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Pakistan Academy of Sciences

Review Article

Solar Energy Potential in Pakistan: A Review

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Abstract: This review paper focuses on the potential of solar energy and its applications in addressing the energy crisis in Pakistan. Currently heavily reliant on non-renewable sources, Pakistan faces severe power shortages and lacks access to electricity in many rural areas. The paper highlighting its geographical position and the availability of solar radiation. The review emphasizes the need to harness the vast solar energy resources available in the country. It was discovered that over 100,000 MW of electricity from sunlight could be generated, particularly in regions within the Sunny Belt. The annual average daily global radiation of Pakistan is: horizontal (GHI) - 16 MJ/m^2, oblique (GTI) - 13 MJ/m^2. The minimum annual average daily GHI in Pakistan is higher than the world average annual average daily GHI. At the same time, in Pakistan, thermal power generation accounts for most of the energy production (61% in 2020, 63% in 2021, and 61% in 2022), while renewable energy sources account for the least (3% in 2020, 2% in 2021, and 3% in 2022). It emphasizes the need for research and development, as well as policy support, to promote the adoption of solar energy technologies. In conclusion, this review paper sheds light on the significance of solar thermal energy as a renewable and sustainable solution for Pakistan's energy dilemma. It underscores the need for increased investments, technological advancements, and policy interventions to harness the immense solar energy potential and pave the way for a greener and more energy-secure future in Pakistan.

Keywords: Solar Energy, Solar Resources, Renewable Energy, Solar Reflector, Pakistan.

1. INTRODUCTION

Oil, gasoline, and coal are the main energy sources and are very commonly used [1, 2] and extremely negatively affect the ecological situation [3]. Oil is used extensively in industry, but the "oil-age" is coming to an end [4, 5]. Oil reserves will only endure for 35 years if current industrial and household use trends continue. There are still 37 years of natural gas' age and 107 years of coal's age left, respectively. Therefore, the only fossil fuel that will still be available after 2042 is coal reserves [6]. In terms of fossil fuels, they help meet 85% of the global energy demand. This causes an increase in CO_2 emissions, which contributes to global warming [7]. Pakistan's entire reliance on hydal power generation has made the energy crisis there critical. Three significant hydal power plants Terbela, Mangla, and Ghazi Brotha are currently producing energy, but their output is far less than the nation's rising need for it. Given the current energy problem, renewable energy sources need to receive more attention [8, 9]. So far as non-renewable energy sources are less ecological and and will run out sooner or later, technologies for sustainable development will become more and more important. This is pushing society to start developing renewable energy technologies now in order to prevent potential problems from arising [10, 11].

Moving toward renewable energy is the greatest way to tackle the energy dilemma. The application of renewable energy technologies can meet

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approximately 14% of the world's needs [12]. The supply of renewable energy sources is abundant, sustainable, and unrestricted [13]. Renewable resources persist over time. The supplies of clean energy include biomass, wind, solar, tidal, and hydroelectric power [14, 15]. Among the primary sources, solar energy shines as the most abundant and environmentally friendly option. The sun radiates on our globe with an estimated output of 175,000 TW [16] and it is four times as much energy as our peak energy consumption [17]. Renewable power resources such as biomass, hydro, solar, and wind are abundant in Pakistan [18]. Pakistan's vast landmass, plentiful sunshine, wind, and water resources provide ideal circumstances for the development of renewable energy. The province of Baluchistan and the deserts in Punjab, Sindh, and Cholistan have been highlighted as areas with high potential for the production of solar energy. The country receives an average of 15.5×10^{14} kW/h of solar radiation each year. However, the development of solar energy is still in its infancy [19]. Particularly in rural areas with a sizable population engaged in agriculture, biomass, which is obtained from organic and renewable sources, offers enormous potential for energy generation. Although there is currently inefficiency in the use of biomass resources, attempts are being made to create biomass energy technology [20]. Pakistan has abundant wind energy resources, especially in areas like Sindh, Baluchistan, and Punjab. Despite its potential, wind power currently makes up a small portion of the energy mix; nonetheless, by 2030, the government wants to produce 30 GW of wind power [21]. With a total capacity of 100 GW and 59 GW of recognised sites, hydroelectricity is the most major renewable energy source in the world and has tremendous potential in Pakistan. In order to achieve its goal of producing 16,000 MW of electricity from hydro sources, the government intends to construct small-scale hydropower projects. Beside this Pakistan boasts significant reserves of natural gas, amounting to approximately 282 trillion cubic feet (Tcf), of which 24 Tcf is considered recoverable. The country also possesses noteworthy hydroelectric potential, with multiple locations earmarked for the establishment of hydel-based power generation facilities. Furthermore, Pakistan possesses considerable coal reserves estimated at 185 billion tons, including proven reserves of 2.07 billion tons, primarily concentrated in the Thar region of Sindh province [22]. The most significant and plentiful sort of revolving resource in Pakistan is solar power. Installing solar power projects demonstrates to be an effective, quick fix for the electricity shortage [18]. Soon, solar applications might be pursued to address the energy dilemma [23]. Solar energy has a wide range of future applications, including rooftop and wall integration in buildings for self-sufficient electricity generation, revolutionizing agriculture through solar-powered irrigation systems, enhancing heating and cooling in commercial and residential sectors, improving agricultural storage with solar thermal refrigerators, optimizing drying processes with photovoltaic technology, supporting plant growth in solar greenhouses, enabling remote electricity supply and electronic device charging, facilitating wastewater treatment and salinity removal, and even powering space technology through solar arrays. With ongoing research and development, solar energy has the potential to address energy needs sustainably and drive advancements in various industries [24]. In 2010, the world's first smelted salt concentrating solar energy facility, known as "Archimede", was constructed on 8 hectares of land in Sicily, Italy. Owned by ENEL, this solar power facility takes the form of a parabolic trough and generates an impressive 9200 MWh of electricity each year during the summer season [25]. The 1600-acre Crescent Dunes Solar Project is situated near Tonopah, Nevada, in the United States. With a 110 MW generation capacity, it is presently active, furthermore uses solar power tower technics. Tonopah Solar Energy, LLC of Solar Reserve is the owner [26, 27]. China is building the Dacheng Dunhuang Molten Salt Fresnel project. It has a 50 MW generation capacity and employs linear fresnel reflector technology. Lanzhou Dacheng Technology Co. Ltd. is the owner [22, 23]. In Utah on 17 acreages The United States' Tooele Army Depot is situated. With a 1.5 MW generation capacity, it is not in use right now. This project, which belongs to Tooele Army Depot, uses Dish/Engine Solar Technology [28].

This research is novel since it examines CEPEC's involvement in renewable energy in Pakistan. Although there has been a lot of research done on solar energy, CEPEC's true capabilities in terms of renewable energy have not yet been examined.

2. PAKISTAN'S SOLAR POWER PROFILE

The geographical position of Pakistan corresponds to 24°-27° north latitude; 61°-76° E [25], which means there is a lot of potential for Pakistan [26]. Over 95% of the sovereign state, Pakistan has a global average solar radiation of 5-7 kWh/m^2/day. The sun shines for above 2300-2700 hours a year in the south-western province of Balochistan and the north-eastern region of Sindh. A few rural regions maked use of primarily photovoltaic (PV) devices with with power generation of 100–500 W/unit to produce electricity. It should be noted that more than 40,000 Pakistani communities lack access to power [29].

Alternative Energy Development Board (AEDB) served all four provinces of Pakistan by supplying energy to about 3000 homes. In the districts of Rawalpindi, Kohat, Turbat/Kalat, D.G. Khan, Tharparkar, 200 kW of PV power generating was made available. Each residence received an 80 W panel with illumination system. More than 500 mosques, schools, and homes received electricity from PCRET using PV power (capable of 80 kW generation). In Pakistan, the private sector has installed around 500 kW of solar power [30].

In the near future, more tiny independent projects are planned rather than any major initiatives. Just like the implementation of the 10,000 solar thermal unit system will be accomplished, the total PV installation appears to be significantly less than 1000 kW [30]. With a minimal number of solar panels, Akhat Solar Limited generated about 2 MW of electric light. Additionally, PCRET produces 26.5 KW through the aid of 13 solar panels that are erected and placed to various rural homes and small size schools [31].

3. THE ENERGY PROBLEM IN PAKISTAN AND ITS DOMESTIC ENERGY SOURCES

Energy problem of Pakistan has a few different primary causes. A significant shortage of electricity usually occurs due to an increase in the needs of the population for electricity, which is associated with its excessive consumption. In Pakistan, there is a severe power outage that can last for about 18 hours daily in countryside and 8 to 12 hours daily in city districts. This is a result of the 5-7 GW power supply deficit [32]. The electricity production system is very old and inefficient when compared to the needs for energy. In Pakistan, renewable resources are not used effectively.

Mixed power scenario for the production of electricity in the pecuniary years 2021–2022 is depicted in Figure 1, Figure 2. Figures 1 and 2 display the contributions of various energy sectors to Pakistan's electricity production in GWh and percentages (%) for the fiscal years 2020, 2021,

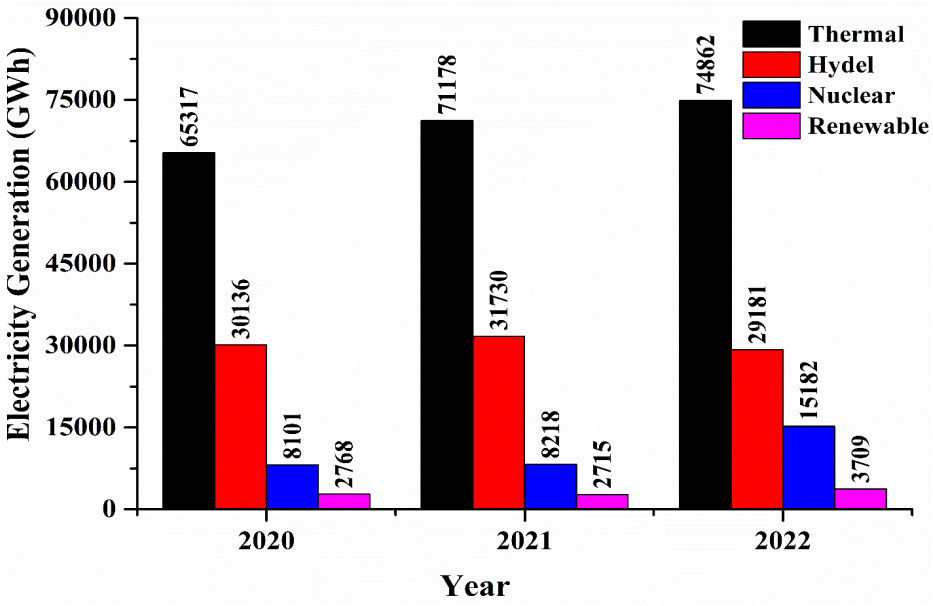

Fig. 1. Shares in electricity generation in GWh (Pakistan Economic Survey, 2021-22).

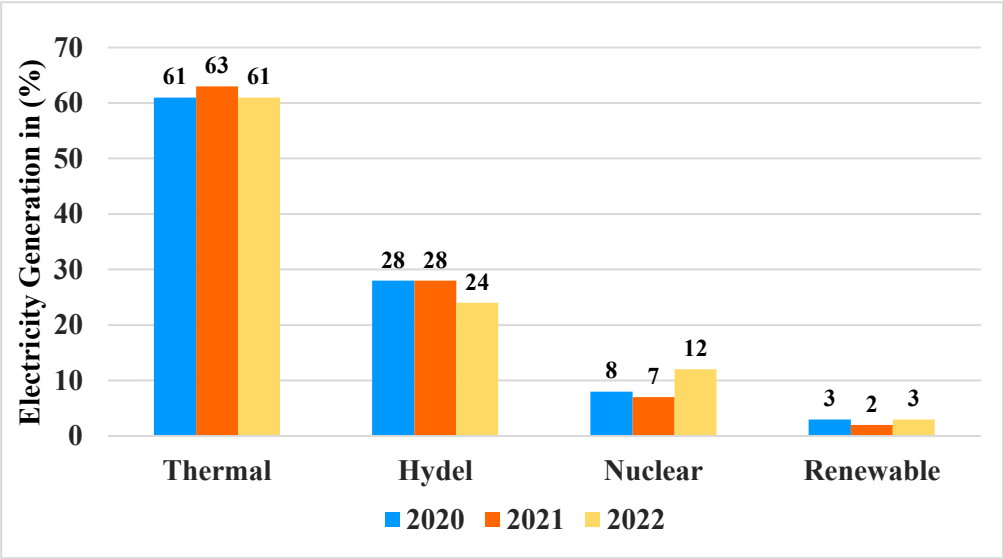

Fig. 2. Shares in electricity generation in percentage (Pakistan Economic Survey, 2021-22).

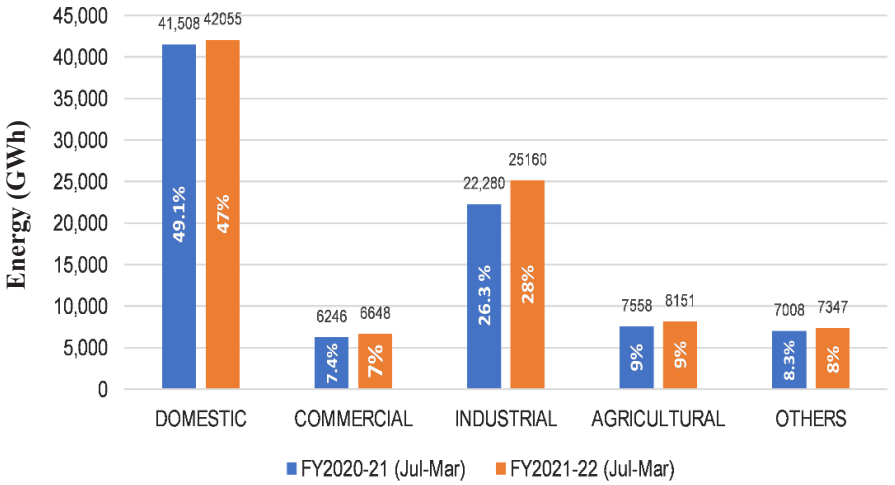

Fig. 3. Energy consumption by sectors (Pakistan Economic Survey, 2021-2022).

and 2022. In Pakistan, thermal power generation accounts for most of the energy production (61% in 2020, 63% in 2021, and 61% in 2022), while renewable energy sources account for the least (3% in 2020, 2% in 2021, and 3% in 2022) (Pakistan Economic Survey, 2021-22). Figure 3 represents the domestic energy use is the largest energy consumption sector in Pakistan for the financial years 2021 and 2022, with a percentage of 49.1% in 2021 and 47% in 2022. The industrial sector is on the second place with a percentage of 26.3% in 2021 and 28% in 2022, and the agricultural sector is on the third place with a percentage of 9% in both financial years 2021 and 2022 (Pakistan Economic Survey, 2021-2022).

Solar power is an abundant revolving resource that can be used for electricity generation. Over 100,000 MW of electricity from sunlight could be generated, particularly in regions within the Sunny Belt [33]. Approximate radiation dose in the Pakistan' provinces like Punjab and Khyber Pakhtunkhwa (KPK) is 400-440 cal/cm^2 daily, while Sindh and Balochistan obtain more than 440 cal/cm^2 daily. The northern regions and Kashmir only receive fewer than 400 cal/cm^2 daily of radiation. Finally, accumulating about 6840-8280 MJ/m^2 daily, provides an average daily exposure of about 00 to 250 W/m^2 [2014]. The annual average daily global radiation of Pakistan is: horizontal (GHI) - 16 MJ/m^2, oblique (GTI) - 13 MJ/m^2. The minimum annual average daily GHI in Pakistan is higher than

the world average annual average daily GHI. This demonstrates the brilliant potential of Pakistan in the field of solar energy [34].

In order to increase the efficiency of the use of sunlight, solar reflectors are placed on the side of the solar panels. Solar Collectors Performance and Gain are also increased by using solar reflectors. The incident sun energy is concentrated onto the receiver via solar reflectors [35]. Since the energy is dispersed, nothing heats up quickly. However, employing solar reflectors to focus the sun's energy can have amazing benefits. Of the actually existing known solar reflectors, the most stable material is a silver/glass thick mirror, which has a reflectance close to 94% [36]. A thin, highly reflective film with self-cleaning capabilities. For the solar front reflectors, this thin film of TiO_2-SiO_2-Ag was successfully created by magnetron sputtering. After a 1200-hour ageing test, this layer still has a high reflectivity of 0.9578.

However, applications for silver polymer solar reflectors tend to use dish concentrators. As a result, there are less solar losses and more solar energy is stored [37]. Reflector material can indeed be made using recycled or locally sourced materials, specifically when utilizing glass. The production of solar reflectors can successfully utilize recycled glass. In the construction of Fresnel mirror reflectors, glass is employed to effectively reflect light. These reflectors utilize flat mirror strips that are adept at reflecting incoming light and can be conveniently adjusted to track the sun's movement [38].

Once the industry is established, both virgin and recycled glass can be employed effectively side by side to lower the whole manufacturing process' capital cost. Aluminium can also be recycled from garbage. Instead of importing such structures from other countries, it will be much more lucrative to produce them with domestic resources. If this industry is able to run smoothly, it will eventually bring in money for the government and employ the harmed workers [38].

4. APPLICATIONS OF SOLAR THERMAL ENERGY

By utilising its thermal qualities, solar power may be applied directly across a wide range of use cases. These technologies are relatively straightforward, inexpensive, and simple to use [39]. In Pakistan, these use cases include cooling and heating buildings, heating and cooking, harvesting agricultural products by drying them, heating water for industrial and domestic purposes [40].

4.1. Sun-Powered Water Heaters

Solar water preheating use in Pakistan has been somewhat restricted despite the fact that the technology is highly developed. This is mostly due to the larger initial investment required for water heaters that run on solar energy in comparison with conventional models that use natural gas. Due to the cold climate and the scarce and challenging natural gas supply in these areas, some government organizations are beginning to actively create low-cost solar water heaters, which are now gaining popularity, mainly in the northern mountainous regions. Solar water heaters will no doubt be adopted due to the ever-increasing cost of electricity and natural gas. Such heaters are already being produced and commercialised in the individual households [40].

4.2. The Solar Panel

Numerous society sector organisations have worked on the manufacturing of affordable and effective designs for both drawers and type of hub solar panels in the past and are actively working on it today. The Pakistan Council for Renewable Energy Technologies (PCRET) regularly organizes educational lectures and seminars on the application and maintenance of such high-tech devices [41].

4.3. Sun Dryers

Agricultural products may be of better quality when dried and at a lower cost if solar dryers are used thanks for the cost savings from not utilising electricity or other heating fuels for the same reason. For example, fruits such as apricots were thrown out by the ton every year in the northern highlands of Gilgit and Sakardu, etc., due to a lack of logistics and fundamental infrastructure. Large amounts of this fruit are now dried using solar dryers before being transported and sold later in the urban market, which has a good impact on the local economy [42].

NGOs are making a concerted effort to increase

the use of these dryers. In the provinces of Punjab and Sindh, solar dryers could be utilised equally efficiently for the process of drying agricultural crops for a higher market value and to create local jobs [43]. Drying processes in developing countries contribute significantly to their overall energy consumption, with fossil fuels like coal and natural gas being the primary sources for meeting these energy needs. For instance, India annually consumes approximately 157 million tonnes of coal, 89 million tonnes of petroleum products, and 233 million tonnes of other conventional energy sources to fulfill industrial, agricultural, and domestic requirements. However, there exists a considerable potential for solar dryers in the agricultural sector, offering an alternative to open sun drying and industrial drying while conserving substantial energy quantities. Solar drying presents advantages such as lower operating costs and the production of higher-quality food. Despite its potential, solar drying faces challenges such as intermittent sunshine, higher initial costs, and the availability of space compared to fossil fuel alternatives, impacting its widespread adoption [44].

5. PAKISTAN'S DEVELOPMENT IN SOLAR ENERGY

Punjab's Bahawalpur is home to the magnificent Quaid-e-Azam Solar Power Park (QASP) project. It is a 1000 MW project that would aid in reducing Pakistan's energy shortage [45, 46]. Solar energy is a requirement for QASP's main energy input. In this Solar Park, conventional PV silicon-based solar panels have been erected. The silicon that makes up the PV module transforms sunlight immediately into direct current electricity. Is used to power electrical appliances after being remaded into AC power by an inverter. This massive plan is installed by organizing three stages. Start-up of Phase-I plant with a capacity of 100 MW is completed, and more than 200 hectares of flat wilderness in Bahawalpur, about 400,000 solar panels were put in place. Phase-II and Phase-III of the QASP each have an installation capacity of 300 MW. The end of Phase-II was in August 2016. As soon as 1000 MW plan is finished and operational, 1530 GWh per year of output is anticipated [47-49].

Power systems fed from the sun do not need special maintenance once it is installed. It anticipates reduced maintenance expenses, resulting in a reduction in staffing needs. Pakistan ranks among the top 15 solar PV countries and accounts for 1% of global PV usage [50]. In 2014, the Pakistani government approved the Quaid-e-Azam Solar Park, a solar photovoltaic project with a capacity of 1000 MW. The first phase of the project, consisting of 100 MW, was successfully completed by the end of 2014 and officially established as the Quaid-e-Azam Solar Power Pvt Ltd. (QASP) in Bahawalpur, Pakistan [51]. Figure 4 shows the electricity produced by the

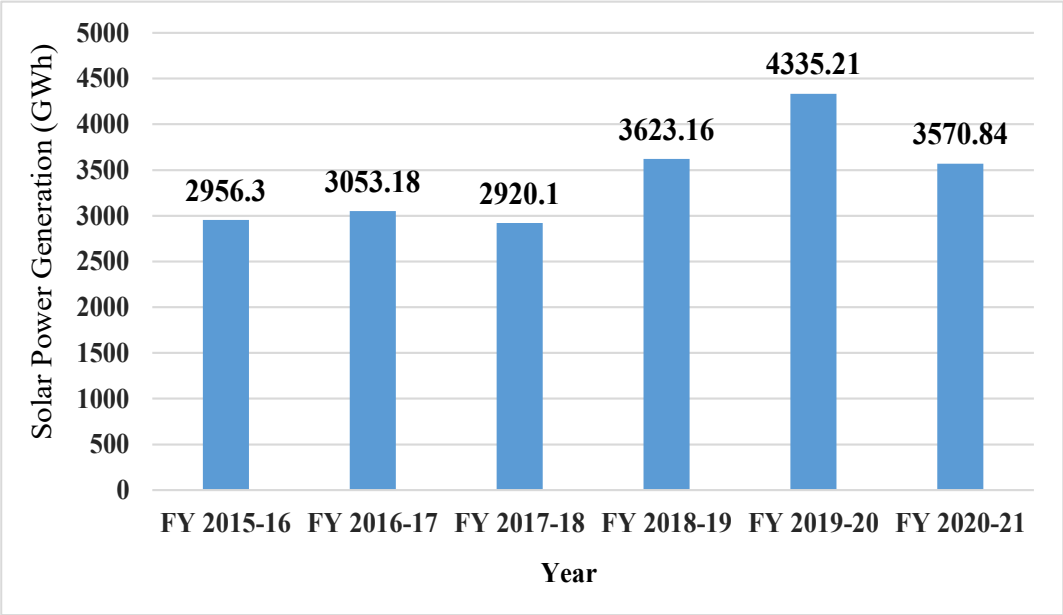

Fig. 4. Quaid-e-Azam Solar Power Generation (Solar Power Plant Bahawalpur, 2021-22).

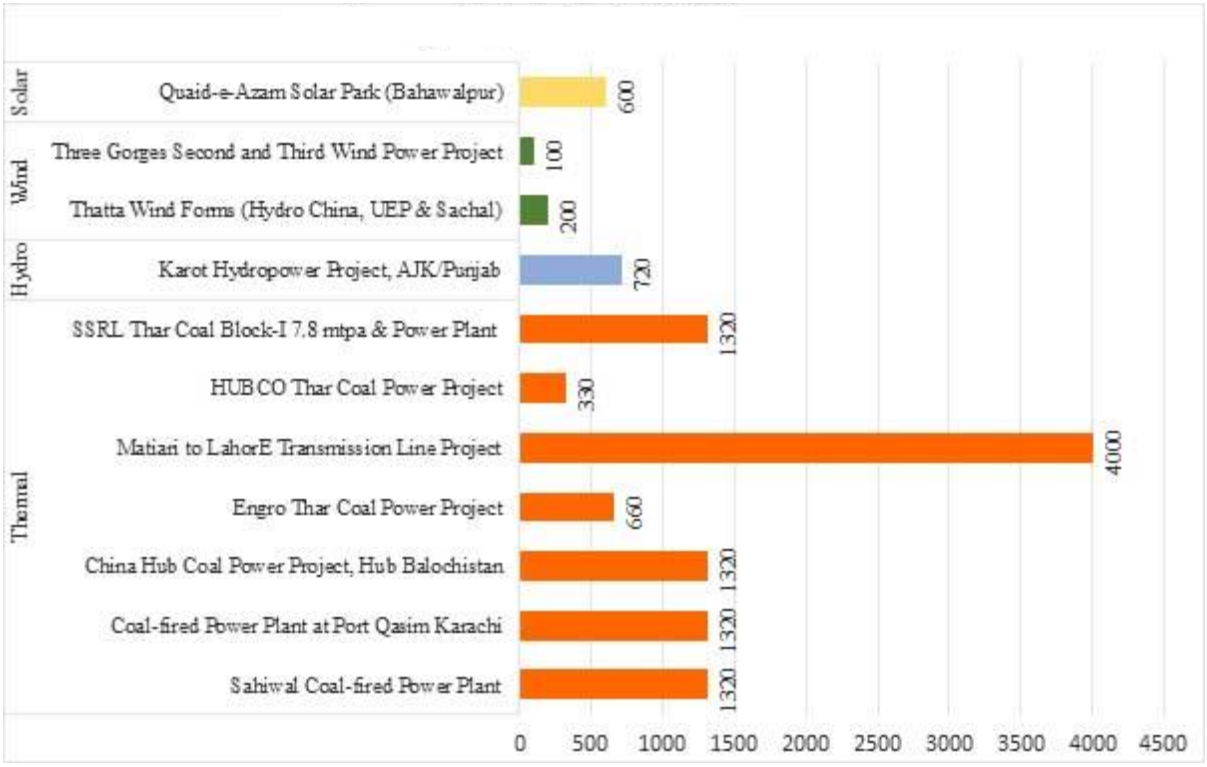

Fig. 5. Renewable energy projects under CPEC (CPEC).

Quaid-e-Azam Solar Plant from 2015 to 2021 (Solar Power Plant Bahawalpur, 2021-22).

Pakistan's Punjab and Sindh provinces are home to a number of active solar energy installations. The entire project of Quid e Azam solar plant is expected to cost US$1302 million. The Ministry of Water and Power organised it. Punjab Power Development Board and AEDB served as the overseeing organisation [52].

6. CONCLUSIONS

One of the best heavily irradiated nations in the world, Pakistan is located on the Sun Belt. Unfortunately, no action has been taken thus far to use such a package of energy for the advancement of the nation. Solar reflectors may turn out to be in concealment for efficiently collecting solar energy from the sun. Since sunshine is abundant in our country, developing indigenous energy technologies like solar reflectors is urgently needed to address this shortfall. The oil import cost, which is truly a big burden on Pakistan's economy, is expected to diminish with increased usage of solar energy technologies. The government must make well-planned and coordinated efforts to encourage solar energy use and inform the public about its advantages. Balochistan has abundant solar radiation, yet this resource is still untapped. It is the largest province in terms of area, but unemployment there is still at an all-time high. A reflector-solar PV industry must be concentrated on the enormous undeveloped region of Balochistan. In Pakistan, CPEC must be more than just a route; if the solar reflector business is established through cooperation, then it will be beneficial for both nations.

7. RECOMMENDATIONS

Against the background of the global energy crisis, associated simultaneously with several problems, such as a shortage of fossil fuels for energy [53, 54] and greenhouse gas emissions from the combustion of fossil fuels [53, 55], Pakistan has a significant advantage in the form of solar energy. As a result, many countries experiencing a lack of solar radiation are engaged in the search and development of other types of renewable energy [56 - 58].

However, there is a great potential for the use of solar energy in Pakistan. If this potential is used, the country will have enough energy by 2050. The

government is currently very interested in utilising the solar energy potential and has begun to set up solar power producing plants in locations where this resource is more abundant. The work on CEPEC projects may move more quickly because CEPEC includes numerous renewable energy projects in Pakistan.

8. INTEREST OF PRIVATE ORGANIZATION IN RENEWABLE ENERGY

The development of the new industry is greatly aided by the privatisation of the new technologies. To establish an industry, solar projects must use solar reflectors. If private businesses and organisations adopt this, there will be a greater likelihood that technology will become affordable and widely accessible to the general people. For commercialize the practical implementation of solar technologies in the citizens homes and government agencies, a consultative and/or consulting council must be established to oversee the solar reflector business, as well as propose creative concepts.

9. EXPLORATION OF BALOCHISTAN SOLAR POWER AND CPEC RENEWABLE ENERGY PROJECTS

In the entire nation, Balochistan has the highest solar resource potential. How to transport the power generated by Balochistan's solar resources to load centres is a critical issue. Presenting plans for utilising the solar energy potential, the China-Pakistan Economic Corridor (CPEC), a significant infrastructural project between the two countries, might encourage R&D in Balochistan. It would mark an important turning point in Balochistan's development, spurring further growth [45, 59]. Figure 5 represents the various renewable energy projects under CPEC.

10. CONFLICT OF INTEREST

The Authors declare no conflict of interest.

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Research Article

Fruit Morphological and Biochemical Characterization of Three Saudi Arabian Date Palm (*Phoenix dactylifera* L.) Cultivars Grown in District Khairpur, Pakistan

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Abstract: In the present study fruit morphological and biochemical characterization of three Saudi Arabian date palm cultivars (Ajwa, Safawi and Ruthana) were carried out, which were cultivated in the district Khairpur, Pakistan. Fruits were collected at four different fruit growth stages (kimri, khalal, rutab and tamar) from fifteen years old trees for morphological (fruit colour, fruit length, fruit diameter, fruit weight, pulp ratio, seed length, seed diameter, seed weight) and biochemical analyses (moisture content, pH, total soluble solids, total sugars, reducing sugars, non-reducing sugars). Results of fruit physical characters showed that significantly highest fruit length (5.5 cm at rutab stage), fruit weight (20.2 g at khalal stage) was observed in cv. Safawi, whereas at khalal stage significantly lowest fruit length (3.4 cm) was observed in cv. Ajwa and fruit weight (13.2 g) was observed in cv. Ruthana. Fruit/pulp ratio (FPR) (90.7%) was higher in the fruits of cv. Ajwa at khalal stage, while at rutab stage FPR (92%) was higher in the fruits of cv. Safawi. Fruits showed significant variations in colour, size and weight from kimri to tamar stage. Biochemical analyses of fruits revealed that highest moisture content (86.2%) was observed in fruits of cv. Ruthana at kimri stage. Total soluble solids (TSS) increased from 8.7% (at kimri stage) to 20.5% (at tamar stage) in cv. Ruthana. pH of the fruits at kimri stage was 3.2 in cv. Ajwa, whereas pH increased to 5.2 in cv. Ruthana at tamar stage. Total sugars (reducing and non-reducing) increased from 7.56% in cv. Ajwa at kimri stage to 69.7% in cv. Safawi at tamar stage as significantly highest values. Findings obtained in the present study described morphological attributes and biochemical constituents of the fruits of three exotic cultivars introduced in climatic conditions of the district Khairpur, Pakistan will open the route and interest of the farmers to introduce other elite date cultivars grown in the world.

Keywords: Biochemical Analysis, Moisture Content, Total Soluble Solids, Morphological Attributes, Rutab, Tamar.

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) belongs to family Arecaceae being diploid (2n = 36), dioecious and having potential varietal collection cultivated in the tropical world [1]. According to Chao and Krueger [2] date palm remain full productive for 40–50 years under normal conditions but generally it may bear fruit up to 150 years in certain circumstances. Maryam *et al*. [3] described that date palm holds 4th position among commercial crops (mango, citrus and banana) playing a key role in the socio-economic development of the country. Pakistan holds 5th position in dates' production with established industrialization, shares 10.2% in world production [4]. Date fruits are enriched in minerals, sugars, vitamins and various compounds used as traditional medicines [5, 6]. Agronomy of date palm play significant role for peoples' income in rural areas, major income source for farmers, important food source to the peoples in rural areas [7]. Date fruits are oblong, berry with thick mesocarp and thin fibrous endocarp, constitute 85-90% of total fruit weight [8]. Fruit developmental stages of dates are categorized into five stages (hababouk, kimri, khalal, rutab and tamar). Hussain *et al*. [9] described that fruits at hababouk and kimri stage remain green in colour and non-edible, while fruits at edible khalal stage acquire proper size, shape and colour, and next to khalal is rutab stage (half ripened) and tamar (full ripened either on the tree in dry cultivars or manually dried

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under sun). Markhand *et al*. [10] mentioned that dates belong to soft type are consumed at khalal stage contain low tannins and sweet taste whereas semi-dry and dry varieties are mostly consumed at tamar stage. Ismail *et al*. [11] reported that dates are consumed frequently at rutab and tamar stages. Baliga *et al*. [12] mentioned that shape and organoleptic characteristics of dates categorize different date varieties. According to Al-Jasass *et al*. [13] colour of dates is important feature, which helps the consumers in selection of dates quality. Additionally, physical properties of dates are key aspects in determination of dates quality based on colour, shape, size and texture, while nutrient content analyses can be assessed with biochemical analyses and assessment of organoleptic characteristics are one of the key factors. Previous studies reported significant variations in fruit colour occur throughout different fruit growth stages in different kinds of date varieties [14]. Physical evaluation (flesh weight and seed weight, length and diameter) of fruits vary significantly from one variety to another often used as criteria helping the suppliers in sorting and grading of dates [15]. The intake of dates in the daily diet may reduce the risk of several chronic diseases like diabetes, cancer and cardiovascular diseases [16]. Al-Mamary *et al*. [17] described that dates retain some biological activities including anticarcinogenic, anti-inflammatory, and antioxidant. In food industries immediate analysis is generally carried out, with regard to food grading to identify biochemical constituents in each category of fruits [18]. Likewise, the concentration analysis of total soluble solids can be identified in fruit juices, which can help to know total soluble sugars (glucose, fructose and sucrose) with other soluble compounds, i.e., minerals and pectin. Identification of biochemical compounds in the dates is of great importance for food industries, ultimately enhance demand of foods in the markets. Carbohydrates are the major components of dates at tamar stage, therefore are the major source of calories, generally in the form of simple sugars, while proteins present in dates provide a little amount of calories. Currently, in fulfilling the demands and preferences of consumers in selecting food and food products that possess health protecting effects, knowledge regarding the type and concentration of health protective compounds available in food is crucial. Present study described the fruit morphology and biochemical constituents of three elite exotic cultivars (Ajwa, Safawi and Ruthana) originally belongs to Saudi Arabia cultivated in district Khairpur, Pakistan. Study confirmed that exotic cultivars of date palm showed acceptable fruit size and food grade compounds beneficial for human health as a source of daily diet, hence the date cultivars can be introduced in the area for commercial cultivation and production.

2. MATERIALS AND METHODS

2.1. Morphological Attributes of Fruits

Fruits were collected at four different fruit growth stages (kimri, khalal, rutab and tamar) from the fifteen years old trees cultivated at Shah Abdul Latif University, Khairpur (latitude 27.490418° N, longitude 68.761593° E). Vernier Caliper was used to measure length and diameter of whole fruits and seeds according to procedures of Moustafa *et al*. [19] at four different growth stages (kimri, khalal, rutab and tamar) as fruit colour, fruit length, fruit diameter, fruit weight, pulp ratio, seed length, seed diameter, seed weight. Fruits were weighed using an analytical balance. All measurements were taken as an average of 10 randomly picked fruits and their seeds.

Pulp/fruit ratio (PFR) was recorded using formula:

$$PFR = \frac{PW}{FW} \times 100$$

Seed weight (*SW*) was taken using formula:

$$SW = FW - PW$$

Where *FW* and *PW* are fruit and pulp weights, respectively.

2.2. Chemical Analysis of Fruits

Chemical characteristics of fruits, i.e., moisture percentage, total soluble solids, pH and total sugars (reducing and non-reducing) were identified at four different fruit growth stages as per method suggested by Association of Official Analytical Chemists (AOAC) [20]. A Spectrophotometer was used to identify biochemical constituents in the fruit samples. Fresh fruits were chopped into small pieces and put in hot air oven at 60 ℃ for 48 hours until it reached to a constant weight. Dried fruit samples were further divided into small pieces and grinded with mortar and pestle. A solution of fruit sample

was prepared by adding 800 ml of water and 300 g of dates' paste, filtered and subjected to analysis of pH, TSS, TS, RS and NRS. Moisture content was identified according to following formula:

$$Moisture\ \% = \frac{Initial\ weight - Final\ weight}{Total\ weight} \times 100$$

2.3. Statistical Analysis

Three varieties were used in this study. Fruits were collected at four different growth stages (kimri, khalal, rutab and tamar). Data were analyzed using Completely Randomized Design (CRD) and two-way analysis of variance (ANOVA) was performed. Each treatment contained three replicates and each replicate was consisted of 10 randomly picked fruits for each replicate of each fruit growth stage. Comparison of mean values in columns was obtained with LSD at 5% using Statistix software.

3. RESULTS AND DISCUSSION

3.1. Morphological Attributes of Fruits at Different Growth Stages

3.1.1. Kimri stage

Kimri is the second fruit growth stage after hababouk which lasts for longest period during fruit growth. Ajwa, Safawi and Ruthana were green at kimri stage (Figure 1a, 1b, 1c), however colour of the fruits slowly turned into a characteristic fruit colour at khalal and tamar stages. Results obtained with two-way-ANOVA showed significant effect of growth stage (< 0.0001), variety (< 0.0001), whereas combined effect of growth stage and variety was non-significant for seed length (0.9972 cm) and seed weight (0.6461 g). Data in Table 1 show that at kimri stage significantly highest fruit length (4.5 cm) was observed in cv. Safawi followed by Ruthana (4.0 cm) and Ajwa (3.2 cm). Ajwa dates exhibited significantly highest fruit diameter (2.4 cm) followed by Safawi (2.3 cm) and Ruthana (2.2 cm). Fruit weight (15.8 g) was noted as significantly highest in cv. Safawi followed by Ruthana (10.2 g) and Ajwa (8.2 g). Comparing the seed length, seeds of cvs. Safawi and Ruthana were equal is length however vary in diameter. Fruits at kimri stage remain green, hard and generally inedible for all date varieties studied; however, fruits in this stage exhibited rapid growth. Al Udhaib [21] noted average fruit length (27.5 mm) and average weight (5.8 g) at kimri stage. Tafti and Fooladi [22] observed rapid fruit growth at kimri stage (90% weekly growth) gradually decreased to 20% at late kimri stage. Abdul-Hamid *et al*. [23] reported significant differences among dates with regard to dimensions particularly for cv. Ajwa which is among elite cultivars, had smallest fruit size (14.6 mm diameter and 26.4 mm length respectively). Dimensions (length, width, thickness) of date fruit were 34.45 mm, 17.52 mm and 16.80 mm, respectively at kimri stage [24].

Table 1. Morphological Characterization of fruits of Ajwa, Safawi and Ruthana at different growth stages (kimri, khalal, rutab and tamar).

