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Review Article

Date Palm Cultivation, Consumption and Export: Current Status and Future Challenges - A Review

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Abstract: The date palm is an important horticultural crop, cultivated in all provinces of Pakistan, particularly in Khairpur and Sukkur districts of Sindh. Khairpur is biodiversity center for dates, with cultivation of elite commercially important cultivars like Aseel (predominant cultivar), Karbalain, Otakin, Kurh, Dedhi, Dhakki, Kashuwari, Kupro and Fasli etc. Aseel dates are consumed locally as table dates as well as exported to Germany, USA, Canada and Japan in the form of pitted and chopped dates by the different dates factories established in the country. Most of the Aseel dates are exported to India as yellow and brown chhuhara (boiled dried dates). Dates are also used to make different value-added products (date syrup, date paste, date pickles, date bars, date powder, date extracts, date jam). Poor pre- and post-harvest management and poor cultural practices are major obstacles in obtaining dates of international standards. Currently, date palms in Sindh face the effects of natural disasters, i.e., floods, post-flood diseases (sudden decline disease in the crown caused by *Fusarium solani*), and pests, such as, Red Palm Weevil (*Rhynchophorus ferrugineus*). Multiplication of elite and rare date palm cultivars by tissue culture technique is pre-requisite for large-scale cultivation which may fill the gap of destruction of indigenous date palm cultivars. Currently, thousands of grown-up trees of date palm have been destroyed with post-flood sudden decline disease. Proper pre- and post-harvest management and cultural practices and in addition cultivation of disease resistant cultivars are need of the time. In this review article, different aspects of date palm problems have been discussed and their solutions are suggested to benefit the date palm growers who can manage the crop in a better way.

Keywords: Horticulture, Cultivation, Biodiversity, Cultivars, Pitted, Value Addition, Red Palm Weevil, Post-harvest.

1. INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is dioecious and diploid (2n = 36) belongs to the family Arecaceae with diverse varietal collection grown in hot and arid regions of the world; where temperature reaches to 50°C during summer [1]. The date palm is called as "Tree of Life" grown in Oasis and arid areas of the world [2]. The date palm tree remains productive up to 40–50 years, and can survive up to 150 years, but with low production in certain conditions [3, 4]. The date palm can be propagated via conventional propagation methods i.e., seeds and offshoots; however, commercially important cultivars of date palm cannot be propagated via seeds due to heterozygosity [5]. The date palm propagated via seeds always show variation and slow growth, which can take 8–10 years to reach up to fruiting stage. Propagation of date palm via seeds is only done when the offshoots are not available [6]. Additionally, the production of limited number of offshoots per tree (10-20) during its whole life, low survival of offshoots after cultivation in the field, slow growth and diseases (Bayoud and Red Palm Weevil) are major hindrances in cultivation of date palm via offshoots [7, 8]. In contrast to conventional propagation methods (seeds and offshoots) of date palm, the tissue culture is an alternative and reliable procedure to produce huge number of true-to-type and disease free plantlets in a short time and space [9-12]. The date palm propagation via tissue culture restricts the spread of

