be the most cost-effective options when compared to the other strategies.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors have no conflict interest.

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Research Article

Evaluation of Protective Clothing against Chemical and Fire Hazards

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Abstract: Workplace accidents are very unpredictable. Protection against these risks and hazards is of utmost importance in every profession. Clothing has always been considered one of the integral parts of personal protective equipment. The present study aims at manufacturing and evaluating protective clothing materials used by chemical workers. It was experimental. Two important resistance characteristics; chemical and fire were assessed against international performance standards. The results highlighted the fact that the right selection of construction parameters was able to make a safe fabric against such hazards. The resultant fabric was evaluated at various washing intervals and was found to be resistant to four selected chemicals through penetration and repellency index. It was also able to resist fire by passing the standard after flame and char length tests. Very little research work has been done in Pakistan regarding the manufacturing and physical assessment of clothing materials used by chemical workers. This study aimed to approach the target area where the protection and safety of the worker should be a priority.

Keywords: After Flame, Aramid, Char Length, Permeation, Repellency, Lamination, Weave, Washing

1. INTRODUCTION

Protective clothing is one of the important fields in technical textiles. It refers to any apparel that is used to protect against environmental hazards such as biological, chemical, or physical [1]. Chemical industry is one of the essential key holders to contribute to the economic growth and development of any country [2]. A wide variety of chemicals such as Sulfuric acid, Ammonia, Sodium hydroxide or Nitric acid, etc. having multiple characteristics are used in this sector for manufacturing different products. These chemicals may pose a great risk to the person dealing with them. The type of danger differs from chemical to chemical depending upon its nature. For example, some chemicals can create skin irritation, others may burn the body. So, it is always needed for the worker to wear a protective ensemble such as coveralls, gloves, and masks for safety purposes [3, 4].

Unfortunately, there is a lack of awareness among the staff and management to wear adequate protective clothing items [1]. It must be worn by personnel working with toxic substances at the workplace [5, 6]. Safety at work is a fundamental right of every employee which should not be ignored by higher authorities [7, 8]. To provide safety to the workers, it is necessary to manufacture an appropriate type of protective clothing against certain hazards. The right selection of textile materials should be made for making such clothing items, as these materials are supposed to offer great protection [9]. This type of clothing serves as a second skin for the wearer against many risks like fire, cuts, abrasives, hazardous liquids, or toxic substances [10]. There is always a margin for improvement in every sphere of business. The performance behaviour of such materials should always be assessed before use and requires innovation in the production of fibers, yarns, fabrics and finishes for better results [11].

Designing personal protective clothing should follow easy-to-care instructions for an end user to reduce the hazards related to self-contamination. This should be effective in terms of performance during the working hours of the wearer to give him/her protection against many hazards [12]. Quality

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of clothing material used for making protective coveralls also plays an important role in providing safety. It was found that poor-quality fabric worn out easily during wear and resulted in rips and holes at various areas of the coverall thus, was unable to protect the end user [13]. In another study, it was investigated that nine tested gowns out of twenty-two did not meet the international performance standards against liquid/fluid penetration. Many studies pointed out the fact that pre-marketing evaluation and post-market survey from the end user should be made for better protection [14]. A lot of research is needed to manufacture PPE which is breathable, comfortable yet functional that does not hinder the activities of workers at work place.

Chemical protective clothing must be taken as a protective measure and the last safety line for workers dealing with toxic substances and chemicals. Although protective gowns and other equipment cannot provide a hundred percent against all types of chemicals, as they vary in nature, severity, and toxicity [15]. It depends on the total amount of chemical penetration, permeation, repellency and breakthrough time. Thus, various kinds of polymers are being tried to bring the best suitable product to the ultimate consumer. There are generally four ways in which interaction can be made between a chemical and a protective ensemble. Firstly, in the process of chemical degradation where a breakdown of the substrate is made. Secondly in the form of penetration of specific chemicals where a chemical flow is being made through wicking or wetting action. Thirdly, permeation of chemicals in the form of molecular flow and fourthly in the form of vapours where a chemical reaches the garment [16,17].

There is very little research done on protective clothing used by chemical workers in Pakistan. This study aims at the manufacturing of clothing materials through a combination of natural and synthetic fibers. It also evaluates the efficiency of protection in terms of chemical and fire resistance after various washing intervals.

2. MATERIALS AND METHODS

The study was experimental in nature and completed in two sections. In the first phase, an experimental clothing material was manufactured and in the second phase, the performance behavior of manufactured fabric was assessed by following international standards.

Opening and cleaning was the first operation in the spinning process. The fibers were blown out through the ducts in a blow room where they were free from any residual dirt and dust. Then these fibers were straightened out to convert them into a sliver. The alignment of fibers was made in the combing stage. Short fibers were separated from long fibers and became parallelized. The twist was inserted into the yarn to make it strong and even. The rovings were stretched out to induce thickness to the prepared yarns. Finally, they were wound onto the bobbins.

Yarns were manufactured through ring spinning to weave a fabric. It consisted of two layers. An inner layer was made of a combination of polyester and cotton with a ratio of 60 / 40. It is the most common blend percentage used in making comfortable and breathable fabrics. This blend was chosen as cotton was a locally produced fiber and was the best choice to provide comfort to the wearer due to its inherent ability to breathe. Polyester content was used to add strength to the material. This blending ratio also helps to make the fabric wrinkle-resistant. The linear density was 17.653 in the tex system for warp and weft directions. The outer layer was composed of an Aramid fiber that has the inherent ability to resist fire. The two ingredients such as m-phenylenediamine and m-isophthalic acid were used to manufacture Meta Aramid fibers. The polymers were extruded through a spinneret at 120 °C. The obtained fibers were then washed, dried, and presented in highly crystalline form with a high modulus. The linear density was set at 15.023 in the tex system for warp and weft directions. Transol FL-20 chemical protector was applied over the surface of the prepared fabric to avoid chemical leakage through padding [18]. Liquid pick-up was 30-70 %. pH was between 4-8. The fabric was dipped in the solution and then squeezed completely. It was then dried for a few seconds at 120 °C. Curing was done for a minute at 155 °C. Plain weave structure was adopted in which each warp yarn was passed alternately above and under one weft yarn. Fabric density was 73 ends and 65 picks per inch. Fabric weight was 127 GSM. The length of the prepared fabric was 7 meters and the width was set at

19 inches in accordance with the width of the loom. It was desized and scoured to make it impurity free. Waxes and pectins were removed by using caustic soda. It was bleached to remove stains and to obtain better results.