Growth Stage	Cultivar	FL (cm)	FD (cm)	FW (g)	F/PR (%)	SL (cm)	SD (cm)	SW (g)
Kimri	Ajwa	3.2 ± 0.11^{d}	2.4 ± 0.1^{bc}	8.2 ± 0.18^{g}	87.8 ± 0.2^{g}	1.9 ± 0.1^{c}	0.7 ± 0.08^{abc}	1 ± 0.08^{a}
	Safawi	4.5 ± 0.11^{b}	2.3 ± 0.1^{bc}	15.8 ± 0.18^{b}	93 ± 0.2^{a}	2 ± 0.1^{bc}	0.9 ± 0.08^{ab}	1.2 ± 0.08^{de}
	Ruthana	4.0 ± 0.11^{c}	2.2 ± 0.1^{cd}	10.2 ± 0.18^{f}	87.2 ± 0.2^{h}	2 ± 0.1^{bc}	1 ± 0.08^{a}	1.3 ± 0.08^{d}
Khalal	Ajwa	3.4 ± 0.11^{d}	3.0 ± 0.1^{a}	15.2 ± 0.18^{c}	90.7 ± 0.2^{d}	2 ± 0.1^{bc}	0.9 ± 0.08^{ab}	1.4 ± 0.08^{cd}
	Safawi	5.4 ± 0.11^{a}	2.4 ± 0.1^{bc}	20.2 ± 0.18^{a}	90.2 ± 0.2^{e}	2.2 ± 0.1^{bc}	0.8 ± 0.08^{abc}	1.6 ± 0.08^{cd}
	Ruthana	4.1 ± 0.11^{c}	2.3 ± 0.1^{bc}	13.2 ± 0.18^{d}	85.6 ± 0.2^{i}	2.1 ± 0.1^{abc}	0.8 ± 0.08^{abc}	1.9 ± 0.08^{a}
Rutab	Ajwa	3.5 ± 0.11^{d}	3.0 ± 0.1^{a}	15 ± 0.18^{c}	91.3 ± 0.2^{c}	2.1 ± 0.1^{abc}	0.8 ± 0.08^{abc}	1.3 ± 0.08^{d}
	Safawi	5.5 ± 0.11^{a}	2.5 ± 0.1^{b}	20 ± 0.18^{a}	92 ± 0.2^{b}	2.3 ± 0.1^{a}	0.5 ± 0.08^{d}	1.6 ± 0.08^{bc}
	Ruthana	4.1 ± 0.11^{c}	2.4 ± 0.1^{bc}	13 ± 0.18^{d}	85.3 ± 0.2^{i}	2.2 ± 0.1^{bc}	0.8 ± 0.08^{abc}	1.9 ± 0.08^{a}
Tamar	Ajwa	3.4 ± 0.11^{d}	2.8 ± 0.1^{a}	13 ± 0.18^{d}	90 ± 0.2^{e}	2.1 ± 0.1^{abc}	0.7 ± 0.08^{abc}	1.3 ± 0.08^{d}
	Safawi	5.4 ± 0.11^{a}	2.0 ± 0.1^{d}	14.8 ± 0.18^{c}	89.1 ± 0.2^{f}	2.3 ± 0.1^{a}	0.5 ± 0.08^{d}	1.6 ± 0.08^{bc}
	Ruthana	4.0 ± 0.11^{c}	2.2 ± 0.1^{cd}	11.3 ± 0.18^{e}	84 ± 0.2^{j}	2.2 ± 0.1^{bc}	0.6 ± 0.08^{cd}	1.8 ± 0.08^{ab}
Variability		Pr > F	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
GS		< 0.0001	0.0005	< 0.0001	< 0.0001	0.0238	0.0017	< 0.0001
V		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0600	0.0931	< 0.0001
GS × V		0.0023	0.0372	< 0.0001	< 0.0001	0.9972	0.0537	0.6461

GS = Growth Stage, V = Variety

3.1.2. Khalal stage

Khalal is the third fruit growth stage after hababouk and kimri, this is distinguished with appearance of a particular fruit colour (yellow or red) based on variety types (Figure 1d, 1e, 1f). Data presented in Table 1 show that fruits of Ajwa and Safawi at khalal stage were red coloured while fruits of cv. Ruthana were yellow. Fruit length significantly increased at khalal stage compared to kimri stage in cvs. Safawi (5.4 cm) followed by Ruthana (4.1 cm) and Ajwa (3.4 cm). Comparing the fruit diameter among studied cultivars at khalal stage, cv. Ajwa revealed with significantly larger fruit diameter (3.0 cm) followed by cvs. Safawi (2.4 cm) and Ruthana (2.3 cm). Fruit weight increased significantly at khalal stage in cv. Safawi (20.2 g) followed by Ajwa (15.2 g) and Ruthana (13.2 g). Seed length, seed diameter and seed weight of studied cultivars similarly increased at khalal stage. Most of the growth of fruits is completed at khalal stage, and after khalal stage fruit dimensions are also increased significantly compared to kimri stage. Different reports [25, 26] mentioned that kimri stage is followed by khalal stage, at which a significant change in fruit colour occurs from green to yellow or red depending on date varieties, and simultaneously length and width of fruits were also increased and average weekly growth was reduced slowly at late khalal stage while average fruit weight was increased due to increase in sugar content. Al-Jasass *et al*. [13] observed the several colours of different Moroccan dates at edible khalal stage. Biglari *et al*. [27] observed that the difference in colours of the dates is mainly due to genetic variations resulting in the development of characteristic colour pigments. Several pigments (chlorophylls, carotenoids, and anthocyanins) are responsible for green, yellow, and red colours in date fruits at khalal stage, respectively [28]. Usually, at khalal stage the fruit dimensions were significantly highest [29]. Several studies done on dates in Tunisia [30] and Iran [31] observed significant increase in fruit dimensions at khalal stage.

3.1.3. Rutab stage

Rutab stage of dates is obtained before harvesting and is distinguished by gradual conversion of khalal into tamar while the dates are attached in bunch on the tree (Figure 1d, 1e, 1f). Physical observations of fruits showed that colour of one half of the fruits of cvs. Ajwa and Safawi remained red and of Ruthana remained yellow at edible rutab stage while another half of the fruits acquired characteristic colour of tamar stage (brown, black or dark brown). Measurement of fruit dimensions presented in Table 1 at rutab stage revealed that significantly larger fruit length (5.5 cm) was observed in cv. Safawi followed by Ruthana (4.1 cm) and Ajwa (3.5 cm). Fruit weight declined at rutab stage was observed due to a significant decline in moisture content in cv. Safawi (20.0 g) followed by cv. Ajwa (15.0 g) and Ruthana (13.0 g). Seed length enhanced in all cvs whereas seed diameter of cv. Safawi decreased at rutab stage. Highest seed weight (1.9 g) was noted in cv. Ruthana at rutab stage followed by Safawi (1.7 g) and Ajwa (1.4 g). Dates at rutab stage turn brown or black slowly from one side, becomes soft, and severe fruit taste due to high concentration of tannins is changed to sweet taste due to increase in sugar content in the fruits [21]. Al-Shahib and Marshall [26] reported that fruits at khalal stage can be harvested and stored in low temperatures to avoid spoilage. According to Ahmed *et al*. [32] the appropriate fruit harvesting stage is rutab compared to khalal stage to avoid fruit ripening failure; however, if the fruit is harvested at khalal stage, consume more time to dry, simultaneously major quantity of dates turns into inferior quality during drying process. At khalal stage significantly highest fruit weight was due to high moisture content, which decreases slowly till tamar stage and results in low fruit weight [33]. Fruit size (except width) is reduced at rutab and tamar stages [24]. Similarly, Tafti and Fooladi [22] observed differences in fruit weight and dimensions during ripening stage in cv. Shamsaei.

3.1.4. Tamar stage

Final stage of fruit growth is tamar stage obtained either on the tree (in dry date varieties) or through drying the dates under sun or in artificial dehydrators. Data in Table 1 show that colour of the fruits of Ajwa and Safawi was black whereas colour of fruits of cv. Ruthana was brown at edible tamar stage. Results showed that fruit dimensions significantly decreased at tamar stage in all date varieties due to a significant decrease in moisture content. The largest fruit length (5.4 cm) was noted

in cv. Safawi followed by Ruthana (4.0 cm) and Ajwa (3.4 cm). Fruit diameter of cv. Safawi (2.0 cm) decreased significantly followed by Ajwa (2.8 cm) and Ruthana (2.2 cm). Similarly, there was a significant decrease in fruit weight compared to rutab stage, it was observed in cv. Safawi (14.8 g) followed by cvs. Ajwa (13.0 g) and Ruthana (11.3 g). Seed length measured in cvs. Safawi (2.3 cm) as largest value, Ruthana (2.2 cm), Ajwa (2.1 cm). Seed diameter and seed weight similarly decreased in all studied cultivars compared to previous rutab stage. Asif *et al*. [34] described measurements of Saudi Arabian date cultivars Ajwa and Safawi without indicating fruit growth stage as fruit length (2.89 cm and 4.02 cm) width (2.17 cm and 2.17 cm), weight 10.39 g and 9.76 g, pulp weight (9.25 g and 8.86 g), seed weight (1.14 g and 0.90 g), respectively. Gasim [35] recorded fruit dimensions at tamar stage in cvs. Ajwa and Safawi as fruit length (3.07 cm and 4.07 cm), width (2.21 cm and 2.45 cm), weight (8.24 g and 9.46 g), seed weight (0.97 g and 1.0 g), respectively. Haider *et al*. [29] described that fruit and pulp weight (5.01 g − 13.40 g) and (4.18 g − 12.33 g), respectively, decrease significantly from khalal to tamar stage was observed in certain cultivars of date palm and in addition, dimensions of dates were also reduced from 1.73 cm − 1.20 cm length, 0.63 cm − 0.38 cm width. Phenological characters are supporting in the studies of different fruit maturation stages and fruit shelf life and useful in sorting, grading, and processing [36]. Dates become brown at tamar (if fruit colour was yellow at khalal stage) or black or dark brown (if fruit colour was red at khalal stage) and the skin is soft in moist dates and hard in dry dates obtained from the trees growing in different soil types and climatic conditions. Similarly, fruit weight continues to reduce if the harvesting is delayed.

3.2. Biochemical Analysis of Fruits at Different Growth Stages

3.2.1. Kimri stage

Kimri stage is characterized by green colour of the dates (Figure 1a, 1b, 1c), occurrence of high tannins, high moisture and decreased sugar content, acidic pH, therefore fruit remain inedible during kimri stage. Results obtained with two-way-ANOVA showed significant effect of growth stage (< 0.0001), variety (< 0.0001), whereas combined effect of growth stage and variety was non-significant for pH (0.4094). Data presented in Table 2 show that significantly highest moisture content was noted in cv. Ruthana (86.2%) followed by Ajwa (80.2%)

Fig. 1. Fruits of cvs. (a) Ajwa (b) Safawi (c) Ruthana at kimri stage, (d) khalal, rutab and tamar stage of cv. Ajwa, (e) khalal and rutab stage of cv. Safawi, (f) khalal and rutab stage of cv. Ruthana.

and Safawi (78.1%). Highest pH value at kimri stage was observed in cv. Ruthana (3.5) followed by Safawi (3.4) and Ajwa (3.2). Total soluble solids were higher in the fruits of cv. Safawi (9.7%) followed by Ajwa (9.2%) and Ruthana (8.2%). Total sugars (reducing and non-reducing) were higher in Ruthana (9.0%) followed by Safawi (8.9%) and Ajwa (7.8%). Khodabakhshian and Khojastehpour [24] observed 84% moisture content, 9.82% TSS, 3.15 pH of dates at kimri stage. Myhara *et al*. [37] described that severe flavor of date fruit at kimri stage is due to the presence of tannins, which decreases at ripening stage (khalal) and almost vanishes at tamar stage.

3.2.2. Khalal stage

The percentage moisture content of fruits of all studied cultivars decreased gradually while the fruits reached at khalal stage and acquired a particular fruit colour (yellow or red) (Fig. 1d, 1e, 1f). Soft type date varieties are edible at khalal stage due to occurrence of high sugar content and low tannins which make the fruit edible. Results showed that significantly highest moisture content percentage was noted in cv. Ruthana (56.7%) followed by cvs. Ajwa (55.6%) and Safawi (51.2%). Comparing the percentage of moisture content at khalal stage with kimri stage, the moisture content was decreased to 30% − 36% once the fruit acquired yellow or red colour depending on the type of date variety. Highest pH value was noted in cv. Safawi (3.8) followed by Ruthana (3.7) and Ajwa (3.6). Total soluble solids increased at khalal stage in the fruits of all cultivars compared to kimri stage. Results showed that highest total soluble solids were observed in cv. Ruthana (14.2%) followed by cv. Ajwa (13.2%) and Safawi (12.2%). Total sugars (reducing and non-reducing sugars) also increased at khalal stage compared to kimri stage were significantly highest in cv. Ajwa (22.5%) followed by cv. Ruthana (17.6%) and Safawi (14.5%). Conversion of khalal stage just into rutab stage in a bunch indicates full maturation of khalal stage containing proper sugar content and ready for harvesting to make tamar or Chhuhara (boiled/dried dates). Dates belong to dry category are not harvested at khalal stage due to high tannins, however dates are left on the tree until reach to tamar stage on the tree. Full ripening of dates was obtained at tamar stage containing health promoting compounds. Total soluble solids (glucose, fructose and sucrose) increase at khalal and tamar [38]. Several reports [39, 40] described that Ajwa dates are a good energy source due to high amount of glucose and fructose (reducing sugars) and low amount of sucrose (non-reducing sugars) making the dates suitable for diabetic patients. Khodabakhshian and Khojastehpour [24] observed 52% moisture content, 12.9% TSS, 3.85 pH in dates at khalal stage.

3.2.3. Rutab stage

Rutab stage is distinguished from khalal by gradual conversion of one side of fruits into tamar which continues until fruits convert into complete tamar if the harvesting is delayed (Figure 1d, 1e, 1f). At rutab stage, further decrease in moisture content compared to khalal was observed; however, a significant increase in sugar content, pH, total soluble solids were noted in fruits at rutab stage. Data presented in Table 2 show that significantly highest moisture content was noted in cv. Ajwa (43.5%) followed by cv. Safawi (40.4%) and Ruthana (38.7%). Highest pH value was observed in cv. Ruthana (4.2) followed by Ajwa (4.1) and Safawi (4.2). Similarly, percentage of total soluble solids increased at rutab stage compared to khalal stage was higher in cv. Ajwa (18.2%) followed by Safawi (17.1%) and Ruthana (17.0%). Significantly highest percentage of total sugars (reducing and non-reducing) was noted in cv. Ajwa (40.6%) followed by cv. Ruthana (38.5%) and cv. Safawi (36.7%). Weight of Ajwa flesh and seed increase at rutab stage and decrease during tamar stage; therefore, Ajwa dates contain 77% sugars (0.5% sucrose, 34.5% glucose and 25.6% fructose) at tamar stage compared to other date varieties [35]. Khodabakhshian and Khojastehpour [24] described the moisture content (43%), TSS (17.23%), pH (4.02) in dates at rutab stage.

3.2.4. Tamar stage

Tamar is the final stage of fruit growth either acquired on the tree or through drying the khalal or rutab stage dates under sun or using solar dehydrators. Tamar stage dates contain significantly lowest moisture content percentage compared to rest of the earlier fruit growth stages (kimri, khalal and rutab); however, sugar content, pH and TSS are significantly increased. Data in Table 2 show that moisture content of fruits of cv. Ruthana (24.6%) was noted as highest value among three cultivars at tamar stage followed by Safawi

(23.2%) and Ajwa (20.6%). Maximum pH value was observed in cv. Ruthana (5.2) followed by Safawi (5.0) and Ajwa (4.9). Percentage of total soluble solids were higher in cv. Ruthana (20.5%) followed by cv. Safawi (20.1%) and Ajwa (19.6%). Total sugars (reducing and non-reducing were significantly highest in the fruits at tamar stage was noted in cv. Safawi (69.7%) followed by cv. Ajwa (68.9%) and Ruthana (63.0%). AlShwyeh and Almahasheer [41] conducted a detailed study on biochemical analysis of thirty-five Saudi Arabian date varieties at tamar stage observed highest moisture content (29.2%) per 100 g sample in cv. Safawi, whereas highest glucose content was observed in cv. Rawthanat Alqasim (83.09%). Abdul-Hamid *et al*. [23] described that at tamar stage highest moisture content (28.6%) was observed in Ajwa dates while lowest moisture content (12.6%) was observed in dates of cv. Berni. Moisture level in dates of Ajwa at tamar stage maintains fruit flesh and results in high preference of consumers [23]. Moisture content of dates at tamar stage is generally 20% or less depending on the variety. Dates with moisture content (10% to 30%) belong to semi-soft, while dates with moisture content less than 10% are included in dry category [42]. Studies done on majority of date varieties holds optimal moisture content level range from 10 − 30% [43]. Ajwa dates can be isolated from other varieties due to occurrence of higher nutritional compounds and also being preferred dates of Holy Prophet (PBUH) [44]. El-Sohaimy and Hafez [45] in a study on biochemical analysis of dates observed 13.8% moisture and 73% carbohydrates. Khodabakhshian and Khojastehpour [24] described the moisture content (20%), TSS (19.45%), pH (4.76) in dates at tamar stage, since increase in pH was due to a decrease in hydrogen ions during ripening process. pH was increased during ripening of nine Iranian date varieties [46]. Carbohydrates content (69.7%) was observed in Safawi as highest level whereas in Ajwa amount of carbohydrates was 63.0% at tamar stage. Jamil *et al*. [47] identified similar carbohydrate levels in dates at tamar stage. Glucose level in different date varieties was observed in Rawthanat Alqasim (83.0 mg/dL), Sukari Alriyad (80.4 mg/dL), Alkhanizi (51.0 mg/dL) had maximum glucose content while, Khadri (19.7 mg/dL), Majdul Almadina (24.1 mg/dL), Shuqara (24.7 mg/dL) had the lowest [41]. Studies suggest that the dates are a good source of soluble sugars required for different metabolic reactions in the body.

4. CONCLUSIONS

Morphological attributes of dates studied at different growth stages revealed that fruits of cv. Ajwa were smallest in length however fruits of cv. Safawi were the longest at rutab stage among the fruits studied. Fruit colour of all date varieties at kimri stage was green, slowly developing into red (Ajwa and Safawi) and yellow (Ruthana) at khalal stage. Fruit length, diameter and weight increased significantly from kimri to rutab stage, while at tamar stage there

Table 2. Biochemical analysis of fruits of Ajwa, Safawi and Ruthana at different growth stages (kimri, khalal, rutab and tamar).

G. Stage	Cultivar	MC (%)	pH	TSS (%)	TS (%)	RS (%)	NRS (%)
Kimri	Ajwa	80.2 ± 0.18^{b}	3.2 ± 0.19^{h}	9.19 ± 0.11^{j}	7.56 ± 0.36^{j}	5.29 ± 0.94^{g}	2.4 ± 0.13^{j}
	Safawi	78.1 ± 0.18^{c}	3.4 ± 0.19^{gh}	9.7 ± 0.11^{i}	8.89 ± 0.36^{i}	6.29 ± 0.94^{g}	2.6 ± 0.13^{j}
	Ruthana	86.2 ± 0.18^{a}	3.5 ± 0.19^{fg}	8.7 ± 0.11^{k}	8.99 ± 0.36^{i}	7.09 ± 0.94^{g}	1.9 ± 0.13^{k}
Khalal	Ajwa	55.6 ± 0.18^{e}	3.6 ± 0.19^{efg}	13.2 ± 0.11^{g}	22.5 ± 0.36^{f}	16.4 ± 0.94^{e}	6.0 ± 0.13^{h}
	Safawi	51.2 ± 0.18^{f}	3.8 ± 0.19^{ef}	12.2 ± 0.11^{h}	14.5 ± 0.36^{h}	14.1 ± 0.94^{ef}	7.4 ± 0.13^{g}
	Ruthana	56.7 ± 0.18^{d}	3.7 ± 0.19^{ef}	14.2 ± 0.11^{f}	17.6 ± 0.36^{g}	12.4 ± 0.94^{f}	5.1 ± 0.13^{i}
Rutab	Ajwa	43.5 ± 0.18^{g}	4.1 ± 0.19^{c}	18.2 ± 0.11^{d}	40.6 ± 0.36^{c}	29.2 ± 0.94^{c}	11.4 ± 0.13^{e}
	Safawi	40.4 ± 0.18^{h}	4 ± 0.19^{cd}	17.1 ± 0.11^{e}	36.7 ± 0.36^{e}	29.83 ± 0.94^{c}	10.2 ± 0.13^{f}
	Ruthana	38.8 ± 0.18^{i}	4.2 ± 0.19^{c}	17.0 ± 0.11^{e}	38.5 ± 0.36^{d}	26.2 ± 0.94^{d}	12.3 ± 0.13^{d}
Tamar	Ajwa	20.6 ± 0.18^{l}	4.9 ± 0.19^{b}	19.6 ± 0.11^{c}	68.9 ± 0.36^{a}	54.2 ± 0.94^{a}	14.7 ± 0.13^{a}
	Safawi	23.2 ± 0.18^{k}	5 ± 0.19^{ab}	20.1 ± 0.11^{b}	69.7 ± 0.36^{a}	56.1 ± 0.94^{a}	13.6 ± 0.13^{b}
	Ruthana	24.93 ± 0.18^{j}	5.2 ± 0.19^{a}	20.5 ± 0.11^{a}	63 ± 0.36^{b}	50.1 ± 0.94^{b}	12.9 ± 0.13^{c}
Variability		Pr > F	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
GS		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
V		< 0.0001	0.0176	0.0009	< 0.0001	0.0010	< 0.0001
GS × V		< 0.0001	0.4094	< 0.0001	< 0.0001	0.0098	< 0.0001

GS = Growth Stage, V = Variety

was slight decrease in fruit weight and diameter due to loss of moisture content at tamar stage. Fruits of all date varieties developed acceptable size which can be utilized for commercialization by making edible tamar dates. Chemical characterization of fruits at different growth stages (kimri, khalal, rutab and tamar) showed that sugar content, pH and TSS level increased gradually from kimri to tamar stage showed acceptable food grade constituents. TS of the fruits increased significantly from khalal to tamar stage. Highest sugar content was observed at tamar stage can be utilized as a good source of human nutrition. Fruits' physical and chemical characterization studied would help the farmers to prefer the cultivation of exotic cultivars in addition to cvs. Ajwa, Safawi and Ruthana in the agro-climatic conditions of the district Khairpur, Pakistan.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Macromoths (Erebidae: Lepidoptera) and Geometer Moths (Geometridae: Lepidoptera) Species Diversity in Central Sindh, Pakistan

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Abstract: Moths are primarily nocturnal, phytophagous, pests of agriculture, night pollinators, and potential bioindicators. The current study is the first to provide information on the diversity, richness, and abundance of moth species in central Sindh, Pakistan. Moths were collected by light traps, light sheets, and insect nets. A total of 517 specimens were collected during four seasons (Winter, Spring, Summer, and Autumn) and sorted out into 2 families. Moreover, Family Erebidae was sorted out into 3 subfamilies (Arctinae, Calpinae, and Erebinae) of 5 genera (*Utethesia, Chalciope, Creatonotus, Grammodes,* and *Eudocima*) with 8 species (*Utethesia pulchella* Linnaeus 1758, *Utethesia lotri*x Cramer 1789, *Chalciope mygdone* Cramer 1775, *Creatonotus transiens* Walker 1855, *Grammodes geometrica* Fabricius 1775, *Grammodes stolida* Fabricius 1775, *Eudocima phalonia* Linnaeus 1763, and *Eudocima maternal* Linnaeus 1767) while family Geometridae was sorted out into 3 subfamilies (Sterrhinae, Ennominae, and Geometrinae) of 3 genera (*Scopula, Isturgia,* and *Microloxia*) with 5 species (*Scopula pulchella* Fabricius 1794, *Scopula minorata* Boisduval 1833, *Isturgia disputaria* Guenee 1858, *Isturgia Pulinda* Walker 1860, and *Microloxia herbaria* Hubner 1800). However, the family Erebidae was found to be the most prevalent with 8 species while the family Geometridae was found to be the least prevalent with 5 species. However, the family Geometridae was found to be highest in abundance with 305 specimens while the family Erebidae was found to be lowest in abundance with 212 specimens. According to the localities, the highest value (0.91) of Simpson's index (D) was recorded for Matiari; while the lowest value (0.86) was recorded for Jamshoro. The survey locations featured varied moth fauna, as evidenced by the fact that Matiari was recorded as the highest value (2.394) Shannon index (H) while Jamshoro was recorded as the lowest value (2.034).

Keywords: Diversity, Erebidae, Geometridae, Moths, Pakistan.

1. INTRODUCTION

Diversity is the first step in creating conservation objectives for all arthropods [1]. In most cases, it pertains to variation within and between species, and ecosystems. The total known species of Lepidoptera comprise about 10% of all animal species, which is comparable to the total known species of flowering plants [2]. There is little information and little study on Lepidoptera due to researchers' propensity for working on less diverse taxa [3]. Nearly 200,000 distinct Lepidopteran species exist [4], of them about 6,000 species are economically significant [5].

Moths are bioindicators that are sensitive to environmental changes [6]. Moths are members of the suborder Heterocera of Lepidoptera order, which plays a significant role in the forest environment as herbivores and a source of food for parasitic and predatory species [7].

Moths constitute several families, from them Erebidae constitutes the largest family [8]. Geometridae is a diverse family of moths that act as dependable sensors of environmental change [9]. A number of moths are pollinators but due to nocturnal activity further, they don't seem to take an interest in research [7]. They are also recognized as crucial for ecosystem services due to their role as pests on a variety of agricultural crops [10], food for mammals [9], bird fauna [11], and their role in night pollination [12]. In the world, there are over 127,000 different species of moths, and

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more than 12,000 of them have been found in India [13]. Moths are among the most stunning creatures in nature, making up 91% of all species in the Lepidoptera order, and they are extremely important to the ecosystem [14].

Morphological characteristics, including labial palpi, reception apparatus construction, and wing venation, have been used to isolate the Lepidoptera taxon since the significance of outer genital morphology has recently become apparent, the scientific categorization and phylogeny of Lepidoptera have been well understood [15]. A few analyses on the overall external morphology of several lepidopteron groups have been conducted: among them systematics importance of the male genitalia in micro-Lepidoptera [16]. However, previously no detailed work was reported from Sindh province except a few reports which are uncompleted so there is a great need to work on this important group of moths, and all the species in this paper are reported first time from central Sindh.

This research assumes significant importance as it addresses a critical gap in scientific understanding. To date, no comprehensive study has explored the diversity, richness, and abundance of moth species in central Sindh, Pakistan. This pioneering study sheds light on the region's moth richness, abundance, and taxonomy, making it the first of its kind in the area. Using a combination of collection methods, 517 specimens were categorized into two families: Erebidae and Geometridae with distinct subfamilies, genera, and species. The findings reveal variations in prevalence and abundance, with the Erebidae family exhibiting higher prevalence but lower abundance compared to Geometridae. The variation in Simpson's and Shannon's indices across survey locations underscores the diverse moth fauna present in different parts of the region. This research enriches agricultural practices by identifying potential bioindicators for pest control and pollination in central Sindh, Pakistan, aiding in sustainable ecosystem management and enhanced crop productivity.

2. MATERIALS AND METHODS

2.1. Study Site

The study was carried out during four seasons of 2020 and 2021 in three localities (Jamshoro, Tandojam, and Matiari) of Central Sindh, Pakistan. The highest temperature in Central Sindh normally ranges from 109 to 120 °F (43 to 49 °C) (Pak Met Department). These localities in Sindh are Agricultural hubs that occupy a diverse fauna of moth species. Latitude longitude and altitude of these localities are also recorded (Table 1).

2.1.1. Collection

Moths were captured by insect net, light trap, and light sheets [17, 18]. Light traps and light sheets were operated from 09:00 pm. to 03:00 am. A mercury vapor lamp was used to power the light trap and light-sheet (7 × 4 meters, and 250 W). In light sheets, the light was positioned so that it illuminated the entire white sheet, which was strung between two neighbouring trees. The captured specimens perished from ethyl acetate vapours. Later, samples were dried, stretched, and pinned before being placed in entomological boxes.

2.1.2. Identification and preservation

For the identification of moth current taxonomic keys, literature, and genitalia dissection was accomplished. The categorization scheme of genital dissection was modified [19, 20]. A stereo binocular Microscope (BD-45T1) was used for identification. The significant diagnostic features were identified, and images were taken. The location of the collection, date of collection, and collector's name was appropriately listed on the labels for the moths. At the Department of Zoology, University of Sindh Jamshoro, the captured specimens were deposited.

2.2. Diversity Index's

2.2.1. Shannon Weiner index (1963)

The Shannon index (1963) (H), was used to determine the moth fauna's diversity.

Table 1. Showing the longitude, latitude, and altitude of sampling sites.

Locality	Longitude	Latitude	Altitude/ Elevation (m)
Jamshoro	67.952363	25.6250777	120
Tandojam	68.530677	25.428073	29
Matiari	68.5008666	25.6430861	29

The following equation used to determine the Shannon index:

$$H' = [\Sigma Pi\ In\ Pi]$$

Where H is the Shannon-Wiener indicator of species diversity.
Pi = the percentage of the entire sample that belongs to the species.
and ln = Natural log in calculating

In order to determine species dominance and evenness, the Simpson's index (D) and the Evenness index (E) are analyzed [21].

2.2.2. Simpson diversity index

SDI = 1- Σn (n-1)
N (N-1)
Where,
Σ = sum of (total)
Where n is the total number of species, while N is the overall number of individuals in each species.

3. RESULTS

Studies on moth diversity were carried out over 8 months in 2020 and 2021. The study of the moth fauna of two distinct families Erebidae and Geometridae (Lepidoptera), from different agricultural areas of Central Sindh has been conducted. During the current investigation, 517 moth specimens represent 13 species, 8 genera, 6 subfamilies, and 2 families (Figure 1). Eight species of the family Erebidae and five species of the family Geometridae were captured from 3 studied localities. Due to the controversial status of some species, all species were identified via genital dissection and external morphology.

Based on the number of species, the family Erebidae had the highest species richness, with 8 species followed by Geometridae with 5 species, while Erebidae recorded as lowest in abundance (Table 2). In family Geometridae, *Scopula pulchella,* was most prevalent specie (15.47%) followed by *Microloxia herbaria* (14.50%), where *Isturgia*

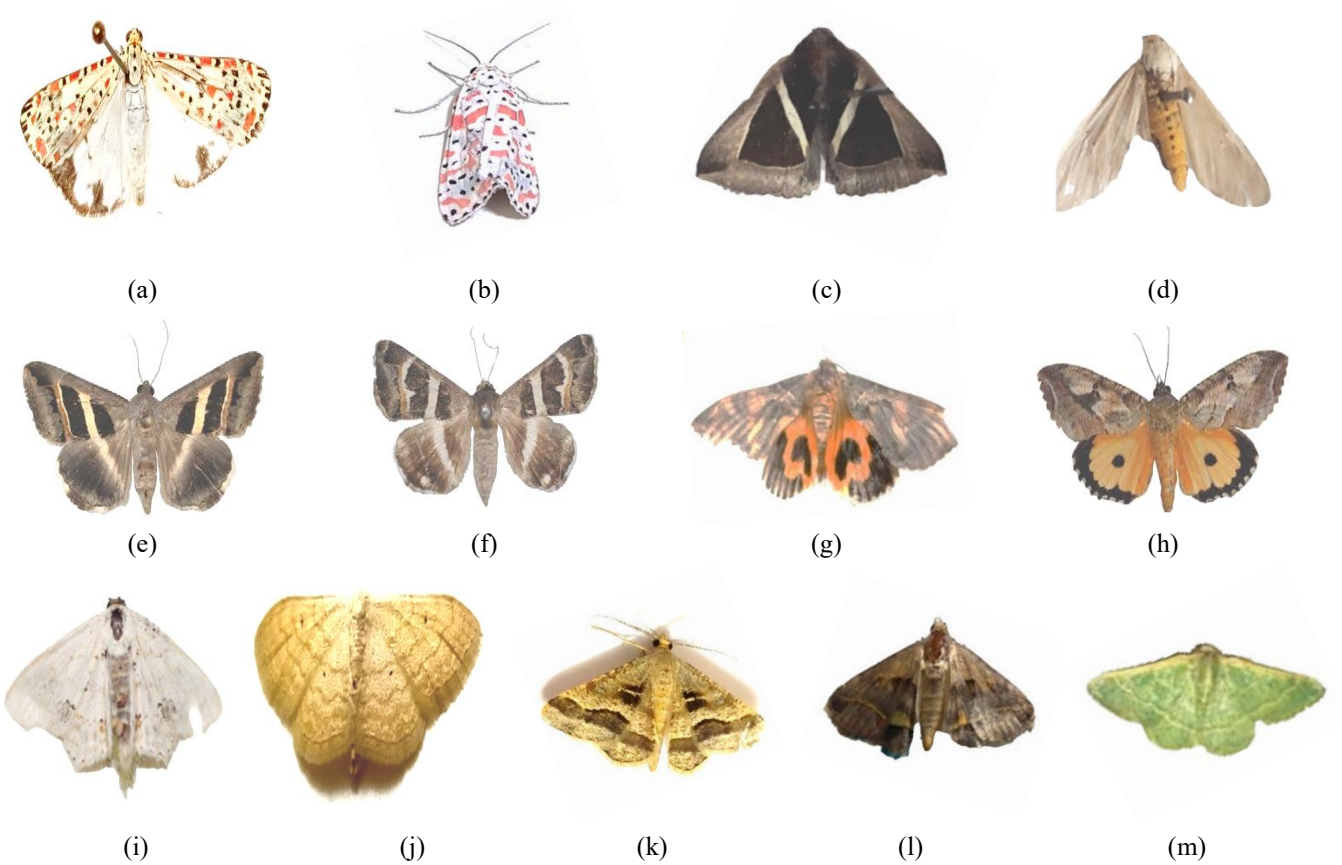

Fig. 1. (a) *Utethesia pulchella*, (b) *Utethesia lotrix*, (c) *Chalciope mygdon*, (d) *Cretonotus transiens*, (e) *Grammodes geometrica*, (f) *Grammodes stolida*, (g) *Eudocima phalonia*, (h) *Eudocima materna*, (i) *Scopula pulchellata*, (j) *Scopula minorata*, (k) *Isturgia disputaria*, (l) *Isturgia pulinda*, and (m) *Microloxia herbaria*.

Table 2. Showing collected species of Moths from three study localities of Central Sindh Pakistan.

Species	No: Specimen	Subfamily	Family	Species Author
Utethesia pulchella	38	Arctinae	Erebidae	Linneaus, 1758
Utethesia lotrix	29	Arctinae	Erebidae	Cramer 1779
Chalciope mygdone	20	Erebinae	Erebidae	Cramer 1777
Creatonotus transiens	24	Arctinae	Erebidae	Walker, 1855
Grammodes geometrica	28	Erebinae	Erebidae	Fabricius 1775
Grammodes stolida	18	Erebinae	Erebidae	Fabricius 1775
Eudocima phalonia	25	Calpinae	Erebidae	Linnaeus, 1763
Eudocima materna	30	Calpinae	Erebidae	Linnaeus, 1767
Scopula pulchellata	80	Sterrhinae	Geometridae	Fabricius 1794
Scopula minorata	45	Sterrhinae	Geometridae	Boisduva 1833
Isturgia disputaria	62	Ennominae	Geometridae	Guenee 1858
Isturgia pulinda	43	Ennominae	Geometridae	Walker 1860
Microloxia herbaria	75	Geometrinae	Geometridae	Hubner 1800
Abundance (N)	517			
Richness (S)	13			

pulinda was the least prevalent species (8.31%). In the family Erebidae, *Utethesia pulchella* was the most dominant (7.35%), while *Grammodes stolida* was recorded as least dominant (3.48%) (Tables 3).

Simpson's Diversity Index (D), Shannon Diversity Index (H), and Evenness (E) were calculated across the survey and were 0.91, 0.394, and 0.964, respectively, (Table 4), indicating that the moth fauna is diversified. The highest value of (D) was measured in Matiari (0.91) followed by Tandojam (0.89) and the least value were recorded from Jamshoro (0.86). Matiari recorded the highest value of H (2.394), followed by Tandojam (2.23), and Jamshoro recorded the lowest value (2.034). Matiari displayed the greatest species richness (12), followed by Tandojam (11), and Jamshoro displayed the least amount of species (9). The maximum abundance was recorded from Matiari (212), followed by Tandojam (162), and the least abundance was recorded from Jamshoro (143). The highest value of evenness was obtained from

Table 3. Showing Locality wise collection of Moth species from three study localities.

Species	Localities			Total	Percentage
	Jamshoro	Tandojam	Matiari		
Utethesia pulchella	10	16	12	38	7.35%
Utethesia lotrix	8	12	9	29	5.69%
Chalciope mygdon	0	8	12	20	3.86%
Creatonotus transiens	0	5	19	24	4.64%
Grammodes geometrica	0	0	28	28	5.41%
Grammodes stolida	0	2	16	18	3.48%
Eudocima phalonia	8	12	5	25	4.83%
Eudocima materna	11	0	19	30	5.80%
Scopula pulchellata	37	22	21	80	15.47%
Scopula minorata	10	18	17	45	8.70%
Isturgia disputaria	28	12	22	62	11.99%
Isturgia pulinda	11	32	0	43	8.31%
Microloxia herbaria	20	23	32	75	14.50%
R=13	N=143	N=162	N=212	517	100%

Matiari (0.964), while Tandojam had the second-highest value (0.93), and the lowest value (0.926) was recorded from Jamshoro (Table 4).

Sindh is blessed with four distinctive seasons (winter, spring, summer, and autumn), 517 moth specimens from 13 variant species have been collected in four seasons of Central Sindh over the course of the research period. It was observed that moths were active throughout the whole research period, with notable variations in abundance and species richness across these seasons. The percentage of family Geometridae (58.99%) was recorded as highest as compared to family Erebidae (41.00%), (Table 5). The highest quantity and diversity of moths were observed during the spring season throughout all families, with 263 individuals representing 13 species and accounting for 50.87% of the total capture. Of these, 147 individuals belonged to the Geometridae and 116 to the Erebidae. The summer season was reported to be the second favorite season, especially for the moth of the family Geometridae with 61 individuals from 5 species representing 20% of the population, followed by 42 individuals from 8 species of the family Erebidae, representing 19.81%. Winter had the third-highest diversity with 77 species of Family Geometridae, accounting for 25.24% of all species, however, in Autumn, this family had the lowest diversity, 20 accounting for 6.55%, while the number of moths of family Erebidae was lower in the winter than the autumn, 26 collected, representing 12.26% and in autumn 28 specimens found representing 13.20%. (Table 5), showing a seasonal abundance of moth families.

4. DISCUSSION

Pakistan is an agricultural country in which the agriculture sector contributes approximately 25.6% of GDP (Gross domestic production) [22]. Among insects, Lepidoptera is the second largest and diverse order of insects. Lepidoptera comprises of more than 180,000 species belonging to 46 superfamilies and 126 families [23]. More than 28000 species are butterflies. In Pakistan approximately 5000 insect species are present among them 400 species are butterflies and moths [24]. Lepidopteran insects are butterflies, moths, and skippers that are widely distributed in agro-environment. The abundance of lepidopteran insects depends upon many factors such as type of vegetation and availability of hosts). The diversity and occurrence of lepidopteran fauna in the cropland ecosystem is mainly due to their feeding habits [26]. In this study, we investigated the moth diversity in the biodiversity-rich areas of Sindh, Pakistan with the intention of recording the macro and geometer moth species found in the agroecosystem, species-rich families, and habitats with high diversity.

Table 4. Showing measurement of Simpson diversity, Shannon diversity index, Species richness, evenness, and abundance of moths from three study localities of Central Sindh.

Locality	Simpson Diversity Index (D)	Shannon Diversity Index (H)	Evenness (E)	Richness (S)	Abundance (N)
L1 (Jamshoro)	0.86	2.034	00.926	9	143
L2 (Tandojam)	0.89	2.23	00.93	11	162
L3 (Matiari)	0.091	2.394	00.964	12	212

Table 5. Showing seasonal variation in abundance of family Erebidae and Geometridae.

Seasons	Months of 2020 and 2021	Erebidae	Geometridae	Total	Percentage
	December 2020 and February 2021	26	77	103	19.92%
	March and April 2021	116	147	263	50.87%
	May and August 2021	42	61	103	19.92%
	October and November 2021	28	20	48	9.28%
	Total	212	305	517	100%
	Percentage	41.00%	58.99%	100%	
Winter	Spring	Summer		Autumn	

The percentage of family Geometridae (58.99%) was recorded as highest as compared to family Erebidae (41.00%). Based on the number of species, the family Erebidae had the highest species richness, with 8 species followed by Geometridae with 5 species, while Erebidae recorded as lowest in abundance. In family Geometridae *Scopula pulchella*, was most prevalent specie (15.47%) followed by *Microloxia herbaria*, (14.50%) where *Isturgia pulinda* was the least prevalent species (8.31%). In family Erebidae *Utethesia pulchella* was the most dominant (7.35%) while *Grammodes stolida* recorded as least dominant (3.48%). The study's findings also highlight variations in species distribution across different survey locations, as demonstrated by the calculated Simpson's index and Shannon index values.

Previous studies on species richness of moths reveal that some are on par with the current study, and some have deviations. Some researchers have revealed that moth species richness is high in agroecosystems and have suggested possible reasons for this observation [26, 27]. Moths are widely distributed in agro-environment, Patil *et al*. [27] described that the abundance of moths depends upon many factors such as type of vegetation, availability of hosts, and presence of predators and parasitoids.

It has been reviewed that diversity patterns suggest that farmland abandonment is likely to positively affect both overall macro-moth diversity and forest macro-moth diversity and to negatively affect species diversity of non-forest macro-moth species [28 - 30]. Our results also show that habitat diversity is important to maintain the diversity of macro and geometer moths.

Several moths are pollinators but due to nocturnal activity of some moths, it is difficult to find them during day time [7]. They are also recognized as crucial for ecosystem services due to their role as pests on a variety of agricultural crops [10], food for mammals [9], bird fauna [11], and their role in night pollination [12]. In our study, we have 7 species (*Utethesia pulchella, Utethesia lotrix, Chalciope mygdon, Grammodes geometrica, Grammodes stolida, Eudocima phalonia,* and *Eudocima materna*) as pest of various agricultural crops.