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pests and diseases. Therefore, the micropropagation of date palm is reliable procedure for large scale production of commercially important cultivars of the date palm [13]. Currently, the micropropagation is applied progressively to produce huge number of disease-free plantlets [14]. The date palm is a major crop mainly cultivated in Khairpur and Sukkur districts of Sindh (85% dates produced in Khairpur) [15]. Full productive age of the tree is 15-30 years [36]; whereas, height of the tree above 15-20 meters increases risk and efforts for pollination and harvest. Several studies [4, 16-21] have been conducted at different times regarding vegetative, flowering and fruit evaluation of the date palm in different regions of the world. The developmental stages of date fruits have been categorized into hababouk and kimri (green and non-edible fruit), followed by khalal stage (fruit reaches to full size is edible and acquire a particular colour, i.e., yellow or red depending on the cultivar), rutab (half ripened) and tamar (fully ripened) obtained either on date palm tree (in case of dry date cultivars) or khalal and rutab dates are sun-dried to make tamar in case of semi-dry date cultivars [4, 22]. The date fruit is eaten fresh or processed into different products (date bars, date syrup, alcohol, breads, date powder, jam, chocolate, paste and sweet candy). Chhuhara dates are generally exported to India, UAE, Bangladesh, Nepal; whereas, fresh dates are exported to Germany, USA, Turkey, UK, Srilanka and Australia [23]. Pakistan is 8th largest exporter of dates in the world and 6th largest producer of dates [24], exporting most of the dates produced in Khairpur and Sukkur districts of Sindh. Nutritional importance of dates [25], physicochemical properties and mineral contents [26], antioxidant activities [27] and bioactive compounds of boiled dates [28] have been described previously by several workers. The dates contain vitamins, proteins, sugar, dietary fibers, flavonoid, minerals and polyphenolics [29]. Holy Quran and other literatures mentioned the ethno-medicinal uses of dates [30, 31]. Several changes occur during growth and ripening stages influencing the quality and nutritive value of the dates [32]. Poor pre- and post-harvest practices are still obstacles in getting attractive prices of dates in the international markets. There is need to maintain quality of dates for export as per requirement of international standards by establishing the factories equipped with latest machines following the international codex quality standards to prepare good quality dates. Quality indices for dates include fruit size, shape, color, texture, cleanliness, and free from defects (such as sunburn, skin separation, insect damage, sugar migration to fruit surface, fermentation and decay-causing pathogens. Codex Alimentarius Commision's standards for dates include three sizes based on the number of dates per 500 g; such as, small (> 110 dates without seeds or > 90 dates with seeds), medium (90-110 dates without seeds or 80-90 dates with seeds). Natural disasters are another threat to date production and export across the country. Thousands of grown-up trees of date palm have been died due to sudden decline disease after floods and heavy rains in the Sukkur and Khairpur districts of Sindh during the year 2022. Micropropagation of the date palm is reliable method to improve the varietal structure of the date palm in the area affected by pests, diseases and natural disasters i.e., floods and heavy rains. The objectives of the current study are to describe the cultivation trends, consumption behavior, export, post-harvest and disease and pests' management, to discuss about the introduction of exotic cultivars, and the role of micropropagation in improving the varietal structure of the date palm in the area.

2. CULTIVATION OF DATE PALM VARIETIES IN KHAIRPUR

Khairpur district is the biodiversity centre of date palm with cultivation of more than 300 commercial and non-commercial varieties [33]. The date palm cultivated on an area of 22310 hectares with production (158775 tons). Major export varieties of date palm have been cultivated in district Khairpur and Sukkur are Aseel, Karbalain, Dhakki, Otakin, Kurh, Dedhi, Kashuwari, Khar, Kupro, Gajar and Fasli [34, 35]. Different varieties of the date palm contain different colour and taste, consumed at different growth stages i.e, khalal, rutab and tamar.

2.1. Commercial Varieties of Dates

Aseel is the predominant cultivar of the date palm cultivated largely in district Khairpur and Sukkur; produces 15-20 bunches each year (Avg. 25 kg per bunch) and size (4.3 cm long × 2.5 cm in diameter), narrow at base, wide in the middle and oblong [36]. Aseel dates are processed in different ways, i.e., it is consumed at rutab stage as fresh dates, or processed to make dry dates and chhuhara. Dates which are consumed at khalal stage are Otakin, Kurh, Dedhi, Mithri are known as soft dates and has the sweet test.

The dates consumed at rutab and tamar stages are Aseel, Karbalain, Dhakki, Fasli, Kupro, Kashuwari. The dates which are consumed at tamar stage only is Khar, and is ripened on the tree. Generally, dates are consumed at rutab and tamar stages (semi-dry date category) [37], or at khalal stage (soft type) date category and the dates which are only consumed at tamar stage and ripened on the tree are placed into dry date category. Early date varieties which are harvested in June include Kashuwari and Gajar (both are semi-dry). Kashoowari is consumed at rutab and tamar stages; while, it is inedible at khalal stage due to occurrence of high tannins; whereas Gajar dates are largely utilized to make tamar dates by shaking the khalal stage dates with little quantity of salt and put for a night. Aseel dates remain green during June (Figure 1(a)). Harvesting time of Aseel (Figure 1(b)) is at the end of the July; whereas, the percentage of rutab dates depends on the rise in temperature upto 50°C, but generally only 10% of dates are turned into rutab during this period. Most of the quantity of Aseel dates is used to make yellow and brown chhuhara (boiled dried dates) [15]. Yellow chhuhara are prepared using sodium formaldehyde sulphoxylate in boiling water in an open pan; however, brown chhuhara are prepared with boiling of khalal stage dates in water without using any type of chemicals.