In the second part of the study, the newly manufactured fabric was evaluated for its chemical and fire resistance properties after multiple washing intervals.

Resistance against chemicals was evaluated by following ISO 6530:2005 test method to know the rate of penetration and repellency of selected chemicals [19]. According to the standard, the fabric was tested against four chemicals i.e. Sulphuric Acid (diluted 30-aqueous), Sodium Hydroxide (diluted 30-aqueous), Xylene (concentrated), and Butanol (concentrated). In order to perform the test, (Fig 1) the test specimen was cut from the fabric with the dimensions of 360 mm / 235 mm. An absorbent sheet with the same dimensions was also cut. Both layers were weighed and turned a little bit along their length before placing them on a collector sheet. They were clipped to the gutter inclined diagonally at 45° where a beaker was put at its bottom to collect the liquid spilled from the test sheets. A dropper was filled with 10 ml of each liquid one by one and poured out at 100 mm for 8-10 seconds on the upper surface of a test sheet. Poured chemical that was not absorbed by the test sheets was dripped in a beaker. After a minute, both layers were separated and weighted. The amount of chemical solution that remained in the test solution was considered as the rate of retention. The amount of chemical retained by the collector sheet was considered as the rate of penetration and the amount of chemical collected in a beaker was the rate of repellency.

The rate of penetration was measured as the amount of chemical on an absorbent sheet divided by the amount of chemical retained by the test specimen and expressed as a percentage. The rate of repellency was evaluated as the amount of chemical in a beaker divided by the amount of chemical retained by the test specimen and expressed as a percentage.

Resistance against flame was determined by following ASTM D 6413-99 known as the Vertical Flame Test [20]. In order to perform this test, five test specimens were cut with dimensions of 3 x 12 inches. Care was taken to avoid any selvedge, creased or wrinkled areas. The samples were conditioned by following instructions stated in ASTM-D1776 [21]. The test specimen was fitted to a holder to maintain its position and exposed to the fire area. The distance between the burner and the specimen was kept at 19 mm. A stopwatch was set to note the time. The specimen was exposed for 10-12 seconds to the burner. The behavior was noted in terms of melting or dripping. The specimen was removed from the fire area. An after-flame time was

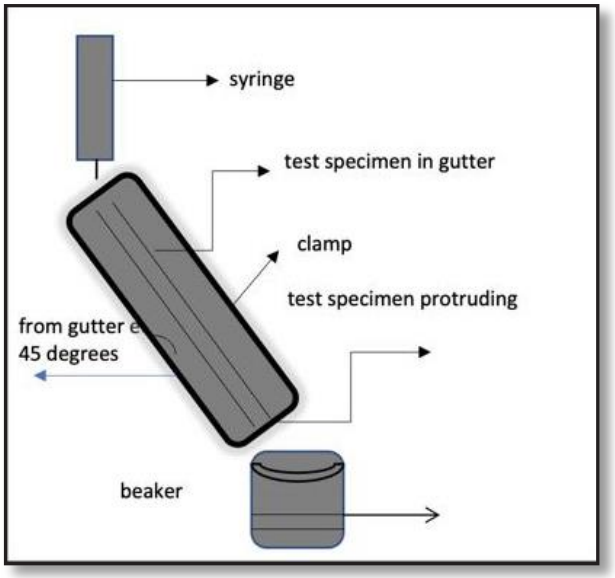

Fig 1. Determination of chemical resistance

recorded and char length was measured.

Washing of specimens was conducted by following Monograph M6 [22]. Front-load automatic machine was used with an agitation speed of 45 rpm. The temperature was maintained at 50-60 °C for 12 minutes. 0.1g/liter AATCC washing detergent was added to the washing cycle. Two rinsing cycles were given, and a final drying was made in tumble form at 65 °C for one and a half hours. The specimens were given a total of 9 washing cycles and evaluated after every 3 cycles.

3. RESULTS AND DISCUSSION

The collected data was recorded and analyzed through SPSS version 25. Mean values along with standard deviation were calculated. ANOVA was applied to analyze the variance at various washing intervals for chemical and fire resistance characteristics of the experimental fabric. P-value ≤ 0.05 was taken as significant.

Table 1 explains the chemical behaviour of fabric in terms of repellency and penetration index. It was observed that all tested chemicals penetrated between 0.00 % to 0.003 % through the test specimens at different washing intervals. Whereas the rate of repellency was more than 95% in each tested chemical at each interval of washing. Repellency rate of 95 % or more and a penetration rate of less than 5 % were considered acceptable criteria as per the guidelines of a test procedure.

Risks associated with chemicals are acute to chronic, depending on the toxicity and duration of exposure. To evaluate their resistance clothing materials must be assessed with the rate of penetration, permeation and repellency [6].

Table 2 describes the difference among each chemical after various washing intervals. The p-value stated as 0.003, 0.001, 0.001 and 0.000 respectively for each liquid chemical. It shows that there is no statistical difference among all tested chemicals after each washing interval against the criterion. The fabric remained stable up to 9 washes. One of the possible reasons is the right selection of polymer to resist chemical penetration through the fabric surface. George and Thomas [23] investigated in their study how kind of polymer can impact the permeation properties of an end product. Selection and ratio of blending fibers greatly affect the molecules to behave against certain hazardous substances and solutions [18]. Aramid fiber showed greater resistance against liquid chemicals. Meta and para-aramid are good options to respond against thermal hazards due to their strong structure. They can be used as the upper layer in protective clothing materials for various industries [24, 25]. Another reason is the application of an effective chemical finish that helped to retain up to 9 washes. Lamination and coating add good protection against chemicals, liquids, and fluids [26]. Good quality lamination or coating adheres to the surface of the material in a uniform way to avoid any leakage, on the other side poor finishing treatments crack away easily to permeate liquids through the surface [27]. Light weight clothing is a better option as compared to thick and heavy fabric. The physical burden adds to the weight of the fabric. It also decreases the effectiveness of clothing items and the performance of a worker [28].