5. CONCLUSIONS

In conclusion, this research provides valuable insights into the diversity, richness, and abundance of macro moths (Erebidae) and geometer moths (Geometridae) species in central Sindh, Pakistan. Through comprehensive collection methods across four seasons, a total of 517 specimens were identified, revealing 8 species within the Erebidae family and 5 species within the Geometridae family. Notably, Erebidae exhibited greater species prevalence, while Geometridae demonstrated higher abundance. The study's findings also highlight variations in species distribution across different survey locations, as demonstrated by the calculated Simpson's index and Shannon index values. This research contributes to the understanding of moth populations in the region and lays the foundation for further ecological investigations and conservation efforts.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

PCR-based Detection and Prevalence of *Theileria* Species in Sheep from Quetta District, Balochistan

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Abstract: Theileriosis is a serious hemoparasitic illness that severely limits the production of small ruminants. The current study aims for early and accurate detection of theileriosis using polymerase chain reaction for treatment as conventional techniques are not specific. Blood samples were collected from sheep between July and October of the year 2020. A total of 100 samples were taken, 69 samples were from tick-infested sheep exhibiting illness signs, while 31 samples were from tick-free sheep. To detect *Theileria* species in sheep, DNA was extracted, and the PCR method was used. The prevalence of *T. lestoquardi* and *T. ovis* was 22.47% and 64.04%, respectively, with a 13.48% frequency of mixed infection with both species. *T. ovis* was detected in around 80% of tick-infested sheep, indicating a significant association ($P < 0.01$) with ticks. *T. lestoquardi* was found in 28.99% of tick-infested sheep, indicating a significant connection ($P < 0.001$) with ticks. The incidence of *T. ovis* was significantly related to the summer season but *T. lestoquardi* was non-significantly related to the summer season ($P > 0.05$). A high prevalence of Theileriosis is found in Quetta. The parameters studied were strongly correlated to the infection except for *T. lestoquardi* with the summer season. Early detection with the help of polymerase chain reaction can accelerate treatment and reduce transmission to increase livestock production in Pakistan.

Keywords: *Theileria* Species, Sheep, Quetta, Balochistan, Pakistan.

1. INTRODUCTION

Livestock is the backbone of Pakistan's economy, accounting for around 61.89% of agriculture and 14.04% of GDP. The national herd population of sheep is 31.9 million [1]. Balochistan contains 34.8 million hectares of land, of which only 4 percent is arable, and the rest is employed to feed small ruminants [2]. In Balochistan 80% of the population is rural and 3/4th of them are associated with livestock somehow. Livestock in Balochistan generates almost 50% of agriculture and 10% of the overall provincial GDP. The herd population of sheep in Balochistan is 15.85 million which is 52% of the national herd [3]. However, numerous unsanitary conditions and parasite infestations have an unfavorable impact on the country's animal sector, where Theileriosis is a prominent parasite infestation that has proven lethal in animals [4].

Pakistan's tropical and subtropical climate facilitates Theileriosis by providing optimum conditions for ticks to infest Bovidae. Insufficient disease management in Pakistan also increases the risk of Theileriosis [5]. Theileriosis occurs due to the members of the genus *Theileria* [6] which is associated to the phylum Apicomplexa and the order Piroplasmorida [7]. *Theileria lestoquardi* (*T. lestoquardi*) which is responsible for malignant ovine theileriosis (MOT) and *Theileria ovis* (*T. ovis*) which is responsible for benign ovine theileriosis, are considered the causative agents for ovine theileriosis in Pakistan. MOT is accompanied by pale and yellow mucous membranes, enlargement of superficial lymph nodes, diarrhea or constipation, listlessness, emaciation, and high fever; whereas benign ovine theileriosis has symptoms of weight loss, fever, reduced production, and ultimately death of infected animals [8]. The sporozoan protozoa "*Theileria*" is transmitted by ixodid ticks mainly by Hyalomma species in the case of *T. lestoquardi* and *T. ovis* [9]. This parasite has three different stages

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in the life cycle, i.e., sporogony, merogony, and gametogony [10]. The parasites belonging to the *Theileria* genus have a digenetic life cycle. *Theileria* species and strains are either classified pathogenic as they have a schizont-associated "transforming" leukocyte stage or benign to mildly pathogenic as they have a schizont-associated "non-transforming" leukocyte stage. The disease occurs in benign cases due to piroplasm-evoked acute hemolytic anemia. *T. lestoquardi* is pathogenic whereas *T. ovis* is a benign species of sheep and goats [11]. *Theileria* can be treated with buparvaquone with high efficacy when used in the early stages of the disease. Imidocarb and oxytetracyclines are also found to be effective but only in the initial stage [12]. As the treatment is effective only in the initial stages, it is necessary to detect it early.

2. MATERIALS AND METHODS

A total of 100 sheep were examined at random from various herds in Quetta city, including seemingly healthy sheep with ticks on their bodies and animals with significant signs of Theileriosis. All individuals' ear veins and jugular veins were sampled for 3ml of blood, then processed for DNA extraction using Grimberg's non-organic approach [13]. The present investigation utilised two sets of oligonucleotide primers. The first set [fwd. 5′-GTGCCGCAAGTGAGTCA-3′ and rev. 5′-GGACTGATGAGAAGACGATGAG-3′] was used to amplify the 785 bp sequence of *T. lestoquardi* and a second set [fwd. 5′-TCGAGACCTTCGGGT-3′ and rev. 5′-TCCGGACATTGTAAAACAAA-3′ was used to amplify the 520 bp sequence of *T. ovis, T. lestoquardi and T. ovis* sequences were amplified in a thermal cycler (Prime thermal cycler: SPRIMEG/02).

For *T. lestoquardi* the PCR was performed in a total volume of 25 µl reaction mixture containing genomic DNA (250 ng), PCR buffer (1X), $MgCl_2$ (2.5 mM), primers (20 pmol), dNTPs (0.16 mM), and Taq polymerase (2.5 U). The PCR was started with a 3-minute denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 45 seconds. Following this, a final 5-minute step at 72 °C was taken. PCR products were visualized on 1.5% ethidium bromide-stained agarose gel. The amplicon size for *T. lestoquardi* was 785 bp.

For *T. ovis* the PCR was performed in a total volume of 25 µl reaction mixture containing genomic DNA (250 ng), PCR buffer (1X), $MgCl_2$ (2.5 mM), primers (20 pmol), dNTPs (250 mM), and Taq polymerase (2 U). The PCR was started with a 3-minute denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute, and extension at 72 °C for 1 minute. A 5-minute final stage followed this at 72 °C. PCR products were observed on a 1% ethidium bromide-stained agarose gel. The amplicon size for *T. ovis* was 520 bp.

2.1. Statistical Analysis

The Chi-square (χ^2) test was used to examine the relationship between the detection of *T. lestoquardi and T. ovis* and the presence or absence of ticks on sheep, as well as the influence of season.

3. RESULTS AND DISCUSSION

The *Theileria* spp. was found in 89 sheep of which the isolated infection of *T. lestoquardi* and *T. ovis* was recorded to be present in 20 (22.47%) and 57 (64.04%) of the infected sheep, respectively. However, 12 (13.48%) of infected sheep exhibited mixed infection, i.e., infested by both the species. 80% of animals were negative for isolated *T. lestoquardi,* 43% of animals were negative for isolated *T. ovis*, and 88% of animals were found to be negative for mixed infection (Table 1).

Several indicators related to the prevalence and transmission of Theileriosis were also calculated in this investigation. The study examined the correlation between the infection rate and variables such as the presence or absence of ticks on the sheep, as well as the months during which the investigation was conducted. The study months showed a statistically non-significant (P = 0.089) correlation with *T. lestoquardi,* whereas tick

Table 1. PCR results for *Theileria* spp.

***Theilaria* spp. Infection**	**Total N=100**	
	Positive (%) n=89(89%)	**Negative (%) n=11 (11%)**
Isolated *T.lestoquardi*	20 (22.47%)	80 (80%)
Isolated *T.Ovis*	56(64.04%)	43 (43%)
Mixed (*T.lestoquardi + T.Ovis)*	12(13.48%)	88 (88%)

presence revealed a statistically very significant (P = 0.0008) correlation with *T. lestoquardi*. *T. ovis* prevalence exhibited a statistically very significant (P = 0.00001) connection between ticks and study months (Table 2).

The *T. lestoquardi* was identified by a specific band size of 785 bp obtained on 1.5% ethidium bromide-stained agarose gel. The absence of the 785 bp band indicated the parasite-negative samples (Figure 1). Whereas *T. ovis* was identified by a specific fragment of band size 520 bp unique to it. The result was obtained on 1% ethidium bromide-stained agarose gel. The absence of the 520 bp band indicated the parasite-negative samples (Figure 2). Microscopic tests cannot distinguish between *Theileria* species since the piroplasms have the same appearance, making the researcher's job more difficult if discovered in mixed infections. It is critical to distinguish these parasites accurately to comprehend their epidemiology. As a result, using PCR to detect distinct species of *Theileria* in carrier animals has shown to be a powerful technique for epidemiologically investigating *Theileria* infection. PCR amplification is a more sensitive and dependable method compared to microscopy when it comes to assessing carrier animals with low levels of parasitemia [14, 15]. Since light microscopy has a lower capacity to detect parasitemia levels than PCR, it may be verified that light microscopy cannot distinguish carrier animals.

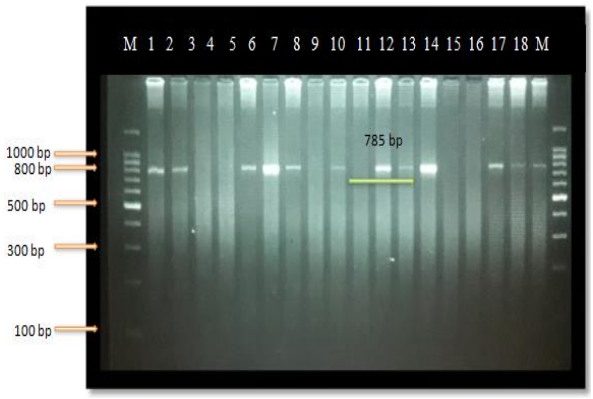

Fig. 1. 1.5% Agarose gel stained with ethidium bromide displaying 785 bp bands of PCR products for the infection of *T. lestoquardi*.

Lane M: 100 bp DNA sequence marker;
Lane 1: Positive control for *T. lestoquardi* DNA;
Lanes 2, 5, 6, 7, 9, 11,12, 13, 16, 17, 18: positive samples *T. lestoquardi* DNA;
Lane 3: (Distilled water) Negative control;
Lane 4, 8, 10, 14, 15: Parasite-free blood samples.

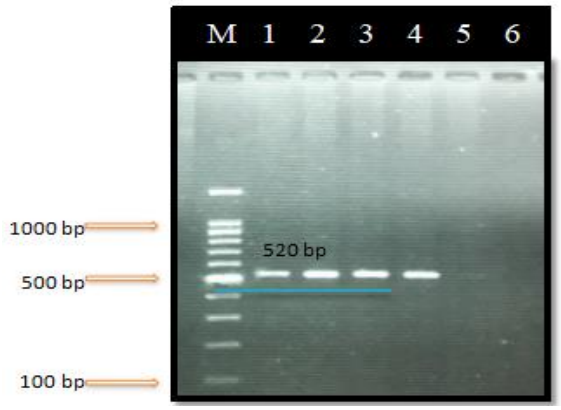

Fig. 2. 1% Agarose gel stained with ethidium bromide displaying 520 bp bands of PCR products for the infection of *T. ovis*.

Lane M: 100-bp DNA marker;
Lane 1: *Theileria ovis* positive control;
Lanes 2, 3, 4: Parasite-positive blood sample;
Lane 5: Parasite-negative blood sample;
Lane 6: Negative control (distilled water).

In sheep, the current study found a higher prevalence of *T. ovis* (64.04%) than *T. lestoquardi* (22.47%), along with mixed infection of both species (13.48%). The frequency of theileriosis in sheep is 89% which is much higher than the findings of Riaz and Tasawar [8], who observed 39.31% (57/145) of sheep in Multan infected with *Theileria* spp. However, the findings are consistent with the frequency of infections observed in infected sheep. They found that 63.16% (36/57) of infected sheep had *T. ovis* infection, 22.81% (13/57) of infected sheep had *T. lestoquardi* infection, and 14.04% (8/57) of infected sheep had mixed infection. But the molecular survey conducted in different regions of Multan in 2013 [16] found that 41.7% (65/156) of sheep had *Theileria* while *T. ovis* was found in 24.6% (16/65), *T. lestoquardi* was found in 57% (37/65) and mixed infection was found in 20% (12/65) of sheep. Tick infestation was also found to have significant relation with the infection in their study. A similar study by Durrani *et al*. [17] in district Lahore, found theileriosis in 70/200 (35%) of sheep while *T. ovis* was identified in 79% (55/70) and *T. lestoquardi* was identified in 21% (15/70) of samples.

In Balochistan, the only large-scale study on theileriosis in small ruminants was conducted between June 2012 to May 2013 in the Northern highlands and Suleiman mountain range of Balochistan comprising 2200 sheep. A significant relation was found between the summer season and

the prevalence of infection. 22.82% of sheep were positive for theileriosis, *T. lestoquardi* accounted for 73.80% (338/458), and the remaining infections 26.20% (120/458) were caused by *T. ovis* [18]. The results contradicted our study this might be due to variations in sample size, herd conditions, and region. In the present study, *T. lestoquardi* accounted for 22.47% (20/89) and *T. ovis* was recorded to be present in 64.04% (57/89) of infected sheep respectively.

Heidarpour *et al*. [19] identified 56% (56/100) of sheep positive for *Theileria* spp. in East and South-East Iran; where 12.5% (7/56) were positive for *T. ovis* and 87.5% (49/56) were positive for *T. lestoquardi*. Similarly, Yaghfoori *et al*. [20.] found 76% (76/100) of sheep infected with *Theileria* spp. in the Fasa Province of Iran. A higher prevalence of 56.58% (43/76) of *T. ovis* was found in infected sheep as compared to *T. lestoquardi* 3.95% (3/76) in sheep. Meanwhile, 39.47% (30/76) presented mixed infection. The findings of Zaeemi *et al*. [10] in the western half of Iran found 32.8% (82/250) of sheep positive for *Theileria,* where 54.8% (45/82) and 40.2% (33/82) were positive for *T. lestoquardi* and *T. ovis*, respectively. Mixed infection was detected in 4.8% (4/82) cases. The first molecular data on *Theileria* infection of sheep in eastern Turkey found 41.2% (90/218) of sheep positive for *Theileria* spp. but *T. lestoquardi* was not detected [21]. Altay *et al*. [22] reported that 58.79% (398/677) of sheep suffering from theileriosis caused by *T. ovis* and *T. lestoquardi* were absent. Altay *et al*. [23] found only 28.90% (37/128) of sheep suffering from theileriosis but *T. ovis* had significantly a higher prevalence of 94.59% (35/37) compared to other *Theileria* species, and the absence of *T. lestoquardi* and mixed infection was observed.

The prevalence of *Theileria* infection is determined by several variables, including tick density and environmental conditions (seasons of the year). From July to August, our study detected a high prevalence of Theileriosis triggered by *T. lestoquardi* and *T. ovis* (24.5% and 73.5%, respectively), as compared to September to October (12.7% and 38.2%, respectively). *T. lestoquardi* showed a non-significant relation between its prevalence and study seasons but a significant association was shown by *T. ovis*. Based on our research, (28.99%) of *T. lestoquardi*-positive sheep were infested with ticks, and (79.7%) of *T. ovis*-positive sheep had ticks. Both species showed a statistically significant association indicating that ticks are involved in parasite spread. Hosseini *et al*. [24] conducted a study in western Iran and their findings proposed that tick infestation is higher during the summer season (June - August) and so is the *Theileria* infection; these findings suggested that higher tick infestation increases the rate of theileriosis. Muhammad *et al*. [25] identified June as the peak season for bovine disease outbreaks in the Faisalabad region of Pakistan because the extreme temperature of the climate promotes tick development and multiplication, making ticks more energetic and thus more likely to transmit Theileriosis. Hegab *et al*. [26], also identified June as the peak month of *Theileria* infection in Egypt but no significant relation was observed between season and infection rate. In the southern

Table 2. *Theileria* spp. prevalence in accordance with investigated parameters.

Parameters		**Ticks**		**Study Months**	
		Absent	**Present**	**July - August**	**September - October**
Sample Count		n=31	n=69	n=53	n=47
T. lestoquardi	(+ve)	0 (0%)	20 (28.99%)	14 (24.5%)	6 (12.7%)
	(-ve)	31 (100%)	49 (71%)	39 (75.4%)	41 (87.2%)
Chi-square		11.23		2.90	
P-value		0.0008^{***}		0.089^{ns}	
T. ovis	(+ve)	2 (6.45%)	55 (79.7%)	39 (73.5%)	18 (38.2%)
	(-ve)	29 (93.5%)	14 (20.2%)	14 (26.4%)	29 (61.7%)
Chi-square		46.84		12.66	
P-value		0.00001^{***}		0.00001^{***}	

Punjab area of Pakistan, Saeed *et al*. [27] found that 0% of tick-absent sheep had *Theileria* caused by *T. lestoquardi*. Iqbal *et al*. [28] discovered a statistically significant positive connection (p = 0.03) between the presence of vector ticks and the occurrence of the disease in the collected samples of goats and sheep.

4. CONCLUSIONS

A high prevalence of Theileriosis is found in Quetta. The parameters studied were strongly correlated to the infection except for *T. lestoquard*i with the summer season. Early detection with the help of polymerase chain reaction can accelerate treatment and reduce transmission to increase livestock production in Pakistan.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Immunity Patterns of Covid-19 Recovered Patients in Gilgit Baltistan, Pakistan

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Abstract: The main purpose of this study was to assess the immunity of recovered patients in COVID-19 patients. The idea was to unveil the nexus between the onset of COVID-19 coupled with the virulence of the disease and the immune responses of the target population in tandem with the intake of local/traditional foods. In the current study antibody presence and complete blood profile of 100 individuals were studied for COVID-19, from the Gilgit area. Immunity against COVID-19 recovered patients was observed during this investigation after more than eight months of their recovery. In this study, 78.2% of people were positive for Immunoglobin G antibody and 23.7% for Immunoglobin M antibody. The most infected age group recorded was 31-40 years. The mean blood glucose level in random was 143.38 mg/dL and 128.18 mg/dL in males and females, respectively. The mean cholesterol level in males was 148.82 mg/dL while the female cholesterol mean was 126.49 mg/dL. White Blood Cells were observed within their prescribed ranges. The mean hemoglobin level was 14.580 g/dL and 12.022 g/dL while mean RBC was 4.7838×10^6 m/µL and 4.2993×10^6 m/µL and the mean platelets were 238.45×10^3/µL and 261.91×10^3/µL in male and female, respectively. It is observed that the A^+ blood group was more susceptible to infection. About 75% of individuals consume highly rich and nutritious food. About 55% of people engaged themselves with exercise. 25% of participants used their normal regular diet during the infection phase on the other hand 75% of individuals treated themselves with a specific and highly nutritious diet for their rapid and healthy recovery. More than 65% of people were re-infected after COVID-19 vaccination but the symptoms were not severe. Due to the strong innate immunity of people from this region, they recovered more rapidly. The dietary habits, high hemoglobin levels coupled with regular exercise might have a positive impact on early recovery of COVID-19-infected patients.

Keywords: IgG, IgM, Vaccination, Immunity, Susceptibility.

1. INTRODUCTION

Outbreak of a novel coronavirus was reported at the end of 2019, causing respiratory diseases in Wuhan, Hubei, China that disease was officially named as Corona Virus Disease 2019 (COVID-19). The coronavirus that is the contributory agent of these respiratory diseases was identified and the genome of this virus was sequenced [1]. Coronaviruses belong to family Coronaviridae and order Nidovirales. These are RNA based viruses, non-segmented and enveloped in their morphology. They are largely scattered in humans and other mammals [2]. The four types of HCoVs (HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1) worldwide spread in the human race and caused around one-third of common cold infections of human population [3]. These four HCoVs can cause life-threatening pneumonia and bronchitis especially in elders, children and immune compromised patients in the severe case of viral infection [4-6]. COVID-19 is considered as the third terrifically pathogenic infection of 21st century among mankind after MARS-CoV and SARS-CoV [7]. The first case of coronavirus was reported on 29th December 2019; Chinese authorities recognized a group of similar cases of pneumonia of mysterious etiology in Wuhan City of China, Hubei Province at Huanan seafood market. At that market several animals such like birds and rabbits were on sale before the outbreak of virus [8-9]. A new strain of coronavirus (2019-nCoV) was isolated on 7th of January 2020 from a patient [8]. Most of the initial cases had the epidemiological associations towards a live animal market (Huanan South China Seafood Market), signifying a probable zoonotic base [10].

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Though, the ultimate source of the coronavirus is mysterious. Occurrence of infection in at least one family chain in healthcare staff verify the human-to-human mode of transmission, nevertheless the extent of this means of transmission was unclear until 21 January 2020, the WHO recommended that there was potential continual human-to-human transmission mode [10].

Since the epidemic of the COVID-19 in January 2020, the virus has attacked most of countries around the world. This outbreak has devastated several thousands of lives worldwide [11]. The World Health Organization (WHO) announced it as a pandemic in March 2020, and proclaimed as emerging threat. Many parts of countries have been locked down and strict social distancing measures have been announced by the states to stop the virus transmission [11, 12]. Li *et al.* [13] and Ong *et al.* [14] stated Novel coronavirus, known as COVID-19 or SARS-Cov-2 has become a global threat and healthcare alarm. Human to human transmission happens through respiratory droplets (coughing or sneezing) and by direct contact with any infected person or indirect contact with fomites in the surroundings Guan *et al.* [15, 16]. According to WHO [10], the pandemic, COVID-19 has affected above 2.5 million people throughout the globe. The reports of the WHO revealed that the epidemic of COVID-19, has affected over 2164111 people and more than 146,198 people were died in more than 200 countries around the world. Sohrabi *et al.* [17] emphasized the coverage of the epidemic with the World Health Organization (WHO) affirming the COVID-19 outburst as a worldwide emergency on January 30, 2020. Huang *et al.* [18] and Guan *et al.* [16] explained that the Covid-19 is clinically identified on the basis of illness expression and the patient's distinctiveness such as epidemiological conditions, interview of sickness, travel history, among associates, and laboratory conclusion. Huang *et al.* [18] showed that Covid-19 disease may produce single or numerous symptoms include dyspnea (shortness of breath), cough, chills, fever, headache, myalgia (muscle pain), sore throat, and loss of taste and/or smell. Hu *et al.* [19] said that the symptoms in the early phase sometimes bear a resemblance to those of the common cold and influenza; thus, health care providers frequently face complexities in distinctive coronavirus infection from the other infections. Furthermore, asymptomatic and subclinical SARS-CoV-2 contagion is comparatively widespread. These factors make satisfactory identification extremely complicated. Intermittently, COVID patients boost pneumonia, which can lead to brutal death and respiratory failure. Arabi *et al.* [20] determined that viral tropism is towards lungs and Community-acquired pneumonia (CAP). Some patients having pneumonia abruptly worsen into severe respiratory failure (SRF) so they require intubation and Mechanical ventilation (MV). This type of patient goes through towering risk of death up to 60% Long *et al.* [21]. Corona Virus 2019 infection explicit IgG and IgM came to top intensities at 17-19 days and 20-22 days after manifestation beginning correspondingly.

In Pakistan, the seroprevalence of SARS-CoV-2 antibodies was 7.1%, according to a research. IgG positivity was found in 6.3% of people, whereas IgM positivity was found in 1.9%. Seroprevalence varied from 0% in Ghotki to 17% in Gilgit. Seroprevalence varied by age category, ranging from 3.9% (0-9 years) to 10.1% (40-59 years) [22]. The purpose of this study was to assess the immunity pattern and reasons behind the Covid-19 recovered patients of mountainous region, to test the blood cell morphology, dietary pattern, age and recovery relationship of recovered patients.

2. MATERIALS AND METHODS

2.1. Study Area and Sampling

This study was conducted at Gilgit Baltistan the very north of Pakistan. A controlled study was conducted on convenient samples of more than 100 recovered patients of COVID-19 by visiting them at declared COVID-isolated centers by Gilgit Government and Isolated patients in homes. All recovered patients of any age group and background were tested. Blood samples were collected from the rehabilitated patients. Sampling was done through proper Biosafety guidelines. The clinical data along with personal data of patients were added in this research. Age, gender, epidemiological history, history of smoking, and co-morbidities, e.g., cancer, blood pressure, high blood cholesterol, hypertension and diabetes were recorded in the personal data of patients. The clinical data included early symptoms, clinical presentation, fundamental signs, remedial drug-use, respiratory assistance, and disease outcome. A pre-planned questionnaire

was also developed to assess the dietary patterns of patients.

2.2. Antibody Test

The Rapid COVID-19 IgM/IgG combine Test Kit is a lateral flow immunoassay proposed for qualitative recognition and discrimination of Immunoglobulin M (IgM) and Immunoglobulin G (IgG) antibodies to SARS-CoV-2 in blood. We used whole blood for antibody tests. "HEALGEN One Step Rapid Testing Kit" was used in this research in order to detect the antibodies presence in recovered patient's blood. About 5 µl whole Blood specimens were taken and transferred the specimen into the sample well. Added 2 drops of sample buffer (80 µl) into buffer well immediately. Observed the red colored lines appeared on test window.

2.3. Complete Blood Count (CBC Test)

Blood was collected in EDTA coated CP tubes. EDTA tubes were used to prevent clotting in blood. About 2 µl blood was drawn from patients using hypodermic disposable syringe. Tubes were placed on shaker machine after 4-5 minutes; the CBC tube was placed in "Sysmex XP-100 Automated Hematology Analyzer" to analyze the sample, as mentioned by Farah *et al.* [23]. The analysis was performed at the Laboratory of hematology Department, District Head Quarter (DHQ) Hospital Gilgit.

2.4. Sugar Test

Portable Glucometer (On Call EZ II) was used to test the blood sugar level of patients at random on the spot. Sugar fasting test was not taken due to different timing of sample collection. Randomly sample was taken during this investigation.

2.5. Cholesterol Tests and Blood Grouping

Cholesterol and blood group testing was done in the Laboratory of DHQ Hospital Gilgit according to their prescribed methods. Cholesterol reagent (Human 100 ml) was added into a simple glass testing tube about 1000 µl and placed the tube in temperature tank for 10 minutes in 37 ºC. Then added 10 µl blood serum and tested blood cholesterol level in MERCK analyzer (Micro-lab 300 by ELI Tech Group).

2.6. Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (Version 22.0, SPSS Inc). Categorical variables were presented as mean and Standard error in each group. ANOVA and LSD was run through the parameters. A p-value less than 0.05 is considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Blood Profile of Recovered Patients of COVID-19

The findings were normal after recovery from infection as this was a post disease study; the survivors regain their normal amount of all blood components during recovery phase. Lu and Wang [24] analyzed the changing in blood parameters and their results showed that all the parameters gradually increased to their normal percentages as leading towards the recovery from the disease. The mean Hemoglobin level shown in current research is 14.580 g/dL and 12.022 g/dL in male and female respectively. The average Hemoglobin level was observed with in standard range the individuals, included in the present research, belong to the high altitude where Hb% is generally high than individuals living in lower altitudes. A study by Liu and Li [25] reported that the Hemoglobin molecules become physiologically inactive and no longer play their role as oxygen carriers during SARS-CoV-2 infection. Lanini *et al*. [26] investigated different blood parameters of individuals suffered from covid-19; They observed average Hb levels were in the normal range in survivors while a mild anaemia was observed at the end in non-survivors.

The average readings of Total leukocyte count (TLC) observed within male individuals was 6913.04/µL and the female reading was 9490.89/µL. The means recorded in this study lies within the prescribed limits of WBCs. After recovery from infection the blood components regain their optimal range. The highest and lowest observed values were recorded in male individuals were 21100/µL and 2420/µL, respectively. Platelets mean for male is 238.45×10^3/µL and 261.91×10^3/µL for female is observed during this investigation. Xu *et al.* [27] observed that SARS-CoV-2 can decrease platelet formation, increase platelet degradation, or more likely, increase platelet consumption due to platelet

activation and thrombosis. Lippi *et al.* [28] found that lower platelet counts are linked to an elevated risk of in-hospital death in COVID-19 patients. The normal platelet counts ranges from 150,000 to 450,000 per micro-liter of blood. The means for both groups (Male and Female), fall within the normal limits of platelets in this research has been observed. The both upper and lower values were observed in female individuals $5.24\times10^3/\mu L$ and $8.0\times10^3/\mu L$, respectively. Lanini *et al*. [26] observed in their study that the platelets' amount was inferior in survivor group than that of the non-survivors group of COVID-19 at early infection, meanwhile the count increased along the way of recovery. This study is on the post infection analysis, therefore investigation showed normality in platelets after infection.

3.2. Blood Glucose and Cholesterol

Guo *et al.* [29] and Zhou *et al.* [30] worked on diabetes and reported that it is one of basic co-morbidities for infection development and mortality by COVID-19. Zaki *et al.* [31] also suggested a strong association between diabetes and severity of disease in COVID-19. Deng and Peng [32] investigation on patients of Corona, reported that 19% patients had diabetes and diabetic patients contributed to 42% of death rate. In this research sugar was also considered as a part of study. Random sugar level was analyzed in male and female recovered patients and the observed mean was 143.38 mg/dL and 128.18 mg/dL, respectively. The highest observed value was 250 mg/dL in male diabetic patient while lowest value was 85 mg/dL in a female at random. Majority of the population were males who had diabetes in the current investigation. A study by Wu *et al.* [1], also revealed that diabetes was the second most common co-morbidity among the patients of COVID-19. The observed Cholesterol mean of male is 148.82 mg/dL while female mean for cholesterol is 126.49 mg/dL in current investigation. The standard cholesterol level must be less than 200 milligrams per deciliter (mg/dL) for adults. The highest recorded cholesterol level in this investigation was 300 mg/dL while the lowest recorded value was 84 mg/dL and both of them are recorded in male patients. Kocar *et al.* [33] elucidated that the cholesterol levels and the amount of lipoproteins in blood are good indicators for examining the status of viral infection in any individual. Another study by Daniels *et al.* [34] revealed that the cholesterol levels are key feature in infections of COVID-19.

3.3. Age of Infected Patients by SARS-CoV-2

During this study the most infected age group was 31-40 years; however, Corona virus attacked older people as well. In this study people nearly 90 years old were also observed to be infected by COVID-19. Ho *et al.* [35] investigated that people with older age are more susceptible to the infection. The condition was observed more severe in people more than 60 years. A similar study by Hu *et al.* [19] also reported that the older age was more affected by this pandemic. Another related study by Mahase [36] reported that aged people are more vulnerable to COVID-19 having multiple co-morbidities and weak immune system.

3.4. Antibodies

This research describes the initial and cross-

Table 1. Blood group and complete blood profile of patients.

Blood Group	%	No	Sugar mg/dL	Cholesterol	Hb g/dL	WBCs/µL	Neutro	Lymph	Eosino	Mono	RBCs m/µL	Platelets /µL
A+	37.60%	38	140.89 ± 7.4263^{B}	138.65 ± 5.1689^{A}	13.457 ± 0.3047	6654.1 ± 241.87^{A}	58.811 ± 1.7782^{A}	33.919 ± 1.6147^{B}	4.4595 ± 0.3906^{A}	2.5946 ± 0.3582^{A}	5435000 ± 1003000^{A}	227412 ± 12163^{AB}
B+	19.80%	20	140.1 ± 9.859^{B}	142.7 ± 7.408^{A}	13.43 ± 0.442	6047.5 ± 359.1^{A}	56.1 ± 2.442^{A}	36.4 ± 1.978^{AB}	5.6 ± 0.613^{A}	2.5 ± 0.500^{A}	4648000 ± 126582^{A}	266400 ± 22686^{A}
B-	2%	2	106.5 ± 2.5^{B}	114.5 ± 18.5^{B}	13.3 ± 0.1	2755 ± 2745^{B}	54 ± 2.0^{A}	39.5 ± 1.5^{AB}	3 ± 2.0^{A}	3 ± 1.0^{A}	4905000 ± 285000^{A}	107650 ± 107350^{B}
AB+	15.80%	16	131.38 ± 8.371^{B}	146.56 ± 12.15^{A}	13.513 ± 0.53^{A}	7181.3 ± 366.8^{A}	55.063 ± 1.84^{A}	39.813 ± 2.02^{A}	3.4375 ± 0.55^{A}	1.625 ± 0.28^{A}	4514000 ± 188120^{A}	231200 ± 20803^{AB}
O+	22.80%	23	124.57 ± 9.815^{B}	128.83 ± 5.33^{AB}	13.191 ± 0.34^{A}	7550.9 ± 786.8^{A}	56.565 ± 2.41^{A}	36.087 ± 2.06^{AB}	4.6957 ± 0.57^{A}	2.7391 ± 0.436^{A}	4342000 ± 209863^{A}	256304 ± 20051^{A}
O-	2%	2	245 ± 5.0^{A}	179.5 ± 3.50^{A}	14.85 ± 1.05^{A}	6950 ± 350^{A}	60.5 ± 2.5^{A}	34.5 ± 2.5^{AB}	4.5 ± 0.5^{A}	0.5 ± 0.5^{A}	4945000 ± 295000^{A}	236000 ± 27000^{AB}

Note: A, B, C, and D showed ANOVA and LSD statistically significant within means at alpha value of $p \leq 0.05$.

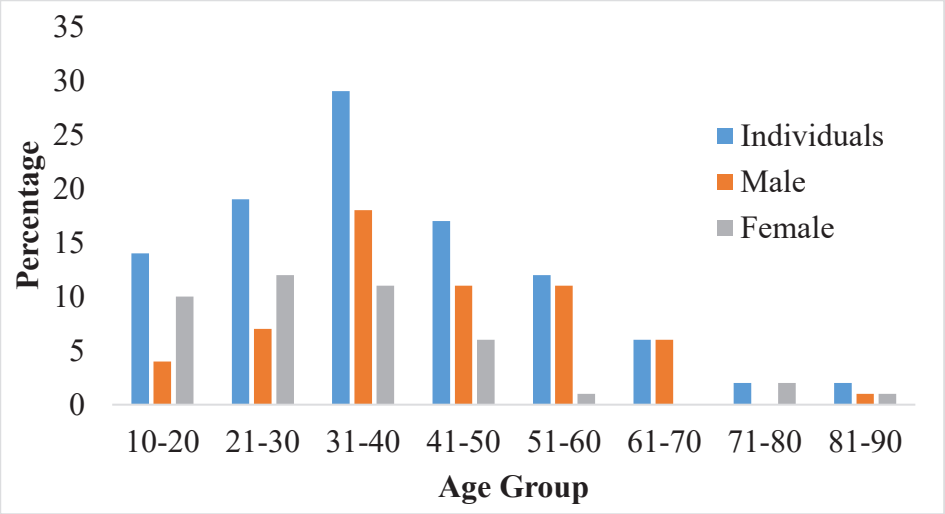

Fig. 1. Total number of individuals and gender wise ratio in different age groups.

sectional results of quantitative and functional SARS-CoV-2 antibodies. The expression of IgM and IgG antibodies varied because of some factors like severity of SARS-CoV-2 infection, and time delay from recovery period started. During this investigation the immunity against COVID-19 was observed up to more than eight to nine months after infection. Huynh *et al.* [37] investigated antibodies against COVID-19 and observed their presence up to 180 days. Bitzogli *et al.* [38] tested for anti-SARS-CoV-2 antibodies in patients survived from ICU found considerably lesser at disease inception, but they gradually lose auto antibodies by onset of pathogens. Antibody IgM and IgG were tested to check the long lasting and chance of re-infection. It was observed that the patients recovered from severe corona infection have higher immunity and presence of antibodies for long time period in their body. Elslande *et al.* [39] have emphasized that after eight months of infection, antibodies stay more commonly positive in those patients who suffered from severe infection contrasted to the patients gone through mild symptoms during their infectious phase. Ibarrondo *et al.* [40] underlined that severity in infection emphasize the importance of response triggered by immune system, with drastically advanced antibody titers within people with severe Corona infection.

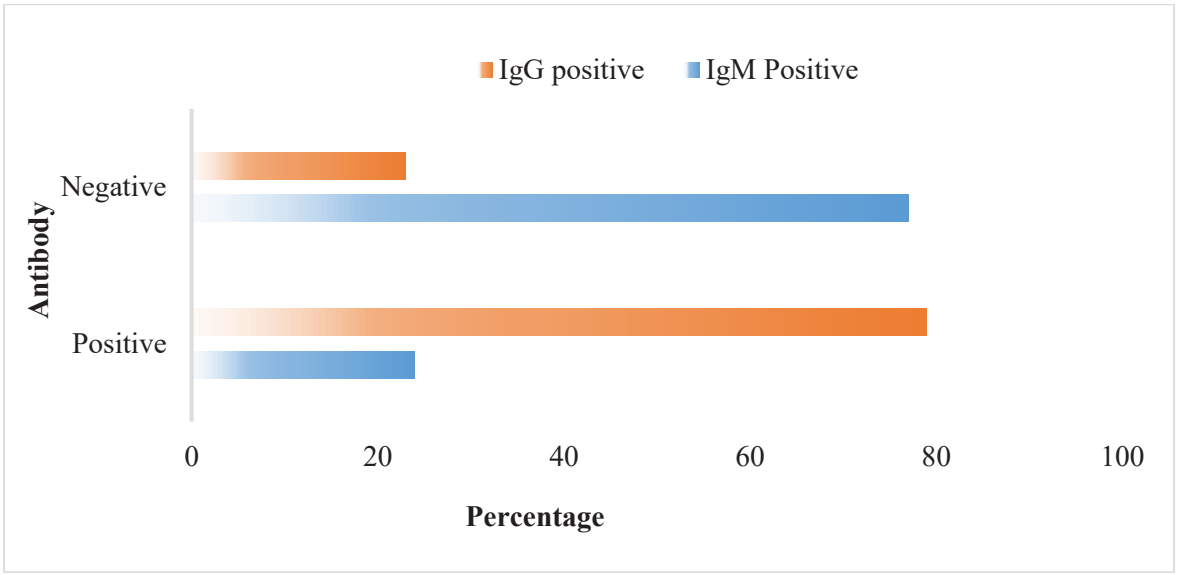

Fig. 2. Antibody status in post recovery stage of individuals.

The observed Immunoglobin G (IgG) positive individuals were 78.23% and 23.6% positive for Immunoglobin M (IgM). Long *et al.* [21], Adams *et al.* [41] Gao *et al.* [42] found during their studies that IgM positive rate was lower than that of IgG rate in patients after recovery Covid infection. Alzaabi *et al*. [43] suggested that the regular up thrust in antibody and tremendous tiers of IgG titers inside the first five months suggest that immunization is feasible, and the probabilities of re-infection is minimum. They also found that the aged individuals are likely to have higher positive IgG antibody reaction and even higher in the amount of IgG antibody levels comparatively the younger individuals. It might be the fact that the aged patients were frequently gone through more severe conditions.

3.5. Gender Differences in Infection

Male participants were observed to be more affected by COVID-19 than female individuals during this research. Total 55.5% male individuals while 44.5% females were included in this investigation. Huang *et al.* [18] investigated that men make up more than two third of the total confirmed cases about 73% vs. 27%. A similar study by Jin *et al.* [44] also reported gender differences in previous studies to affect where male individuals were at a higher infection risk than that of females. Another similar investigation supported the findings of gender difference, and reported that the number of male subjects were double of female patients of COVID-19 [45]. Furthermore, the diabetic patients were observed to be older aged, therefore having another risk that could boost their vulnerability to corona infection.

3.6. Blood Group Susceptibility

The observed prevalent blood group was A^+ with 37.6% followed by O^+ with 22.8%, B^+, AB^+, O^- and B^-. This investigation showed that the blood group A^+ is more susceptible towards the COVID-19 infection. Zhou *et al.* [30] results indicated that the antigen A carrier has drastically high possibility of developing severe or critical infectious cases than that of other blood types. In current investigation A^+ blood type was most susceptible and O^+ was second most susceptible group while on contrary most of researches revealed that O blood type is at very low risk of infection. Zhou *et al*. [30] evaluated ABO blood types distributions in 2173 patients of Corona, and found that blood type O was associated with a lower risk while A was related with higher risk of infection. A similar investigation done by Solmaz and Araç [46] investigated that having blood type O might be defensive, and blood type A might have greater vulnerability towards infection, but this does not affect the way of the disease and is not linked with mortality. Wu *et al.* [1] found similar results in their research on 187 patients of COVID-19 and Goker *et al.* [47] in their study on 186 COVID-19 patients.