3. POST-HARVEST MANAGEMENT OF DATES

3.1. Harvest of Dates

Generally, the harvest of Aseel dates is done in the last week of July in Khairpur district; while harvesting of Begum Jangi originally belongs to Balochistan province of Pakistan is done during third week of August. Khalal stage dates of Begum Jangi were observed as rain tolerant in district Khairpur because the fruit skin does not break during heavy rains; therefore, such varieties can be cultivated on commercial level in the area. Post-harvest management of dates is crucial stage during which the harvested khalal stage dates are processed differently, i.e., either to make dry dates or chhuhara (brown and yellow). After harvesting, first step is the picking of rutab dates in the bunches which are sold as fresh as well as dried under sun to make tamar dates to keep for off-season. Date varieties of soft type consumed at khalal stage include Otakin, Kurh, Dedhi, Mithri are sold immediately as fresh dates after harvest [38], or processed to make chhuhara to preserve for off-season. Date varieties which are known as semi-dry are sold as fresh (Kashuwari, Naqul Kurh, Khar, Fasli etc.) or processed to make chhuhara and tamar dates (Aseel, Karbalain and Dhakki) after drying under sun [4]. Harvested khalal dates (without rutab dates) are spread on the mats under sun for drying and processing for 4-5 days. After harvest (Figure 2(a)); first step is separation of khalal stage dates carefully from bunches using wooden forked instrument (Figure 2(b)). Dates are immediately washed and kept in a large pan for boiling (Figure 2(c)), and kept under sun for 4-5 days to make chhuhara (Figure 2(d)), or khalal dates are directly spread on mats under the sun for 4-5 days for making tamar dates.

4. DATES CONSUMPTION BEHAVIOR

4.1. Soft Dates

Dates which can be consumed at khalal stage contain low tannins and high sugar concentration, and are not further processed to make tamar are

Fig. 1. (a) Date palm cultivation behavior in Sindh and (b) Date palm cultivar Aseel with fruit showing the number of bunches with dates at harvesting time.

Fig. 2. Processing of khalal stage dates for preparing chhuhara and tamar dates. (a) bunches just after harvest, (b) fruit separation from bunches, (c) boiling of khalal dates for preparation of chhuhara, and (d) spread of boiled dates on mats for sun drying process.

classified as soft dates. Dates are consumed at different ripening stages except kimri, depending on the variety and chemical composition of dates [4]. Some date varieties known as soft contain low tannins and 50-85% moisture content are usually consumed at khalal stage such as Otakin, Dedhi, Kurh and Mithri grown in district Khairpur, Pakistan [33]. Soft dates contain high sugar content and are crispy and sweet in taste. Dates at khalal stage are harvested earlier during the season, and therefore, are a good source of income for the growers [38]. Dates with low tannins at khalal stage can be consumed, but soft type date varieties are rarely found in Pakistan. International commercial date varieties consumed at khalal stage are Zaghloul, Barhi, Hayany and Khalas. Barhi is sold in France, England and Australia; whereas, other three date varieties are mainly consumed locally [39].

4.2. Semi-dry Dates

Dates consumed at rutab and tamar stages are classified as semi-dry dates. Rutab is the fourth stage of the fruit growth after khalal. Most semi-dry dates cannot be consumed at khalal stage due to high tannins such as Aseel, Kashuwari, Kupro etc. Rutab dates are a good source of income when sold as fresh, but in Pakistan poor storage conditions and packaging are big hindrances to obtain attractive prices. Aseel and Dhakki dates are mostly consumed in three forms (rutab, tamar and chhuhara) (Figure 3(a-d)) and date varieties grown in Balochistan, Pakistan are consumed as tamar (Figure 3(e)). Selected tamar dates of Aseel are consumed as table dates contain high amount of sugars and other food supplements required in daily diet. Dates are immediate source of energy,

Fig. 3. (a) Rutab stage dates of Aseel (semi-dry), (b) Rutab stage dates of Kashuwari (semi-dry), (c) Tamar stage 'A' grade dates of Aseel consumed as table dates, (d) Yellow chhuhara of cv. Dhakki consumed locally as well as exported to India, and (e) Different date varieties grown in Balochistan, Pakistan which are consumed at tamar stage. (Photos of (e) were taken by Summar A. Naqvi: University of Agriculture, Faisalabad, Pakistan).

generally in the form of rutab and tamar [40-43]. Kashuwari is the early date variety contains high tannins at khalal stage and is inedible; while, it is consumed at rutab and tamar stages [44] (Figure 3(b)). Kashuwari dates are picked from bunches at rutab stage, and directly sold in the markets. Dates are eaten as fresh, dried (tamar and chhuhara), or processed to make different products. Generally, the dates are consumed fresh after picking at rutab stage. Dates of some cultivars are consumed at rutab and khalal stages. Mainly dates are consumed at fully ripened stage (tamar). Dates at tamar stage contain very low moisture and can be stored for long period to be consumed during off-season [45].