An experimental fabric comprised of an inner and outer layer with the addition of lamination to provide better protection and safety to the worker against toxic liquids/chemicals. This fact is also highlighted by many researchers that multilayered

Table 1. Chemical penetration and repellency

Washing Interval	Sulphuric Acid		Sodium Hydroxide		Xylene		Butanol	
	P(%)	R(%)	P(%)	R(%)	P(%)	R(%)	P(%)	R(%)
0	0.02	96.35	0.02	97.28	0.00	98.25	0.00	97.63
3	0.02	96.12	0.02	97.11	0.00	98.11	0.00	97.01
6	0.03	95.33	0.02	96.98	0.01	97.15	0.01	96.51
9	0.03	95.01	0.03	96.15	0.01	97.12	0.01	95.89

Table 2. Tests of within-subjects contrasts for various chemicals

Source	Sulphuric Acid	Sodium Hydroxide	Xylene	Butanol
Washes	Linear	Linear	Linear	Linear
Mean Square	0.138	0.121	0.151	0.231
Frequency	17.897	45.754	74.321	22.781
p-value	0.003	0.001	0.001	0.000

materials are a better option for protective clothing as thick fabrics resist chemical permeation through its surface for a longer time as compared to single-layered or thin clothing materials [29-31]. Now-a-days multilayered materials are used for making protective clothing items. The textile layers with different functions/benefits are used against various types of hazards such as environmental, physical, thermal, or ballistic, etc. The added layers are also used to release the heat stress for physio thermal comfort [17].

Openness and closeness of the weave structure are also important factors to consider against the permeation behaviour of materials [32]. An increased number of open areas in the fabric structure presents a high rate of penetration and low rate of repellency. An inadequate manufacturing process can open the pores with each washing cycle and let the liquid pass through more readily [33]. Insertion of high twist in a material has a twofold function, inducing strength and making a compact structure. So, another important aspect to consider while making protective clothing [34]. The time of exposure to a chemical with a particular fabric structure and the nature of the chemical itself can either increase or decrease the associated risks [35].

Table 3 explains the rate of fire resistance in terms of after flame measured in seconds and char length measured in inches. According to the test method, the time for after flame should not exceed 2 seconds. One possible reason is the compact structure that supports less exposure to oxygen [36]. Aramid fiber has an inherent ability to resist fire as the polymer plays an important role in determining performance behaviour [37].

Some fibers such as nylon drip and melts upon exposure to flame and can cause severe dangers. It is a synthetic fiber made of petroleum as a major ingredient. It rapidly burns and shrinks away from fire. It produces black smoke and hazardous fumes around the environment. Aramid becomes swollen when interacting with fire and becomes thicker in its mass, thus providing resistance against fire hazards [1]. Char length should not exceed 4 inches; the obtained results fall within the acceptable range.

Chemical protective clothing should provide complete protection against flame, as it needs to provide at least a few precious seconds of escape to the wearer. Repeated washing must not affect the fire resistance characteristics of tested fabrics [38]. P-value 0.002 was recorded for after flame and 0.001 for char length which clearly explains that there is statistically an insignificant difference between experimental fabric and the standard criterion (Table 4).

Aramid fiber helps in minimizing the burning process and even laundering did not affect much

Table 3. Fire resistance of tested fabrics

	After flame (sec)		Char length (inches)	
Washing cycle	Mean	S.D	Mean	S.D
0	0.12	0.02	0.56	0.01
3	0.13	0.01	0.66	0.02
6	0.23	0.02	0.79	0.02
9	0.35	0.03	0.85	0.04

Table 4. Tests of within-subject contrasts for fire resistance

Source	After flame	Char length
Washes	Linear	Linear
Mean Square	0.024	0.213
Frequency	5.765	9.546
p-value	0.002	0.001

of their durability [37]. Multilayered fabric poses better protection against many mechanical and thermal hazards by proving self-extinguishing properties [25]. Lamination and coating on the surface of yarns and fabrics are better able to provide protection and safety to the wearer in response to many physical and chemical hazards. The fact was also supported by another research that the best coating of fibers plays an important role in presenting 100 % accuracy against fire hazards [39, 40].

In this world of technological advancements, aramid fibers (meta and para) are used in technical textiles for providing thermal protective clothing materials along with stable mechanical properties [41]. It is necessary to produce an outer layer of protective clothing with aramid fiber to protect against flames that may encounter the worker at the workplace. The aramid fiber is an inherently fireproof material that helps to protect the inner layer of fabric and save the skin of the wearer [42]. The structural behavior of fabrics has a strong impact on mechanical characteristics such as fabric mass, thickness, flexibility, elasticity, linear density, porosity, penetration, and permeation through them. Protective clothing materials are usually multilayered. Woven structures are followed by usual interlacing patterns such as plain, twill, satin, or sateen weaves along with a combination of non-woven layers. lamination and coating should be applied to give maximum protection against thermal, physical, biological, or chemical hazards [43, 44]. The composition of different blends makes the breathable and protective against thermal effects.

It can be said that additional layers give better protection against fire, chemicals, and certain other hazards. Weaving as a fabric construction technique not only provides a clothing material with protection against risks but also gives comfort to the wearer [45].

4. CONCLUSION

Workplace safety and protection are directly associated with the health and well-being of an employee. So, it should not be ignored. It is concluded from the current study that an appropriate type of clothing material can be able to protect against chemical and fire hazards. The results depicted that an experimental fabric made with multiple layers of cotton, polyester, Aramid, and lamination can assist in passing the required international standards against such risks. The construction parameters used for manufacturing the material were able to serve as a safety measure. The statistical analysis clearly explains that manufactured fabric passed the internationally accepted criterion against all tested four liquid chemicals. Moreover, the char length and after flame depicted that the experimental fabric can able to provide adequate safety against fire hazards. This research can assist textile manufacturers and technologists to alter their key indicators for manufacturing chemical protective clothing materials. Follow-up studies can focus on the evaluation of mechanical and resistance characteristics of clothing materials used for making chemical protective clothing. Moreover, future studies can be made to evaluate the performance behavior of protective items such as headgear, footwear, masks, or gloves.