3.7. Symptoms during Infection Phase

The symptoms of COVID-19 may different from person to person. The most common symptoms are fatigue, fever, cough, headache, and nasal congestion, shortness of breath, nausea, and diarrhea [48]. The current study reveals that 50.5% people showed moderate symptoms, 31.7% and 13.9% individuals were observed with mild and severe symptoms respectively during

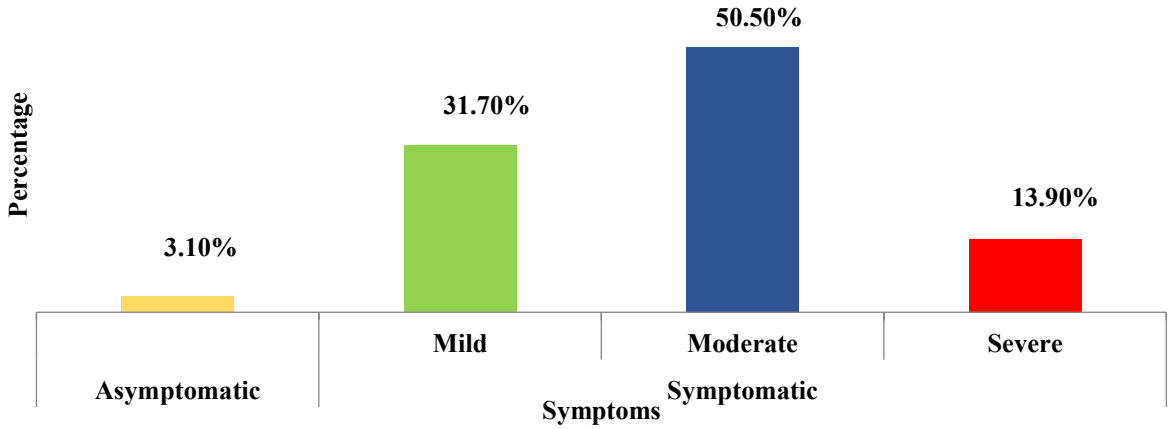

Fig. 3. Percentage of symptoms in people suffered from COVID-19.

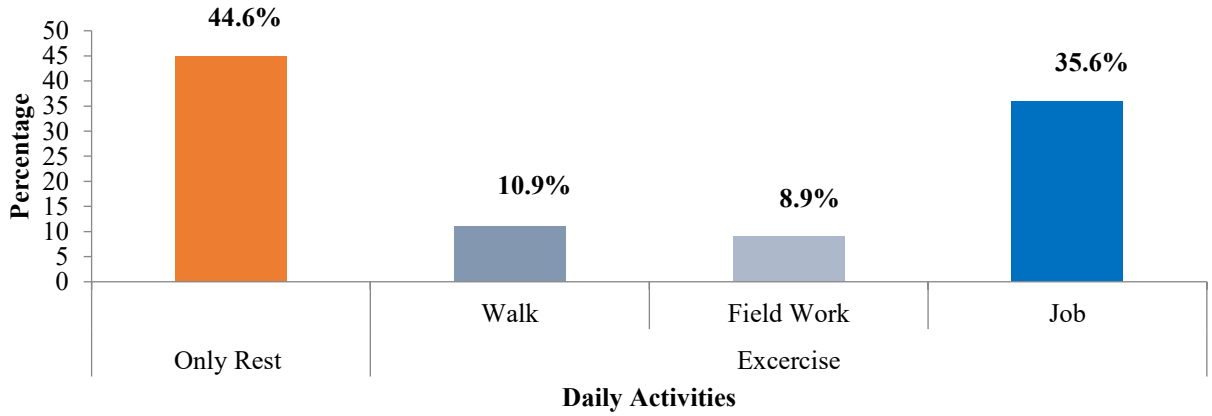

Fig. 4. Daily Life activities of patients during infection period.

infection. About 3.10% people were examined to be asymptomatic. The current study showed that infected people were gone through moderate symptoms more than the other symptoms. Very few were observed to be asymptomatic. During current research the most common observed symptoms were: fever, cough, sneezing, difficulty in breathing, chest pain, headache, nausea, body pain, flu, sour throat, muscle pain, fatigue, loss of taste and Smell. Some of symptoms were observed unusual within this targeted group of people. Those unusual symptoms were: elevated heart beats, digestion issues, abdominal pain, vomiting, shivering, anxiety and depression. Guan *et al.* [15] and Qin *et al.* [49] analyzed the symptoms and reported that all those mild or moderate symptoms could steps forward into harsh lung infection and ARDS (Acute Respiratory Distress Syndrome) that will go together with by neutrophilia, lymphopenia, and thrombocytopenia.

3.8. Daily Life Activities and Exercise

Regular exercise boosts up the immunity system and person stay healthy. In broader level the routine and moderate exercise provides protection against any disease or illness and is favourable for healthy immune system. Individuals who are habitual to exercise usually stay fit and active in daily life. An investigation by Walsh *et al.* [50] explained that routine exercise influences the immunity along with the antiviral defense mechanism of immune system. Another similar study by Vina *et al.* [51] considered exercise as one of the most important and repeatedly given treatments in diseased condition as well as in healthy conditions.

The results of the current study revealed that about 44.6 % people get proper rest during their infection phase. Whereas, remaining 55.4% people did different form of exercises of their daily life. From exercise group 35.6% individuals used to continue their jobs, 10.9% used to do their routine walk for exertion while 8.9% people did their field work properly. A study by Sallis *et al.* [52] analyzed the patients of Corona virus and observed minor danger and having severe outcomes in patients engaged in fewer physical activities comparatively those who were constantly inactive. Davies *et al.* [53] highlighted that exercises and all other types of physical activities are very important in conserving mass of muscles by making active the process of muscle protein synthesis.

3.9. Dietary Patterns during Infection

Taking balance amount of nutrients and proper diet boost up the immune system, and is essentially required in order to maintain health and appropriate functioning of cells. A healthy immune system can easily defend body against pathogens. Appropriate quantity of highly nutritious food is taken during any infection. Aman and Masood [54] reported that the optimum intake of healthy diet and dietary nutrients influence the immune system of the body. An appropriate diet can make sure that body is in good condition to overcome the viral attack. Afshin *et al.* [55] highlighted that a deprived or bad diet causes more demises worldwide than that of smoking or hypertension.

In the present study, we have investigated the types of food consumed during COVID-19 infection. Individuals are divided into two groups:

Regular diet group and Special diet group. About 25% participants used their normal regular diet during their infection phase, on the other hand 75% individuals treated themselves with specific diet for their rapid and healthy recovery. Individuals among these 75% consumed protein rich food in form of meat, meat stock, chicken and fish. Secondly, most intakes were of functional foods (nuts and dry fruits) then vegetables and fresh fruits along with milk and milk products. Deutz *et al.* [56] investigated in their study and suggested that higher intake of protein possibly will be advantageous to aged people particularly those having any chronic disease. According to WHO [10] a healthy nutrition and water consumption are very essential. A diet in balanced proportion keeps person well and in good physical shape, boost the immune system, and decreases infections and chronic diseases risk. Diet consisting of fresh and organic foods is suggested for daily intake, in order to supply the needed nutrients for the body.

3.10. Reinfection after Vaccination and Types of Vaccines

Results of the current study revealed that more than 65% people get infected even after COVID-19 vaccination. Reinfection was also observed in recovered patients of COVID who are not vaccinated. The vaccinated individuals did not have any severe symptoms. Prado-Vivar *et al.* [57] reported reinfection in an immuno compromised patient of 46 years and a similar study by Tillet *et al.* [58] reported reinfection in 25-year-old immuno-deficient patient.

3.11. Pregnancy Effected by COVID-19

Baud *et al.* [59] reported an instance of unsuccessful labor during the second trimester of pregnancy in a lady with COVID-19, which gave off an impression of being identified with placental disease with SARS-CoV-2. During the current study, among the target group, there were some pregnant females who recovered from infection, one of them gone through miscarriage at first trimester. We also tested child for COVID-19 infection transfer from mother to baby by testing antibody's presence in child after birth as mother suffered from COVID-19 during pregnancy. The new born was not found to be symptomatic for infection. Antibody test by rapid kit was negative.

In a research, Wang *et al.* [60] reported that coronavirus was not spotted in the blood of umbilical cord. Another related study by Schwartz [61] studied pregnant women suffering from COVID infection and reported, that there is no reference of transplacental or intrauterine transmission of virus. Zeng *et al.* [62] investigated immunoglobulin M (IgM) in babies and revealed a high level of antibody IgM in their blood. A contradictory study by Farhat *et al.* [63] reported that about four neonates were positive for PCR test, as their mothers were symptomatic and doubtful for COVID-19 infection during pregnancy.

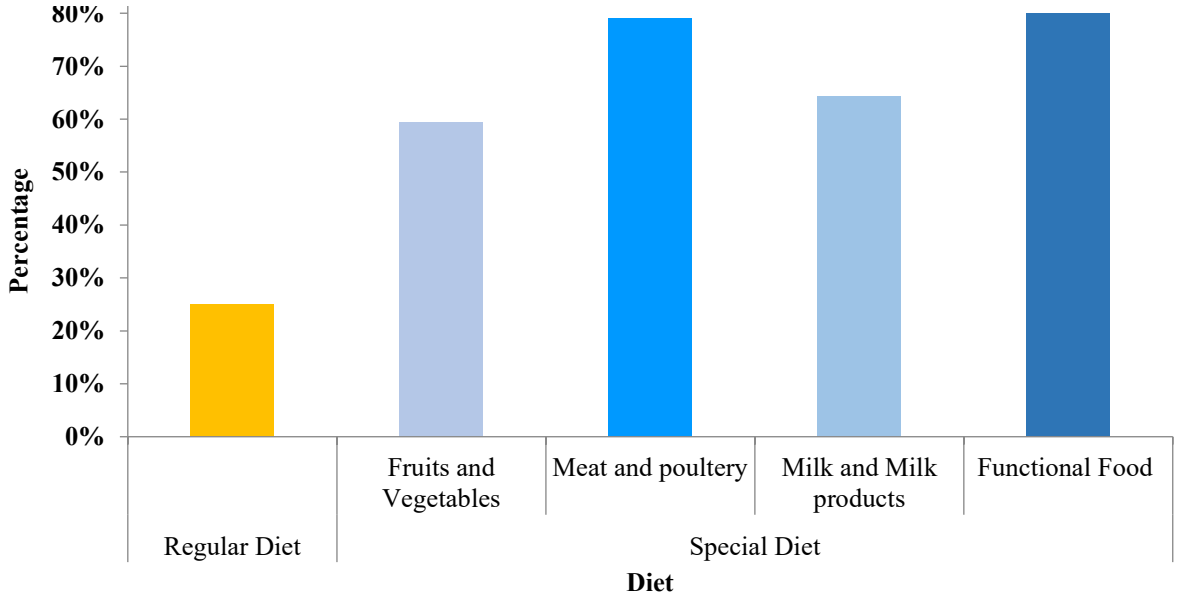

Fig. 5. Diet taken by individuals during their Infection period.

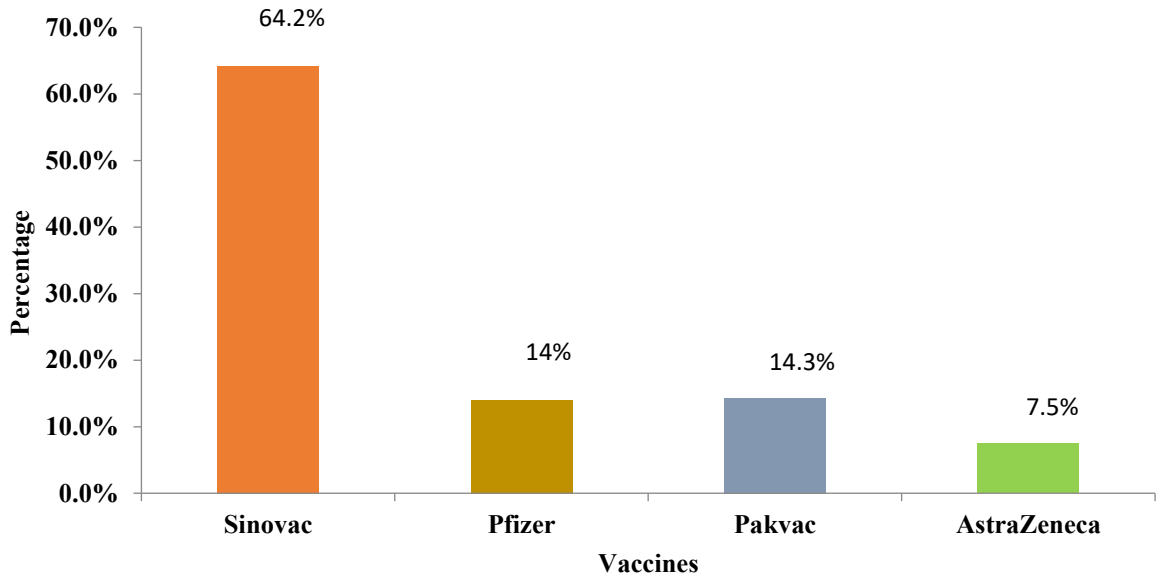

Fig. 6. Types of vaccines given to the followed-up patients.

4. CONCLUSIONS

In conclusion, the results of the present study indicate a sustained and extended positive immune response among recovered patients of COVID-19. Immunity against COVID-19 observed during this investigation was more than eight months after infection. Aged people got severe conditions but the most infected age group recorded was 31-40 years. Males were more infected than female individuals. A+ blood group was observed to be more susceptible towards infection. The high Hb levels being the inhabitants of areas having higher altitudes, may be a factor in retaining higher immunity levels. This is coupled with pure intake of traditional and organic diet and strenuous pattern of life (in terms of life style in vogue). Vaccination prevented people from severity in disease. Due to strong innate immunity of people from this region, they recovered more quickly. The co-morbidities and immuno-compromised condition along with dietary habits and regular exercise was revealed where results showed high hemoglobin levels coupled with healthy and nutrition rich diet, regular exercise. It has a positive impact on early recovery of Covid infected patients. All these factors collectively might have a positive impact on early and rapid recovery of Covid infected patients of this area.

5. RECOMMENDATIONS

According to our knowledge, there are no other studies regarding corona conducted in Gilgit Baltistan, therefore, more research is needed. Specifically, on the recurrent infection on those patients who already recovered from Covid during 1st, 2nd and 3rd wave. Multi-dimensional analysis should be performed related to Covid from this area in broader level in context of the genetic/molecular characteristics. The transfer of Corona virus to neonates needs to be further investigated. No studies suggesting an ethnic preponderance to COVID-19 infection in this area although the disease behavior has been diverse. The increased prevalence in people having blood group A is needed to be explored further in terms of the detailed body immune pre-cursors and their subsequent role portraying higher susceptibility rates.

6. CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest concerning research, authorship, of this article.

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Research Article

Prevalence of Microplastic Pollution in Freshwater Ecosystem: A Case Study of Thal Canal

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Abstract: Microplastic pollution in the aquatic ecosystems is a hot global debate due to wide spread effects on human life and environment. This study aims to identify microplastic pollution presence in the Main Line (ML) Thal canal, its distributaries and provide an overall estimation of microplastics concentration in surface water of Thal Canal. Six major sampling points at ML canal and distributaries were assessed for microplastic prevalence out of which five locations showed microplastic contamination. Size, structure and type of microplastic were assessed using light microscopy and FTIR. The study provided baseline information about the prevalence of microplastics in Thal Canal and evaluated their categories according to their size, color and type of polymers. Microplastics concentration in the canal ranged from 6.4 ± 0.5 to 8.8 ± 0.5 particles/m^3. Films, mostly transparent in coloration, were the most prominent microplastic type appeared in this study. Polyethylene with 55% presence was the most prevalent type of microplastics found in the canal and the distributaries. This study provides a better understanding of the extent of microplastic pollution assessment in Thal canal with equal emphasis on Microplastic presence in distributaries which may be beneficial in identifying the introduction of microplastics at sources.

Keywords: Microplastic, Sampling, Thal Canal, Assessment, Characterization.

1. INTRODUCTION

Synthetic products of petroleum made up of repeating macro molecules having high molecular weight are known as plastic. There are more than 200 families of plastics [1]. These are more durable, convenient and due to their increasing demand, unchecked proliferation and skyrocketing production is causing their accumulation in the environment. Macro and microplastic have a very smaller boundary to separate that is the size of microplastic < 5 mm [2]. Microplastic (MP) itself has two categories depending upon the origin. Primary microplastics come as pellets and used as feedstock of plastic industry or abrasive. Secondary microplastic are broken fragments of larger plastic materials [2].

Since 1970, plastic pollution in the marine environment is being studied. The freshwater system is least understood, but is getting attention with every passing day. In oceanic ecosystem, microplastics are present in surface water column, near shores and in deep sea sediments [3]. Microplastics can be tossed to thousands of miles away with the tides and oceanic currents [4]. Microplastics due to their resemblance in size with planktons and food of marine fish can be ingested by these marine biotas. Thus, transfer of these pollutants in upper food chain is also a debating arena [1, 4].

Freshwaters may amass a large number of microplastic particles and fibers; yet freshwater microplastics have received less attention than those in seawaters. Microplastics can be found in such fresh waters as sources (such as wastewater plants), transfer media (such as rivers), and sinks (such as isolated lakes), which may differ from those found in seawaters due to huge changes in amount [5]. In the fresh water ecosystem, microplastic addition is a terrestrial factor influenced by roads, agriculture

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plastics, atmospheric dust and industrial effluents [6]. In freshwater ecosystem, microplastics are mostly present on the surface forming a layer or in deposited sediments form, and are settled at the very bottom. Microplastics serve as the vessels for the pollutants adhesive to their micro surface and also as transporter of invasive species to the remote areas, creating threat to native fauna and flora [7]. The micro spores adhesive to microplastics, survive longer time than in the aquatic environment and transferred successfully. Aquatic biota like fish engulf these plastics, which often cause digestive tract damage and decline to the population. These are often transferred from lower trophic levels to the upper trophic levels causing toxic effects due to toxic pollutants adhesive to their surface [4].

Microplastic on the surface of water also form a microlayer which put different undescribed effects to the aquatic environment, their accumulation increases as sediments deposition, slow degrading material and causing pollution by leaching of toxic compounds [8, 9]. Therefore, the canal irrigation water has no exception from microplastic pollution, as it comes from fresh water bodies, i.e., rivers. Microplastic pollution is causing severe harm to food crops decreasing their yield. Pesticides and fertilizers adhesion to microplastic reduces their bioavailability and causes environmental pollution. Possible sources of microplastic pollution in canal water could be due to (i) fragments of plastic debris present in the environment, (ii) runoff and deposition from the surroundings, and (iii) from agricultural practices. Therefore, major source includes fragmentation of intentionally or unintentionally discarded plastic debris. Canal water sampled near urban areas contained many types of suspended riverine particles and different types of microplastics similar to those in wastewater and sludge [10, 11].

The unavailability of data about microplastic pollution in canal irrigation water is presenting a gap of knowledge for global understanding. This comparatively new research area would get benefit from more quality assurance/quality control design, as there is a current dearth of data relevant to reference materials, proficient testing and training. The skyrocketing production, increased usage and insufficient waste management of plastics are causing microplastic introduction in freshwater environments globally. Most of documented work about microplastic pollution in aquatic ecosystems is about marine environment. The indication of inflow mechanism and pointing out sources is as important as the assessment of microplastics concentrations in freshwater. The objective of current research was the provision of first hand quantitative and qualitative data of micro plastic contamination in Thal canal irrigation water and creating a base line data for future studies.

2. MATERIALS AND METHODS

Assessment of micro plastics in Thal Canal was conducted to check their prevalence and distribution. Their size, color and types were analyzed to see their physical and chemical characteristics. Sampling site is the Main Line Thal Canal and its different distributaries. Thal canal originates from Indus River at the location of Jinnah Barrage in Kalabagh, district Mianwali. The coordinates for its origin point are 32.916069° N at longitude and 71.527647°E latitude. Sampling was done from the major distributaries located in the Bhakkar District. The schematic map of irrigation network showing Thal Canal is presented in Figure 1.

2.1. Phase 1: Walk Through Survey and Sample Site Selection

Thal canal is the main sources of irrigation water in Thal region distributing water from the Indus river system to quench the thirst of Thal desert. The supply of 8000 cusec water to three lac acre cultivated land is made possible by different distributaries originating from ML Thal canal at different head-works. Therefore, walk through surveys in the Bhakkar district's main areas provided the insight to select the correct sampling site. It was kept in focus that the site was easily accessible, less turbulent and fragile to MPs contamination by being in public reach or at the prime vicinity of population, roads, farms and agriculturally active areas. For data collection, six sampling points were selected.

2.2. Phase 2: Sample Collection from Surface Water of Thal Canal and its Distributaries

The sampling started at tail and moved towards the head area in Bhakkar district. The Trawl was

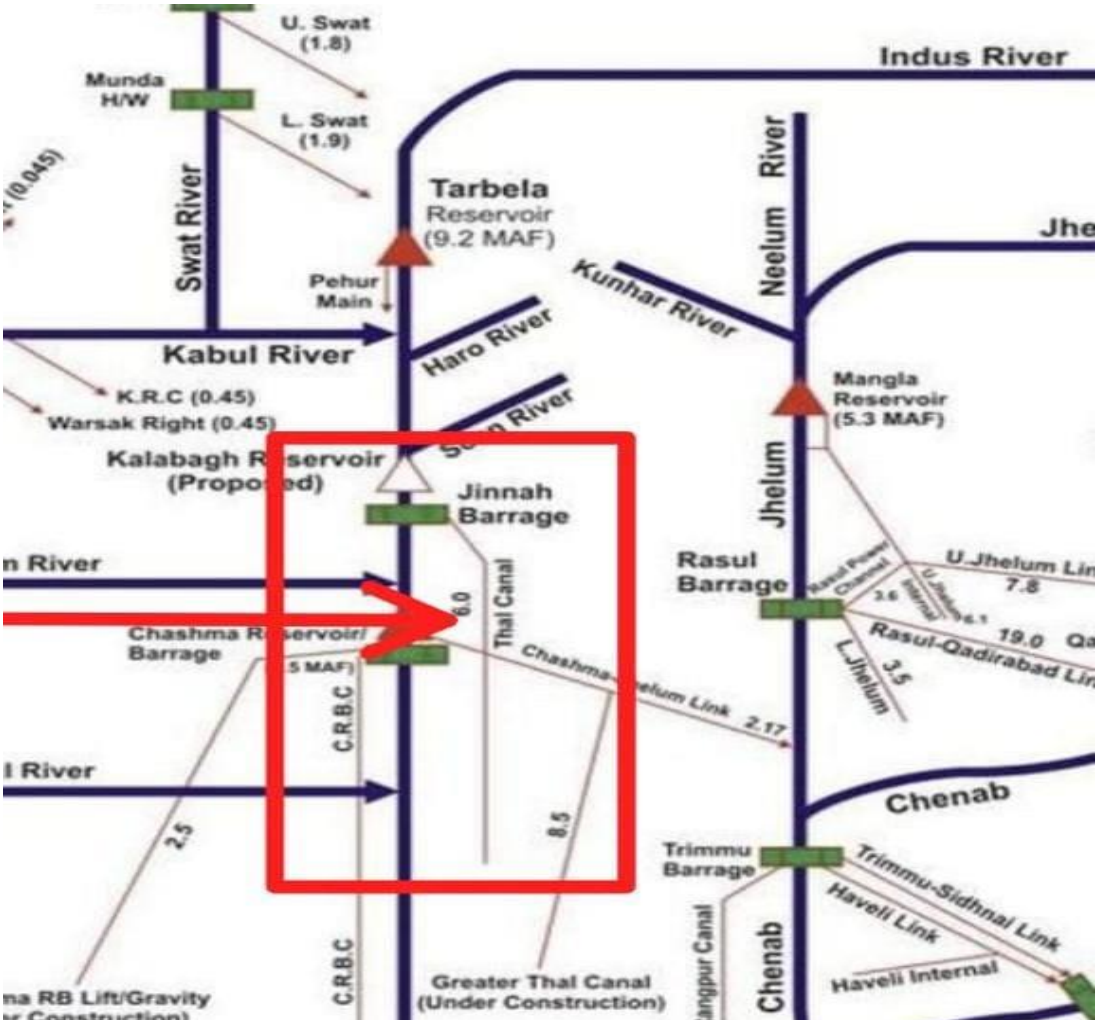

Fig. 1. Schematic map of irrigation network showing Thal Canal.

manufactured according to equipment specifications for sampling [12]. The location of sites is presented with coordinates given in the Table 1.

Samples of microplastic were collected by using microplastic-sampling protocols [12]. Samples from surface of canal water was acquired by using manta trawl (Table 2) with mesh size ~330 µm. From each site, at least 2-3 samples were collected during 5-15 minutes of water flow for every sample collection. Microplastics collected in the trawl net were washed from outside and collected on a standard sieve with mesh sized 2 mm to get the concise sample. The larger particles were manually segregated from the main sample. Table 2 depicted the specifications of trawl.

Sediments sample collection was done by metal spatula and sieve. Wet sieving was performed to remove clay and get clean and categorized sample.

Table 1. Sampling points coordinates.

S. No.	Sampling Site	No. of Samples	Coordinates (Latitude, Longitude)	
1	Firdous Head (main line Canal)	3	31.57064	71.135295
2	Khokhar disty (Distributary)	3	31.568358	71.133897
3	Thalla Sarein (ML Canal)	3	31.925071	71.231438
4	Hukam Minor (Distributary)	3	31.931255	71.227804
5	Tinda Section (ML Canal)	3	31.956893	71.233641
6	FG Kallurkot	3	32.12025	71.31067

Table 2. Manta Trawl specifications.

Trawl frame part	Length	Width	Height
Wings	23 inches each side	5 inches	2 inches
Aperture	20 inches	7 inches (From inlet towards outlet)	11 inches inlet 6 inches outlet
Meshing Net	95 inches (2.4 meters)	Fixed to aperture box	Fixed to aperture box
Towing ropes	8 meters	-	-

The material on the sieve was photographed and documented, while the material on the larger sieve was rinsed until the filtrate is practically transparent and free of clay [12]. These samples were transferred to jars rinsed with distilled water covered with aluminum foil and placed for drying in oven for 24 hours at 65 °C.

2.3. PHASE 3: Manual Sorting and Sample Preparation

The samples collected from all sampling sites were transferred to lab in air tight jars. Every sample was placed into a wash basin for removing contamination and sorting the larger particles, gravel and dregs from smaller particles. This was done in sieve washing and the gravel and solid non plastic materials were discarded.

2.4. PHASE 4: Lab Processing and Analysis: Sample digestion

The next step was to remove organic matter and non-plastic materials stuck with plastic particles, which were accomplished by digesting both the water and sediment samples. The washed, purified, sorted sample after drying in oven for 24 hours was settled for digestion. The sample in jar was shifted to a beaker in the presence of 20 ml distilled water and digestion chemical (Fenton reagent) [12]. It comprises of 30% hydrogen peroxide (H_2O_2) in the presence of 0.05 M Fe (II) solution. The beaker was covered with aluminum foil and placed on magnetic hot plate at 65 °C temperature.

In the whole digestion process the gas bubbles in the beaker were observed and the process continued until the bubbles stopped appearing in the beaker. It was the sign of digested sample, free of organic and non-plastic materials. The whole process took 53 minutes to complete and removal of bubbles. The digested material was then left to be cooled at room temperature and allowed to settle down.

2.4.1. Density separation

Following digestion, a homogeneous solution containing various types of suspended particles was utilized for density separation, with low-density materials being separated from high-density particles. The saturated solution of NaCl was used for density based separation of microplastics according to the method of Wang et al. [13] with some little changes. NaCl weighing 337 g was dissolved in 1 L of distilled water to prepare a saline solution. The NaCl method has a good separation rate and been used widely to separate microplastics and other materials [13-16].

The homogenized sample was properly shifted into a clean 500 ml glass beaker (washed with deionized water). After that, 500 ml of the NaCl filtered solution was introduced and stirred with rod for 2 to 5 minutes. The sample was left for settling for 5 hours. In a 150 ml beaker, the suspension was decanted carefully. With this process, the lower density microplastics floated into the upper water, and the higher density non-plastic particles sank to the bottom, thus recovering the micro plastics in the supernatant.

2.5. Counting and Morphological Identification of Microplastics

The oven dried sample placed for counting of particle type by their size, color and appearance. The pictorial representation of separated Microplastics is shown in Figure 2. All collected samples were observed for their size, structure, color and type under light microscopy and FTIR spectroscopy

Fig. 2. Pictorial representation of separated Microplastics.

technique. Microplastics were also divided into five categories depending on their morphology (fibres, sheets, pieces, foams, and beads).

3. RESULTS AND DISCUSSION

The present study was intended towards the estimation of microplastic pollution in Thal Canal and highlighting the sources contributing to this pollution by assessing the microplastic inputs and identifying the potential contributors to the microplastic contamination.

3.1. Counting and Morphological Identification

Altogether, microplastic detected had the size range of 0.045 to 5 mm as previous studies reported [17-19] with 74.5% falling in the range of under 0.33 mm and 25.5% within 0.33 to 5 mm range (Figure 3), larger than 5 mm plastic particles were discarded [20]. The majority of the detected microplastic fractions consisted of finer proportions. The high proportions of secondary finer particles point out to the degradation of large plastic fragments [21] which provide endorsement to our previous statement of biotic and abiotic breakdown of plastic to smaller fractions that results in underestimation of microplastics. These smaller and finer plastic proportions together with other apparent features (color and shape) may tempt and persuade aquatic biota and resulted in plastic ingestion [22-26].

The most abundant type of microplastic in our results was transparent extending to 60% (Figure 4), which may points out the transparent plastics' extensive use for packaging of food and other materials, and single use shopping bags. The remaining proportion of identified microplastics comprised of colored particles majorly blue, green and white that may be linked to packaging material, cleaning products, bottles, cosmetics, and clothing [27]. In modern times, single use plastic goods have become an integral part of our daily lives which started the competition of making products more alluring through coloring [25, 28] not considering the prospects of toxic effects [24, 29, 30]. Though lack of evidence exists but coloration may spoil the polymer characteristics [31], the weathering of plastics may give rise to microplastic pollutions in both terrestrial and aquatic environment [21].

According to results by shape the most identified plastics were irregular hard fragments (Figure 5), which originate mostly as a result of weathering of larger plastic waste [32]. Second most found shape on the list was fibers and could have been the result of sewage and garbage from household in the vicinity of canal, air deposition

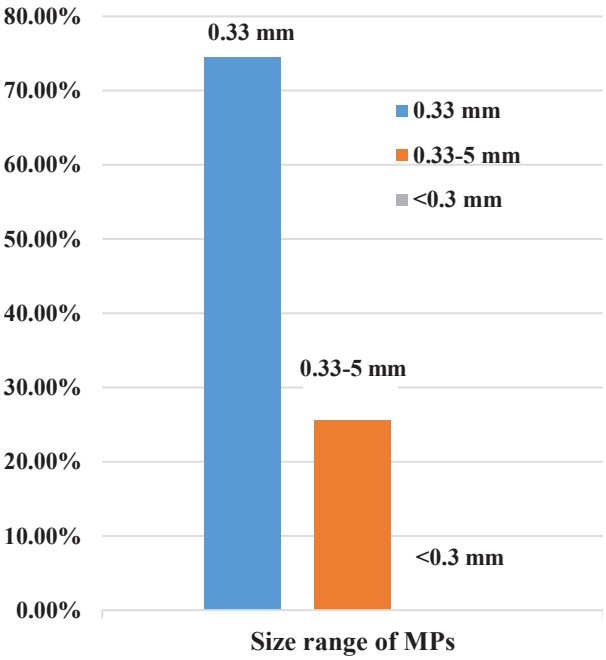

Fig. 3. Size range percentile of collected microplastics.

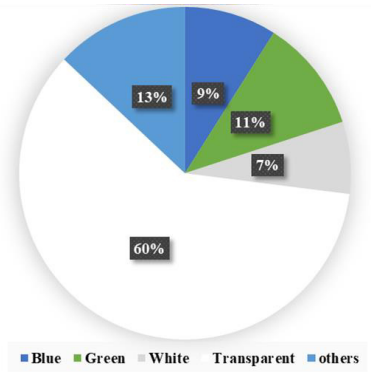

Fig. 4. Percentage distribution of Microplastic by color.

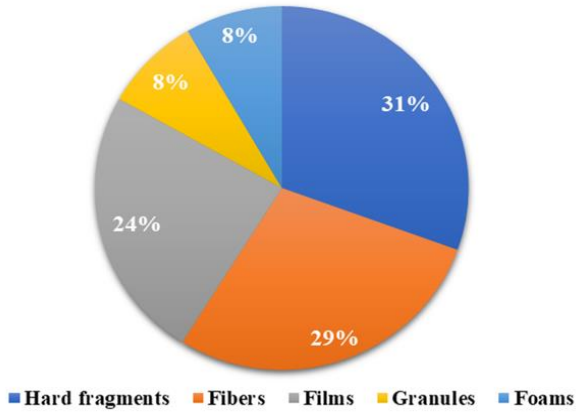

Fig. 5. Microplastic percentage by morphological

from roads and nearby farms or disintegrated fertilizer and seed bags [25, 33, 34]. Films were also the major contributor in the list and this could also be the result of breakdown of larger plastic waste. Small numbers of granules were also present in the samples, which pointed towards cleaning products and cosmetics as their source [34], while presence of foams in the samples might have a source origin from the packaging and building materials [30].

3.2. Categorization of Polymers

In our analysis, we identified total four types of polymers (Table 3). The predominant type of microplastic identified was Polyethylene in Thal Canal and its distributaries which is coherent with previous studies on freshwater ecosystem [35, 36]. Total 95 particles of varying shape, color and sizes were carefully selected as representative of each sampling visit's visually identical 50 fractions [25, 37]. Polyethylene (PE) was most recognized type among the selected particles as followed by polypropylene (PP) and polystyrene (PS) which is testimony to the previous literature and the fact that PE, PP and PS are single-use types of plastics largely produced [30, 38-41].

3.3. FTIR Spectrum of Identified Polymers

Each peak showing in Figure 6 at specific frequency range in the absorption spectra tells about the specific compound class and chemical group. The first two sharp peaks show the frequency range of compounds of alkane which are polypropylene. The second peak is medium peak and the frequency

Table 3. Polymer types of Microplastic.

	Polyethylene (PE)	Polyester (P)	Polystyrene (PS)	Polypropylene (PP)
Granules	8			
Fibers	13	9		5
Films	17		1	5
Foams			8	
Hard fragment	20			7
Percentage (%)	62	10	10	18

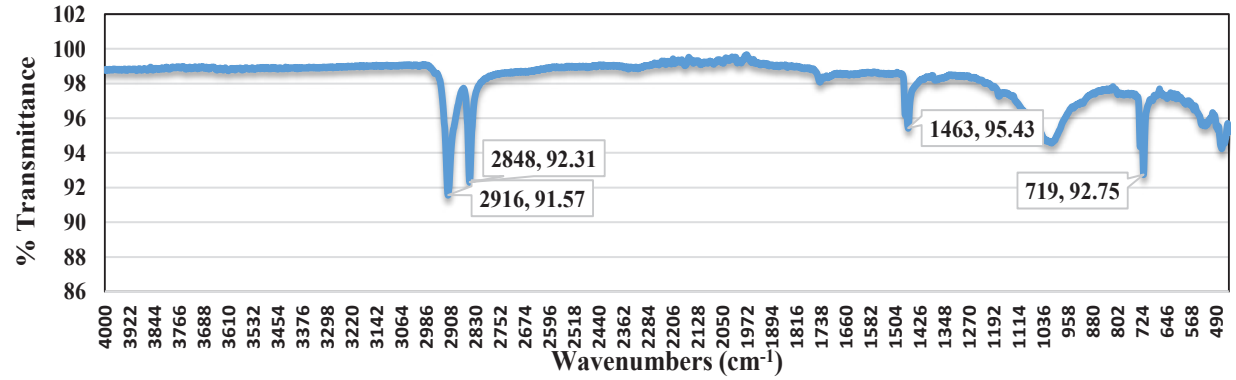

Fig. 6. FTIR spectra of MPs sample.

range represents the Methylene group, those of polyethylene plastic which are often referred as engineering plastic. The third and last peak is a medium peak and falls in the frequency range of Benzene derivative compounds group which are polystyrene compounds mostly synthetic rubber and food packaging materials.

4. CONCLUSIONS

The study assessed the microplastic pollution prevalence and abundances in Thal Canal and its distributaries. Consistent with our assumption, the Canal's surface water was found contaminated with microplastics. The concentrations of microplastics were between 6.4 ± 0.5 to 8.8 ± 0.5 particles/m³. Household dumping and sewage waste along the distributaries and main line canal proved to be the primary sources of pollution. In distributaries, microplastics were abundant at the points closer to the high population areas. Polyethylene was found as the dominant type of microplastic, almost 55% of the total microplastics collected from the canal. The dominant microplastics in the canal were fine sized microplastics, which together with the prevalence of secondary microplastics, indicated about the possible breakdown of the larger plastic waste. The major characteristics of sampled microplastics were films and transparent nature. Subjected to polymer identification, polyethylene and polypropylene dominated the mega proportion of microplastics. This preliminary study can notify policymakers for being concerned about microplastic pollution and articulating a suitable microplastic controlling and management system. Further studies should be conducted on the Thal Canal and its distributaries to evaluate the microplastic concentration in the outflow water. Similar studies must be conducted for soil and crops in the Thal region mostly irrigated by Thal Canal water to predict the effect of microplastic on these areas of concern as well.

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Price Distortions and Competitiveness of Cotton Production in Pakistan

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Abstract: The research is conducted to determine the cotton price distortions, as well as competitiveness at national and international levels. National competitiveness has been measured by using the nominal protection coefficient (NPC) and benefit-cost ratios for the period 2008-09 to 2018-19. To gauge international competitiveness, trade base indices under the revealed comparative advantage approach are used. A comparison of the international competitiveness of Pakistan with major cotton-exporting countries has also been made. The results of this research revealed that at the national level cotton producers in Pakistan faced implicit taxation for most of the years during the study period, as NPC_i averaged at 0.87 as an import substitution crop. Export parity prices were slightly less or equal to the domestic prices by varying degrees as NPC_e averaged at 1.06. The production cost grew at the rate of 12.3% per annum, while cotton output prices grew at the rate of 8.4 percent per annum. Consequently, a decrease was registered in the benefit-cost ratio during the study period. Pakistan has experienced comparative and competitive advantage for cotton as indicated by results of revealed comparative advantage (RCA) indices. A comparison of Pakistan's indices with main exporters/competitors demonstrated that Pakistan has a relatively higher comparative and competitive advantage for cotton. However, Pakistan's international competitiveness exhibited a declining pattern since 2011-12. This result is consistent with the national level scenarios as the benefit-cost ratio has declined over this period. It can be concluded that productivity growth and a reduction in input costs are needed to improve overall competitiveness in cotton production and trade.

Keywords: Cotton, Competitiveness, Revealed Comparative Advantage, Price Distortions, Protection, Implicit Taxation.

1. INTRODUCTION

Pakistan is fifth largest producer, third largest consumer of cotton and second largest cotton yarn exporter [1]. Cotton production supports Pakistan's largest industrial sector by providing domestic consumption of final textile products. Cotton and cotton products have 10 percent contribution in agricultural GDP and 55 percent of the total exports' earnings [2]. Agriculture is sensitive not only to climate change, but also to globalization of agriculture. To mitigate the sensitivity of agriculture, governments intervene in agriculture to achieve desired policy goals of food security and agriculture competitiveness [3].

This study on price distortions demonstrates the extent to which domestic prices of cotton deviate from its corresponding free trade reference prices, i.e., export parity in case of exportable and import parity prices in case of importable. The distortions induced from the market failure, and/or trade and price related policies prevailed in the country. These distortions may be biased towards farmers and discourage the agricultural production. To maintain the competitiveness there are

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interventions in the agricultural sector in different economies. These interventions may increase the government revenue through implicit taxation; however, these are counter balanced by the loss of earning in term of foreign exchange. The cost of market failure or policy distortions to incentive in term of resource misallocation tends to be greater in production substitution [4]. Cost efficiency in production and marketing is prerequisite for maintaining competitiveness. So, there is a need to reduce per unit production and marketing cost by focusing productivity growth. There is serious threat to farmers due to climate change, which frequently cause failure of crop with outcome of huge financial losses [5].

Pakistan being one of the fifth larger producer and 3rd largest consumer of cotton in world, hence market outcomes in Pakistan is expected to have an impact on global cotton trade [1]. Having this in background, this research seeks the answer of prices distortion, i.e., how far domestic prices of cotton deviate from its corresponding free trade reference prices (import and export parities) to gauge the level of distortions. By addressing the question, it has been determined that whether the country's trade and prices pattern (or policies) have built-in bias towards producers or consumers. As in less developed countries (LDCs) price distortion arises from government policy interventions or market failure effects. The current market system in Pakistan has no capacity to serve the farmers and consumers to save them from boom and bust cycles of the commodity prices [4]. This research study has been planned to exam cotton prices distortions and its implication for farmer's incentives and country's competitiveness of the strategic commodity, in international market. Findings of the present research investigation are important to determine the level of protection and incentive structure prevailing in the country. Existing literature reveals that in developing countries agricultural sector is often taxed, while consumption is subsidized which discourage the production [4]. This approach has been criticized, with the argument that increases in tax revenue to government is counterbalanced by a loss of agriculture foreign exchange earnings. Farm input and output markets in Pakistan have long history of state interventions. The public inter interventions in commodity market such as export monopolies in export of rice and cotton and wheat procurement system are few examples [6], however state trading monopolies have been abolished in agricultural products since 2003 in trade liberation move [7].