4.3. Dry Date Varieties

Dates are classified as dry dates can be consumed only at tamar stage due to high amount of tannins at khalal and rutab, whereas, the moisture content of dry dates can be 20% at tamar stage, whereas, moisture content can be reduced depending on the soil type and climate. Dry date variety grown in Sindh, Pakistan is Khar. Ajwa cultivated in Al-Madinah, Saudi Arabia belong to dry date variety which can be consumed at tamar stage only is cultivated in Shah Abdul Latif University, Khairpur and some other places in Pakistan for field performance. Ajwa trees are fruiting well, but the moisture content of the fruits is higher than the moisture content of the Ajwa dates in its original place, i.e., Al-Madinah, Saudi Arabia. Dry date cultivars are less important due to heavy monsoon rains in Sindh which destroy the 30-50% of the crop every year. Hence, it is difficult to save the dry date cultivars up to ripening stage.

4.4. Chhuhara (Brown and Yellow)

Chhuhara is an alternative source of long-term dates storage for consumption, mostly in the form of brown chhuhara; whereas yellow chhuhara are mainly exported to India. Brown chhuhara is also used to make halwa (pudding) locally. Chhuhara can be stored for long periods due to low moisture content compared to tamar dates. Chhuhara are of two types (yellow and brown). Yellow chhuhara are prepared from khalal dates using sodium formaldehyde sulphoxylate in boiling water (Figure 3(d)) for fifteen minutes. Brown chhuhara are prepared with boiling of khalal stage dates only in water without using any type of chemicals.

5. DATES EXPORT AND VALUE-ADDED PRODUCTS

5.1. Dates Export

Dates production of Pakistan is about 5328795 tonnes cultivated on 102676 hectares and average yield per hectare is 518 tonnes [46]. In the year 2022; Pakistan exported 12124446 million tonnes of dates to different countries. Countries importing the dates from Pakistan are Canada, Malaysia, India, USA, Denmark, UK, and Germany [47]. Pakistan is one of the active exporters of dates and ranked 8th in export of dates to different countries like India, Germany, USA, UK, Turkey, Japan, Australia, Srilanka, Nepal and Bangladesh as fresh, dried, pitted and chopped dates. Different dates factories have been established in Therhi, Khairpur, Pakistan exporting mostly pitted and chopped dates of Aseel and Begum Jangi throughout the year. Tamar dates are packed in 40 kg wooden box by the farmers and sold in the local markets in Khairpur, Sukkur, Lahore and Faisalabad in Pakistan. Large wooden boxes are filled by pressing the dates which cause damage of dates' skin and cause the change in natural shape of dates, results in low prices in the market. Several factors, such as physical, physiological, pathological disorders and insect infestation are involved in post-harvest losses in quantity and quality of dates [48, 49]. It is recommended that packing boxes of dates (made of paper) should be limited to 10 kg which will definitely save the dates to be damaged or pressed. Grading of dates (Figure 4(a)) is an important aspect for getting higher prices at each date consumption stage. The graded dates were filled in the 10 kg paper boxes covered with plastic sheet for fumigation for 3 days (Figure 4(b)) before export. Quality factors in the codex standard for dates include the following: (i) dates should have the characteristic color and flavor for the variety, be of proper stage of ripeness, and be free of live insects and insect eggs and mites; (ii) moisture content should be 26%-30% depending on the variety; (iii) minimum fruit size should be 4.75g (unpitted) or 4.0g (pitted); (iv) absence of defects, such as blemishes, mechanical damage, unripe, unpollinated, embedded dirt or sand, damaged by insects and/or mites, souring, mold and decay. Consumers always prefer the selected fresh or dried dates used as table dates. Maintaining the organoleptic characteristics of dates are of great importance in dates industry regarding export. Poor

Fig. 4. (a) Women making grading of dates at Therhi, Khairpur (b) Fumigation of pitted dates for three days before export, (c) Dates paste balls with Sesame seeds, (d) Dates halwa with almonds, (e) Date bars with pistachio, and (f) Date paste balls with chopped almonds.