5. CONFLICT OF INTEREST

There is no conflict of interest.

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Research Article

Prospects and Constraints of Onion Production and Marketing: A Case Study of District Tando Allahyar, Sindh-Pakistan

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Abstract: This study aimed to identify the existing onion production practices and constraints at smallholder farms and profitability of onion in the Tando Allahyar district of Sindh during the year 2018-19. The primary data were collected from 24 randomly chosen onion-producing farmers in village Ibrahim Shah using a pre-tested systematic questionnaire. Descriptive statistics were used to evaluate percentages, frequency, and mean, while the benefit-cost analysis (BCA) method was used to determine onion growers' profitability. Results of the study revealed that the overall average total cost and gross revenue of onion production were (Rs. 115120/acre, Rs. 154875/acre) respectively. The benefit-cost ratio was found to be 1.52 for (variable costs) and 1.35 for (total cost), indicating that onion cultivation benefits farmers in the Tando Allahyar district. The most common and local onion variety grown was Nasarpuri, which was cultivated by 87 % of farmers, along with Hazari and Phulkara. However, the farmers were reluctant to use hybrid seeds due to their inability to withstand high temperatures. The main production constraints for onion growers were high input prices, particularly seed costs, and high labour charges for hoeing and weeding. Similarly, low product prices and high transportation costs were major marketing constraints. Due to a lack of technical expertise with current technology, such as a shortage of cold storage and skilled labor, the marketing of excess produce throughout the year was a significant problem for onion growers. It is therefore suggested that a comprehensive marketing capacity building and Global GAP will improve the quality, productivity and profitability of the farmers and open the channel of export for Pakistan.

Keywords: Onion Profitability, Production & Marketing, Constraints, Tando Allahyar

1. INTRODUCTION

The onion (*Allium cepa L.*) is a monocotyledonous crop that belongs to the Amaryllidaceae family. The onion has been a valuable vegetable crop for people all across the world from ancient times. Onions are seen in an Egyptian mural from around 3000 BC, so it may be concluded that onions were a key food source for Ancient Egyptians. The word "onion" is derived from Latin and means "large pearl." The onion was compared to a pearl not only because of its shape but also because of its high nutritional value [1]. The onion is a widely grown and popular vegetable crop around the world. It is one of the top 15 most widely produced vegetables on the planet [2]. The cultivated onion is one of the most extensively used spices in all households. It is grown in over 170 countries throughout the world and more than 90 % of onions are consumed in the countries where they are grown [3]. Overall onion production in Pakistan is rising; it increased by 35 % from 1.70 million tonnes in 2010 to 2.30 million tonnes in 2021. The major onion-producing districts are Khanewal, Rajanpur, Bahawalpur, Vehari, and Rahim Yar Khan in Punjab; Mirpur Khas, Umerkot, Jamshoro, Sanghar, Matiari, Shikarpur, Nawabshah, Ghotki, Noushero Feroz and Hyderabad in Sindh; Swat, Dir, and Malakand in Khyber Pakhtunkhwa and Nasirabad, Khuzdar, Awaran, Mastung, Kalat and Chaghi in Balochistan [4]. About 104.55 million tonnes of onions were produced worldwide, and Pakistan ranked sixth with a 2.03 % production share. The other seven top onion-producing nations with the production share were India (25.57 %), China (22.69 %), USA (3.65 %), Egypt

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(3.02%), Turkey (2.18%), Iran (1.97%), Bangladesh (1.87 %), Sudan (1.66 %), and Russian Federation (1.74 %) [3].

Onion yield is known to be influenced by a variety of factors such as cultural practices, growing environment, insect pests, and diseases [5]. Poor transportation, a large number of middlemen, inadequate storage facilities, and a lack of farmer's organizations are some of the issues that prevent efficient onion marketing in the research area [6]. The lack of adequate storage facilities, high transportation costs, and market price fluctuations were all major issues in onion marketing [7]. Instability in onion production was caused by erratic weather, variable market prices, and a lack of suitable storage and market facilities, which prevented farmers from making the best decisions on area allocation and increasing farm productivity [8]. To maximize yield, onion seeds are often sown in a nursery and transplanted with a 15 cm row-to-row spacing and a 7.5 cm plant-to-plant spacing [9]. Because onion production is proportional to yield per hectare and area, efforts must be made to boost yields through the use of modern technologies and higher-quality seeds, fertilizers, and other inputs [10]. Onions are generally grown traditionally, with technology passed down from generation to generation. As a result, the focus should be placed on increasing production by replacing traditional processes with more advanced technologies [11]. *Thrips tabaci* (L.) is a significant pest of the crop whose control is necessary for the growth and profitability of onions. Damage from onion thrips can lower bulb output by 23-85 % if they are not removed. It attacks a variety of vegetable crops, inflicting significant economic harm to onion crops. Young leaves are usually chosen, but buds and blossoms are also affected [12]. The key issues of onion production are the absence of HYV onion seed at the appropriate time, onion growers' lack of technical expertise, high prices throughout the cultivation period, and the non-availability of certified fertilizer at the appropriate time [13]. The use of weedicides, better planting and harvesting tools, and other labor-saving techniques are all urgently needed due to the high cost of labor [14].

Tando Allahyar is one of the most fertile agricultural areas in the country. Farmers are crucial to the production of vegetables. Like other cash crops, onions are grown across a wide area, and this district has some of the highest yields in the province. Given the importance of onions in terms of crop output and area, the research was conducted in the Tando Allahyar district with the following specific goals in mind: (i) to identify the existing onion production practices and constraints at smallholder farms; (ii) to find out the costs of onion production and profitability at selected project sites; and (iii) to explore the marketing issues and opportunities of onion growers.

2. MATERIALS AND METHODS

2.1 Description of the study area

This research was conducted in the village Ibrahim Shah of Tando Allahyar district in Sindh province. Tando Allahyar is located 25 miles (40 kilometers) northeast of Hyderabad and serves as a railway hub for the Sindh region (Fig.1). According to the 2017 census, it is the 56th largest city in Pakistan in terms of population. The primary languages spoken are Sindhi, Siraiki, and Urdu. Mangoes are the most popular plantation in Tando Allahyar, and a vast variety of mangoes are grown here. It is one of the most agriculturally productive places in the country. Sugarcane, wheat, onions, and cotton are among the best yielding cash crops grown in the province.