Historically, in Pakistan implementation of support prices for major crops was aimed to provide a floor for market prices in the harvesting season [8]. State monopoly of exports and imports, restrictions on commodity movement and issuing of wheat to flour mills at subsidized prices have prime objective of provision of cheap food to consumers. Similarly, public sector monopoly in cotton and rice exports has also been maintained to develop export markets. Input subsidies are provided to encourage adoption of agricultural technology. The impact of these policy interventions was a source of distorted agricultural prices and incentives to producers. The policy interventions not only distort agricultural prices but also a source of lowered real prices of tradable agriculture produce [6]. In Pakistan, Rani *et al*. [9] suggested that government should ensure stability in prices that will also helpful in reducing area instability for cotton in the country.

Ender [10] used producer and consumer subsidy equivalent to gauge government intervention in agriculture. He concluded that in case of reduction in domestic support to export in other countries of the world prices go up and Pakistan can reap the benefit of liberalization. By using the same methodology Longmire and Debord [11] measured impact of policies on selected crops including cotton. The result revealed that Pakistan has strong comparative advantage in cotton production, whereas the authors suggested that inputs and output prices of cotton should be near to social cost [11].

Similarly, Akhtar *et al.* [12] studied incentive structure of rice and concluded that, to ensure the farm level competitiveness there is strong need to remove policy distortions and market failure effects. Moreover, Akhtar *et al*. [13] identified that distortions in inputs and output markets result in deviation in economic and private profitability. Javed *et al.* [14] reported that the cotton is losing its competitive position when it's cost of production increases and domestic prices go up. The prime objective of this research is to estimate degree of protection and economic incentives in cotton

production in Pakistan and to identity measures to enhance incentive structure for the cotton farmers in Pakistan to achieve the global competitiveness.

2. MATERIALS AND METHODS

In this study secondary data have been used for the analysis. The analysis covered the period through 2008-09 to 2018-19. Nominal protection coefficients (NPCs) and Benefit Cost Ratio (BCR) are used to quantify extent of protection and dis-protection and incentive structure for farmers prevailing in Pakistan. In the growth analysis compound growth rate is used to gauge the growth in different variables to determine pattern in the cotton competitiveness with respect to market and prices distortions in Pakistan. A well-recognized methodology as used by Anderson *et al.* [15], has been employed to study the market and prices distortions in the cotton. Tsakok [16] described the methods of measuring distortions based on world reference prices for a country that provides a measure of opportunity cost. He further stated that this measure also provides an indication of efficiency in production. Appleyard [17] argued that for tradable commodity efficiency can be measured by using a well-known indicator namely Nominal Protection Coefficient (NPC).

2.1. Nominal Protection Coefficient (NPC)

NPC is the empirical measure of price distortions reflected due to the taxation or protection in the commodity, this indicator also gauges the incentive structure for domestic producer of the traded commodity [18]. The nominal protection coefficient (NPC_{ijt}) as import substitution crop at time year t is given:

$$NPC_{ijt} = \frac{(Pd_{ijt})}{(Pb_{ijt})} \quad (1)$$

Where, Pd_{ijt} is Domestic price of cotton, Pb_{ijt} is Import parity prices of cotton, and Pb_{ejt} is Export parity price of cotton.

The nominal protection coefficient (NPC_{ejt}) as export promotion crop at time year t is given:

$$NPC_{ejt} = \frac{(Pb_{ijt})}{(Pb_{ejt})} \quad (2)$$

When NPC = 1, it demonstrates that there is no distortion and situation is neutral, showing neither an incentive nor disincentives in domestic production. Whereas, if NPC > 1, it shows a protection or subsidy for domestic production and finally, when NPC < 1, there is negative protection (i.e., a tax) for domestic production.

2.2. Benefit Cost Ratio

The benefit cost ratio with R_t as referred the total revenue and C_t reported as the total cost. Total revenue of cotton crop determines the benefits generated through the production of cotton crop. Total cost encompasses all the expenditures on inputs regarding cotton cultivation.

$$BC_{ratio} = \sum_{t=0}^{n} \frac{R_t/(1+i)^t}{C_t/(1+i)^t} \quad (3)$$

Where, R_t stands for revenue at time year t, C_t stands for cost at time year t, n stands for number of periods years, and i stands to discount rates.

When greater than 1, benefit cost ratio indicates that crop is suitable, because the benefits measured by the present value of the total revenues (inflows), are greater than the costs, measured by the present value of the total outflows.

2.3. Compound Growth Rate

To capture the analysis for whole out-going decade and to calculate the compound growth rate, year 2008-09 was set as starting/base year while following formula was applied as used by Rani *et al*. [9].

$$Y_t = Y_0/(1+r)^t \quad (4)$$

Where, Y_t stands for Area/ production/ Yield/ import/exports in year t, Y_0 stands for base year Area/ production/ Yield/ import/exports, r is the compound rate of growth of Y_t.

2.4. Revealed Comparative Advantage Index

Rumankova *et al*. [18] has shown that numerous aspects determine export competitiveness of crops. Actual export performance of any country is demonstrated by country's comparative advantage analysis by using a recognized measure called revealed comparative advantage (RCA) index. RCA based indices are used to measure country's international competitiveness in large number of empirical studies in the literature. RCA indicator as

used in previous studies, i.e., Sossa [19] and Ballasa [20], premeditated as follows has been applied:

$$RCA_{ijt} = \left(X_{ijt}/\sum X_{ait}\right)/\left(X_{iwt}/\sum X_{awt}\right) \qquad (5)$$

Where, RCA_{ijt} is Revealed Comparative Advantage index value for product i in country j in year t, X_{ijt} is Export of product i in country j in year t, X_{iwt} is Total world exports of product i in year t, $\sum X_{ajt}$ is Total exports in country j in year t, $\sum X_{awt}$ is Total world exports in year t, and Product "i" mean cotton.

2.5. Revealed Symmetric Comparative Advantage (RSCA) Index

To make the RCA index symmetric, Laursen [21] and Dalum *et al*. [22] adjusted the RCA index values, which is called adjusted value. The adjusted index values are between –1 and +1. This index is called Revealed Symmetric Comparative Advantage index (RSCA) which enables symmetric index value of RCA. The RSCA is calculated as follows:

$$RSCA_{ijt} = \left(RCA_{ijt} - 1\right)/\left(RCA_{ijt} + 1\right) \qquad (6)$$

The positive (or negative) values of RSCA show a competitive advantage (or disadvantage) in exporting product *j*. In the economics literature, the RSCA is often interpreted as an index of specialization.

2.6. Relative Export Advantage Index (RXA)

The RXA has been used previously, where it is considered that this indicator is more sophisticated and comprehensive measure of international competitiveness [23, 24]. The index is defined as the ratio of a country's export share of a certain product in the world market to the same country's share in world export of all other commodities. While estimating this indicator, the world "total" must be always taken as the sum of all countries except the country under study. This avoids double counting of countries and commodities in both the numerator and the denominator. This aspect is especially relevant if a country is fairly important in trade on international markets, and/or if the commodity considered is important in total trade [25]. RXA is defined as:

$$RXA_{ij} = \frac{\left(x_{ij}/\sum_{l,l\neq j} x_{il}\right)}{\left(\sum_{k,k\neq i} x_{kj}/\sum_{k,k\neq il}\sum_{l\neq j} x_{kl}\right)} \qquad (7)$$

If greater than 1, RXA value shows competitive advantage; while less than 1, it shows competitive disadvantage.

3. RESULTS AND DISCUSSION

In the present study the growth in cotton area and production has been recorded (Table 1), which revealed that the area under cotton in Pakistan has decreased by 1.72 percent per year, whereas production came down to the tune of 0.92 percent per year during the period under analysis, i.e., 2008-09 to 2018-19. The production and area of the cotton is dependent on output prices of competing crops along with prices of inputs [26]. As sugarcane is a competing crop of cotton and due to the guaranteed price of sugarcane there is a better profitability prospects for the farmers as compared to cotton production. Hence, the area under sugarcane has grown at the rate of 0.84 percent per year in the period under analysis. While the productivity and production has grown by 2.29% and 3.15%, respectively, for the period under analysis (Table 1). Thus, due to decrease in the area and production of cotton in Pakistan the dependence of country's textile sector on imported cotton has increased. A study by Shabbir and Yaqoob [27] revealed that root cause of decrease in cotton area in Pakistan is inefficient allocation of resources that resulted in stagnant growth. While, textile manufacturers/ industry opposes to fix minimum guaranteed prices for the local farmers, rather link these with international market for cheap availability of raw cotton in the country.

Growth rate analysis in production cost, output price and benefit cost ratio have been used to determine the farmer's level competitiveness during 2008-09 to 2018-19 (Table 2). The present research was undertaken to investigate growth in different parameters of cost, yield and prices to gauge the pattern of competitiveness in cotton production during the outgoing decade. The analysis identified that highest growth rate in cost of production (12.3% per annum) was registered, whereas less growth in productivity remained -0.55% per annum and output prices grew at the rate of 8.4% per annum. High and rising cost of production of cotton in Pakistan is a major challenge to national competitiveness. Despite increase in, variable input cost (11.29%) there was also sharp increase in the fixed cost, i.e., land rent 14.9% per annum.

Table 1. Area and production of Cotton and Sugarcane in Pakistan.

Crop	Cotton			Sugarcane		
Year	Area (million hectare)	Production (Cotton Lint Prod. 'million' bales* of 375 lbs each	Cotton yield (Mound/acre)	Area (million hectare)	Production (million tonnes)	Sugarcane Yield (Maund/acre)
2008-09	2.82	11.82	20.35	1.03	50.05	526.9
2009-10	3.11	12.91	20.19	0.94	49.37	569.5
2010-11	2.69	11.5	20.88	0.99	55.31	605.7
2011-12	2.83	13.6	23.26	1.06	58.4	597.3
2012-13	2.88	13.03	21.98	1.13	63.75	611.7
2013-14	2.81	12.77	22.10	1.17	67.46	625.1
2014-15	2.96	13.96	22.89	1.14	62.83	597.6
2015-16	2.90	9.92	16.60	1.13	65.48	628.3
2016-17	2.49	10.67	20.82	1.22	75.48	670.8
2017-18	2.70	11.9	19.00	1.34	82.13	664.5
2018-19	2.37	10.78	19.25	1.12	68.25	660.7
Growth	-1.72	-0.92	-0.55	0.84	3.15	2.29

Source: GoP, Economic Survey of Pakistan 2018-19

In conclusion high and rising production costs remained a threat to maintain farmers' profitability as revealed by BCR analysis. BCR have registered a negative growth to the tune of 2.12% per annum. These results are also consistent with the findings of Maqbool *et al*. [29], which suggests that there should be more emphasis on infrastructure, reduction in the cost of production, use of modern technology, investment in agricultural sector and enhancing trade in international market to boost the exports volume of cotton. Balassa [23] argued that in addition to production cost, water shortage and high temperature are noticeable factors affecting cotton production.

The result revealed that high growth in cost and low growth in yield and output prices make the cotton less competitive. BCR was high (2.09) during 2010-11, after that cotton production is found to be economically inefficient and reached as low as level 0.78 BCR ratio in the year 2014-15 (Table 2).

International trade pattern of cotton has been analyzed to determine growth in different trade variables over the period 2008-09 to 2018-19. The result indicates that during the period under analysis there is negative growth in raw cotton export in quantity and value term to the tune of -16.64% and -8.83% per annum, respectively; however, export unit value is positive to the tune of 9.38% for the study period. The results indicate that

Table 2. Production cost, Yield, prices of output Benefit Cost Ratio of cotton in Pakistan.

Year	Variable cost	Fixed cost	Total cost	Yield per acre (kgs)	Gross Income	BCR	Domestic Prices (Rs./ Mound)
2008-09	17346.1	6500.0	23846.1	696.0	27092	1.14	1557
2009-10	18051.2	10000.0	28051.2	696.0	33338	1.19	1916
2010-11	21390.0	12000.0	33390.0	696.0	69652	2.09	4003
2011-12	26701.6	20000.0	46701.6	748.0	47835	1.02	2558
2012-13	31973.0	20000.0	51973.0	748.0	47722	0.92	2552
2013-14	32807.2	21000.0	53807.2	748.0	56923	1.06	3044
2014-15	37366.1	25000.0	62366.1	760.0	48431	0.78	2549
2015-16	37945.2	25000.0	62945.2	760.0	49894	0.79	2626
2016-17	38198.4	25000.0	63198.4	752.0	58092	0.92	3090
2017-18	43153.1	25000.0	68153.1	760.0	59945	0.88	3155
2018-19	50121.1	26000.0	76121.1	770.0	69801	0.92	3626
Growth (%)	11.29	14.9	12.3	1.01	9.93	-2.12	8.4

Source: Authors analysis by using GOP data, i.e., various issues of Cotton Policy Reports

Table 3. Raw Cotton trade in Pakistan (qty thousand tonnes), Value Million Rs., Unit Value (Rs. /Mound).

Year	Export quantity (Thousand tonnes)	Export value (Million Rs.)	Export Unit Value (Rs./ Mound)	Import quantity (Thousand tonnes	Import value (Million Rs.)	Import Unit Value (Rs./ Mound)	Pakistan Net cotton trade quantity (Thousand tonnes)	Pakistan Net cotton trade Value (Million Rs.)
2008-09	78.2	6826.5	3492	397.2	50320.3	5068	-319	-43494
2009-10	160.1	16365.5	4089	342.8	50997.5	5951	-183	-34632
2010-11	144.3	31168.4	8640	344.69	83724	9716	-200	-52556
2011-12	265.54	41393	6235	172.52	43943	10188	93	-2550
2012-13	92.5	14882	6435	429.755	85666	7973	-337	-70784
2013-14	114.79	21353	7441	266.245	59231	8899	-151	-37878
2014-15	95.017	14937	6288	154.521	34880	9029	-60	-19943
2015-16	49.55	7948	6416	436.173	78494	7198	-387	-70546
2016-17	24.976	4484	7181	460.308	84315	7327	-435	-79831
2017-18	35.347	6183	6997	610.149	122010	7999	-575	-115827
2018-19	12.665	2709	8556	414.7	107874	10406	-402	-105165
Growth (%)	-16.64	-8.83	9.38	0.43	7.92	7.46	-	-

Source: Various Policy Analysis Reports of Agriculture Policy Institute, Islamabad

during the period under analysis, growth rates of import quantity, value and unit value are positive by 0.43%, 7.92% and 7.46% per annum, respectively (Table 3). Growth rates of cotton export and import unit values in the study period are 9.38% and 7.46%, respectively. Pakistan remained a consistent net-importer of cotton in quantity term during 2008-09 to 2018-19 except in 2011-12 (Table 3). This result is consistent with previous findings [28]. In that study it has been stated that Pakistan has become net importer of cotton in recent years. The results are in line with Maqbool *et al*. [29] that during 2013 to 2017 cotton exports of Pakistan has decreased [2]. They reported that as Pakistan has comparative and competitive advantage in cotton exports, with comparative advantage in cotton imports, thus Pakistan had net competitive advantage in cotton sector from 2013-17.

Present research has been conducted to determine cotton prices distortion and its effects on competitiveness in national and international market during 2008-09 to 2018-19. These results revealed that during last decade the cotton producers faced implicit taxation for most of the years, as NPCi averaged at 0.87 as import substitution crop. The

Table 4. Domestic Market and International Prices of Cotton in Pakistan: 2008-09-to 2018-19.

Year	Domestic prices	Export parity	NPCe	Import Parity	NPC_i
2008-09	1557	1406.7	1.11	2001.7	0.78
2009-10	1916	1647.2	1.16	2350.5	0.82
2010-11	4003	3480.7	1.15	3837.8	1.04
2011-12	2558	2512.0	1.02	4024.5	0.64
2012-13	2552	2592.6	0.98	3149.5	0.81
2013-14	3044	2997.6	1.02	3515.0	0.87
2014-15	2549	2533.3	1.01	3566.5	0.71
2015-16	2626	2584.8	1.02	2843.4	0.92
2016-17	3090	2893.1	1.07	2894.1	1.07
2017-18	3155	2818.8	1.12	3159.5	1.00
2018-19	3626	3446.8	1.05	4110.4	0.88
Average	2788.73	2406.05	1.06	2949.01	0.87

Source: Authors' calculations by using GOP data

import parity prices of cotton have been generally higher than corresponding domestic prices, while export parity prices were by and large slightly less or equal to the domestic prices by varying degree as NPCe averaged at (1.06) (Table 4). This conclusion for cotton is backed by Valdes [7]. NPCe and NPCi are nominal protection coefficient estimated in relation to export and import parity prices, respectively. The results showed that prices of seed cotton in domestic and international markets are characterized by fluctuations during the period under analysis.

Based on competitiveness indicators provided in Table 5, the results revealed that Pakistan has relatively higher comparative and competitive advantage for cotton relative to its main competitors. RCA values of Pakistan have consistent decrease from 35.34 in 2011-12 to 23.9 in 2018-19 (Table 5). RXA index has more fluctuating pattern during the period under analysis. China and Turkey also registered decrease in comparative advantage slight over the investigated period, and China have fell in comparative disadvantage in 2018-19. United State of America (USA) did not have comparative advantage over the period under investigation except in 2010-11. India, Vietnam and Turkey have secured comparative advantage in cotton with fluctuating trends (Table 5). The research findings by Khalid *et al*. [30] revealed that in cotton exports Pakistan has comparative and competitive advantages. Based on these finding the authors suggested that Pakistan should explore new markets to strengthen comparative advantage in cotton exports. Ahmad and Afzal [31] reported that cotton prices and output are positively, while cost of inputs is inversely related to profitability of cotton.

4. CONCLUSIONS

Evaluation of national and international competitiveness of Pakistani cotton production revealed that low productivity, high and fast-growing production costs and low output prices are important factors of decreasing competitiveness of cotton production at national level in Pakistan. Dependence on imported cotton for local industry is increasing, as exhibited by the positive growth in raw cotton imports in term of quantity and value with a positive growth in import unit value for the period under analysis.

Area and production of the cotton in Pakistan have registered a negative growth; while, a sharp

Table 5. International Competitiveness Indicators of major cotton exporters.

Indicator	Country/ year	2008-09	2009-10	2010-11	2011-12	2012-13	2013-14	2014-15	2015-16	2016-17	2017-18	2018-19	Average
RCA	Pakistan (6)	30.37	31.19	33.46	35.34	35.33	31.84	30.43	28.34	26.64	24.79	23.90	30.15
RSCA		0.75	0.77	0.84	0.83	0.87	0.93	0.93	0.92	0.91	0.93	0.89	0.87
RXA		48.6	58.1	64.0	67.8	68.0	69.3	70.0	64.0	69.3	79.3	76.3	66.79
RCA	China (1)	1.33	1.38	1.36	1.21	1.32	1.16	1.16	1.19	1.11	1.03	0.94	1.20
RSCA		0.14	0.16	0.15	0.09	0.14	0.07	0.07	0.09	0.05	0.01	-0.03	0.09
RXA		2.68	2.23	2.37	2.40	2.29	2.57	2.73	2.50	2.41	2.26	2.46	2.45
RCA	USA (2)	0.78	0.98	1.24	0.89	0.81	0.67	0.65	0.65	0.82	0.84	0.80	0.83
RSCA		-0.13	-0.01	0.11	-0.06	-0.11	-0.20	-0.21	-0.21	-0.10	-0.09	-0.11	-0.10
RXA		1.26	1.52	1.50	1.53	1.53	1.62	1.41	1.62	2.10	1.53	1.31	1.54
RCA	India (3)	3.01	5.20	4.30	4.92	5.58	4.66	4.71	3.99	3.89	4.17	3.23	4.33
RSCA		0.50	0.68	0.62	0.66	0.70	0.65	0.65	0.60	0.59	0.61	0.53	0.62
RXA		6.74	6.50	6.13	7.41	8.96	8.32	5.70	9.33	7.50	9.34	10.62	7.87
RCA	Vietnam (4)	1.07	1.56	1.34	1.21	1.46	1.72	1.75	1.98	2.04	1.94	1.61	1.61
RSCA		0.03	0.22	0.15	0.09	0.19	0.26	0.27	0.33	0.34	0.32	0.23	0.22
RXA		0.48	0.36	0.34	0.46	0.54	1.24	1.89	2.46	2.10	2.00	2.34	1.29
RCA	Turkey (5)	2.08	2.12	2.37	1.95	2.11	1.98	1.97	2.01	1.82	1.81	1.61	1.98
RSCA		0.35	0.36	0.41	0.32	0.36	0.33	0.33	0.34	0.29	0.29	0.23	0.33
RXA		2.07	1.97	1.96	1.83	1.64	1.46	1.32	1.24	1.02	1.02	1.19	2.83

Source: Authors Calculations, Figures in parenthesis are ranking of the countries in exports of cotton in world market. RCA=Revealed Comparative Advantage, RSCA=Revealed Symmetric Comparative Advantage, RXA=Relative Export Advantage Index

and consistent increase in cost of production has been recorded. However, a slow growth in cotton output prices was registered that resulted in decrease in the benefit-cost ratio at substantial rate during 2008-09 to 2018-19.

Pakistan has relatively higher comparative and competitive advantage for cotton relative to its main competitors. However, the country has registered a consistent decrease in revealed comparative advantage position during last five years. The present analysis revealed that Pakistan is losing its competitive position and its share in international market.

Due to the low competitiveness, there is negative growth in raw cotton export in quantity and value terms, however a positive growth in export unit value is registered. It can be concluded that productivity growth is vital for improving competitive position of Pakistan at international level. Policy support is needed to maintain and improve the national and international competitiveness of cotton production in Pakistan.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Efficacy of Drumstick Tree (*Moringa oleifera*) Leaves Powder on Lipid Profile and Hematological Indices in Chickens on a High Fat Diet

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Abstract: *Moringa oleifera* belongs to the Moringaceae family and genus Moringa. *Moringa oleifera* has many beneficial pharmaceutical and nutritional properties. The present study aimed to investigate the effects of dietary supplements of *Moringa oleifera* leaves powder after consumption of a high-fat diet on cholesterol TG, HDL, LDL, VLDL and hematological parameters such as Hb, MCV, MCH, MCHC, PCV, RBCs, WBCs, PLT neutrophil, heterophil and lymphocytes count in chicks. Golden Misri Chickens were divided into 4 groups: Group 1 (the Control group) was given Feed-13. Group 2 (Experimental Group) was given Feed-13 and mustard oil (high-fat diet) Group 3 (experimental Group) was given Feed-13 + mustard oil and 1.5% *Moringa oleifera* leaves powder. Group 4 (experimental Group) was given Feed-13 and 1.5% of *Moringa oleifera* leaves powder for four weeks. The results of the current study showed a significant ($p < 0.05$) increase in body weight in Experimental Group 3. Experimental group 3 showed a significant increase in levels of HDL, TG, RBCs, and Hb, while significantly decreased ($p > 0.05$) in LDL, TLC, WBCs, MCHC, MCV, MCH, TC, TP, Monocytes count, neutrophil count, and platelet counts. *Moringa oleifera* supplementation showed a significant reduction in cholesterol, LDL, and TC levels. The findings of this study demonstrated that powdered *Moringa oleifera* is a beneficial and effective dietary supplement that increases HDL, TG, Hb, and RBCs, and decreases cholesterol levels.

Keywords: Triglyceride, High-Density Lipoprotein, Low-Density Lipoprotein, Very Low-Density Lipoprotein, Hematological Indices.

1. INTRODUCTION

Poultry production is the most popular livestock for small and medium-scale farmers in both rural and urban areas. The main aim of the poultry is to attain the greatest earnings at the smallest amount of construction cost. It greatly contributes to the gross domestic production (1.3%) [1]. Commercial poultry Production in Pakistan began in the 1960s and has made significant contributions. Even though the business of poultry production is facing different problems, such as, high cost of feed and disease. Poultry meat production is increasing day by day, and consumers are very concerned about food safety issues, such as high-quality eggs and meat without any antibiotic residues [1]. Poultry meat is making a significant contribution to the delivery of the best protein. All poultry industries have played a central role in employment. Approximately 1.5 million individuals in Pakistan are involved in these enterprises for their income. Poultry enterprises supply approximately 18% of meat to the public. The annual growth rate 8-10% is very fast-growing rate in Pakistan. Poultry farming is a starting point of income in rural areas around the world, especially in Pakistan. Desi chicks are always a source of higher income [2]. Pakistan has not succeeded in producing specific-pathogen-free (SPF) chickens and poor-quality feed, contains high levels of mycotoxins. The industry needs to control fluctuating prices and maintain a constant income. Poultry health and production are major challenges for future poultry growth. A significant portion of the daily protein (meat and eggs) has been provided to the Pakistani people. Poultry products have been added, but more sustained efforts are needed to raise

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awareness among consumers regarding the overall quality and safety of value-added products [2].

Moringa oleifera is a fast-growing cultivated plant species that belongs to the order Brassicales, Moringaceae family and genus Moringa [3]. Due to its high nutritional values, content in amino acids, flavones, and other factors, *Moringa oleifera* has earned the title of "Miracle Tree" and received viable attention. Phytochemicals, minerals, and vitamins, are present in large amounts in leaves and can be employed in cosmetic and dietary supplements [3]. Every component, including flowers, fruit, pods, and leaves, is used for both dietary and commercial purposes. *Moringa oleifera* aids in milk production. *Moringa oleifera leaves* powder can be used to protect the liver heart, kidney, and lungs; reduce pain, and belly fat [4].

Moringa oleifera Lam is used for pharmaceutical, dietary, and antibacterial purposes. *Moringa oleifera* has many nutrients, proteins, vitamins, and amino acids present in various parts of the plants. Leaf meal shows considerable beneficial remuneration, no adverse effects, and improved development and meat quality at various nutritional incorporation levels (5%–10% in chick ration and 10% in broiler ration) [5]. *Moringa oleifera* leaves supplement increase meat weight, high density lipoprotein (HDL) and decreased the level of LDL, VLDL, cholesterol, blood glucose intensity in egg laying hens and broiler hens *Moringa oleifera* leaves can improve blood chemistry, increase RBC and WBC counts as well as an increased in haemoglobin in broiler chickens [5].

2. MATERIALS AND METHODS

The experiment was performed at Minhaj University Lahore to examine the effect of powder Drumstick Tree *Moringa oleifera* leaves on lipid profile and haematological indices after consumption of high fat diet in chickens. Disinfectants were sprayed to disinfect the chicken wooden coops.

2.1. *Moringa oleifera* Leaves Supplement Preparation

Moringa oleifera leaves were used for experiment, these leaves were collected from the village Sehjowal in the District Kasur; the leaves were dark green in colour. After collecting from trees, leaves were carefully picked off from the branches, washed carefully and dried for seven days in direct sunlight to get rid of moisture completely and easy for grinding to make powder. To make leaf powder dry leaves were grinded by hammer mill to attain the leave residue. For further use the plastic bags were employed to store the powdered leaves as shown in Figure 1. The Feed-13 was bought from Islamabad Feeds (Pvt) Ltd. Rawat Rawalpindi. Feed-13 contains (soyabean oil, canola meal, fish meal, mineral mixture, wheat, maize, rice polish). Nutrient composition: (Crude Protein 15-17%, Crude Fiber 3.0-6.5%, Crude Fat 2% Minimum, Total Ash 13%).

2.2. Collection of Animals

Ethical approval was taken prior to study from ethical approval committee of Minhaj University Lahore. Twenty chickens were bought from the poultry research facility Lahore when they were 7.5 months (around 30 weeks) old. The Golden Misri breed of chicken was chosen for the experiment. Before the experiment, they underwent two weeks of acclimatization. These chickens were kept in the animal house of Minhaj University Lahore at room temperature (22 °C ± 2 °C). The animals were separated into 3 Experimental and one Control group, after six days of acclimation. 5 animals in each group (N = 5) were weighted before the experiment.

Group 1: Control Group (CG-1) was given Feed-13 (1.96 g only. **Group 2:** Experimental Group (EG-2) was given 5 ml of mustard oil which was mixed with simple diet Feed-13 (1.96 g). **Group 3:** Experimental Group (EG-3) was given 5 ml of

Fig. 1. Powdered Moringa oleifera leaves as a dietary supplementation.

mustard oil + 1.08 g of powdered *Moringa oleifera* leaves which were combined with the Feed-13 (1.96 g). **Group 4:** Experimental Group (EG-4) received treatment with 1.08 g of *Moringa oleifera* leaves powder + Feed-13 (1.96 g) to monitor weight utilizing blood chemistry, and lipid profile.

2.3. Blood Sample Collection

At the time of dissection after the experiment of 30 days the mean body weight of each group of chickens was recorded. Blood samples (about 5 ml each) were obtained from the jugular vein of each hen with the help of disposable syringe. Blood was collected in EDTA test tube for measuring haematological parameters, Hb, RBCs, WBCs, Lymphocytes, MCV, MCH, HCT, platelets count, and lipid profile TG, TC, HDL, LDL, and VLDL. After collection, blood was centrifuged for 15 minutes at 3000 revolutions per minutes, carefully collecting the serum require the use of pasture pipette. Serum was stored in Eppendorf tube at 20 ^{o}C in refrigerator. Serum was used for lipid profile and Haematological assessments.

2.4. Lipid Profile Tests

These tests were performed at University of Veterinary and Animal Sciences, Lahore, state of the art Roche Cobas c 111 Chemistry Analyzer was used for the determination of lipid profile by enzymatic calorimetric method **(**Electro Chemiluminescence Technology).

2.5. Haematology Tests

These tests Hb, RBCs, WBCs, lymphocytes, MCV, MCH, HCT, PLT, were performed at University of Veterinary and Animal Sciences, Lahore, using fully automated Haematological analyzer (Sysmex, Japan) by using immunofluorescence techniques.

2.6. Statistical Analysis

Data analysis was done by using Graph pad prism version 5. Student t-test was used to analyze the numerical data. The data was considered significant when the P–value was less than 0.05, ($p \leq 0.05$). Mean ± Standard error mean was used to express the values.

3. RESULTS

In this study 20 Golden Misri chickens were used for experiment and examined the effect of *Moringa Oleifera* on lipid profile and Haematological indices in chickens. The following parameters were compared to examine the statistically identifying similarities and differences among them.

3.1. Body Weight

Statistically, significant differences were observed in the initial, final and mean body weight gain of control, and *Moringa oleifera* treated groups. There is a significant $p < 0.01$ increase in the final weight and weight gain $p < 0.05$ of EG-2 compared to control group vs dosage group (Figure 2 and Table 1).

3.2. Lipid Profile Analysis

The mean of total Cholesterol and high-density lipoprotein concentration was increased. High density lipoprotein is good cholesterol. There was a significance ($p < 0.05$) and ($p < 0.001$) distinction occur increase in Mean value of *Moring*a treated groups as compared to control group (Figure 3). Total protein and low-density lipoprotein show significance ($p < 0.05$) distinction decreased in mean value of *Moringa oleifera* treated groups as compared to control group (Figure 4 and Table 2).

Table 1. Effect of *Moringa oleifera* and combination of oil, at the final body weight and weight gain of 9 months old Golden Misri chickens.

Groups	Initial Weight (kg)	Final Weight (kg)	Weight Gain (kg)
CG-1	1.85 ± 0.1	2.15 ± 0.13	0.3 ± 0.05
EG-2	1.6 ± 0.20	1.5 ± 0.15a*	0.1 ± 0.06
EG-3	1.6 ± 0.15	2.15 ± 0.12b**	0.55 ± 0.18b*
EG-4	1.75 ± 0.07	2.15 ± 0.18a*	0.35 ± 0.06

Mean ± SEM is expressed values (a) = Control vs Experimental group (b) = diet + Oil vs diet + Oil + *Moringa oleifera* powder, (c) = diet + Oil + *M. oleifera* powder vs. diet + *M. oleifera* powder, P < 0.05*, P < 0.01**.

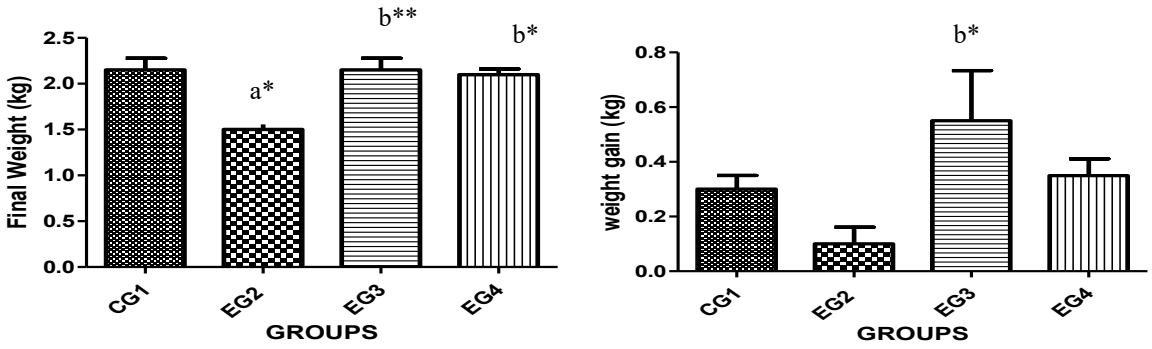

Fig. 2. *Moringa oleifera* and combination of oil on the final weight and weight gain of 9 months old Golden Misri chickens Mean ± Standard Error Mean are used to express data. There is a significant $p < 0.05$ increase in the final weight and weight gain of EG-2 compared to control group vs. dosage group.

Table 2. *Moringa oleifera* and combination of oil, effect on Lipid profile of 9 months old Golden Misri chickens.

Groups	TG	TP	TC	HDL	LDL
Units	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)
CG-1	64.4 ± 6.34	205 ± 2.12	100.8 ± 7.67	48.8 ± 3.39	22.8 ± 2.63
EG-2	67.6 ± 9.91	200 ± 13.15a*	82.2 ± 10.73	58.2 ± 1.31	13. ± 1.39*
EG-3	54.2 ± 5.89	191.4 ± 7.29	112.2 ± 6.98a***	76.6 ± 5.66a**b*	18.4 ± 1.96
EG-4	68.4 ± 8.58	199.8 ± 4.28	166 ± 4.13a***b***	69.8 ± 5.54a*	11.8 ± 2.03b**

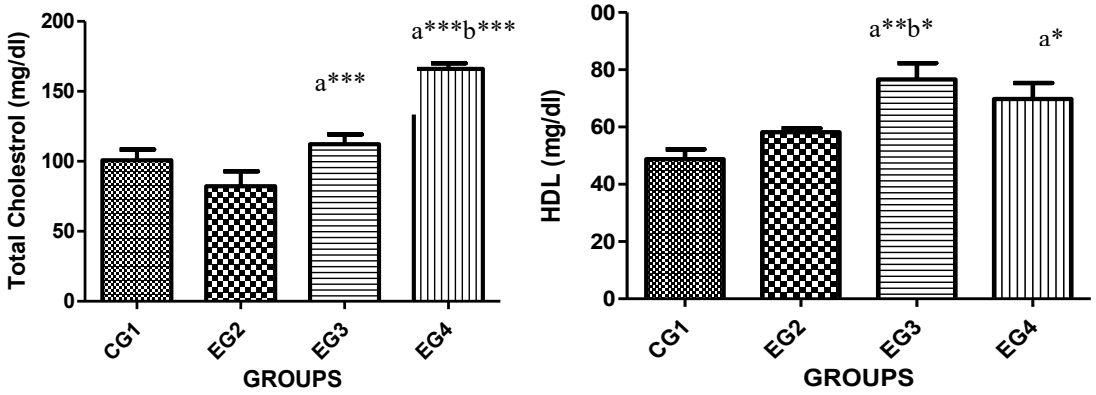

Fig. 3. *Moringa oleifera* and combination of oil effect on total Cholesterol and high-density lipoprotein concentration was increased. Mean ± Standard Error Mean are used to express data. There is significance ($p < 0.05$) and ($p < 0.001$) increased in cholesterol and high-density lipoprotein in Mean value of Moringa treated groups as compared to control groups in 9 months old Misri chickens.

3.3. Haematological Analysis

The mean of haematological parameters Haemoglobin and total red blood cells count show significance ($p < 0.05$) distinction in the Mean value increase red blood cells and haemoglobin of *Moringa* treated group 3 as compared to control group 1 (Figure 5). Lymphocytes count and monocytes level observed and shows significantly ($p < 0.05$) distinction in Mean value increase monocytes level of *Moringa oleifera* treated group 3 as compared to control group. In Lymphocytes count show significance ($p < 0.05$) increase in EG-3 and EG-4 *Moringa oleifera* treated group (Figure 6 and Table 3).

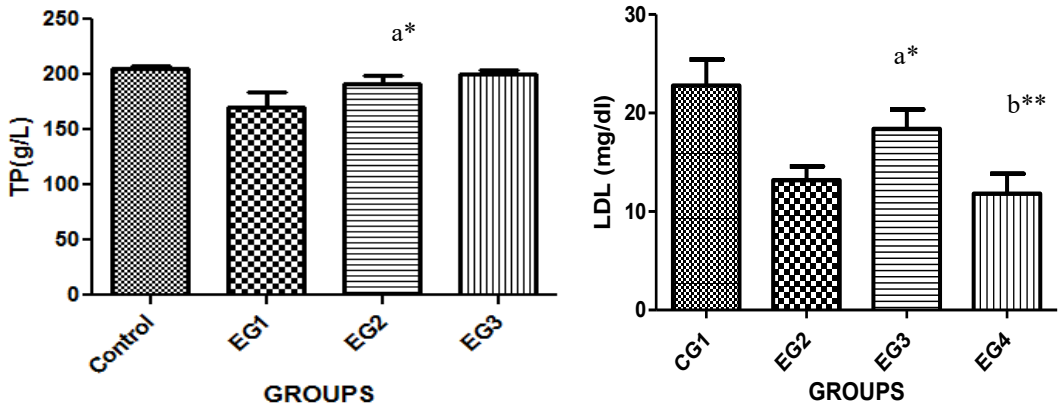

Fig. 4. *Moringa oleifera* and combination of oil effects on total protein and low-density lipoprotein in 9 months older Golden Misri chickens Mean ± Standard Error Mean is used to express the data. There is significance ($p < 0.05$) decrease in mean value of *Moringa oleifera* treated groups as compared to control groups.

Table 3. *Moringa oleifera* and combination of oil, effect on Haematological indices of 9 months old Golden Misri chickens.

Parameters	Units	CG1	EG 2	EG 3	EG 4
Haemoglobin (HB)	(g/dl)	6.82 ± 0.87	3.86 ± 0.53a*	3.2 ± 0.33a**	2.9 ± 0. 51a**
Hematocrit (HCT)	(%)	8.18 ± 2.61	19.14 ± 3.29	16.06 ± 3.03a*	18.4 ± 1.86
TOTAL RBCs	($\times 10^{12}$/L)	2.24 ± 0.23	1.84 ± 2.20	1.62 ± 0. 19a*	1.08 ± 0.25
MCV	(fl)	119 ± 7.82	110.2 ± 10.26	105 ± 7.53	109.6 ± 8.95
MCH	(pg)	28.2 ± 1.019	24.4 ± 2.42	21.4 ± 1.91	22.6 ± 2.89
MCHC	(g/dl)	30.2 ± 1.62	24.4 ± 2.20	22.4 ± 1.86a*	26 ± 2.34
Platelets Count	($\times 10^9$/L)	7.6 ± 1.16	4.94 ± 1.36	4.94 ± 0.75	4.6 ± 1.04
TLC (WBCs)	($\times 10^9$/L)	7.56 ± 0.95	7.32 ± 0.76	5.04 ± 1.34	5.62 ± 0.65
Neutrophil	(%)	64.2 ± 5.48	39.8 ± 6.09	47.6 ± 9.98	38.6 ± 6.56
Lymphocytes	(%)	58.2 ± 3.99	43.8 ± 6.18	34 ± 3.18a**	33.2 ± 4.21a**
Monocytes	(%)	3.76 ± 0.28	1.92 ± 0.32a*	2.14 ± 0.56	1.92 ± 0.41a*

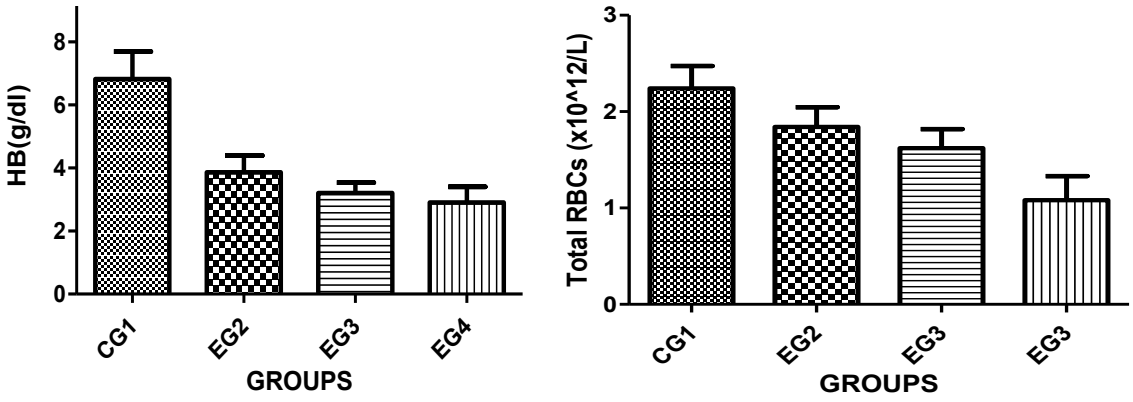

Fig. 5. Moringa oleifera and combination of oil on Haemoglobin and total red blood cells count of 9 months old chickens. Mean ± SEM are used to express data. There is significance ($p < 0.05$) distinction in the Mean value increase red blood cells and haemoglobin of Moringa oleifera treated EG-3 as compared to CG-1.