post-harvest practices of dates processing result in low quality of dates which should be improved to meet the international standards. Heavy winds and monsoon rains result in inferior quality of dates. However, during normal circumstances when there is no rain and winds, the quality of dates can be improved as per international standards by keeping the dates free from dust and undamaged skin during processing and packaging. Active dates exporter in Khairpur is "Khairpur Foods International" exporting dates to USA, UK and Germany as pitted and chopped dates. Quality of dates can be improved through proper management at each stage of processing. Utilization of latest technologies in post-harvest processing of dates can improve the quality of dates like solar tunnels on large scale but small farmers cannot afford to process the dates in solar tunnels due to high cost and some technical issues in drying of dates under the tunnel; which are difficult to handle like high humidity collects inside the tunnel. Pakistan earns lowest rates of dates in international markets due to lack of latest technologies compared to other countries earning five times extra revenue than Pakistan in dates sector. Tunisia, produces 2.7% of the world dates production and earns $2433/ton; compared to Pakistan earns only $565/ton. Inappropriate post-harvest practices and lack of knowledge about post-harvest technologies result in earning small revenue in international markets. Date palm growers are being faced by several hurdles in boosting the dates sector regarding dates export. Dates sector can have a significant contribution in boosting Pakistan's economy; improving the export by facilitating the farmers with latest technologies, sharing information to the farmers in major dates producing areas [34]. Annual dates festival in district Khairpur arranged by the district government in collaboration with several agencies and institutions such as, Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Pakistan related to the agriculture is one of the key platforms for knowledge concerning post-harvest techniques of dates management and processing. It is worth mentioning that Pakistan organized 1st Pakistan International Date Palm Festival during 4-6 October, 2024 at expo centre Karachi in collaboration with United Arab Emirates to showcase different date varieties of Pakistan and to discuss the several problems related to dates and date palm.

5.2. Value Added Products

Dates can be utilized as fresh as well as different value-added products [50], such as, date paste balls with sesame seeds (Figure 4(c)), date paste halwa with almonds (Figure 4(d)), date paste bars with pistachio (Figure 4(e)), date paste balls with chopped almonds (Figure 4(f)). In Khairpur, Pakistan there is no any factory working on value added products of dates as per international standards. However, other countries like USA, UK, Germany and Japan importing dates from dates producing countries for utilization in different products like date juice, date paste, alcohol and in different bakery products. In Therhi, Khairpur dates are used to make date halwa, date pickle and date juice are prepared in small shops locally which do not ensure storage conditions and quality of international standards.

Government should focus on the establishment of factories for manufacture of different value-added products from dates.

5.3. Date Paste

Date pastes are characterized by their high sugar content in the form of glucose and fructose (reducing sugars). The amount and type of sugar change according to variety and ripening stage. The absence of sucrose in some cultivars has been explained by the environmental and genetic factors that may affect the qualitative and quantitative composition of the sugar by altering the activity of the enzymes involved in the synthesis and breakdown processes.

6. DATE PALM PROPAGATION (SEEDS AND OFFSHOOTS)

6.1. Seed Propagation

Easiest and quick way of date palm propagation is by naturally occurring seeds. Seed propagation does not ensure production of true-to-type plants; hence, the seed grown date palm is utilized only for breeding. The selection of date palm propagated via seeds is an economical way which may show some desirable traits such as rain or salt tolerance and increased production in the newly selected trees propagated via seeds. Date palm is dioecious; therefore, always show variation in plants propagated through seeds [8]. Generally, the seed grown date palm develops into either male or female trees; which produce mostly fruits of inferior quality due to its heterozygous nature [5]. Current genetic resources of the date palm obtained through selection are challenging due to dioecious nature i.e., seed grown date palm always produce fruits of inferior quality [51]. Therefore, the selection of elite commercial varieties via seed propagation is very difficult and time consuming but simultaneously, seeds can be exploited to get new varieties. Generally, the farmers prefer the propagation of date palm through offshoots which are produced around each tree during early age. Beside these difficulties some farmers are trying to select varieties of date palm grown through seeds (selection). Date palm farm established in Sukkur, Sindh by active farmer Mr. Imam Bux Jatoi who did selection of seed grown date palm at the time of fruiting. Varieties which produced good quality fruits (Figure 5(a-i)) were left to grow further, while the varieties which produced fruits of inferior quality were cut down and were utilized only for landscaping wherever required. Date fruits shown in the Figure 5(a, c-d) are brown at tamar indicate that these fruits were yellow-coloured at khalal stage. Date fruits shown in the Figure 5(b) and Figure 5(e-i) convert into black/dark brown coloured fruits at tamar stage; indicate that these fruits were red-coloured at khalal stage. Selection of the date palm trees was obtained through twenty years of continuous efforts. Further research should be conducted at genetic and biochemical level to check genomic constitution and food grade nutrients in the dates obtained through selection. There is need to work further on selection of date palm propagated through seeds; because the natural disasters due to climate change such as, floods and heavy rains are the big threats towards the cultivation of date palm in Pakistan.