2.2 Sample and sampling size

The sampled area was selected for the study due to a major onion growing area. MOJAZ Foundation (a local NGO), which was a partner in the project titled “Strengthening Vegetable Value Chains in Pakistan (SVVCP), has randomly selected 24 onion-producing farmers at the village, Ibrahim Shah, Tando Allahyar district of Sindh province. The social scientists conducted in-person interviews with the chosen onion farmers.

2.3 Data collection

The study is entirely based on primary data of the crop year 2018-19, which was gathered through personal interviews with chosen respondents using a pre-tested structured questionnaire to obtain the necessary information. Comprehensive data was gathered on production costs, ranging from land

Fig.1. Map of the study area (District Tando Allahyar, Sindh)

preparation to harvest, as well as the materials required in the process. Cultivation costs include both variable and fixed expenditures. Nursery preparation, land preparation, transplanting, farmyard manure, fertilizers, irrigation, plant protection material, and its application, picking, and operation-wise labour utilization are considered variable expenses, whereas land rents are considered fixed costs. Several questions about production and marketing difficulties that farmers face are also included in the questionnaire. Future crop development recommendations and grower appraisals of prospects are also sought.

2.4 Data analysis

The information gathered included both qualitative and quantitative information. The percentages, frequency, and mean were analyzed using descriptive statistics [15]. To achieve the study's aims, the obtained data was edited, summarized, tabulated, and evaluated. Onion production profitability was investigated using gross margin, net return, and benefit-cost analysis [16].

To estimate the cost of onion production, the following equations were used:

$$VC = \sum (X_i P_i)$$
$$TC = TVC + TFC$$

Where,

TC = Total cost of production (Rs. /acre)
TVC = Total variable costs (Rs. /acre)
TFC = Total fixed costs (Rs. /acre)
Xi = Quantity/Number of inputs per acre
Pi = Price of inputs (Rs. /acre)

To estimate the profitability of onion production, the following equations were used:

$$GR = \sum Y_i P_i$$
$$NR = GR - TC$$
$$GM = GR - VC$$

Where,

GR = Gross return (Rs. /acre)
NR = Net return (Rs. /acre)
GM = Gross margin (Rs. /acre)
Yi = Quantity of output (Kg/acre)
Pi = Price of onion (Rs. /kg)

Benefit-cost ratio (BCR)

The benefit-cost ratio (BCR) is a metric that quantifies the relationship between the financial costs and benefits of a project. It is determined by dividing total revenue by total expenditures.

A higher BCR indicates a higher rate of return on investment. If the BCR exceeds the cost, the project is considered a profitable investment. The benefit-cost ratio was calculated using the following formulas.

When we use the total cost,

$$BCR = TR/TC \quad (1)$$

When we use the variable costs,

$$BCR = TR/VC \quad (2)$$

3. RESULTS AND DISCUSSION

3.1 World onion production status

Around 170 countries cultivate onions for domestic use, with some also growing onions for trade. On a global basis, around 5.48 million acres of onions are harvested each year, with just 8 % of this production being traded internationally. The top-ten producing countries contribute 67 % of global production, with Pakistan ranking sixth while India, China and the United States of America produce about 54 % of the world's total onion production followed by Egypt, Turkey, Pakistan, Iran, Bangladesh, Sudan, and the Russian Federation. The world's highest yield per hectare is in the United States of America, followed by Iran and Egypt (Table 1) [3].

3.2 Pakistan onion production status

During 2020-21, Pakistan produced approximately 2305.7 thousand tonnes of onion on 153.8 thousand hectares of land which is higher than in the past, and the production of onion increased by 8.7 percent over the last year [17]. However, the average yield per hectare is still lower than in the past. The main causes of low yield per hectare are low planting density, insufficient fertilizer use, weed infestation, poor seed quality, and low output prices. Similarly, the absence of plant protection measures exacerbates the situation by lowering yields and lowering quality. Pakistan's climatic conditions have a significant impact on onion farming and despite seasonal changes; the overall acreage and production of onions in Pakistan have increased during the last 12 years (Table 2).

Table 1. Leading onion-producing countries in the world

Rank	Country	Area (Million Hectares)	Production (Million Tonnes)	Yield (Tonnes/ha)
1.	India	1.434	26.738	18.6
2.	China	1.085	23.724	21.9
3.	United States of America	0.054	3.821	70.8
4.	Egypt	0.089	3.156	35.5
5.	Turkey	0.07	2.280	32.6
6.	Pakistan	0.148	2.122	14.3
7.	Iran	0.053	2.064	38.9
8.	Bangladesh	0.185	1.954	10.6
9.	Sudan	0.106	1.950	18.4
10.	Russian Federation	0.06	1.738	29.0

Source: FAOSTAT, 2020

Table 2. Status of onion in Pakistan (2010-2021)

Year	Area (000,ha)	Production (000,tonnes)	Yield (tonnes/ha)
2009-10	124.7	1701.1	13.6
2010-11	147.6	1939.6	13.1
2011-12	129.7	1691.8	13.0
2012-13	126.0	1660.8	13.2
2013-14	143.9	1740.2	12.1
2014-15	130.5	1671.0	12.8
2015-16	136.0	1736.5	12.8
2016-17	137.9	1833.3	13.3
2017-18	149.0	2115.2	14.2
2018-19	148.4	2076.0	14.0
2019-20	148.2	2122.0	14.3
2020-21	153.8	2305.7	15.0
Average	**139.6**	**1882.8**	**13.5**

Source: Agricultural Statistics of Pakistan, 2020-21

3.3 Province-wise onion yield status

Table 3 shows the onion yield by province based on ten years of average data (2010-2021). Balochistan has the highest annual onion output, averaging 18.0 tonnes per hectare, followed by Khyber Pakhtunkhwa, Sindh, and Punjab, which had yields of 16.9, 13.0, and 8.7 tonnes per hectare, respectively [17].