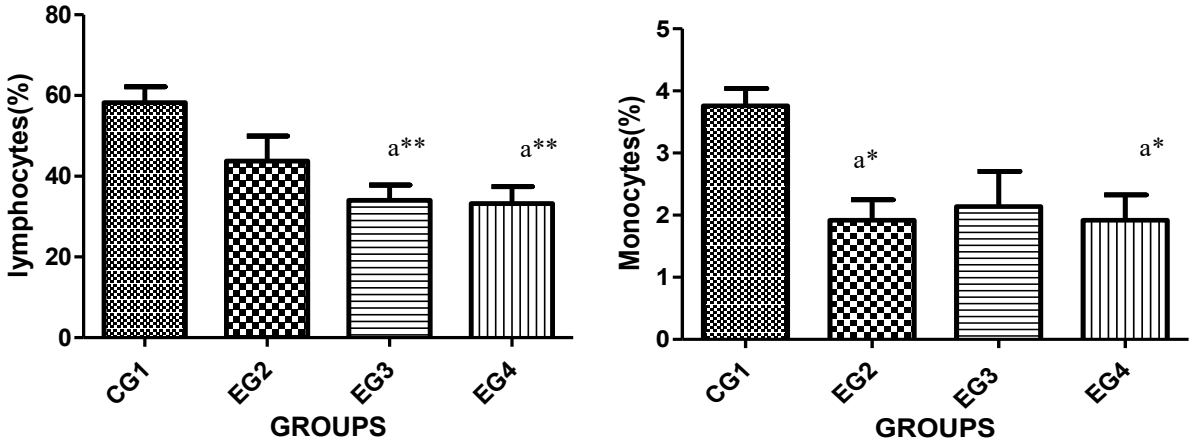

Fig. 6. *Moringa oleifera* and combination of oil on Lymphocytes count and monocytes level of 9 months old Golden Misri chickens. Mean ± SEM is used to express data. There is significantly ($p < 0.05$) distinction in Mean value increase monocytes level of *Moringa oleifera* treated group1 and treated group 3 as compared to control group. In Lymphocytes count show significance in EG-3 and EG-4 *Moringa oleifera* treated group.

4. DISCUSSION

The present study was conducted to inspect the effect of Drumstick tree (*Moringa oleifera*) leaves powder on lipid profile and hematological indices after consumption of high fat diet in chickens at the age of 30 weeks. Healthy and vaccinated chickens were used for the experiment. Lipid profile parameters included triglyceride, TC, high density lipoprotein, low density lipoprotein, and very low-density lipoprotein. The blood parameters included hematocrit, erythrocyte, neutrophil leukocyte, hetrophil, lymphocyte, and monocytes. They also include HB, MCV, PCV, MCH, MCHC, WBCs, RBCs, and PLT. Mean ± standard error mean was used to express the data. Initial and final weights were measured for all chickens in control and experimental groups before and after the experiment. The equal amount of diet was given to all the chickens. The experimental *Moringa oleifera* treated group has additional weight (2.15 ± 0.13 kg) as compared to CG-1 (1.6 ± 0.15 kg). Villarruel-López *et al.* [6] concluded that the *Moringa olifera* leaves play a significant role in enhancing the weight ratio in rats. *Moringa oleifera* helps with weight gain in treated groups but not in control groups.

The chickens were intaking high fat diet oil after a 30-days experiment. Comparing the initial body weight of the chicken to the weight rise in experimental group EG-2 is shown in Figure 3. The chickens were intaking mustard oil diet shows a significant change in the body weight as compared to the control group, and the body showed a larger size appearance because of the high consumption of fatty diet weight is increased Kilany *et al.* [7]. In the present study, lipid profile parameter measurements demonstrated the impact of mustard oil and high fat diet on *Moringa oleifera*. The mean value of *Moringa oleifera* treated groups in comparison to Control groups does not differ significantly ($p > 0.05$). The triglyceride level of 9-month-old Golden Misri chickens was measured using a mixture of mustard oil and *Moringa oleifera* on lipid profile. There was no significance in any of the groups compared to CG-1 vs EG-2 giving (*Moringa oleifera* + Feed-13) compared to EG-3 (Feed-13 + *Moringa oleifera* + oil). Elbashier and Ahmed [8] observed that triglyceride levels increased by decreasing the treatment of supplementation of *Moringa oleifera* diets by 20% compared with the normal diet percentage of 10%. The high percentage of *Moringa oleifera* increased the level of triglycerides and showed significance in p-value. In current study a low dose of *Moringa oleifera* was used.

In the present study, high density lipoprotein (HDL) dose dependence was shown to be extremely significant ($p < 0.05$) and ($p < 0.01$) enhanced high density lipoprotein and dose dependent *Moringa oleifera* combination of high fat diet group compared to CG-1 in 9-month-old Golden Misri chickens. In the previous studies, Basmacioglu and Ergul [9] observed the *Moringa oleifera* leaf meal ratio showed significant difference as compare to

CG-1. Elkloub *et al.* [10] observed an increase in HDL level in broiler chickens treated 4 weeks with three different doses of *Moringa oleifera* (0.2%, 0.4%, and 0.6%). In current study an increase in high density lipoprotein was observed in *Moringa oleifera* treated groups. In the present study, low density lipoprotein (LDL) showed dose dependent increase among *Moringa oleifera* treated groups. In current study in *Moringa oleifera* treated group 9-month-old Golden Misri chickens were given high fat diet in combination with *Moringa oleifera*. Low density lipoprotein is called bad cholesterol Mobolaji *et al.* [11] observed a decrease in low density lipoprotein levels in broiler chickens treated with three different Moringa oleifera doses (0.40%, 0.60%, or 0.80%) over three weeks. Zanu *et al.* [12]. found a decrease in LDL concentration after treatment with 0.20% *Moringa oleifera* for 4 weeks.

In this study, an increase in the total protein (TP) was found in *Moringa oleifera* and a combination of mustard oil treated groups. The difference between EG-2 and CG-1 is statistically significant ($P < 0.05$). This increase in total protein concentration might be due to mustard oil increasing in the activity of 9 months old Golden Misri chickens. Stanley [13] found that increasing total protein concentration by giving a *Moringa oleifera* leaf meal diet for 32 days, showed a significant difference in p-value.

In the present study, highly significantly ($p < 0.001$) reduced cholesterol levels ($p < 0.001$) in *Moringa oleifera* and mustard oil treated groups compared to the control groups in 9-month-old Golden Misri chickens. Alnidawi *et al.* [14] found that total cholesterol was decreased with an increase in the level of *Moringa oleifera* diet of 20%. The decreased level of *Moringa oleifera* was 5%, 10%, and 15% compared with the control group. Ghasi *et al.* [15] found that different levels of extract from *Moringa oleifera* leaves used in a fat diet reduced blood cholesterol levels significantly in Wister rats. In our study, a low dose of *Moringa oleifera* was used.

In a previous study, Gasmalbari *et al.* [16] found that Moringa treated groups caused a reduction in RBCs as compared to control groups, when *Moringa oleifera* leaf meal, 0.25%, was given to 24-day old albino rats for four weeks. In the present study, there was no significant ($p > 0.05$) difference in WBCs. In the current study, there was a considerable rise in Hb level ($p < 0.05$). *Moringa oleifera* and mustard oil treated groups were compared to control groups. EG-3 and EG-4 have revealed an optimistic outcome in haemoglobin level as compared to the Control Group. Verma *et al.* [17] observed an increase in haemoglobin (Hb) level of 9 weeks old chickens fed by *Moringa oleifera* and high fat diet mustard oil for three weeks. In the current study, the hematocrit (HCT) demonstrated that the mean values of *Moringa* treated group EG-2 showed a significant ($p < 0.05$) difference increase (HCT) level compared to the control groups in 9 months old Golden Misri chickens. Similarly, Zanu *et al.* [12] observed an increase in Hematocrit concentration in *Moringa oleifera* treated group at 5% compared to the control group. At 9 weeks old the *Moringa oleifera* treated group caused highly significant change in p-value. In previous studies, a high level *of Moringa oleifera* was used and showed a high significance, Ahmed *et al.* [18]. However, in the current study a low dose of Moringa oleifera was used. Onu and Aniebo [19] discovered no significant difference in Hematocrit level after treatment of *Moringa oleifera* leaf meal 0.75% given for three weeks.

In the present study, no significant ($p > 0.05$) difference in platelet count (PC) and feed intake has been observed in experimental group EG-2 compared to control group CG-1 in 9 months old Golden Misri chickens. When compared to the control group CG-1 experimental group EG-3 and EG-4 showed no significance. Adegbite *et al*. [20] found a significant ($p < 0.05$) increase in the mean value of *Moringa oleifera* treated groups as compared to control groups. In a previous study by Saleem *et al.* [21] using a high dose of *Moringa oleifera* changed the mean value.

In the current study, the group treated with *Moringa oleifera* and mustard oil had a higher total RBCs count. The increase was statically significant ($p < 0.05$) in all treated *Moringa oleifera* and oil groups compared to the control group in 9-month-old Golden Misri chickens. Hafsa *et al*. [22] observed highly significant values of WBCs count treated with three doses (1%, 1.5%, 2%) of *Moringa oleifera* compared with Control Group caused change in P-value show increase in significance.

In the current study, *Moringa oleifera* and a

high fat diet caused no significant rise ($p > 0.05$) in MCH and MCV levels compared to the control group. In 9-months-old Golden Misri chickens, Verma *et al.* [17] observed no considerable change ($p > 0.05$) in MCH and MCV levels, when given *Moringa oleifera* leaf supplementation diet. In previous studies, Osman *et al*. [23] found an increase in MCH and MCV due to the large amount of *Moringa oleifera*. In the present study, there was a significant ($p < 0.05$) change in MCHC and monocytes count. *Moringa oleifera* and mustard oil treated groups were compared to control groups CG-1. EG-4 (Feed-13 + oil + *M. oleifera* powder) and EG-2 (Feed-13 + *M. oleifera* powder) has shown positive results in 9-month-old Golden Misri chickens. Jiwuba *et al.* [24] found an increase in the mean corpuscular haemoglobin (MCHC) and a decreased concentration of monocytes count at 30% of the inclusion level of *Moringa oleifera* diet. In the current study, no certain change ($p > 0.05$) in neutrophil count and feed intake has been observed in experimental group EG-3 (*Moringa oleifera* + Mustard oil) compared to the control group. In comparison to the control group, Experimental Group-4 (Feed-13 + high fat diet + M. powder) and Experimental Group EG-2 (Feed-13 + *M. oleifera*) showed no significance. Tijani *et al.* [25] observed a highly significant ($p < 0.05$) increase in Neutrophil count in the 15% *Moringa oleifera* leaf meal treated group compared to control group. In the present study**,** the mean value was raised significantly ($p < 0.05$). There was a significant difference in lymphocyte count between all groups treated with *Moringa oleifera*, and control group show significance in EG-2 and EG-3. In a Wister rat fed a high fat diet, Ghasi *et al.* [15] discovered that varying doses of leaf extract from *Moringa oleifera* had no discernible effect on lymphocyte count.

5. CONCLUSIONS

Present study explores the effectiveness of *Moringa oleifera* after consumption of high-fat diet in Golden Misri chickens. The *Moringa oleifera* has increased the total body weight of the Chickens. The weight gain effect was positively correlated with the dose of *Moringa oleifera* in the groups. *Moringa oleifera* showed significant results with an increase in high-density lipoprotein (HDL), triglyceride (TG), RBCs, and Hb causing decrease in TC, MCHC, MCV, monocytes count, total protein count, LDL, Tlc WBCs, neutrophil count, MCH, and platelet count. *Moringa oleifera* intake is more effective in the treatment after consumption of high fat diet. In Pakistan, the use of plants for beneficial purposes or as an herbal drug is still ignored. Therefore, awareness about the beneficial effect of *Moringa oleifera* should be increased. The cultivation of *Moringa oleifera* should be increased in Pakistan. *Moringa oleifera* supplements can be good with feed for meat gain and egg production in chickens. Fortification of the diet with *Moringa oleifera* extract can be beneficial for the health of animals as well as human.

6. ETHICS APPROVAL

The experimental protocols and procedures used in this study were approved by the Ethical Committee of the School of Zoology, Minhaj University Lahore, Pakistan with reference number: MUL/ZOOL/235.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Performance and Instability of Oilseed Crops in Pakistan

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Abstract: This comprehensive study spanning from 1971-72 to 2021-22 consistently discerned distinct patterns of variability and instability within Pakistan's oilseed crops. Analyzing instability in the cultivation area, yield, and production of oilseed crops is crucial for effective planning and strategy formulation. The study encompasses the analysis of nine oilseed crops, namely cotton, rapeseed-mustard (including canola), sesame, groundnut, sunflower, castor seed, linseed, soybean, and safflower. The Coefficient of Variation (CV) and the Cuddy-Della Valle Instability Index (CDVI) were employed to assess fluctuations and instability in the cultivation area, production, and yield of these crops. The findings highlighted that cotton exhibited a high degree of instability in production and yield, while rapeseed-mustard (including canola) consistently displayed a high degree of production volatility, underscoring the critical need for a sustainable and steady supply of these commodities. Sesame output revealed frequent instability, demanding prompt and effective mitigation actions. Groundnut production consistently demonstrated mild inconsistency, emphasizing the need for vigilant monitoring to ensure supply stability. Sunflower cultivation faced substantial insecurity, necessitating comprehensive stabilization techniques. Soybean production continually grappled with significant insecurity across all factors, emphasizing the importance of robust risk management. Safflower production consistently posed challenges due to extreme instability, requiring ongoing solutions. Linseed regularly manifested moderate instability, indicating room for improvement with better management. Castor seed production showed considerable volatility in cultivation area and production but low yield instability, underscoring the significance of targeted stability solutions. Addressing insecurity in Pakistan's oilseed crop sectors is crucial for food security, requiring proactive measures like improved forecasting, resource allocation, and informed policy-making for long-term stability.

Keywords: Oilseed Crops, Coefficient of Variation, Cuddy-Della Valle Instability Index, Food Security, Pakistan

1. INTRODUCTION

Oilseed crops have served as the foundation of numerous agricultural economies since ancient times, playing a significant role in global agricultural industries and trade [1]. Internationally, oilseeds are celebrated for being a plentiful source of food, feed, energy, and employment. The by-products derived from oilseeds not only contribute to livestock nutrition but also function as valuable fertilizers for crop production. Poor productivity of oilseed crops can be attributed to several major factors, including a lack of technologies, cultivation in conditions with insufficient inputs, and the need to address both biotic and abiotic stresses [2]. Pakistan produced its own edible oil entirely up to the 1950s. As the demand for edible oils increased, imports were introduced to supplement the native supply. As a result, imports became a considerable component of consumption by the middle of the 1970s, accounting for 41% of it in the years 1974–1975 [3]. Local production in Pakistan encompasses eight oil-bearing seed crops, which can be categorized into traditional crops (cottonseed, rapeseed-mustard, groundnut, sesame, and linseed) and non-traditional crops (sunflower, safflower, and soybean). Among these, cotton, rapeseed-mustard, sunflower, and canola stand out as the primary contributors [4]. During the Green Revolution, non-traditional oilseed crops

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such as sunflower, soybean, and safflower were introduced. However, the area under cultivation for these oilseed crops is still quite small. Because of the steady increase in edible oil consumption, the oilseed sector has grown significantly in importance in Pakistan's economy [5]. Palm oil, the primary imported edible oil, significantly influences edible oil and ghee prices in Pakistan. Sunflower, canola, cotton, and rapeseed-mustard constitute the major oilseed crops in the country [6]. Pakistan's domestic demand for edible oils has predominantly relied on imports, with a substantial proportion of the nation's edible oil requirements being met through foreign sources. Only 20% of the total domestic edible oil consumption is satisfied by local production. The primary oilseed crops cultivated within Pakistan comprise cottonseed, sunflower, canola, and soybean. Historically, cottonseed, rapeseed-mustard, and sunflower have stood out as the primary oilseed crops in the country [7].

The oilseed crops play a crucial role in Pakistan's economy by meeting more than 17% of the domestic demand for edible oil. Additionally, significant biodiesel crops such as rapeseed and mustard, soybean, sunflower, and safflower are cultivated [8, 9]. Despite its fertile land, an efficient canal system, and an economy deeply rooted in agriculture, Pakistan continues to heavily rely on imported edible oil due to insufficient domestic oilseed production [10]. Pakistan is the eighth-largest consumer and fourth largest importer of edible oil in the world. Rapid urbanization and the fast-food industry have tripled per capita consumption from 6 kg to 24 kg per annum [11]. Pakistan imported 2.754 million tons of edible oil with a total value of Rs 662.657 billion in 2022; while the estimated overall availability of edible oil in the country for this period stands at 3.214 million tones [12]. Hybrid varieties have the capacity to produce yields ranging from 3500 to 3900 kg per hectare, representing a significant increase, potentially two to three times the typical yield [13]. The widespread use of advanced technologies, such as high yielding varieties and stress-resistant crops, along with expanded irrigation and crop insurance, has successfully stabilized oilseed crop production and productivity [14].

Pakistan's reliance on imported edible oils underscores the critical need for a coordinated effort to boost domestic oilseed production, increase yields through hybrid types, and encourage the cultivation of unconventional oilseed crops. The study aims to achieve the following objectives: (i) To assess the Coefficient of Variation and Cuddy-Della Valle Instability Index for analyzing fluctuations in oilseed crops cultivation, production, and yield in Pakistan; and (ii) To identify crops with high production volatility, and propose risk management strategies for these crops. The ultimate aim of this research addresses Pakistan's significant dependence on imported edible oils by employing a dual-pronged strategy. It involves analyzing fluctuations in the sector and proposing risk management strategies specifically designed for high-production crops. The study provides a comprehensive toolkit for policymakers, equipping them with the necessary insights to navigate the complexities of the edible oil sector.

2. MATERIALS AND METHODS

The study used secondary time series data from Pakistan's Agricultural Statistics and the Pakistan Economic Survey from 1970-71 to 2021-22 to examine agricultural production instability across a 52-year period. Agricultural insecurity was measured using the coefficient of variation (CV) and the Cuddy Della Valle Index (CDVI). The coefficient of variation may overstate instability when there is a noticeable trend, especially in regions with continual production increases. In contrast, the Cuddy Della Valle Index overcomes this constraint by using the coefficient of determination (R^2) to de-trend the CV. This method provides a more comprehensive measure of agricultural production insecurity, with lower CDVI suggesting less instability and higher scores indicating greater instability. The combination of both the coefficient of variation and the Cuddy Della Valle Index allows for a more thorough understanding of crop production volatility during the study period.

2.1. Coefficient of Variation Estimation

The Coefficient of Variation (CV) is a statistical measure used to assess the relative variability or risk in a dataset, commonly in finance, investment, or quality control. It is a dimensionless figure expressed as a percentage, computed by dividing the standard deviation (which measures the spread of

data) by the mean (the average) and then multiplying the outcome by 100. A higher CV indicates greater relative variability, implying heightened risk or uncertainty, whereas a lower CV suggests reduced relative variability, indicating lower risk or uncertainty. This metric aids in making informed decisions across various domains. Researchers such as Hazell [15], Singh and Ranjan [16], and Abdullah *et al*. [17] have used the coefficient of variation to estimate the instability of agricultural production and poverty.

- The calculation of the Coefficient of Variation for the mentioned variables was carried out using the formula provided below:

$$CV = \sigma / \bar{x} \times 100 \qquad (1)$$

Where;
σ = standard deviation
$\bar{x}$ = mean

2.2. Cuddy-Della Valle Index Estimation

The instability index is a metric employed for assessing the fluctuations or variability of a particular variable over a period. In 1978, the Cuddy-Della Valle Instability Index was introduced as a method to de-trend data and accurately portray the direction of instability. This index effectively captures both the explained and unexplained variations in the variable of interest, offering a more accurate depiction of the true state of instability. Previous studies by Sihmar [18], Kumar *et al*. [19], and Ramoliya *et al*. [20] have adopted the instability index in their work to gauge the variability in the area, production, and yield of agricultural crops. To analyze instability in the area, production, and productivity of oilseed crops in Pakistan, the Cuddy-Della Valle Index, a statistical tool, was employed.

- The mathematical expression for this Cuddy-Della Valle Instability Index (CDVI) formula is as follows:

$$I = CV \times \sqrt{1 - Adj.R^2} \qquad (2)$$

Where,
I = Instability index (percent)
CV = Coefficient of variation (percent)
Adjusted R^2 = Coefficient of determination

The ranges of the Cuddy-Della Valle Instability Index are as follows:

Low instability: 0-15
Medium instability: >15 and < 30
High instability: >30

3. RESULTS AND DISCUSSION

In this study, the primary emphasis is on the variability of three key variables: area, production, and yield. The coefficient of variation, which is the standard deviation divided by the mean and given as a percentage, was used to measure relative variability. This calculation offers valuable insights into the range to which data points deviate from the mean. The instability index, on the other hand, serves as a metric designed to depict the variability of a specific variable across time. It classifies this variability into distinct levels: high, medium, and low instability. This classification provides valuable insights into the temporal consistency or volatility exhibited by the variable under consideration.

3.1. Oilseed Crops Statistics in Pakistan

Table 1 summarizes Pakistan's oilseed crop statistics for the agricultural year 2021-22. The data show the cultivated area, production quantity, and yield of various oilseed crop. Cotton dominates the environment, with a cultivated area of 1936.9 thousand hectares, producing 8328.8 thousand tones and yielding 4300 kg/ha. Rapeseed/Mustard and canola has an area of 368 thousand hectares and yields 557.4 thousand tones at a rate of 1515 kg/ha. Sesame, grown on 199.9 thousand hectares, produced 128.1 thousand tones at a yield of 641 kg/ha. Groundnut and Sunflower occupy significant areas of 153.7 thousand hectares and 53.38 thousand hectares, respectively, producing 145 thousand tons and 83.52 thousand tons, with yields of 943 kg/ha and 1565 kg/ha, respectively. The table underscores the diverse cultivation landscape of oilseed crops in Pakistan, providing valuable insights into the agricultural sector's productivity and distribution.

3.2. Cotton Crop Instability in Pakistan

Cotton (*Gossypium spp*.), a prominent natural fiber, is a key cash crop grown commercially in over 50 nations worldwide. China, India, the United States, Pakistan, and Uzbekistan are the five largest cotton-growing countries. Cotton is a key economic driver for Pakistan, contributing 0.8% to the GDP and representing 4.1% of the total value

Table 1. Oilseed Crops Statistics in Pakistan (2022).

Oilseed Crops	Area (000 ha)	Production (000 tons)	Yield (kg/ha)
Cotton	1936.9	8328.8	4300
Rapeseed-Mustard & Canola	368.00	557.40	1515
Sesame	199.90	128.10	641
Groundnut	153.70	145.00	943
Sunflower	53.38	83.52	1565
Castor seed	4.95	4.54	917
Linseed	2.19	1.56	713
Soybean	0.06	0.05	873
Safflower	-	-	-

Source: Agricultural Statistics of Pakistan, 2022 [6].

addition in the agricultural sector. As the fifth-largest cotton producer globally, Pakistan holds a 6.0% share in the world's cotton production and ranks as the third-largest consumer of cotton. The statistics for the cotton crop in Pakistan during the 2021–22 period reveal a substantial production of 8328.8 thousand tons over an extensive area of 1937 thousand hectares, with a productivity rate of 4300 kg per hectare [6]. However, a deeper analysis of the Variability Index for Cotton in Pakistan spanning from 1971 to 2022 raises concerns about the instability within the cotton sector. The Coefficient of Variation (CV) for area stands at 17.05%, indicating relatively low instability in terms of geographical coverage. In contrast, both production and yield exhibit considerably higher instability with CV values of 55.03% and 50.35%, respectively. The corresponding Instability Index further underscores this volatility, with production and yield registering 34.80% and 31.44%, signifying high instability (Table 2). These findings suggest that while the geographic extent of cotton cultivation remains relatively stable, the actual production and yield of the crop are characterized by significant fluctuations over the years, posing challenges for the sustainability and predictability of the cotton industry in Pakistan. Efforts to address and mitigate the factors contributing to this high instability in production and yield are crucial for the long-term resilience of the cotton sector in the country.

Table 2. Variability Index (%) for Cotton in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	17.05	13.64	Low instability
Production	55.03	34.80	High instability
Yield	50.35	31.44	High instability

Source: Author's own calculations.

3.3. Rapeseed-Mustard and Canola Crop Instability in Pakistan

Rapeseed-mustard (*Brassica campestris* L.), a significant and traditional oilseed crop, is grown in 53 countries across six continents globally. In Asia, it is particularly prominent in India, Pakistan, China, and Bangladesh [21]. Mustard and its various varieties stand as the second-largest source, contributing about 38% to the country's edible oil production. Mustard is renowned for its high oil content, ranging from 40% to 44%, making it an appealing choice [22]. In Pakistan, the rapeseed-mustard including canola crop's statistics for the 2021–22 period were 368 thousand hectares in terms of area, 557.5 thousand tones in production, and a productivity rate of 1515 kg per hectare [6]. Table 3 indicates a complete summary of the variability of major agricultural characteristics (area, production, and yield) for rapeseed-mustard and canola crop in Pakistan from 1971 to 2022. These components coefficients of variation and instability index are as follows: area (28.4% and 18.9%), production (31.4% and 30.9%), and yield (27.2% and 13.4%), respectively. In terms of the "area" under cultivation, the instability index (18.9), indicating "moderate instability". This indicates that changes in cultivated land area are visible but not extremely unpredictable. The "production" component, on the other hand, has a substantially higher instability score of 30.9, suggesting "high instability". This shows that the annual yield of these crops in Pakistan

Table 3. Variability Index (%) for Rapeseed-Mustard and Canola in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	28.4	18.9	Medium instability
Production	31.4	30.9	High instability
Yield	27.2	13.4	Low instability

Source: Author's own calculations.

Table 4. Variability Index (%) for Sesame Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	54.6	35.4	High instability
Production	74.2	55.0	High instability
Yield	14.7	12.7	Low instability

Source: Author's own calculations.

varies significantly from year to year. Meanwhile, the "yield" variable has an instability score of 13.4, indicating "low instability". This means that agricultural production should remain relatively steady over time. The report emphasizes that crop production in rapeseed-mustard and canola was noticeably volatile, showing a sustained tendency of volatility. This insecurity can be attributable to decreases in both cultivated area and output levels.

3.4. Sesame Crop Instability in Pakistan

Sesame (*Sesamum indicum* L.) belongs to the family Pedaliaceae, is an autogamous plant and is categorized as a short-day plant. It is renowned as the "queen of oilseeds" [23]. The seeds of sesame contain high-quality edible oil (43-55%), characterized by its high stability and resistance to rancidity [24]. Additionally, sesame serves as a valuable source of vitamins and minerals, including calcium and phosphorus [25]. In Pakistan, sesame cultivation spans an area of 199.9 thousand hectares, resulting in an annual production of 128.1 thousand tons with an average yield of 641 kg per hectare [6]. Table 4 presents the variability of three critical characteristics of sesame crop production (area, production, and yield) in Pakistan from 1971 to 2022. The coefficient of variation and instability index are as follows: area (54.6% and 35.4%), production (74.2% and 55.0%), and yield (14.7% and 12.7%), respectively. The instability index reveals different patterns within these variables. The "area" has "high instability" with a 35.4 rating, showing significant year-to-year variations in land allocation for sesame cultivation. Similarly, "production" demonstrates "high instability" with a 55.0 index, indicating significant volatility in sesame output levels over the given period. Yield, on the other hand, displays "low instability" with an index of 12.7, signifying a relatively stable sesame crop yield per unit area from year to year. These data highlight the significant diversity and instability observed in both sesame crop cultivation area and production, while simultaneously emphasizing the generally constant character of sesame crop yields.

3.5. Groundnut Crop Instability in Pakistan

Groundnut (*Arachis hypogaea* L.), commonly known as peanut, is a key 'kharif' oilseed crop with a high 50% oil content, primarily cultivated in rain-fed area of Punjab and irrigated areas of Sindh and Khyber Pakhtunkhwa. Over the last two decades, both cultivation and production have consistently increased in these regions [4]. In 2022, the cultivation area of groundnut spanned 135.7 thousand hectares, yielding a total production of 145 thousand tons at an average rate of 943 kg per hectare [6]. Table 5 shows the variability of three critical variables related to groundnut crop (area, production, and yield) in Pakistan from 1971 to 2022. The coefficient of variation and instability index for these components are as follows: area (33.3% and 17.3%), production (26.8% and 21.6%), and yield (19.2% and 11.0%), respectively. In the case of "area", the instability index states 17.3, indicating "medium instability". This means that the area of groundnut production fluctuates noticeably but not dramatically from year to year. Similarly, the instability score for "production" is 21.6, suggesting "medium instability". This implies that groundnut production in Pakistan exhibits considerable fluctuation throughout the provided time period. The "yield" variable, on the other hand, has an instability score of 11.0, indicating "low instability". This implies that groundnut crop production per unit area is generally steady year after year. These findings suggest that, despite both acreage and production vary moderately, groundnut crop yield remains rather consistent across time. According to the instability indexes, both area and production are experiencing medium degrees of instability, indicating variations without significant volatility.

Table 5. Variability Index (%) for Groundnut Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	33.3	17.3	Medium instability
Production	26.8	21.6	Medium instability
Yield	19.2	11.0	Low instability

Source: Author's own calculations.

3.6. Sunflower Crop Instability in Pakistan

Sunflower (*Helianthus annuus* L.) a member of the Asteraceae family, originated in eastern North America and was domesticated by Native Americans around 3000 B.C., with 65 species, including 14 annual plants. Sunflower holds a crucial position among oilseed crops cultivated globally, serving as a primary provider of high-quality oil and dietary fiber. Its substantial nutritional contributions play a significant role in promoting human health [26]. Sunflower ranks as the third major crop worldwide, following soybeans and groundnuts [27]. Sunflower seeds increased an oil content up to 40-50% and a digestible protein content up to 30%, making them a significant food source for humans [28]. In the 2021-22 period, the cultivated area for sunflower in Pakistan was 53.4 thousand hectares, resulting in a total production of 83.5 thousand tons and a productivity rate of 1565 kg per hectare [6]. Table 6 presents the variability of three critical variables (area, production, and yield) relevant to sunflower crop production in Pakistan from 1971 to 2022. The coefficient of variation and the instability index for these factors are as follows: area (109.1% and 85.2%), production (114.2% and 89.9%), and yield (27.4% and 18.6%), respectively. The "area" dedicated to sunflower agriculture stands out with an extraordinarily high instability rating of 85.2, classifying it as "high instability". This label emphasizes that the allocation of land for sunflower cultivation fluctuates dramatically and unpredictably from year to year. Similarly, sunflower "production" reflects this high degree of unpredictability, with an instability rating of 89.9 indicating "high instability". This shows that sunflower production in Pakistan had considerable and irregular fluctuations over the period. The "yield" parameter, on the other hand, has a significantly smaller instability index of 18.6, putting it in the category of "medium instability". This shows that, while sunflower crop yield per unit area fluctuates noticeably, it does not reach the extremes found in area and production.

3.7. Castor Seed Crop Instability in Pakistan

The castor bean (*Ricinus communis* L.), which belongs to the Euphorbiaceae family, originated in East Africa and eventually spread to warm-temperate, subtropical, and tropical climates around the world. Castor beans, with their high fatty content, are extracted for use in pharmaceutical and industrial applications [29]. The cultivated land dedicated to castor seed has decreased from 45.9 thousand hectares in 1978-79 to 4.95 thousand hectares during 2021-22 [6]. Table 7 depicts the significant dynamics of castor seed crop variability across major agricultural variables (area, production, and yield) in Pakistan from 1971 to 2022. The coefficient of variation and instability index for three critical components are: area (104.0% and 87.6%), production (110.2% and 97.9%), and yield (24.0% and 22.0%), respectively. Notably, the instability score for "area" is very high (87.6), suggesting "high instability". This shows extreme and unpredictable variation in the land area allocated for castor seed production year after year. Similarly, the instability score for "production" is far greater, reaching 97.9, indicating "high instability". This suggests that castor seed production in Pakistan is highly erratic, with sharp changes across time. "Yield," on the other hand, has a steadier profile.

Table 6. Variability Index (%) for Sunflower Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	109.1	85.2	High instability
Production	114.2	89.9	High instability
Yield	27.4	18.6	Medium instability

Source: Author's own calculations.

Table 7. Variability Index (%) for Castor Seed Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	104.0	87.6	High instability
Production	110.2	97.9	High instability
Yield	24.0	22.0	Medium instability

Source: Author's own calculations.

3.8. Linseed Crop Instability in Pakistan

Linseed (*Linum usitatissimum* L.), also known as common flax, is a significant global crop, ranking as the third-largest fiber crop and fifth major oilseed crop [30]. It is suitable for temperate zones [31], and is used in varnishes, linoleum, putty, leather preparation, and medicinal purposes [32]. Linseed is cultivated in Pakistan, primarily in Punjab and Sindh provinces, covering 2.19 thousand hectares and producing approximately 1.56 thousand tons annually with an average yield of 713 kg per hectare [6]. Table 8 depicts the dynamics of linseed crop variations in key agricultural variables (area, production, and yield) in Pakistan from 1971 to 2022. The coefficient of variation and the instability index for three important components are: area (39.3% and 20.0%), production (34.4% and 19.5%), and yield (14.4% and 10.8%), respectively. An instability score of 20 indicates "medium instability" in terms of cultivable land area. This demonstrates that the area dedicated to linseed cultivation varies considerably but not significantly from year to year. Similarly, linseed "production" has a "medium instability" rating of 19.5, indicating a moderate level of variation over time. In comparison, "yield" has a lower instability rating of 10.8, suggesting "low instability". This suggests oscillations in linseed crop productivity.

Table 8. Variability Index (%) for Linseed Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	39.3	20.0	Medium instability
Production	34.4	19.5	Medium instability
Yield	14.4	10.8	Low instability

Source: Author's own calculations.

3.9. Soybeans Crop Instability in Pakistan

Soybean (*Glycine max* L.) has evolved as an important and adaptable field crop, accounting for 80% of global legume area and 68% of legume production worldwide [33, 34]. Because of their low fertilizer requirements, they are commonly referred to as "golden beans" or a "miracle crop". Soybeans were introduced into Pakistan in the 1960s and are primarily grown in Sindh and Khyber Pakhtunkhwa [35]. However, soybean cultivation and production have decreased dramatically in 1997-98, from 6.2 thousand hectares to 0.06 thousand hectares, and from 8.1 thousand tones to 0.05 thousand tones [6]. The coefficients of variation and instability index for these variables are as follows: area (102.7% and 84.0%), production (152.0% and 149.7%), and yield (59.0% and 52.8%), respectively. The instability index values are unusually high, at 84% for area, 149.7% for production, and 52.8% for yield. These data combined indicate that soybean agriculture in Pakistan is characterized by dramatic and unpredictable changes in cultivation area, production volumes, and crop yield per unit area. In summary, our findings highlight the critical need for strong measures to effectively manage and reduce the observed instability in numerous elements of soybean farming practices across the country.

Table 9. Variability Index (%) for Soybean Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	102.7	84.0	High instability
Production	152.0	149.7	High instability
Yield	59.0	52.8	High instability

Source: Author's own calculations.

3.10. Safflower Crop Instability in Pakistan

Safflower (*Carthamus tinctorius* L.) is an important oilseed rabi crop in semi-arid regions such as India, Iran, Egypt, Pakistan, and the Mediterranean countries [36]. In Pakistan, it's known as "kusum" and is largely cultivated for dye and oil [37], with Sindh and Baluchistan being important cultivation areas. As of 2021-22, Pakistan's safflower cultivation area was 31.7 thousand hectares, yielding 0.72 thousand tones at a production rate of 22.7 kg per hectare [6]. Table 10 summarizes the fluctuation of key agricultural indicators (area, production, and yield) for Pakistan's safflower crop from 1971 to 2022. The coefficient of variation and the instability index are as follows: area (132.5% and 105.1%), production (150.6% and 152.0%), and yield (187.3% and 167.5%). These findings emphasize the enormous variation and insecurity of safflower farming. The instability index supports this finding, designating both area and production as "highly unstable" with values of 105.1 and 152, respectively. Furthermore, safflower crop production has a "high instability" rating of 167.5, signifying harmful and irregular variations.

Table 10. Variability Index (%) for Safflower Crop in Pakistan (1971-2022).

Variables	CV	Instability Index	Inference
Area	132.5	105.1	High instability
Production	150.6	152.0	High instability
Yield	187.3	167.5	High instability

Source: Author's own calculation.

Managing this instability is crucial for successful agricultural planning and risk management in Pakistan's safflower farming sector.

3.11. Overall Instability in Pakistan's Oilseed Crops

Table 11 demonstrates the comprehensive variability of oilseed crops in Pakistan. The collective analysis of oilseed crops in Pakistan reveals a pervasive instability spanning from 1971 to 2022. Cotton, a significant oilseed crop in Pakistan, shows moderate variability in area, production, and yield. However, it also shows significant fluctuations in both production and yield, indicating its susceptibility to factors like climate, pest attacks, and agricultural practices. Rapeseed-Mustard and Canola, another vital oilseed crop, also shows significant variability in the cultivated area but also faces challenges in maintaining high production levels and yields. Sesame, characterized by its high oil content, also shows considerable variability across all parameters. Groundnut, another major oilseed crop, shows moderate variability in area, production, and yield, but relatively stable trends. Sunflower, known for its high oil content and adaptability to various soil types, also shows high variability across all parameters. Castor seed, with its high oil content and versatility, also shows high variability in cultivation area, production, and yield. Linseed, though less prominent, also shows moderate variability in cultivation area, production, and yield. Soybean, known for its high protein and oil content, also faces challenges in achieving consistent output levels. Safflower, characterized by its drought tolerance and high oil content, also shows high variability in cultivation area, production, and yield.

3.12. Perceptual Map (Positioning Map) based on Instability Index of Oilseed Crops

Figure 1 presents a perceptual map addressing the instability in the area, production, and yield of various oilseed crops in Pakistan. Cotton, rapeseed-mustard and canola, linseed, and groundnut show moderate instability, while sunflower, castor seed, soybean, and safflower demonstrate significant fluctuations in all three parameters. Safflower is the most volatile, with a significant increase in cultivation area, production, and yield. Similarly, Sunflower, Castor seed, and Soybean also depict notable instability in their production and yield metrics. These findings are consistent with the results reported by Jainuddin *et al.* [14] and Ramoliya *et al.* [20]. To tackle these challenges, it is crucial to invest in research, technology, and market development for climate-resilient crops prone to high instability. Implementing price support systems and capacity-building programs for farmers can further aid in managing the risks associated with crop instability. Adapting policy guidelines to meet the specific demands of crops and regions is essential for fostering sustainable agriculture in Pakistan.

Table 11. Overall Instability Index (%) of Oilseed Crops in Pakistan (1971-2022).

Oilseed Crops	Area	Production	Yield
Cotton	13.64*	34.80***	31.44***
Rapeseed-Mustard & Canola	18.9**	30.9***	13.4*
Sesame	35.4***	55.0***	12.7*
Groundnut	17.3**	21.6**	11.0*
Sunflower	85.2***	89.9***	18.6**
Castor seed	87.6***	97.9***	22.1**
Linseed	20.0**	19.5**	10.8*
Soybean	84.1***	149.5***	52.8***
Safflower	105.1***	152.1***	167.5***

Source: Authors' own calculations; (Low instability*, Moderate instability**, High instability***).