Fig. 5(a-i). Fruits of the different seed grown varieties of date palm obtained through selection cultivated in district Sukkur, Sindh, Pakistan.

6.2. Offshoot Propagation

Date palm propagation via offshoots ensures production of true-to-type fruits [8]. There is no risk of genetic variation in the trees propagated via offshoots obtained from the parent tree. However, a tree produces 10-20 offshoots in its life [7]. In case of rare cultivars of date palm, the offshoots cannot fulfil the need due to the limited availability [1]. Offshoots can be easily available for the predominant cultivar Aseel but simultaneously most of the elite commercial date cultivars belong to soft (Otakin, Kurh and Dedhi) or semi-dry types (Kashuwari) are rarely found in different orchards in the area; therefore, is difficult to get offshoots of these varieties. The homogeneity of fruiting depends mainly on the uniformity of the initial offshoots during an orchard establishment. Nursery can provide farmers with uniform plants, their required number, complete root and free from symptoms of disease and insects. Farmers at Khairpur used to cultivate the detached offshoots at the same orchard among the adult date palms which reduced the distance between the trees and increased the moisture. The ideal distance between the trees should be 24 feet. Intercropping the vegetables result in devastation of offshoots by Diplodia disease (*Diplodia phoenica*) infection causes a substantial proportion of the new cultivation mortality. In order to establish a date palm nursery, few specifications and precautions should be taken in consideration such as, selection of an ideal offshoot which includes the age must be not less than 3 years, weight should be 10-25 kg and diameter of the wider part of stem should be 0.5-1.0 foot. Healthy and having a separate root system, and clear from any disease or pest symptoms. Further, offshoots should not obtain from an infected area.

7. OFFSHOOT DETACHMENT AND CULTIVATION IN THE FIELD

7.1. Offshoots Selection

Numerous factors regarding offshoot selection (size and weight of an offshoot), upper or lower offshoots produced around a tree, origin, offshoot removal method, preparation for cultivation in the field, post-cultivation treatments to save from the disease and pests [52]. Offshoots are produced around each date palm tree during its early stage of growth in the field (Figure 6(a)). 2-3 years old offshoots (10-25 kg) with wider part of stem (0.5-1.0 feet) (Figure 6(b)), can be detached from the parent tree for cultivation in the field (Figure 6(c)), and in this way each newly cultivated offshoot produce 10-20 offshoots once established in the field. During detachment of offshoots, it should ensure first that tree should be free from any type of disease and pests. During detachment extra fronds of offshoots should be cut down from the base. Offshoots are removed using chisel made of iron which is used to cut down the side of offshoot attached to the parent tree (Figure 6(a)). Roots and base of offshoots should not be damaged during removal from the tree; because the offshoots with damaged roots will not survive in the field. Considerations related to offshoot selection and detachment include, cutting-off the non-erect outer fronds and trim other fronds and ties them with a rope. Dipping or spraying the

Fig. 6. Offshoot detachment from parent tree and cultivation in the field. (a) Removal of offshoot from parent tree, (b) Preparing offshoot for cultivation in the field, (c) Cultivating offshoot in the field, (d) Offshoot cultivated in the field, and (e) Well developed offshoots in nursery, shifting for cultivating in another field.

offshoot with copper-based fungicides such as, Benomyl (Bavistin), Thiophanate Methyl (Topsin M) and Bordeaux mixture has been found effective against the Diplodia disease. Immerse the offshoot base in the fungicide solution 3-5 grams per liter for 4-5 minutes then leave for a while. Cover the base of detached offshoots with wetted piece of cloth and covering the fronds if possible. Spraying with pesticide to the cut surfaces which made in the mother palm or the offshoot; is important to avoid Red Palm Weevil attack; since, the smell of fresh date palm tissues attract the insects, and enter through any injured area of the tree trunk.

7.2. Irrigation

After removal from parent tree, the offshoots should immediately cultivate in the field (Figure 6(d)) and should be irrigated regularly as per need until it starts to grow properly in the field and its roots go deep inside the soil. Regular irrigation of the planted offshoots can be managed well by drip irrigation systems [15]. Irrigation to newly cultivated offshoots should be carried out as needed regularly; otherwise, the growth of roots will be stopped, which will suddenly cause drying of whole plant gradually. Irrigation requirement of offshoots depends on the soil types. Daily irrigation should be carried out to the offshoots planted in sandy soils during the first summer. Offshoots planted in clay soils should be irrigated once a week; whereas, irrigation is carried out every second or third day in most of the soils. Planted offshoots should be monitored regularly during first six weeks of planting until start of new growth and make sure that soil surface should remain wet around the planted offshoot and should not shrink away. Offshoots should be covered with a mulch of hay or straw may enhance moisture, control weeds and improve humus.