3.4 Province-wise share of onion production, yield, and area

Onion is an important crop in all continents with a world area of about 5.48 million hectares and a production of 104.55 million tonnes per year respectively [3]. In Pakistan, when compared to the previous year, there has been a slight decrease in the area but an increase in onion production. In terms of area and production, Sindh has the highest proportion of onions. The average shares of the provinces in the overall area and production are given in Table 4.

3.5 Socio-economic characteristics of onion growers

Farmers' socio-demographic characteristics have a significant impact on farm performance. Age is a very important demographic factor that shows the ability to do work and willingness to make progress and an attitude towards various social and economic aspects of life. Table 5 shows that the average age of farmers was 36.5 years, implying that the majority of farmers in the study area were youths. However, the literacy rate among farmers was extremely low, with the majority (54.2 percent) of onion growers in the study area being illiterate, with an average formal education of 4.9 years. In agriculture, experience is crucial for making the best use of resources, and onion growers had an average of 13.9 years of farming experience. Household size is one of the most important indicators of a farmer's social status, which influences his or her ability to adopt modern farming practices. In the study area, it was discovered that the majority of onion growers had a large family size (12.5 people). The size of a farm has an impact on the efficient use of resources and cropping patterns; 20.8 % of sampled farmers were tenants with no land of their own, while 45.8 % had farms of less than 5 acres, with an average farm size of 4.96 acres. The majority of respondents (67.7 percent) were living in a joint family system, while farming is the mainstay of the people living in the rural areas. Similarly, the

Table 3. Province-wise yield of onion in Pakistan (2010-2021)

(Yield in tonnes per hectare)

Year	Punjab	Sindh	Khyber Pakhtunkhwa	Balochistan
2009-10	8.0	13.4	17.0	18.7
2010-11	8.2	13.6	16.5	18.4
2011-12	8.4	13.3	16.6	18.2
2012-13	8.7	13.9	16.6	18.1
2013-14	7.7	9.4	16.7	21.0
2014-15	8.6	14.0	16.8	18.0
2015-16	6.8	12.9	15.1	18.9
2016-17	8.1	12.1	17.0	18.5
2017-18	7.1	12.4	18.5	14.4
2018-19	8.1	13.6	15.3	14.9
2019-20	10.3	13.5	17.6	19.2
2020-21	14.1	13.5	18.5	17.2
Average	**8.7**	**13.0**	**16.9**	**18.0**

Source: Agricultural Statistics of Pakistan, 2020-21

Table 4. Share of onion acreage and production by province (2020-2021)

Province	Area		Production	
	(000, ha)	(% share)	(000, tones)	(% share)
Punjab	41.7	27.1	587.6	25.5
Sindh	61.2	39.8	826.7	35.9
Kyber Pakhtunkhwa	11.8	7.7	219.3	9.5
Baluchistan	39.1	25.4	672.2	29.2
Pakistan	**153.8**	**100.0**	**2305.7**	**100.0**

Source: Agricultural Statistics of Pakistan, 2020-21

majority (83.3 percent) were full-time farmers while others engaged in occupations aside from farming, such as laborers (12.5 percent) and traders (4.2 percent) respectively.

3.6 Varieties planted by onion growers

Table 6 shows that 87.5 % of onion growers in the study area grew Nasarpuri, followed by Hazari (8.33 percent), and Phulkara (4.6 percent). Due to high consumer demand, as well as high yielding and heat resistance, growers preferred the Nasarpuri variety for cultivation in the study area.

3.7 Onion growers' primary seed sources

Table 7 presented that 37.5 percent of sampled farmers purchased onion seeds from a local market, followed by 29.2 % who obtained seeds from their harvest, and 33.3 % percent who purchased onion seeds from co-farmers in the study area.

3.8 Onion growers' planting and harvesting schedules

Planting time has a big impact on crop yield and growth. The onion crop is sensitive to temperature and rainfall, and the farmers in this study grow onions during the Kharif (rainy) season. Table 8 depicts the study area's nursery sowing, transplanting, and harvesting months. Farmers planted onions in May and June, transplanted them in July and August, and harvested them from October to December. Farmers harvest onions in December to get better prices when market prices for onions are low.

3.9 Onion growers' information sources

The main information sources used by the sampled farmers are depicted in Table 9. More than half of

Table 5. Socio-economic characteristics of onion growers

Variables	Categories	Frequency	Percentage	Mean
Age (years)	≤ 30	08	33.3	36.5
	31-40	11	45.8	
	41-50	02	8.3	
	Above 50	03	12.5	
Educational Level	Illiterate	13	54.2	4.9
	Primary School	02	8.3	
	High School	04	16.7	
	Graduate	05	20.8	
Farming Experience (yrs)	≤ 10	13	54.2	13.9
	11-20	06	25.0	
	Above 20	05	20.8	
Household Size (no.)	≤ 5	05	20.8	12.5
	6-10	07	29.2	
	Above 15	12	50.0	
Farm Size (acres)	Nil	05	20.8	4.96
	≤ 5	11	45.8	
	6-10	04	16.7	
	10 -15	03	12.5	
	Above 15	01	4.2	
Family Type	Joint Family	16	66.7	-
	Single Family	08	33.3	
Occupation	Farming	20	83.3	-
	Laborer	04	16.7	

Source: Field survey data, 2019

Table 6. Percentage distribution of onion growers by variety planted

Onion Varieties	No. of Farmers	Percentage
Nasarpuri	21	87.5
Hazari	02	8.33
Phulkara	01	4.16
Total	**24**	**100**

Source: Field survey data, 2019

Table 7. Percentage distribution of onion growers by seed sources

Source of Seeds	Frequency	Percentage
Own seed	7	29.2
Fellow farmers	8	33.3
Local market	9	37.5
Total	**24**	**100.0**

Source: Field survey data, 2019

the farmers (58.3 %) indicated that they get their information from fellow farmers for agricultural and marketing purposes. Pesticide companies and middlemen were also significant sources of information for the farmers, accounting for 25 % and 16.7 % of the total, respectively.

3.10 Economic analysis of onion production

All forms of costs, such as fixed and variable costs, are included in the cost of production. The level of production/output affects these variable costs. They rise in response to rising output and fall in response to falling output. Fixed costs do not fluctuate in response to changes in output. They only change when the output increases significantly.