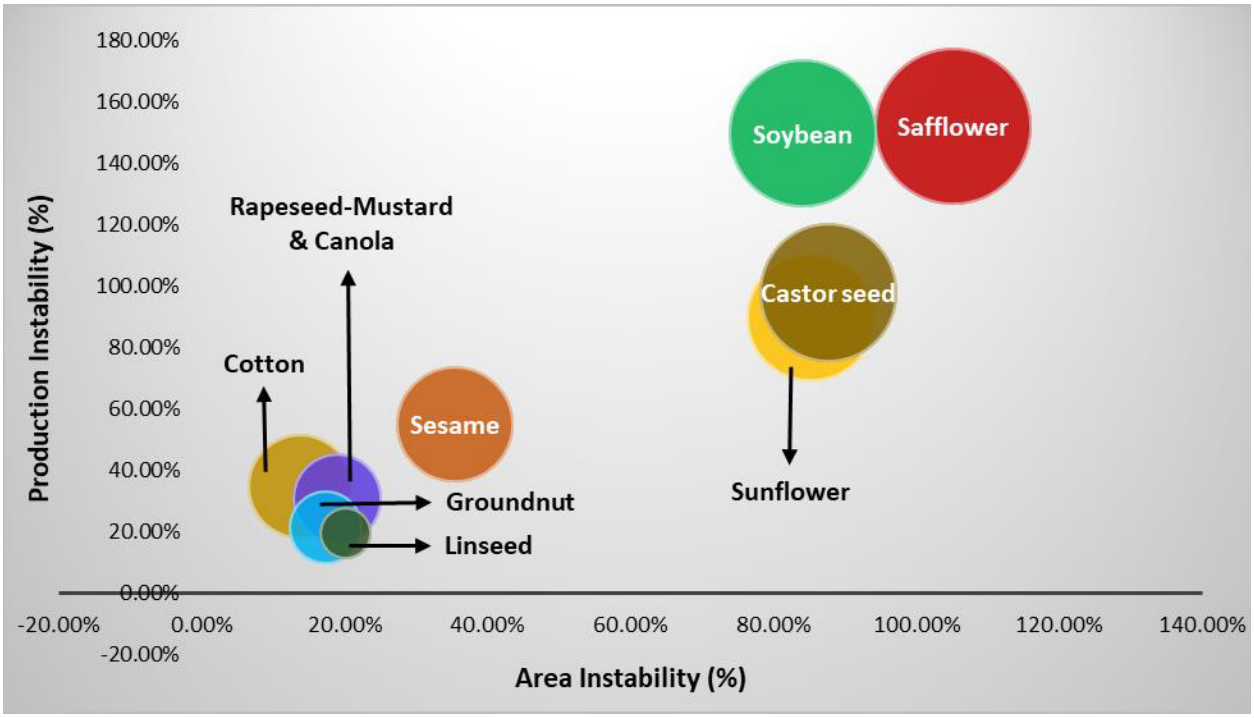

Fig. 1. Perceptual Map of Oilseed Crops based on Instability Index.

4. CONCLUSIONS AND RECOMMENDATIONS

The study on the variability and instability in oilseed crops in Pakistan, covering the period from 1970–71 to 2021–22, demonstrates significant fluctuations in cultivation area and production. Specifically, sunflower, soybean, safflower, and castor seed crops show severe and unpredictable changes and high degrees of instability. The greater values of the instability index point to a higher degree of instability, emphasizing the need for effective agricultural planning and risk management in these sectors. The findings of this analysis provide valuable insights for policymakers and stakeholders in Pakistan's agricultural industry. Based on the study's results, the following suggestions are proposed:

- Implement innovative crop forecasting techniques for informed decision-making.
- Develop resource allocation strategies for improved efficiency.
- Formulate stabilization policies for diversification, crop insurance, and price stability.
- Invest in research for resilient crop varieties, capacity-building programs, market access improvement, and sustainable agricultural practices.

5. DECLARATION

The authors acknowledged that the manuscript submitted in their own original work. The manuscript has not been published and nor considered for publication elsewhere. If accepted for publication, the article's copyright will be transferred to The Pakistan Academy of Sciences.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Phenotypic and Genotypic Characterization of Sheep Breeds in Diverse Habitats of Baluchistan Province through the Analysis of the vertnin Gene

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Abstract: This study was conducted to identify the vertnin gene in four different sheep breeds of Balochistan by studying their genetic characteristics and analyzing molecular markers. For this purpose, four different sheep breeds, Balochi, Rakhshani, Harnai, and Bibrik, were selected. A total of 10 animals from each breed were chosen to study the impact of breed and the vertnin gene on carcass features. The selection of animals was based on their phenotypic characteristics such as age and teeth. Blood samples of 10 ml were collected and transported to the Laboratory of Molecular Genetics at the Faculty of Veterinary and Animal Sciences, LUAWMS Uthal, Balochistan. Genomic DNA (gDNA) extraction was carried out using a modified inorganic method. The results showed that Balochi had a change in amino acid cytosine (C) > into thymine (T) mutation, Rakhshani had a change in guanine (G) > into adenine (A) mutation, Bibrik had a change in adenine (A) > into cytosine (C) mutation, and Harnai had a change in thymine (T) > into cytosine (C) mutation. The maximum divergence was 0.15715 bp in relation to the comparison in all studied sheep breeds. The phylogenetic results showed that the Balochi breed has 95–97% similarities with the other breeds when sequences were compared. The results revealed that the gel-electrophoresis bands of the Balochi breed were slightly higher, respectively. It was concluded that the vertnin gene exists in different breeds of sheep and might show a broad range of differences, even with similar DNA sequences. However, the purity based on the vertnin gene is at high risk, as a notable difference was found between the Balochi and Harnai sheep.

Keywords: Vertnin Gene, Genotype, Phenotype Sheep Breeds, Phylogeny, Balochistan.

1. INTRODUCTION

Sheep (*Ovis aries*) were thought to be one of the earliest domesticated animals. Since the Neolithic agriculture period, they have provided a farmed source of milk, meat, wool, and hide. Archaeological data suggest that sheep were possibly first domesticated in the Fertile Crescent region of the Near East. Before present base pairs (bp) around 8000–9000 years ago, their domestication spread from the center in Asia, Africa, and Europe over the following few thousand years [1, 2]. For animal breeding and the protection of biodiversity, the application of molecular methods is very useful. It is of utmost importance to investigate the genetic potential using various biotechnological and molecular techniques to evaluate the genetic structure of sheep breeds [3]. The Balochi breed of fat-tailed sheep is able to adapt to extreme harsh environmental conditions in Baluchistan as well as the eastern part of the Islamic Republic of Iran [4, 5]. The Birbik and Harnai breeds are also fat-tailed and raised for mutton and wool purposes, commonly found in Dera Bugti district [6]. The Rakhshani breed is also famous for meat and milk, a fat-tailed breed habitat in the Rakshan valley as well as in Kalat, Janagal, and Makran cities of Balochistan. Rakhshani sheep are white in color but occasionally

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black and brown, spotted with a Roman-type nose and small ears [4]. With information regarding carcass traits and molecular genetic research on the *vertnin* gene; there is a vast variation in the number of thoracolumbar vertebrae bone among various species, including pigs (*Sus scrofa*) 21–23, [7, 8] sheep (*Ovis aries*) 19–21, and humans (*Homo sapiens*) 23–38 [9, 10]. It is reported that the *vertnin* gene has a highly influenced the lumbar and thoracic vertebral number, directly affecting carcass width, length, and height in ruminant carcasses [11]. Zhang *et al.* [2] has been reported that the *vertnin* gene is responsible for improving carcass quantity and quality. Various studies have been conducted on pigs and reported that vertebral variation in the thoracic region is considered a commercial choice in mammalian breeding. Similarly, confirmation has been done by observing the sheep vertebral variation increased in the potential of meat production positively [12]. However, no research has been seen on the influence of the *vertnin* gene on the breed carcass traits of sheep. This is one of the first studies conducted based on Pakistan sheep breeds to compare the effect of breed and *vertnin* gene on Balochi, Rakhshani, Harnai, and Birbik sheep breeds in Baluchistan, Pakistan.

2. MATERIAL AND METHODS

2.1. Experimental Animals

To observe the effect of breed on carcass traits of different sheep breeds (Balochi, Rakhshani, Harnai, and Bibrik), 10 male animals of 8 months of age were selected from each breed. In order to analyze and observe the characterization of the *vertnin* gene, the selection of animals followed phenotypic standards and a methodology similar to previous studies [13-15] conducted for morphological characterization.

2.2. Blood Collection and DNA Extraction

The 5 ml blood samples were collected from each animal's jugular vein. The collected blood samples were taken to the Laboratory of Molecular Genetics at the Faculty of Veterinary and Animal Sciences, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Baluchistan. Before processing, the samples were refrigerated and stored. DNA extraction was carried out using inorganic procedures recommended by [16] to study the genetic diversity and genotypic characterization of different sheep breeds in Baluchistan based on the *vertnin* gene. The PCR and DNA quality were assessed using standard markers in gel electrophoresis [16-2]. The details of primers of *vertnin* gene designed for this study given in (Table 1).

2.3. Genotype, Allele Frequency and Phylogenic Tree and Expression of Evolutionary Direction

The construction of the phylogenetic tree and the analysis of the protein sequences of the *vertnin* gene in four distinct sheep breeds from Balochistan were performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean). A sample clustering procedure to determine an accurate percentage of evaluation (molecular clock hypothesis) requires a distance matrix to observe the taxa, which can be calculated from multiple alignments as described by Lori *et al*. [17]. After removing non-coding regions and editing the sequences, the phylogenetic tree was generated utilizing the NJ (neighbor-joining) procedure. This method employed the Q matrix, considering all branches, with a preference for selecting the most similar sequences with the lowest range values.

2.4. Sequencing

After preparation of the PCR products, prepared samples of the *vertnin* gene were sent for sequencing purposes to the Center of Applied and Molecular Biology (CAMB), University of Punjab, Lahore.

2.5. Statistical Analysis

The recorded data was typed to the computer in Microsoft Excel, and regression analysis was performed using the computer software (SAS-8.1).

3. RESULTS

3.1. Genotype and Allele Frequency

The analysis of genotype frequency for the *vertnin* gene in sheep breeds in Balochistan presented in (Table2). In this study, we analyzed the polymorphism distribution of the Balochi, Rakhsani, Harnai, and Bibrik sheep breeds. The results showed that Balochi had a change in amino acid cytosine (C) > into thymine (T) mutation, Rakhshani had a change

Table 1. Primer Sequences for *vertnin* gene used for current study.

Product	Primer sequence 5-3	Chromosomes	Position OAR 0.4	Size of product bp
1	Fwd AGTGTCATCCAGGTACCCGTTA	7	82533260	1,478
	Rvrs GCGGGACAATGGCACCTA	7	82534737	
2	Fwd AAAAGCTCTCCGAAGGAACCC	7	82534539	1,351
	Rvrs GCACCAAGCAGAACTTATGACC	7	82535889	

in guanine (G) > into adenine (A) mutation, Bibrik had a change in adenine (A) > into cytosine (C) mutation, and Harnai had a change in thymine (T) > into cytosine (C) mutation. However, based on the genotype and allele frequency, the loci of the *vertn* gene within the four sheep breeds of Baluchistan were in Hardy-Weinberg equilibrium (P 0.05) in the Balochi, Rakhshani, Bibrik, and Harnai breeds. The overall results for the Balochi breed showed that the CC genotype was more prevalent than the CT genotype at different levels. However, there was no significant difference in allele frequencies (Table 3).

3.2. Estimation of the Divergence

The divergence among breeds was observed with the help of the pair contrast procedure and base numbers. The data displayed in (Table 4) showed the number of base substitutions on each side of the sequences. The highest level of divergence was 0.15715 bp, which is similar in contrast to the different sheep breeds of Balochistan.

3.3. Phylogenetic Tree

Based on the molecular phylogenetic tree analysis, the *vertnin* gene sequences were divided into two major branches. The terminal nodes, representing present-day sequences, were used for classification, while internal nodes indicated ancestral sequences. The tree displayed a branching pattern with nodes bifurcating into two branches, reflecting distinct genetic patterns within the *vertnin* gene sequences. Comparative analysis of the sequences revealed significant taxonomic diversity, allowing for the exploration of phylogenetic relationships among different sheep breeds based on their *vertnin* gene sequences. Despite some genetic similarities, Balochi and Rakhshani sheep showed a distinct genetic distance, with divergence of 95% and 73%, respectively. The sequencing of the Balochi breed was aligned using MEGA6 software, known for its progressive algorithmic approach to sequence alignment and analysis are given in Figure 1.

Table 2. Genotype frequency analysis for vertnin gene in sheep breeds of Balochistan.

Breed	Genotype Frequency			Chi-Square Test (p-Value)
Balochi	TT	TG	GG	0.213
	0.565	0.35	0.28	
Rakhshani	AA	AG	GG	0.251
	0.45	0.35	0.27	
Bibrik	CC	CT	TT	0.236
	0.355	0.25	0.25	
Harnai	CC	CT	TT	0.291
	0.285	0.23	0.2	

Table 3. Allele frequency analysis for vertnin gene in sheep breeds of Baluchistan.

Breed	Allele Frequency		Chi-Square Test (p-Value)
Balochi	C	T	0.241
	0.775	0.75	
Rakhshani	A	G	0.233
	0.72	0.73	
Bibrik	A	G	0.254
	0.7	0.73	
Harnai	C	T	0.209
	0.65	0.695	

Table 4. The estimated divergence among the sequences of *VRTN* gene in four sheep breeds of Balochistan.

Breed	Balochi	Rakhshani	Bibrik	Harnai
Balochi	0.09555	-	-	-
Rakhshani	0.0982	-	-	-
Bibrik	0.11905	0.1065	-	-
Harnai	0.15715	0.1221	0.14415	-

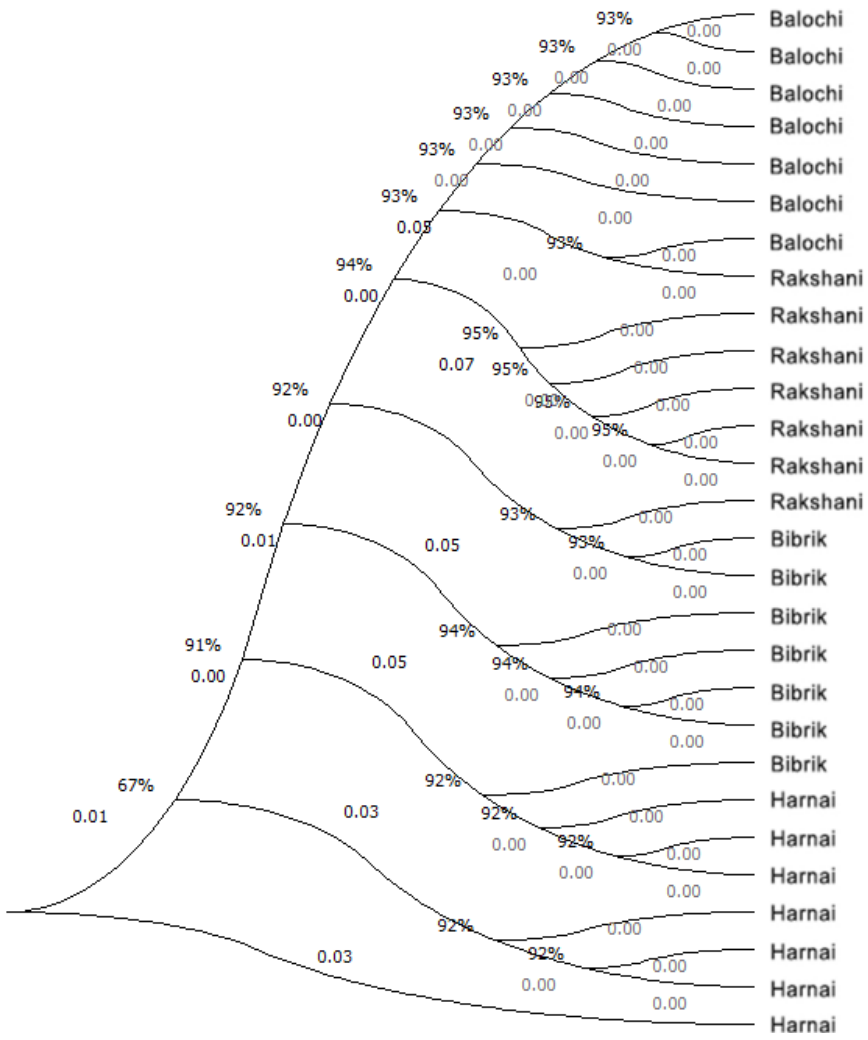

Fig. 1. Phylogenetic tree of estimated genotypic characterization of four sheep breed of VRTN gene sequencing.

3.4. PCR-Gel-Electrophoresis

PCR-gel electrophoresis was used to evaluate the bands of the *vertnin* gene in four different sheep breeds. The D-loop fragment of 778 bp (from position 1,478 to 1,351) was amplified with primers for *vertnin*. The results revealed that the bands of the Bibrik, Rakhshani, and Balochi sheep breeds were slightly higher than those of the Harnai breed (Figure 2).

3.5. Phenotypic Appearance

The results for thoracolumbar vertebral bone number, carcass height (cm), and carcass length (cm) in Balochi, Rakhshani, Harnai, and Bibrik sheep breeds were as follows. In the Balochi breed, the thoracolumbar vertebrae bone number was 20, with a carcass height of 76.12 ± 0.67 cm and a carcass length of 22.32 ± 0.18 cm. In the Rakhshani breed, the thoracolumbar vertebrae bone number was 18, with a carcass height of 71.61 ± 0.51 cm and a carcass length of 21.15 ± 0.19 cm. The Harnai breed had a thoracolumbar bone number of 18, a carcass height of 71.43 ± 0.93 cm, and a carcass length of 20.71 ± 0.31 cm. In the Bibrik breed, the thoracolumbar vertebrae bone number was 19, with a carcass height of 68.21 ± 0.7 cm and a carcass length of 18.13 ± 0.46 cm. The results showed a significant ($P < 0.05$) change in the region of thoracolumbar vertebrae has a positive influence on the carcass weight and carcass length of sheep meat details given in Table 5.

4. DISCUSSION

In our study, the genotype frequency analysis for the *vertnin* gene in different sheep breeds was

Table 5. Effect of thoracolumbar vertebral bone number on the carcass weight and carcass length of sheep breeds in Balochistan.

Breed	TVLN	Carcass height (cm)	Carcass length (cm)
Balochi	20a	76.12 ± 0.67a	22.32 ± 0.16a
Rakhshani	18c	71.61 ± 0.51b	21.15 ± 0.19b
Harani	18c	71.43 ± 0.93c	20.71 ± 0.31c
Bibrik	19b	68.21 ± 0.74	18.13 ± 0.46

*(P-Value is 0.003) The value with various superscript uppercase in same table showed significantly different ($P < 0.05$). The values with various superscript in lowercase in similar table showed highly significant different ($P < 0.05$).

conducted. In the Balochi breed, the genotype frequencies for the *vertnin* gene are TT: 0.211, TG: 0.565, and GG: 0.35. The Chi-Square test results indicate that these frequencies are significantly different from the expected frequencies ($P < 0.05$). Similar studies conducted previously and reported the least deviation among the expected frequencies

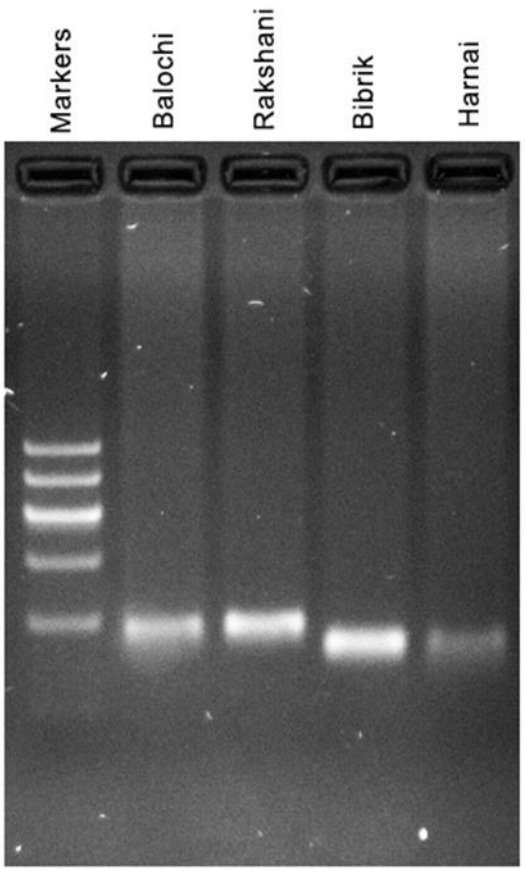

Fig. 2. PCR-amplified product of *vertnin* gene fragments from sheep breeds of Baluchistan (Marker: 1478bp to 1,351bp).

of breeds in the *vertnin* gene [18, 19]. Variations in the frequencies of different genotypes for the *vertnin* gene may indicate genetic diversity or potential breed-specific variations in the sheep populations of Baluchistan. [17-20] stated that to further understand these findings and put them into context, it would be beneficial to compare them with similar research conducted on other sheep breeds, both within the country and in other regions [21, 22]. By examining the genotype frequencies of the *vertnin* gene in other studies, we can gain a broader perspective on the genetic variations in sheep populations and potentially identify any patterns or trends. In conclusion, the genotype frequency analysis of the *vertnin* gene in sheep breeds of Baluchistan reveals significant variations in genotype frequencies across different breeds. Further research comparing these findings with other studies and exploring the functional implications of these genotypes can enhance our understanding of the genetic diversity and potential breed-specific characteristics of sheep populations. The result of allele frequency analysis for the *vertnin* gene in different sheep breeds of Baluchistan includes information on the gene, sampling breed, allele frequencies, and analysis of the Chi-Square test results. We observe that the p-values for all breeds are above 0.05, indicating that there is no significant association between the allele frequencies and the breeds at the specified level of significance. It has been reported that this means that the observed distribution of alleles in each breed is not significantly different from the expected distribution [12, 23, 24]. It's important to note that this analysis is specific to the sheep breeds of Baluchistan and the *vertnin* gene. Researchers and geneticists interested in this specific gene and breed combination can refer to this table to understand the allele frequencies and test results. The phylogenetic tree for the Balochi, Rakhshani, Bibrik, and Harnai sheep breeds was used in our study by narrow regions of axon with maximum association among the breed [25, 26]. The phylogenetic tree was formed on the base of the exon of the v*ertnin* gene, which was again confirmed through the classification of different mammalian species. The comparative study of sequences showed a large level of taxonomic analysis to explore phylogenetic linkage between various sheep breeds among the sequences of the *vertnin* gene. Although having similar characteristics among the genetic scattering rate, the phylogenetic tree and sequence percentage expressed the distance between Balochi

and Rakhshani sheep in the *vertnin* gene with percentages of 98 and 73, respectively (Figure 2). Furthermore, the sequencing of the Balochi breed was aligned using MEGA6 software, which is commonly used for layout. This is mainly utilized as the progressive algorithm layout for sequences. The results have shown that the Balochi breed has 98% similarities with the other three sheep breeds when sequences were compared (Figure 3). In the Balochi breed, the thoracolumbar vertebrae bone number was 20, with a carcass height of 76.12 ± 0.67 cm and a carcass length of 22.32 ± 0.18 cm. In the Rakhshani breed, the thoracolumbar vertebrae number was 18, with a carcass height of 71.61 ± 0.51 cm and a carcass length of 21.15 ± 0.19 cm. The Harnai breed had a thoracolumbar vertebrae number of 18, a carcass height of 71.43 ± 0.93 cm, and a carcass length of 20.71 ± 0.31 cm. In the Bibrik breed, the thoracolumbar vertebrae number was 19, with a carcass height of 68.21 ± 0.7 cm and a carcass length of 18.13 ± 0.46 cm. The results showed that a change in the thoracolumbar vertebrae region has a positive influence on carcass weight and length of sheep meat. Our study was supported by [1], who have reported that the number of thoracolumbar vertebrae is directly linked with body length, height, and carcass weight and length, which are economically important traits in farm animals' small ruminants. Another study was conducted by that carcass weight and carcass length is associated with body height and carcass traits in farm animals [10-26].

5. CONCLUSIONS

This study provides basic information on the genetic divergence between sheep breeds in Baluchistan based on the sequences of the *vertnin* gene. Researchers can use these estimates to study the genetic relationships, population structure, and evolutionary history of these sheep breeds, which can have implications for breeding programs, conservation efforts, and understanding the genetic diversity within Baluchistan's sheep populations.

6. FUNDING

Higher Education Commission of Pakistan provided funding for this study through PSDP project titled: Establishment of National Center for Livestock Breeding, Genetics and Genomics" at Lasbela University of Water Agriculture and Marine Sciences, Uthal, Balochistan.

7. ETHICAL STATEMENT

The Ethical Research Committee of the Directorate of Advanced Studies and Research Board of Sindh Agriculture University, Tandojam, Pakistan, approved all procedures of this study in its 131st meeting held on 13-11-2018. The approval was notified by the Directorate of Advanced Studies via letter No. DAS/5528 dated 28-11-2018.

8. CONFLICT OF INTEREST

All the Authors declared no conflict of interest.

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Research Article

Caffeine-Containing Local Products and Their Effects on Liver and Kidney Histopathology: A Comparative Study

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Abstract: Caffeine has several modified natural and synthetic forms and it is the most abundantly consumed drug around the globe. It is present in fruits, seeds and leaves of many plants but coffee contains highest content of caffeine and is considered its richest source. It is also found in much high ratio in non-alcoholic beverages, i.e., tea and carbonated drinks like coke. The aim of current mammalian model based investigation was to evaluate the possible histopathological variations influencing the function of kidney and liver due to the intake of selective caffeinated products: Arabica and Robusta beans' blend based coffee and coke. The 30 albino mice of male gender and 1.5 month age were acclimatized for ten days and later on, categorized as group I (control group which was treated with standard mice feed), experimental group II (treated with coffee blend) and experimental group III (treated with coke). The renal and hepatic histological sections were cut and stained for weekly observations. Hematoxyline and eosin staining was used and microscopic observations were recorded at 40X. The ANOVA based statistical analyses showed that significant variations occurred in body weight, diameter of renal blood vessels, glomeruli and of necrotic areas of both experimental groups ($p \leq 5\%$). Moreover in hepatic tissues, vein wall thickness, diameter of bile ducts and liver lobules also indicated significant variations. The conclusion is excessive and regular intake of above mentioned composition having coffee and coke may result in diverse physiological disturbances and a programmed general public awareness is required regarding their limited intake.

Keywords: Caffeine, Coffee, Coke, Liver, Kidney.

1. INTRODUCTION

In daily life, exposure to caffeine products is quite common for people. Among these products, coffee (family Rubiaceae) has its 2 major species: *Coffea arabica* and *Coffea robusta* [1]. Though coffee is consumed round the globe but it also causes serious side effects on vital organs like in liver, disturbances in serum enzymatic activities, hepatic cirrhosis, hepatocellular carcinoma and other liver injuries and extent of damage varies from type of coffee and frequency of its consumption [2]. Similarly, excessive use of coffee causes nephrotoxicity, reduction of kidney volume and of glomerular diameter along with disturbances in blood serum urea and nitrogen levels and body weight loss [3]. Moreover, a soft drink usually consists of many ingredients, for example, a sweetener, carbonated water and either oragnic or synthetic additive. Additionally, caffeine, food colours and stabilizers like constituents may be included in soft drinks. Similarly, a worldwide frequently consumed and much demanding soft drink is Coca-Cola (Coke). It metabollically boosts the ecstatic effect but its regular in take may results in metabolic disturbances espacially related to renal and hepatic finstions [4]. The effects of carbonated soft drinks on health are not clear; however, they are considered as a link with obesity, osteoporosis, renal and hepatic disorders along with other severe health problems which are linked and directly proportional to the usage of these drinks. Numerous compounds are present in carbonated soft drinks which consist of caffeine that is behaviorally active substance and has the highest consumption rate throughout the world [5].

In this regard, recent data suggests that

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excessive intake of coke may cause metabolic syndrome, hyperuremia, atherosclerosis, elevated level of uric acid and creatinine in blood and chronic kidney disease [6]. However, the average lethal dose of caffinated products is 200 mg/kg body weight and casue hepatic and renal toxicity [14, 15], like swelling of hepatocytes, renal vascular congestion, focal autotophy, epithelial degeneration [16]. In addition to this, liver toxicity was also reported but its comparative severity is not known yet in selected caffinated products [4]. That is why, coke and blended composition having coffee were selected in current research design to know the range of its possible renal and hepatic side effects spectrum.

2. MATERIALS AND METHODS

2.1. Sample Collection

The 30 albino mice (*Mus musculus*) of male gender and 1.5 months age (body weight around 28 g/ mouse) were bought from UVAS, Lahore.

2.2. Dose Preparation and Optimization

For dose preparation and optimization, 19.95 ml of water + 0.05 g of coffee (*C. arabica* and *C. robusta* blend) and its 0.1 ml was orally administrated as dose during experimentation, whereas 0.1 ml of coke was given without any dilution. Moreover, for control group 0.1 ml distilled water was given.

2.3. Study Design

Mice were acclimatized for 10 days in the animal house of Minhaj University Lahore at room temperature (25 °C ± 2 °C) and were fed locally available standard food and clean water. Then mice were grouped and tagged in control group labeled as (Control) while experimental group was distributed into experimental group A (treated with coffee: *C. arabica* and *C. robusta* blend) and experimental group B (treated with coke). The body weights of all mice were measured at the start and end of the experimentation [13].

2.4. Histopathological Observations

The renal and hepatic tissues of mice of both experimental groups were collected on weekly basis and for their fixation 10% formalin solution was used. Histological sections of liver and kidney were prepared and Haematoxyline and Eosin (H&E) staining was done. The microscopic readings were noted at 40X while microphotographs were captured at 10X with PixelPro software.

2.5 Statistics

The data was statistically anlyzed through single factor analysis of variance using SPSS (Statistical Package for the Social Sciences) version 20 at $p \leq 5\%$ [7].

3. RESULTS AND DISCUSSION

The tabulated data is presenting the ANOVA based results of variations after intake of selective caffeinated products in mice body weight (Table 1). The mice body weight was significantly increased due to intake of coke than coffee (*C. arabica* and *C. robusta*) blend on completion 2nd week and a pattern of gradual body weight decline was noticed till 4th week, 15.33 g, 15 g and 14 g, respectively. Because presence of high sugar content in composition of coke results in body weight gain but in coffee the concentration of caffeine is high which boosts up body weight loss [8].

Whereas, variations based on vasodilation and vasoconstriction were noticed in histopathological studies of renal blood vessels diameter and wall thickness (Figure 1) and in this regard, significant rise was observed at the end of 2nd week in diameter

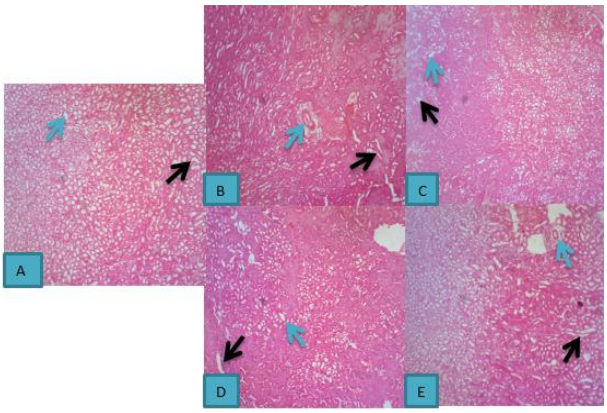

Fig. 1. Diameter of blood vessels of kidney, A = Control group, B = group A at the end of 1st week, C = group B at the end of 1st week, D = group A at the end of 4th week, E = group B at the end of 4th week presenting the diameter of vein (µm) with black arrows while blue arrows are indicating diameter of artery (µm). All microphotographs are taken at 100X magnification, H & E staining was used.

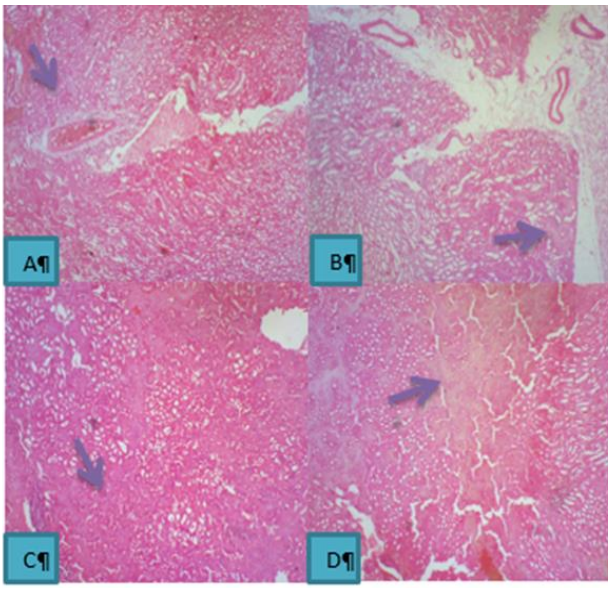

Fig. 2. Diameter of necrotic area of kidney, A = group A at the end of 1st week, B = group B at the end of 1st week, C = group A at the end of 4th week, D = group B at the end of 4th week presenting the diameter of necrotic area (µm) with purple arrows. All microphotographs are taken at 100X magnification, H & E staining was used.

of veins with 176.5 µm (Table 2) after exposure to coke (group B) and it happened due to biochemical and genetic alterations [9]. However, impact of coffee (*C. arabica* and *C. robusta* blend) intake compared to coke was found more influential on glomerular diameter, and maximum increase was of 120.46 µm on completion of 3rd week. Similarly, the diameter of kidney damaged area (Table 2) due to necrosis was significant (111 µm) in histological sections of group A at 2nd week (Figure 2) which might be due to over stressed blood filtration in glomeruli because of vasodilatory influence leading to bursting effect of selected caffeinated products [10].

Table 1. Effect of caffeinated products on body weight (g).

Weeks	Mean (µm) ± S.EM(n)		
	Control	Group A	Group B
1	1.33 ± 0.33 (3)	12 ± 1 (3)	12.67 ± 0.33 (3)
2		8 ± 3 (3)	*15.33 ± 0.33 (3)
3		6.67 ± 0.882 (3)	15 ± 1.155 (3)
4		7 ± 1 (3)	*14 ± 2.082 (3)

*Values of Mean ± SEM (n). Data of respective columns were compared by employing single factor analysis of

Moreover, the histopathological observations for blood vessels in liver tissues indicated variations but a prominent increase in arterial diameter of 198.7 µm in group A than group B (120.5 µm) at 3rd week stage (Table 4). But in case of veins the pattern of gradual vasoconstriction was noted in group A, while in group B at 3rd week stage (Figure 3) maximum diameter of 159.6 µm was recorded due to biochemical alterations at cell and molecular levels [9]. Table 5 is presenting significant results of diameter of bile duct till 28th day (83.47 µm) for group A compared to group B (70.8 µm) but overall considerable variations in bile duct diameter were observed in group B treated with coke. For the diameter of liver lobule significant results of 384.63 µm in group A and 151 µm in group B at 1st week, and 286.35 µm in group B (treated with coke) and 165.89 µm in group A (treated with selected coffee) at 4th week [11]. Another histopathological change was due to necrosis in hepatic tissue (Figure 4) which was noticed in both experimental groups but comparatively more tissue (164.84 µm) was damaged after intake of coke than coffee treated group of 142.65 µm [12] and and such effects of tissue hemorrage and necrosis have also been repoted in other recently examined caffiniated products [17].

Table 2. Effect of caffeinated products on renal artery and vein diameter (µm).

Weeks	Mean (µm) ± S.EM(n)					
	Renal Artery Diameter			Renal Vein Diameter		
	Control	Group A	Group B	Control	Group A	Group B
1	126.8 ± 63.51 (3)	110.95 ± 12.003 (3)	238.81 ± 26.48 (3)	117.29 ± 13.11 (3)	94.0433 ± 33.9 (3)	89.82 ± 17.4 (3)
2		102.5 ± 20.16 (3)	127.86 ± 5.88 (3)		90.87 ± 22.9 (3)	*176.5 ± 15.6 (3)
3		97.213 ± 5.9 (3)	225.41 ± 62.31 (3)		126.8 ± 6.6 (3)	130 ± 19.02 (3)
4		116.23 ± 5.6 (3)	90.63 ± 9.62 (3)		164.84 ± 16.8 (3)	146.88 ± 6.93 (3)

*Values of Mean ± SEM (n). Data of respective columns were compared by employing single factor analysis of variance and results were found significant at 5% (*).

Table 3. Effects of caffeinated products on glomerular and renal necrotic area diameter (µm).

Weeks	Mean (µm) ± S.EM (n)				
	Diameter of Glomeruli			Diameter of Renal Necrotic Area	
	Control	Group A	Group B	Group A	Group B
1	57.06 ± 5 (3)	66.57 ± 3.7 (3)	61.3 ± 14.22 (3)	152.16 ± 6.6 (3)	150.05 ± 46.7 (3)
2		78.2 ± 4.3 (3)	75.023 ± 1.06 (3)	* 111 ± 24.21 (3)	181.8 ± 5.6 (3)
3		*120.46 ± 12.68 (3)	106.72 ± 6.43 (3)	209.7 ± 6.33 (3)	209.22 ± 6.34 (3)
4		104.61 ± 3.17 (3)	103.6 ± 4.61 (3)	207.73 ± 8.8 (3)	206.05 ± 8 (3)

*Values of Mean ± SEM (n). Data of respective columns were compared by employing single factor analysis of variance and significant results were found at 1% (*).

Table 4. Effect of caffeinated products on diameter of liver artery and vein (µm**).**

Weeks	Mean (µm) ± S.EM(n)					
	Hepatic Artery Diameter			Hepatic Vein Diameter		
	Control	Group A	Group B	Control	Group A	Group B
1	95.1 ± 11.43 (3)	155.33 ± 47 (3)	144.8 ± 26.54 (3)	110 ± 35.5 (3)	262.0533 ± 178.45 (3)	108.834 ± 50.6 (3)
2		134.2 ± 33.4 (3)	82.42 ± 5 (3)		223 ± 62 (3)	105.7 ± 11.032 (3)
3		198.7 ± 72 (3)	120.5 ± 11.43 (3)		172.24 ± 9.4 (3)	159.6 ± 34.84 (3)
4		90.9 ± 16.61 (3)	137.7 ± 25.53 (3)		183 ± 34.4 (3)	108.84 ± 18.42 (3)

*Values of Mean ± SEM (n). Data of respective columns were compared by employing single factor analysis of variance and no significant results were found.

Table 5. Effect of caffeinated products on bile duct, liver lobule and necrotic area diameter (µm).

Weeks	Mean (µm) ± S.EM(n)							
	Liver Bile Duct Diameter			Liver Lobule Diameter			Liver Necrotic Area	
	Control	Group A	Group B	Control	Group A	Group B	Group A	Group B
1	63.4 ± 3.7 (3)	69.74 ± 5.5 (3)	58.12 ± 2.8 (3)	85.6 ± 3.17 (3)	*384.63 ± 77 (3)	151.003 ± 76.8 (3)	117.3 ± 22.3 (3)	108.84 ± 8.3 (3)
2		65.51 ± 2.8 (3)	62.34 ± 2.8 (3)		150.05 ± 28.5 (3)	99.33 ± 19.05 (3)	137.4 ± 9.4 (3)	124.7 ± 11.032 (3)
3		74.5 ± 20 (3)	73.44 ± 8 (3)		153.22 ± 13.77 (3)	158.5 ± 41.6 (3)	106.33 ± 8.84 (3)	105.7 ± 8.66 (3)
4		*83.5 ± 2.8 (3)	70.8± 4.23 (3)		166 ± 23 (3)	**286.4 ± 8.3 (3)	142.65 ± 13 (3)	164.84 ± 40 (3)

*Values of Mean ± SEM (n). Data of respective columns were compared by employing single factor analysis of variance and results were found at 5% (*) and 1% (**) significant for liver bile duct and lobule diameter. Whereas liver tissue necrosis based results were not found significant.

4. CONCLUSIONS

In the present study, hepatic and renal histopathological effects due to intake of selective caffeinated products were investigated and it can be concluded that the intake of *C. arabica* and *C. robusta* blend based coffee and coke imparted many physiological and histopathological variations in the body weight, diameter and thickness of blood vessels, glomeruli, bile duct, liver lobule and necrotic area. Comparatively more side effects were observed in experimental group treated with selected composition having coffee than coke and the intake of this blend induces synergistic but damaging effects on liver and kidney of mice. The way, mammalian model of current study faced side effects; similarly, the regualr consumers of these local caffinated products may also suffer from hepatic and renal physiologocal disturbances which are usually overlooked yet due to lack of general

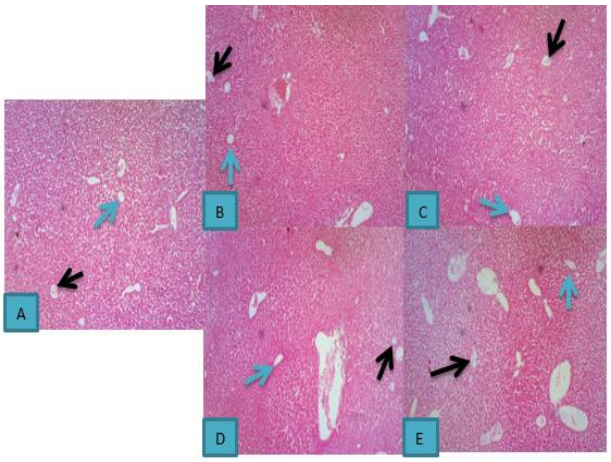

Fig. 3. Diameter of blood vessels of liver, A = Control group, B = group A at the end of 1st week, C = group B at the end of 1st week, D = group A at the end of 4th week, E = group B at the end of 4th week presenting the diameter of vein (µm) with black arrows while blue arrows are indicating diameter of artery (µm). All microphotographs are taken at 100X magnification, H & E staining was used.