7.3. Field Preparation

Hole in the field for cultivation of offshoot is prepared according to offshoot size. Basal side of offshoot up to 1.5 feet should be submerged in the field. Fertilizer treatments should start when the offshoot start to grow well in the field.

7.4. Tree Management after Offshoot Excision

After removal of offshoots the damaged side of parent tree should be sprayed with systemic fungicide (Carbendazim) to save the tree from all types of fungal infections, and insecticide (Polytrin-C) to protect the tree from the attack of Red Palm Weevil and other types of insects. The injured side of the tree then must be covered completely with mud. Pesticide treatments should be repeated 2-3 times after every week on the open side of trunk of parent tree after offshoot removal [15].

7.5. Date Palm Nurseries

Offshoots exhibit lower survival rate in the field, when cultivated directly without growing further in the small nurseries (Figure 6(d)). During detachment of offshoots from the tree results in damage to the offshoot's base resulting in the lower survival rate in the field. However, growing the newly detached offshoots in nurseries before planting in the fields resulted in the maximum survival rate when shifted to another field. Recently, most growers applying such techniques (Figure 6(e)). In nurseries, offshoots develop better roots and canopy in two years, ready to cultivate in another field (Figure 6(e)) to get 100% survival rate.

8. MICROPROPAGATION AND FIELD EVALUATION OF TISSUE CULTURE DERIVED DATE PALM PLANTS

Micropropagation of date palm is the production of plants from cell, tissue or organ culture under sterile conditions inside the laboratory [53] (Figure 7(a)). Plantlets about 15 cm long can be acclimatized in the greenhouse for further growth (Figure 7(b)), and after growing in the greenhouse for two years, the plants were shifted in the open field (Figure 7(c)) for further vegetative growth and fruiting (Figure 7(d-i)). It is worth mentioning that Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Pakistan successfully produced three thousand plants of three elite local cultivars of date palm (Dedhi, Kashuwari and Gulistan) originally belonged to Pakistan and three exotic cultivars (Samany, Bertamoda and Barhi) through micropropagation process using offshoot and inflorescence explants [11, 12]. All cultivars of date palm showed normal vegetative growth and produced true-to-type fruits [38]. Tissue cultured plants were disseminated among the active date palm farmers for cultivation in different fields in

Fig. 7. (a) Tissue culture derived plantlets of date palm, (b) Micropropagated plants in the greenhouse ready for shifting in open field, (c) Tissue cultured plants growing in the field, (d) Tissue cultured plants at fruiting stage in the field, (e-f) Tissue culture obtained date palm cultivars Gulistan and Kashuwari respectively with fruits, (f, g, h, i) True-to-type fruits of date palm cultivars Samany (Egypt), Dedhi, Kashuwari and Gulistan respectively.

districts Khairpur and Sukkur, Pakistan. Tissue cultured cultivars include belong to soft type (Dedhi, Samany, Barhi), semi-dry (Kashuwari, Gulistan) and dry (Bertamoda) cultivars [44, 11]. More than 70 date palm growers cultivated tissue cultured plants of different varieties produced in Date Palm Research Institute which are currently ten years old and all the trees are bearing normal fruits (Figure 7(e-i)).

8.1. Field Performance of Exotic and Local Cultivars of Date Palm

8.1.1. Exotic cultivars

Field performance of different exotic cultivars of date palm was carried out in Research Orchard of Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Pakistan. Orchard includes eight cultivars of date palm belong to Balochistan, Pakistan are Aab-e-dandan (Figure 8(a)), Begum Jangi (Figure 8(b)), Gogna (Figure 8(c)), Halini (Figure 8(d)), Kooznabad (Figure 8(e)), Muzawati (Figure 8(f)), Pashna (Figure 8(g)) and Shakri (Figure 8(h)), and three cultivars of Saudi Arabia, i.e., Ajwa (Figure 8(i)), Safawi (Figure 8(j)) and Ruthana (Figure 8(k)). Offshoots of Saudi Arabian date palm cultivars were brought from Al-Madinah, Saudi Arabia in the year 2005, and planted in orchard [54]. Studies conducted on the tree and fruit evaluation confirmed that all three varieties of Saudi Arabia showed normal vegetative and fruit growth including number of bunches, bunch weight, fruit size, shape, weight and taste [4]. All fruit characteristics were similar to the fruits of parent trees as in Saudi Arabia.