3.10.1 Variable costs

Variable costs account for the vast majority of the total cost of any agricultural production system. This includes expenses such as land preparation, seed or seedlings, plant protection materials, manure and fertilizers, harvesting, and labour. All of the inputs were calculated on a per-acre basis. Total variable costs (Rs. 102120/acre) accounted for 88.7 % of the total cost of production, while fixed costs (Rs. 13000/acre) accounted for 11.3 percent. The total cost of production includes land preparation (6.91 percent), seed and sowing operations (9.27 percent), farmyard manure (4.35 percent), fertilizers (12.35 percent), hoeing and weeding (8.95 percent), pesticides and insecticides (5.61 percent), irrigation (7.83 percent),

Table 8. Percentage distribution of onion growers by planting & harvesting months

Months	No of Farmers	Percentage
Nursery Sowing		
May	6	25
June	18	75
Total	**24**	**100**
Transplanting		
July	14	50.0
August	10	41.7
Total	**24**	**100**
Harvesting		
October	10	41.7
November	10	41.7
December	04	16.7
Total	**24**	**100**

Source: Field survey data, 2019

accounting for 25% and 16.7% of the total, respectively.

Table 9. Percentage distribution of onion growers by sources of information

Sources of Information	Frequency	Percentage
Fellow farmers	14	58.3
Pesticides companies	06	25.0
Middle-men	04	16.7

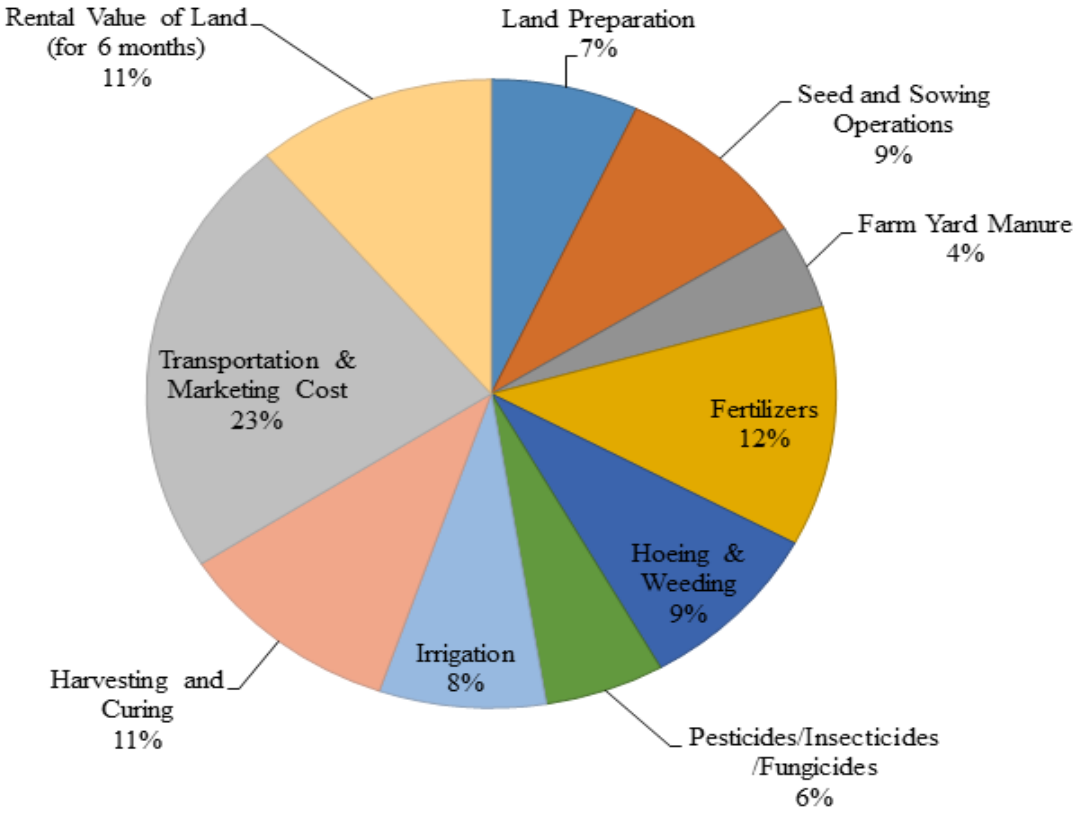

Fig. 2. Percentage distribution of production cost (Rs. /acre) by ingredients Source: Field survey data, 2019

transplanting (5.06 percent), harvesting and curing (10.64 percent), and transportation and marketing cost (22.80 percent). Farmers' highest cost of cultivation was Rs. 14215/acre for fertilizer purchases, followed by Rs. 12250/acre for harvesting and Rs. 10674/acre for seed and sowing operations (Table 10).

3.10.2 Fixed costs

The major component that was considered a fixed cost was the rental value of owned land. Fixed costs are costs that are not affected by the quantity of goods or services produced. They are usually time-related, such as taxes and rent that are paid once a year. Variable costs, on the other hand, are volume-dependent. The average fixed cost of onion production was Rs. 13000/acre in land rent (Table 10).

3.10.3 Total cost

The entire cost of producing onions includes both fixed and variable expenses. The total average cost to produce an acre of onions was Rs. 115120. The average fixed cost was 11.3 %, and the average variable cost made up 88.7 % of the overall cost of producing onions. In per acre onion production, variable costs were eight times higher than average fixed costs (Table 10).

3.11 Gross return, gross margin, net return, and benefit-cost ratio

Table 11 revealed that the total revenue per acre was Rs. 154875 whereas; the total cost amounted to Rs.115120/acre. The average onion yield in the study area was 8260 kg/acre (20.4 tons/ha), which was nominally less than the potential yield (22 tons/ha). The gross margin was found at Rs. 52755/acre while the net return on the total cost per acre was found at Rs. 39755. The gross profit margin was 34 %, which means that for every rupee generated, Rs. 0.66 would go into the cost of goods sold while the remaining Rs. 0.34 would be used to pay back expenses, taxes, etc. The benefit-cost ratio over variable cost was found to be 1.52 and the over total cost was found to be 1.35, indicating that the

onion crop is a profitable enterprise for the farmers and emphasis should be laid on overcoming the constraints impeding the growth of the enterprise. Other researchers discovered similar results with benefit-cost ratios of 1.30 and 1.43 respectively [18, 19].