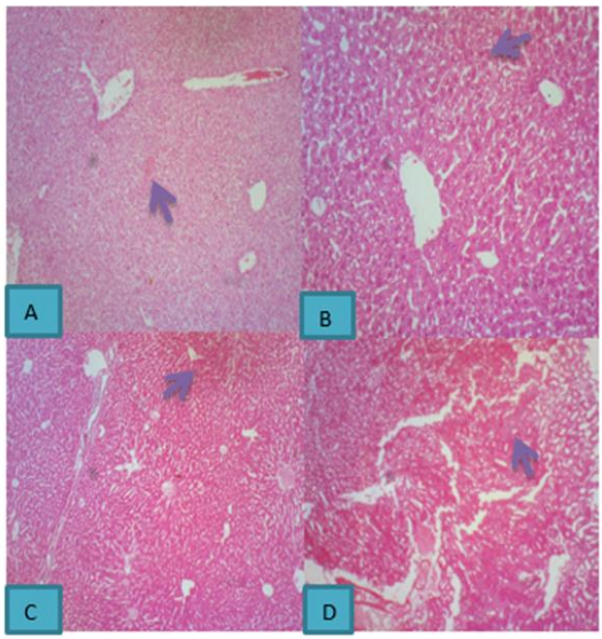

Fig. 4. Diameter of necrotic area of liver, A = group A at the end of 1st week, B = group B at the end of 1st week, C = group A at the end of 4th week, D = group B at the end of 4th week presenting the diameter of necrotic area (µm) with purple arrows. All microphotographs are taken at 100X magnification, H & E staining was used.

public awareness and such health related awareness should be provided to consumers through different research and elctronic media.

5. ACKNOWLEDGEMENTS

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6. ETHICAL STATEMENT

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.

7. CONFLICT OF INTEREST

All authors have no conflict of interest.

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Research Article

Impact of Perceived Servicescape on Patient Satisfaction through Mediating Role of Service Credibility and Moderating Role of Received Word of Mouth in Rehabilitation

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Abstract: The aim of this study was to explore the effects of perceived servicescape (PS) and credibility of the healthcare service provider (CHSP) on patient satisfaction(PtS) in rehabilitation settings of public and private hospitals of Islamabad and Rawalpindi, Pakistan. Mediating effect of CHSP and moderating effect of received word of mouth (WOM) have also been studied in this relationship. A sample of 300 patients receiving rehabilitation care was selected by applying convenience sampling technique. The design of this study was cross-sectional in nature. Self-administered questionnaire was designed to be utilized. Application of the correlation and regression were executed to analyze the collected quantitative data. The cross-sectional study method, limited geographical area coverage, and small sample size did not allow the detailed testing of the causal relations among study variables. Results of this study have indicated that there was a significant direct positive relationship between perceived servicescape and patient satisfaction and this relationship was also mediated positively by CHSP. There was a substantial direct positive relationship between perceived servicescape and CHSP and, between CHSP and patient satisfaction. However, the moderating effect of received WOM was found to be insignificant. It is recommended that healthcare managers should develop a credible service framework that focuses on better affordability, competitiveness, awareness, empathy, service expertise, pleasant interactions with patients through centeredness communication and beliefs and, subsequently, it will contribute to patient satisfaction and success of the rehabilitation settings. This is the first-ever study of the impact of perceived servicescape on patient satisfaction through CHSP as a mediator and the moderating influence of received WOM in rehabilitation.

Keywords: Perceived Servicescape, Service Credibility, Received Word of Mouth, Rehabilitation, Healthcare Service Environment.

1. INTRODUCTION

World Health Organization (WHO) reported in 2023 about the disabled population that more than 1 billion people live with some form of disability and are increasing in recent years due to demographic trends and chronic health conditions [1]. According to the disability statistics report till 2021 provided by the Pakistan Bureau of Statistics, 371,833 people living in Pakistan are experiencing some form of disability. Out of 371,833 disabled people, majority (147,539) of them are living in Punjab and majority (295,093) of them have a physical disability [2]. Early rehabilitation is crucial in physical and neurological disabilities for functional outcomes [3]. The major challenges faced by persons with disabilities in Pakistan have been poor-quality rehabilitation services. The developing country "Pakistan" still lacks a registry of national disability to estimate the total burden of people with disabilities accurately [4].

Rehabilitation settings is defined as the place where the therapists provide therapeutic services to address both physical and mental health conditions after surgical interventions or illnesses [5]. There are few physical rehabilitation centers located in the urban areas of Pakistan, whereas a large percentage of the disabled population lives in rural parts. Moreover, rehabilitation settings have been in the developing stage [6]. Kotler [7] introduced the concept of servicescape by using

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the term 'atmospherics' for the first time and stated that buying environment of the organization produces specific emotions in the customers that increases the likelihood of purchase. The term 'servicescapes' was first used by Bitner [8] in a service environment,where three servicescape dimensions, such as ambient condition, spatial layout/functionality and sign, symbol and artifact were explained. All three servicescape dimensions have shown the significant positive impact on patient satisfaction [8, 9]. DCunha *et al*. [10] stated that service quality perception by customers depends on ambient, design and social factors of the servicescape of the hospital that contributes to customer satisfaction, enhances the patient experience, retains existing customers and attracts new ones.

Credibility of the healthcare service provider (CHSP) is the capability of the healthcare professional to meet a level close to expectations of the patient [5]. Jeng [11] stated that the service is regarded as reliable when provider of that service has both expertise and trustworthiness. When patients personally experience the servicescape that meets their expectations, it contributes to the credibility of a service provider and trust of the patients [12]. CHSP enhances pleasure-feeling. The image of the healthcare organization is influenced by both the servicescape of the hospital and service credibility [13]. Lai and Chong [5] found that servicescape is a significant driver that shapes the behavior of patients and has significant impact on service credibility.

According to Ngoma and Ntale [14], customers spread either negative or positive word of mouth (WOM). Word of mouth shapes attitudes and behaviors of the customers resulting in patient satisfaction. Positive WOM recommendations remove uncertainty, create customer excitement and act as a switching barrier resulting in long-term relationships with the healthcare service providers. Fatima *et al*. [15] concluded that the better servicescape of the hospital contributes to patient satisfaction and loyalty intentions, resulting in positive word of mouth recommendations. Limbu *et al*. [16] found that the decision-making of the patient is extremely affected by positive word of mouth recommendations.

The overarching theory supporting this study was the Expectancy-Disconfirmation theory. According to this theory, customer relates his experience of the service received with his own expectations. If the perceived service encounter of the customer is equivatent to or higher than his expectations, he/she will be more satisfied [17]. In this study, this theory suggests that if the perceived servicescape experience of the patient in the rehabilitation settings meets equal or higher than his/her expectations, it will enhance the CHSP which, subsequently, contributes to patient satisfaction. The role of the Stimulus–Organism–Response (SOR) Model in explaining the behavior of disabled patients has also provided the theoretical foundation for this research [18]. In this study, this SOR model suggests that stimulus from the service environment (servicescape) affects organism (i.e., CHSP), which, subsequently, affect patient response behavior (patient satisfaction). Directed by the SOR model and Expectancy-Disconfirmation Theory, the research model of this study will give a more comprehensive assessment of how the servicescape of the rehabilitation settings will influence CHSP and their consequent effects on patient satisfaction. The servicescape of the rehabilitation settings in Pakistan have got limited attention by healthcare managers. Nowadays, the challenege is the increasing demand for better rehabilitation services which also includes servicescape cues [19]. There were gaps in the healthcare literature that how patients' perceptions of the servicescape of rehabilitation settings were related to the CHSP and patient satisfaction; and whether received WOM would strong or weak the relationship between the CHSP and patient satisfaction (Figure 1). This study addressed these gaps in the existing literature and extended the existing literature on the study variables that was the significance of this study. To my knowledge, this study is a unique attempt and it is relevant to the study of Lai and Chong [5]. This study will also extend the understanding of patient satisfaction by proposing the importance of received word-of-mouth in Pakistani environment where collectivism is promoted over individualism that contributes to social bonding capital and trust; and people share their thoughts, beliefs and feelings [20]. There is a necessity to understand the mediating role of CHSP because it influences the decision making and emotional states of the patients as when a patient has faith in the claims

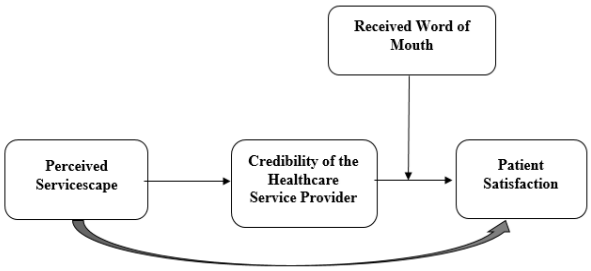

Fig. 1. Research model: Impact of perceived servicescape on patient satisfaction with mediating role of CHSP and received word of mouth as a moderator.

of the hospital credibility, he/she may experience pleasure-feeling and satisfaction towards the hospital [13].

This study would guide the local healthcare managers to better understand how cues of servicescape influence satisfaction of the disabled patients in the rehabilitation settings. This study has also extended the literature on received word of mouth, CHSP and patient satisfaction that will help local healthcare managers and marketing agencies when targeting, attracting, engaging, and retaining patients in the rehabilitation settings. The findings of this study would be fruitful for the local healthcare managers.

The research objectives of this study are to find out the effects of perceived servicescape on patient satisfaction, perceived servicescape on CHSP, and CHSP on patient satisfaction. Moreover, mediating effect of CHSP on perceived servicescape, patient satisfaction relationship and the interactive effect of received WOM and credibility of the healthcare service provider on the dependent variable (patient satisfaction) in the rehabilitation have also been analyzed.

Based on the preceding discussion, hypotheses are proposed that:

H1: Perceived servicescape will have a significant positive impact on patient satisfaction in the rehabilitation settings.

H2: Perceived servicescape will have a significant positive impact on credibility of the healthcare service provider in the rehabilitation settings.

H3: CHSP will have a significant positive impact on patient satisfaction in the rehabilitation settings.

H4: CHSP will mediate the relationship between perceived servicescape and patient satisfaction in the rehabilitation settings.

H5: Received word of mouth will moderate the relationship between credibility of the healthcare service provider and patient satisfaction in a manner that this causal relationship will be stronger when received word of mouth is highly positive.

2. MATERIALS AND METHODS

2.1. Sample and Data Collection

Primary data were collected from study participants (disabled patients) who were receiving rehabilitation care for some form of disability at rehabilitation settings in Rawalpindi and Islamabad, Pakistan. These participants were both male and female patients aged between 18-80 years receiving rehabilitation care for some form of disability such as cerebral palsy, spina bifida, polio, congenital talipes equinovarus, genu-valgum, genu-varum, stroke patients, musculoskeletal disorders, knee osteoarthritis and amputees. Patients having current symptoms of psychosis, dementia and other psychological illnesses were excluded. For data collection, participants receiving rehabilitation care were reached out to fill the questionnaire through traditional field survey technique such as hard copy questionnaires as study tool. A cover letter was attached with the questionnaire ensuring the participants about strict confidentiality of the information, consent, and providing details about the purpose of the study. In addition, data collection was cross-sectional and voluntary participation was ensured in this study. This study was grounded on convenience sampling technique. Out of 324 questionnaires distributed in hardcopy, the researcher received 312 responses. Useable responses were 300 resulting in a response rate of 93%.

2.2. Scales and Measures

All four variables were measured by utilizing the five-point Likert scale. In this Likert scale, 1 denoted the low value of the variable and 5 denoted the high value of the variable and it has a range from 1=strongly disagreed to 5=strongly agreed. The questionnaire was formed in the English language. English is taught as a compulsory subject in Pakistan. Therefore, the translation of the questionnaire into the native language was not required.

2.2.1. Perceived servicescape (PS)

Perceived servicescape was measured using 11 items of the scale developed by Reimer and Kuehn [21]. One sample item of the scale was "The building's architecture is visually appealing". The scale reliability as measured by Cronbach's alpha was 0.946.

2.2.2. Credibility of the healthcare service provider (CHSP)

Credibility of the healthcare service provider was measured using 8 items of the scale developed by Jeng [11]. One sample item of the scale was "The healthcare service in this center delivers what it promises". The scale reliability as measured by Cronbach's alpha was 0.945.

2.2.3. Received word of mouth (RWOM)

Received WOM was measured using 3 items of the scale developed by Schumann *et al*. [22] and modified by Sundermann [23]. One sample item of the scale was "People I know have recommended this rehabilitation center to me". The scale reliability as measured by Cronbach's alpha was 0.927.

2.2.4. Patient satisfaction (PtS)

Patient satisfaction was measured using 3 items of the scale developed by Choi and Kim [24]. One sample item of the scale was "Overall, I am satisfied about the experience with this physical environment". The scale reliability as measured by Cronbach's alpha was 0.947.

2.3. Control Variables

A one-way ANOVA was executed to manage the deviation in the analysis of the CHSP, received word of mouth, and patient satisfaction caused by the demographic variables. Results (Table 1) showed substantial differences in patient satisfaction across gender ($F = 42.52$, $p < 0.05$), marital status ($F = 4.99$, $p < 0.05$), and type of hospital ($F = 184.61$, $p < 0.05$). Therefore, these demographic variables (gender, marital status, and type of hospital) were put as control variables in the regression analysis for patient satisfaction.

However, results (Table 1) presented no substantial differences appeared in the mean values of the credibility of the healthcare service provider (CHSP-mediating variable) among groups on the basis of age, gender, qualification, marital status, type of hospital, and frequency of visitation. Similarly, results presented (Table 2) that no substantial differences appeared in the mean values of received word of mouth (moderating variable) among groups on the basis of age, gender, qualification, marital status, type of hospital, and frequency of visitation.

2.4. Measurement Model

This section includes confirmatory factor analysis (CFA) level 1 and level 2, and measurement model estimates involving the items of scale of each study variable, factor loadings, Cronbach's alpha reliability, composite reliability, and average variance extracted for analyzing the validity and reliability of the scales of the study variables

Table 1. One-way ANOVA executed to manage the variation in the CHSP, received word of mouth, and patient satisfaction due to demographic variables.

	Credibility of the Healthcare Service Provider		Received Word of Mouth		Patient Satisfaction	
	F value	**p-value**	**F value**	**p-value**	**F value**	**p-value**
Age	1.06	0.39	1.09	0.36	1.1	0.33
Gender	64.68	0.06	44.79	0.06	42.52	0*
Qualification	4.24	0.06	4.95	0.06	5.31	0.24
Marital status	2.33	0.07	2.73	0.06	4.99	0*
Type of hospital	122.18	0.06	107.53	0.06	184.61	0*
Frequency of visitation	0.313	0.73	0.43	0.65	0.25	0.78

N = 300, *p value < 0.05.

performed in the Smart-PLS 3. According to Hair *et al*. [25], the minimum threshold of factor item loadings is 0.6. According to Fornell and Larcker [26], the minimum threshold of average variance extracted (AVE) is 0.50, composite reliability (CR) is > 0.60, and Cronbach alpha is > 0.6 for assessing the convergent validity of the scale. The values of Cronbach alpha, composite reliability, and AVE had met the threshold levels (Table 2) indicating the reliability (alpha and composite reliability) and validity (AVE) of the scales used and showing good model fit. Figure 2 and Figure 3 illustrated the factor loadings of all items of the study variables and relationships between them. As the factor loadings of all items were 0.6 or greater than 0.6 except the PS-9 as shown by the results (Figure 2). Therefore, the PS-9 item was removed due to low factor loadings, i.e., 0.574 for performing further data analysis and slight improvement was observed (Figure 3). These figures proved the convergent validity of each scale item used and model was ready and adequate. Moreover, results have shown that the values of good model fit were within acceptable ranges (SRMR= 0.05, and NFI = 0.91). According to Hair *et al*. [27], the value of SRMR must be less than 0.10 and NFI > 0.90 for good model fit.

3. RESULTS

The results of the frequency distributions of demographic variables are given in Table 3. Out of 300 respondents, most of the participants (41%) receiving rehabilitation care aged 18 to 30 years. 41.7% were male and 58.3% were female. 30% were unmarried, 68% were married and 2% were divorced and widow. Most of the respondents (45.3%) completed nursery to matric education. 150 (50%) of them were drawn from the public rehabilitation settings while 150 (50%) of them were drawn from the private rehabilitation settings. 48.7% of respondents visited the rehabilitation center 1-2 times, 21.7% of them visited the rehabilitation center 3-5 times and 29.7% of them visited the rehabilitation center above 5 times

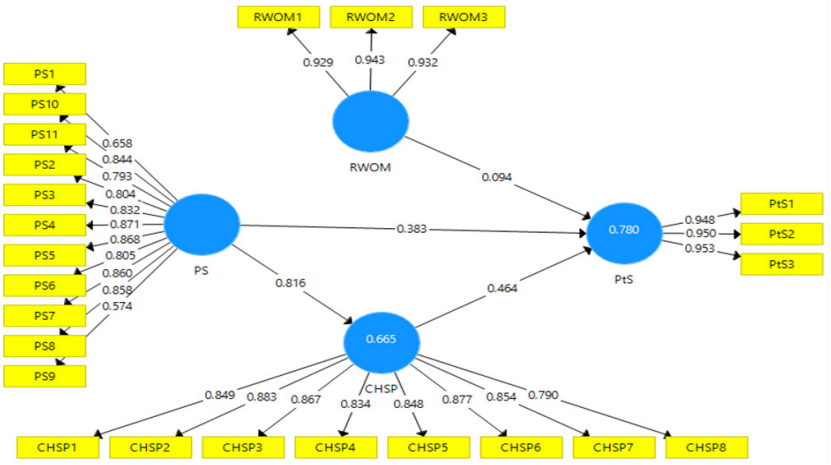

Fig. 2. Confirmatory factor analysis level 1.

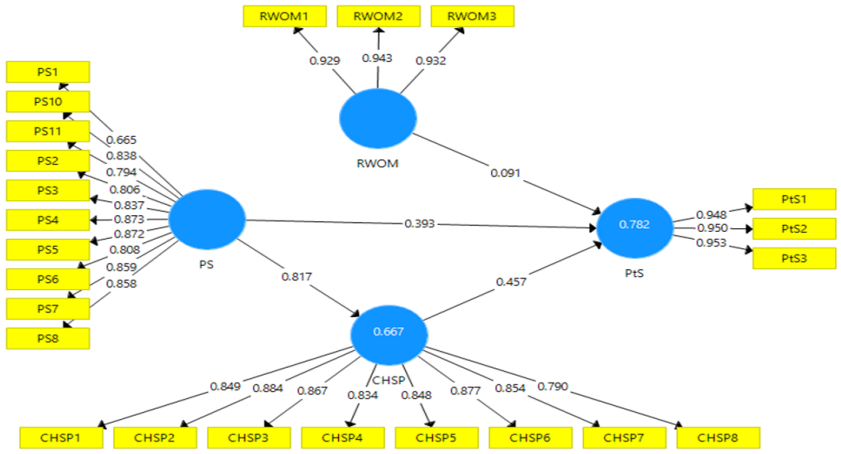

Fig. 3. Confirmatory factor analysis level 2.

Table 2. Measurement model (estimates) for analyzing the validity and reliability of the scales of the study variables.

Constructs	Items	Loadings	Alpha	Composite reliability (CR)	Average variance extracted (AVE)
PS	PS 1	0.665	0.946	0.954	0.678
	PS 2	0.806			
	PS 3	0.837			
	PS 4	0.873			
	PS 5	0.872			
	PS 6	0.808			
	PS 7	0.859			
	PS 8	0.858			
	PS 10	0.838			
	PS 11	0.794			
CHSP	CHSP 1	0.849	0.945	0.954	0.724
	CHSP 2	0.884			
	CHSP 3	0.867			
	CHSP 4	0.834			
	CHSP 5	0.848			
	CHSP 6	0.877			
	CHSP 7	0.854			
	CHSP 8	0.790			
RWOM	RWOM 1	0.929	0.927	0.954	0.873
	RWOM 2	0.943			
	RWOM 3	0.932			
PtS	PtS 1	0.948	0.947	0.966	0.904
	PtS 2	0.950			
	PtS 3	0.953			

Abbreviations: PS = Perceived Servicescape, CHSP = Credibility of the Healthcare Service Provider, RWOM = Received Word of Mouth, PtS = Patient Satisfaction.

in the last 12 months. Quantitative data for each demographic variable is presented in Table 3.

3.1. Descriptive Statistics and Correlation Analysis

Descriptive statistics and correlation coefficients are presented in Table 4 and Table 5, respectively. The study findings unveiled that all variables are positively correlated to each other. Table 5 indicated that perceived servicescape exhibited a positive correlation with patient satisfaction ($r = 0.83$, $p < 0.05$); thus, providing initial confirmation of the hypothesis H1. Perceived servicescape was positively correlated with CHSP ($r = 0.82$, $p < 0.05$). CHSP was positively correlated with patient satisfaction ($r = 0.85$, $p < 0.05$). Received WOM also exhibited a significant positive correlation with patient satisfaction (0.73, $p < 0.05$). The skewness values were between the range of +1 and -1 and kurtosis was between the range of +3 and -3, as mentioned by Hair *et al*. [28], the data of this study was normally distributed.

3.2. Regression Analysis

3.2.1. Simple linear regression analysis

In step one, Demographic variables such as gender, marital status and type of hospital were put as control variables. The results indicated that perceived servicescape had a significant positive direct effect on patient satisfaction in rehabilitation settings ($\beta = 0.833$, $p < 0.05$) as shown in Table 6. The hypothesis H1 was validated, as R-square

Table 3. Frequency distributions of demographic variables.

Demographic Variables		Frequency	Percentage %
Age (Years)	18-30	123	41
	31-40	57	19
	41-50	60	20
	51-60	32	10.7
	61-70	26	8.7
	71-80	2	0.7
Gender	Male	125	41.7
	Female	175	58.3
Marital Status	Unmarried	90	30
	Married	204	68
	Divorced	4	1.3
	Widow	2	0.7
Qualification	Uneducated	27	9.0
	Nursery school to 8th grade	76	25.3
	Matric	60	20.0
	Intermediate	35	11.7
	Bachelor	60	20.0
	Master	37	12.3
	Doctorate Degree	5	1.7
Type of hospital	Public	150	50
	Private	150	50
Frequency of visitation	1-2 times	146	48.7
	3-5 times	65	21.7
	Above 5 times	89	29.7

Table 4. The results of descriptive statistics.

	Minimum	Maximum	Mean	SD	Skewness	Kurtosis
PS	1.30	5.00	3.13	1.00	-.10	-1.26
CHSP	1.25	5.00	3.15	.95	-.19	-.99
RWOM	1.33	5.00	3.33	.97	-.19	-.82
PtS	1.00	5.00	3.19	1.22	-.23	-1.07

N = 300, *p-value < 0.05, PS = Perceived Servicescape, CHSP = Credibility of the healthcare service provider, RWOM = Received Word of Mouth, PtS = Patient Satisfaction, SD=Standard Deviation.

Table 5. The outcomes of Pearson correlation.

	1	2	3	4
PS	(.95)			
CHSP	.82*	(.95)		
RWOM	.72*	.77*	(.93)	
PtS	.83*	.85*	.73*	(.95)

n = 300, *p-value < 0.05, alpha reliabilities as in parentheses, PS = Perceived Servicescape, CHSP = Credibility of the healthcare service provider, RWOM = Received Word of Mouth, PtS = Patient Satisfaction.

Table 6. Simple linear regression analysis.

Hypothesis	Relationship	Beta Coefficient	R-square	Standard deviation	T statistics	P-Values
Step 1						
	Control variables		0.475			
Step 2						
H1	PS-PtS	0.833	0.693	0.017	48.068	0.000*

n = 300, Dependent variable: PtS = Patient Satisfaction, Independent variable: PS = Perceived Servicescape, Control variables: Gender, Marital status, Type of hospital, *p value < 0.05.

value was 0.693 showing the variation of 69.3% in patient satisfaction due to perceived servicescape as shown in Figure 4.

3.2.2. Mediated regression analysis

The specific indirect effects through CHSP between perceived servicescape and patient satisfaction for mediation regression analysis were reported in Table 7 and demonstrated in Figure 5, indicating the beta values and R-square values, respectively. The results indicated that perceived servicescape had a significant positive direct influence on CHSP in rehabilitation settings ($\beta = 0.817$, $p < 0.05$), thus, hypothesis H2 was accepted. The results indicated that CHSP had a significant positive direct influence on patient satisfaction in rehabilitation settings ($\beta = 0.507$, $p < 0.05$), hence, hypothesis H3 was also accepted. Mediating effect of CHSP between perceived servicescape and patient satisfaction in the rehabilitation settings was also found to be significant ($\beta = 0.414$, $p < 0.05$), thus, hypothesis H4 was also confirmed. As R-square value was 0.667 showing the variation of 66.7% in the CHSP due to perceived servicescape. Moreover, as the value of R-square was 0.779 showing the variation of 77.9% in the dependent variable owing to perceived servicescape and CHSP.

3.3.3. Moderated regression analysis

In step one, demographic variables (gender, marital status, and type of hospital) were put as control variables. In step 2, direct effects of CHSP and received word of mouth on patient satisfaction were evaluated. In step 3, the interactive effect of received word of mouth and CHSP towards the dependent variable for moderated regression analysis was calculated as presented in Table 8 and shown in Figure 6. Moderating effect of RWOM on the relationship between credibility of the healthcare service provider and patient satisfaction was found to be insignificant ($\beta = -0.003$, $p = ns$), therefore, hypothesis H5 was not accepted.

4. DISCUSSION

In the present study, we have investigated the impact of servicescape of the rehabilitation settings on patient satisfaction as patient satisfaction is a key to the success of rehabilitation settings today. As, patients and visitors are more focusing on the

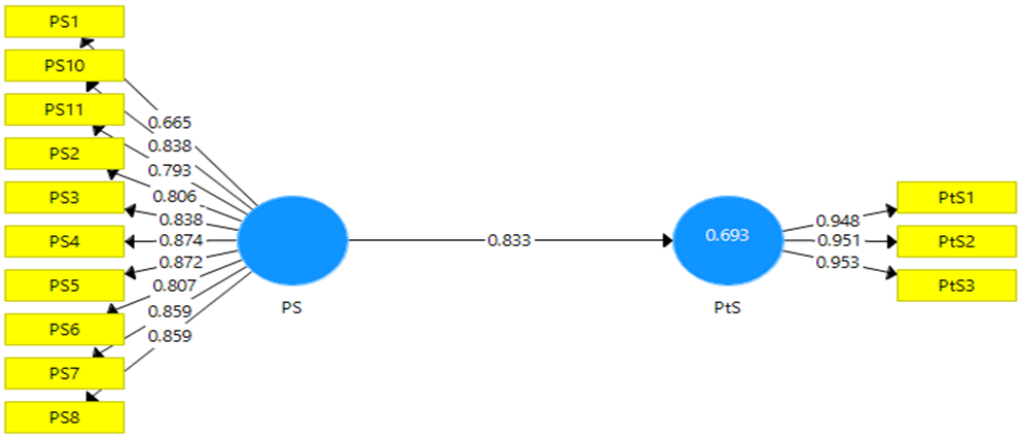

Fig. 4. Structural model (simple linear regression analysis).

Table 7. Mediated regression analysis.

Hypotheses	Relationship	Beta Coefficients	Standard deviation (STDEV)	T statistics	P-Values
H1	PS-PtS	0.833	0.693	48.068	0.000*
H2	PS-CHSP	0.817	0.018	45.951	0.000*
H3	CHSP-PtS	0.507	0.051	9.936	0.000*
H4	PS-CHSP-PtS	0.414	0.042	9.761	0.000*

n = 300, Dependent variable: PtS = Patient Satisfaction, Independent variable: PS = Perceived Servicescape, CHSP = Credibility of the Healthcare Service Provider, *p value < 0.05.

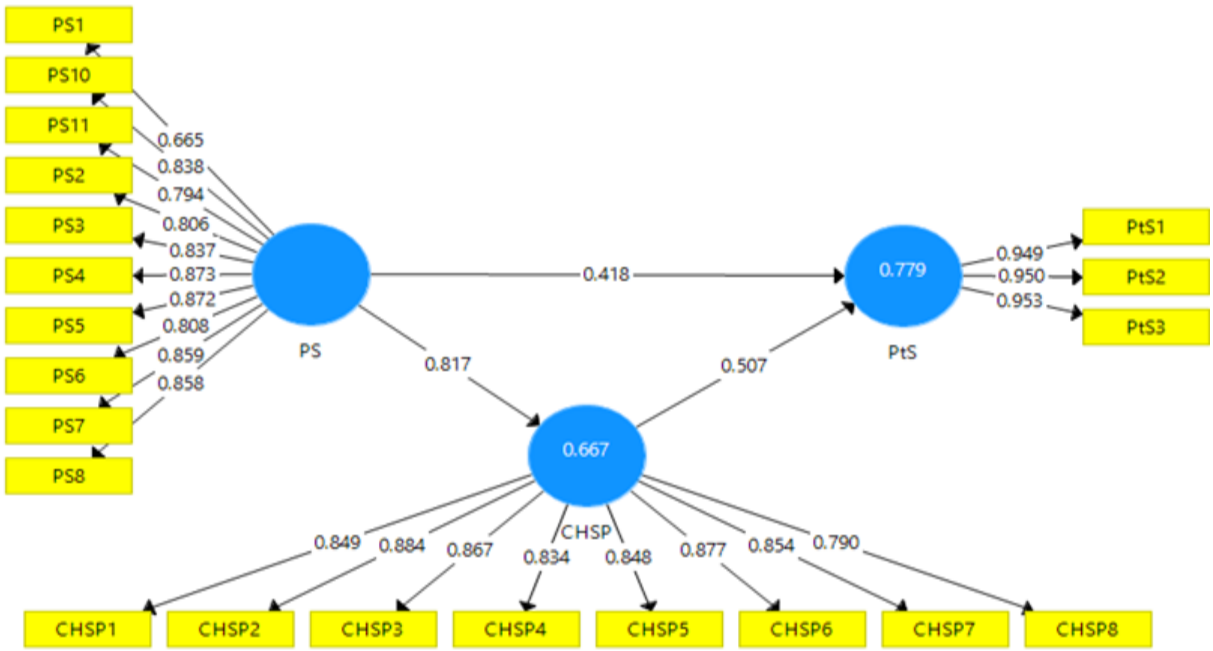

Fig. 5. Structural model (mediated regression analysis).

Table 8. Moderated regression analysis.

Hypotheses	Relationship	Beta Coefficients	R-square	Standard deviation	T statistics	P-Values
Step 1						
	Control variables		0.475			
Step 2						
	CHSP-PtS	0.710	0.735	0.045	15.92	0.000*
	RWOM-PtS	0.179		0.049	3.637	0.000*
Step 3						
H5	CHSP*RWOM-PtS	-0.003	0.735	0.039	0.068	0.940 ns

n = 300, Dependent variable: PtS = Patient Satisfaction, Independent variable: CHSP = Credibility of the Healthcare Service Provider, Moderator: RWOM: Received Word of Mouth, Control variables: Gender, Marital status, Type of hospital, *p value < 0.05, ns = not significant.

aspects of the physical environment now-a-days, healthcare managers of the rehabilitation centers can boost patient satisfaction by paying attention to the physical service environment.

In this study, a significant positive direct relationship was observed between perceived servicescape and patient satisfaction that supported the formulated hypothesis H1. It also aligned with the results of Fatima *et al.* [15], Lee and Chuang

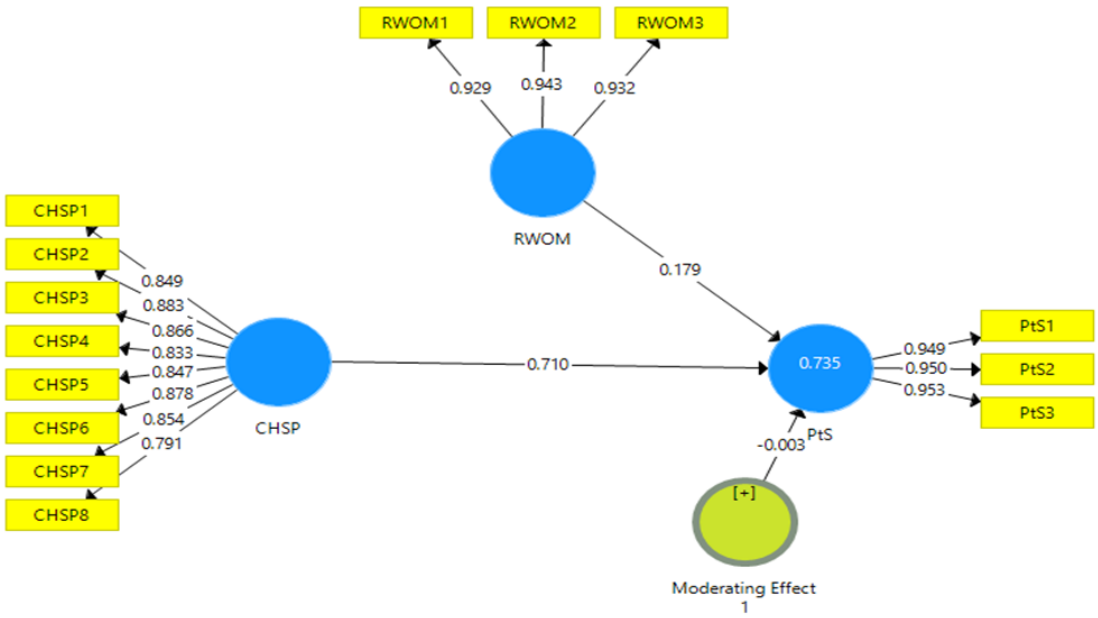

Fig. 6. Structural model (moderated regression analysis).

[29], Peng *et al.* [30], Rosenbaum *et al.* [19], Suess and Mody [31], and Vigolo *et al.* [32]. This may be due to the fact that if the physical environment of the rehabilitation center is well-presented, it will communicate about its professionalism to the patients, and create a sense of affiliation and good rapport between the patients and healthcare service provider. Firstly, the positive attributes of servicescape seem to be more important in building servicescape perception in the minds of the patients as these attributes create the first impression to attract patients [10]. Secondly, tangible aspects of the physical environment help new patients to form their evaluation towards the rehabilitation settings. Moreover, servicescape of the rehabilitation settings also affects the physiological states of patients and influences patient satisfaction. So, all physical and mental dimensions of the physical surroundings must be balanced to provide better experience to the patients receiving rehabilitation care [33]. Therefore, the patient-oriented servicescape has impact on patient satisfaction visiting the rehabilitation settings of Pakistan similarly as in the developed nations.

In this research, a significant positive direct relationship was observed between perceived servicescape of the rehabilitation settings and CHSP that supported the formulated hypothesis H2. It also aligned with the conclusion of Lai and Chong [5]. The reason is that perceived servicescape cues increase their trust in the healthcare service provider and shape the decision-making of the patients visiting the rehabilitation settings that, consequently, contributes to the CHSP. In addition, when the services perceived by the patients are credible, trustworthiness in the service provider will rise. As a result, when patients have a greater level of trust and perceive that the service is delivered with assurance and reliability, they will become more confident and consequently enable them to develop positive feelings. Consequently, perceived servicescape experienced by the patients visiting rehabilitation settings of Pakistan has direct impact on CHSP.

According to this research, a significant positive direct relationship was observed between CHSP and patient satisfaction that supported the formulated hypothesis H3. It also aligned with the results of Sia *et al.* [12]. Reason for this is that when healthcare service providers deliver what they promised, patients and their family members will trust in the service providers and create favorable impression in the minds of the patients and their families. Moreover, when the promises of the healthcare service providers are credible and reliable, perceived risk and uncertainty about services will reduce. Consequently, patients receiving rehabilitation care may feel happy and even satisfied. Hence, the CHSP has primary worth and has direct impact on patient satisfaction in the rehabilitation settings of Pakistan.

Mediating effect of CHSP between perceived servicescape and patient satisfaction was also found to be significant that supported the formulated hypothesis H4. It also aligned with the outcomes of Lai and Chong [5], Loureiro [13], and Sia *et al*. [12]. The reason is because when the patients receiving rehabilitation care believe that the promises of the rehabilitation centers are trustworthy and credible, they may develop trust, affective commitment, happiness, and satisfaction towards the rehabilitation settings. Moreover, CHSP will help in building a long-term relationship with the patients visiting rehabilitation settings. CHSP is also a good interpreter of rehabilitation center image and pleasure-feeling. As a result, CHSP has primary worth and perceived servicescape has indirect impact on patient satisfaction through CHSP. This study explored the mediating role of CHSP between perceived servicescape and patient satisfaction to address the current research gap.

Moderating impact of received WOM between CHSP and patient satisfaction relationship was proved to be insignificant and it was also contrary to the formulated hypothesis H5. This may be due to the fact that the patients may remain doubtful of the WOM received by the people they know. As health is a vital aspect of the human life, it is difficult for the patients to do decision-making about the selection of the healthcare service providers based merely on word of mouth received from friends, family, colleagues, opinion leaders, influencers, online evaluation platforms and social media. Furthermore, there is a high level of uncertainty in received WOM about the healthcare service providers when the real experiences are lacking by the patients. It also depends on the sources by which the people are receiving WOM such as either traditional or e-word of mouth. Moreover, there may be differences in patients' perceptions about the physical environment of the rehabilitation settings and CHSP when it comes to actual experiences faced by them. Therefore, received word of mouth is not a good predictor of patient satisfaction and it is least effective in satisfying the patients. It was in opposition to the outcomes of Ruswanti *et al*. [34], Sanjaya and Yasa [35], and Kuo and Nakhata [36]. To the best of my knowledge, the present study has investigated the moderating effect of received word of mouth for the first time to address the current research void in Pakistani culture.

4.1. Practical Implications

Healthcare managers should implement measures and devise strategies for patient oriented servicescape of the rehabilitation settings which will contribute to patient satisfaction and success, profitability, and authority of the rehabilitation settings. They should also provide such nurturing atmosphere to their patients where trust and credibility are given top priority. Managers should also put emphasis on ambient condition, spatial layout/functionality and sign, symbol, artifact factors in the rehabilitation settings. Managers should develop a credible service framework for enhancing trustworthiness and affective commitment that focuses on better affordability, competitiveness, awareness, empathy, service expertise, pleasant interactions with patients through centeredness communication and beliefs.

4.2. Limitations of the Study

There are a few constraints concerning this study that require attention. This study employed a cross-sectional design and utilized convenience sampling. This study was restricted by its geographical coverage. The sample size was also small due to limited time duration. This study employed a quantitative approach. Another limitation was the patient's perspective of the servicescape of the rehabilitation settings. Moreover, moderation results of this study were unsupported.

4.3. Directions for Future Research

Future studies are advised to employ larger sample sizes and adopt a prospective approach. Subsequent investigation should perform the qualitative study. Furthermore, it should also explore the impacts of additional moderating factors such as personality traits, emotional intelligence, social support, price, appointment procedures, and cultural factors. Potential research can also be performed by finding the relative importance of each dimension of the servicescape in the rehabilitation settings. Employees can be further investigated to explore their satisfaction with the servicescape of the rehabilitation settings.

5. CONCLUSIONS

This study has provided a clear understanding about perceived servicescape of rehabilitation settings

of Pakistan and how perceived servicescape and credibility of the healthcare service provider contribute to patient satisfaction in order to attract and retain the patients receiving rehabilitation care. Outcomes of this study demonstrates that perceived servicescape influences positively patient satisfaction with the physical environment of the rehabilitation settings in Pakistan justifying the findings of different studies performed in the western countries. Moreover, perceived servicescape influences positively CHSP and CHSP influences positively patient satisfaction. When the patients receiving rehabilitation care experience the better servicescape, they would be more likely to be satisfied and have intention to revisit the rehabilitation settings for the same services or some other services.

As CHSP has direct effects on patient satisfaction, it also acts as a mediator between perceived servicescape and patient satisfaction. So, it is treated as a key determinant in developing patient satisfaction in the rehabilitation settings. This is noteworthy for both theory and practice in the framework of the healthcare industry. However, results of the interactive effect of CHSP and received word of mouth on patient satisfaction suggested that highly positive received word of mouth does not influence patient satisfaction and it is a poor predictor of the patient satisfaction.

As patient satisfaction is central to the accomplishment of rehabilitation settings, healthcare managers can boost patient satisfaction by considering the patient's perceptions that will give insights about how to improve the different aspects of physical environment of the rehabilitation settings. Explicitly, this study may guide the management, investment and marketing decisions that will direct the development of patient-oriented service environment in the rehabilitation settings. Finally, this study will also be useful about how to recruit and train new employees/managers in the rehabilitation settings.

6. ETHICAL STATEMENT

The study was conducted under the Declaration of Professional Ethics and Code of Conduct and the protocol was approved by the FMS Ethics Committee of the Riphah International University.

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Instructions for Authors

Manuscript Format

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

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

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

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

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

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

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

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

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

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

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

Manuscript Formatting

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

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

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

List of REFERENCES must be prepared as under:

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

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

b. Books

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

c. Book Chapters

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

d. Reports

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

e. Online references

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

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

Tables and Figures

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

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

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

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• Full address of Institute/organization

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• All figure captions

• All tables (including title, description, footnotes)

Further considerations

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

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

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