3.12 Problems and constraints of onion production

The onion growers face some problems with the production and marketing of onion in the study area. To identify the relative importance of the problems and constraints in onion production and marketing, the Simple Ranking Technique has been applied. All the 24 sampled farmers were asked to assign ranks to these problems in order of importance. Each farmer was instructed to indicate the importance of the problem by giving Rank-I to the most important problem, rank II to the second important one, and so on.

3.12.1 Production constraints

During onion cultivation, the farmers in the study areas encountered a variety of issues. To examine the production constraints, eight factors are taken into consideration. Table 12 (a) shows that three major production constraints are high prices of seeds and fertilizers, high labor costs, and weeds mentioned by sampled farmers representing 83.3 %, 79.2 %, and 62.5 % respectively. The other problems faced by onion growers were infestation of insects/pests and diseases, lack of storage facilities, availability of quality seed, lack of technical knowledge, and availability of canal water.

3.12.2 Marketing constraints

The producers' top eight marketing constraints have been identified and presented in Table 12 (b). The problems were ranked based on their priority. The three major marketing constraints are low prices of onions, high charges for transportation, and lack of market information indicated by onion growers representing 95.8 %, 70.8 %, and 54.2 % respectively. The other problems were high packing material cost, exploitation by a middleman, perishability of the product, lack of markets, and transportation problems.

Table 10. Costs of onion production (Rs. /acre)

S. No.	Operations/Inputs	Cost /Acre	Percent
A	**Variable costs**		
1	Land preparation	7956	6.91
2	Seed and sowing operations	10674	9.27
3	Farmyard manure	5007	4.35
4	Fertilizers	14215	12.35
5	Hoeing & weeding	10306	8.95
6	Pesticides/insecticides/fungicides	6458	5.61
7	Irrigation	9010	7.83
8	Harvesting and curing	12250	10.64
9	Transportation & marketing cost	26244	22.80
	Total variable costs (item 1 to 9)	**102120**	**88.71**
B	**Fixed costs**		
1	Rental value of land (for 6 months)	13000	11.29
	Total fixed cost	**13000**	**11.29**
C	**Total cost of production (C= A+B)**	**115120**	**100.00**

Source: Field survey data, 2019

Table 11. Profitability of onion production (Rs. /acre)

S. No.	Particulars	Mean
A	Total cost (Rs./acre)	115120
B	Total variable Cost (Rs./acre)	102120
C	Returns/yield (kgs/acre)	8260
D	Average sale price (Rs./Kg)	18.75
E	Gross returns (Rs./acre) **(C*D)**	154875
F	**Gross margin** (Rs./acre) **(E-B)**	**52755**
G	Gross profit margin (%) **(E-B/E)*100**	34 %
H	**Net return** (Rs./acre) **(E-A)**	**39755**
I	**Benefit-cost ratio** (over variable cost) **(E/B)**	**1.52**
J	**Benefit-cost ratio** (over total cost) **(E/A)**	**1.35**
K	Unit cost of production (Rs./kg) **(A/C)**	13.94
L	Profit (Rs./kg) **(D-K)**	4.81

Source: Field survey data, 2019

Table 12 (a) Onion production constraints faced by onion growers

Production Constraints	Respondents (N=24)		
	Number	Percentage	Rank
High price of seed and fertilizer	20	83.3	1
High labor cost	19	79.2	11
Weeds	15	62.5	111
Infestation of insect/pest and diseases	12	50.0	1V
Lack of storage facilities	10	41.7	V
Availability of quality seed	9	37.5	VI
Lake of technical knowledge	7	29.2	VII
Availability of canal water	7	29.2	VIII

Source: Field survey data, 2019

Table 12 (b) Onion marketing constraints faced by onion growers

Marketing Constraints	Respondents (N=24)		
	Number	Percentage	Rank
Low price	23	95.8	1
High charges of transportation	17	70.8	11
Lack of market information	13	54.2	111
High packing material cost	12	50.0	1V
Exploitation by brokers/middlemen	10	41.7	V
Perishability	10	41.7	VI
Lack of markets	9	37.5	VII
Transportation problems	2	8.3	VIII

Source: Field survey data, 2019

4. CONCLUSION & RECOMMENDATIONS

Pakistan ranked sixth in terms of onion cultivation with about 148.2 thousand hectares and contributes 2.7 % of the onion area and 3.1 % of onion production worldwide. Sindh produces 36 % of the nation's total onion output, followed by Balochistan and Punjab with 29 % and 26 %, respectively. Onions make up less than 10 % of all exports from Pakistan, indicating a large amount of untapped potential.

The majority of farmers grew native varieties, with Nasarpuri being the most common in the study area. Onion growers avoided using hybrid varieties because these varieties were less heat resistant. Despite the outstanding onion yield in the study area, the farmers' ability to keep a high-profit margin was limited by the lower output prices. According to the benefit-cost ratios of 1.52 (for variable costs) and 1.35 (for fixed costs), indicated that onions is advantageous for the farmers in Tando Allahyar district of Sindh.

Production and marketing are difficult for onion growers and the marketing of onions was also hampered by low product prices, high transportation expenses, and a lack of market knowledge. Similarly, weed infestation, labor costs, and fertilizer costs were all mentioned as significant productivity challenges. Furthermore, the growers had no interaction with extension agents and were reliant on the information and advice of other farmers and pesticide agents. The following recommendations can be made based on the study's findings:

1. Market pricing uncertainty contributes to regional instability; the government should intervene to alleviate it.
2. New hybrid varieties that can thrive in hot climates and are pest resistant should be developed.
3. Onion growers must be trained in storage facilities to improve their technical expertise.
4. Comprehensive capacity building program on marketing and Global GAP standards will improve productivity and profitability of the farmers by fetching high prices in the market and leads to export oriented product for Pakistan.
5. Developing smart value chain for onion producers coupled with global market integration will increase efficiency and ultimately profitability.

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6. DECLARATION

The study findings are original; the same material is neither published nor under consideration elsewhere, and if the article is approved for publication, its copyright will be assigned to the Pakistan Academy of Sciences.

7. CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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