Fig. 8. (a) Aab-e-dandan, (b) Begum Jangi, (c) Gogna, (d) Halini, (e) Kooznabad, (f) Muzawati, (g) Pashna, (h) Shakri, (i) Ajwa, (j) Safawi, and (k) Ruthana.

8.1.2. Local cultivars

Field evaluation performed for eight date palm cultivars of Balochistan, Pakistan include Aab-e-dandan, Begum Jangi, Gogna, Halini, Kooznabad, Muzawati, Pashna, Shakri (Figure 8(a-h)) showed that all trees produced normal fruits as fruiting in their native place (Balochistan). Due to variations in soil and climatic conditions of Khairpur district of Sindh and Balochistan; fruits produced in trees in climate of Khairpur were healthier than fruits in their native place; reason could be raised water table in this area; which provides enough water to the trees for vegetative growth and fruiting. This phenomenon is general for all date cultivars in the area except those growing in mountainous soils at Shadi Shaheed, Khairpur, Pakistan. Fruits of date palm growing in mountainous soils contain less moisture due to lower water table; hence, such fruits have long shelf life compared to fruits obtained from all non-mountainous soils [33]. Dates of Ajwa (Figure 9(a)) and Safawi (Figure 9(b)) are red at khalal stage, while Ruthana (Figure 9(c)) is yellow at khalal [4]. Two date palm cultivars of Balochistan (Muzawati (Figure 8(f)) and Shakri (Figure 8(h)) were red-coloured fruits at khalal stage, while rest of the cultivars including Halini (Figure 9(d)) and Begum Jangi (Figure 9(e)) were yellow coloured at khalal stage. Field performance of different exotic cultivars recommends introducing same and other date cultivars in the area in addition to existing cultivars of date palm. New varieties of date palm may have resistance to decline disease which is one of the major threats in this area. Additionally, there are few commercial varieties; and most of them belong to soft types which are consumed as fresh at khalal stage and cannot be stored in the form of tamar dates. Growers have established date palm orchards of exotic cultivars at Dera Ghazi Khan, Pakistan in the supervision of Agriculture University, Faisalabad, Pakistan. Trees are at fruiting stage in orchard. Orchard includes elite cultivars of date palm like Sukkary, Medjool, Shishi, Zaghlool, Khalas and Sagai. Additionally, there are several other orchards of the exotic cultivars of date palm in Sindh contained international commercial cultivars, but on small scale.

Fig. 9. Different exotic cultivars of date palm cultivated in district Khairpur, Pakistan: (a) Ajwa, (b) Safawi, (c) Ruthana, (d) Halini, (e) Begum Jangi, and (f) Shakri.

8.1.3. Field evaluation of tissue culture derived date palm

Solangi *et al*. [11] conducted a comprehensive study on micropropagation and field transfer of micropropagated plants of cvs. Samany and Bertamoda, and confirmed that all plants produced true-to-type fruits in the field. Solangi *et al*. [12] also conducted another comprehensive study on micropropagation of date palm cv. Barhi using shoot tip explants; evaluated tissue cultured plants in open field, and observed production of true-to-type fruits in open field trials conducted in agro-climatic conditions of district Khairpur, Sindh, Pakistan.

9. INTERNATIONAL VARIETIES OF DATES

9.1. Ajwa

Date palm cultivar Ajwa cultivated largely in in Al-Madinah, Saudi Arabia. Fruit is oval-shaped, medium sized (3.38 cm long and 2.68 cm in diameter). Fruit colour is red at khalal stage which turns into black at tamar. Ajwa cultivation surrounds Al-Madinah, yield thousands of tons of dates which are consumed locally as well as exported to Pakistan and other countries. Moisture content range of Ajwa dates at tamar stage is 10%-25% (Figure 10(a)).

9.2. Medjool

Date palm cultivar Medjool or in Arabic “majhul” which means unknown is a sweet and large in size (3-6 cm long and 2-3 cm in diameter), yellow colour at khalal stage and brown colour at tamar stage, cultivated originally in Tafilalt, Morocco. In addition, it is also grown in USA, Saudi Arabia, Israel, Jordan. Moisture content percentage of

Medjool dates range from 17%-25% at tamar (Figure 10(b)).

9.3. Mabroom

Mabroom dates fall into dry date cultivar similar to Ajwa dates but are narrow and large in size, are mainly grown in Saudi Arabia. Mabroom dates are brown colored. Mabroom dates may have size (6-8 cm long). Dates are sweet in taste and chewy. Mabroom dates are long and slender (Figure 10(c)).

9.4. Lulu

Lulu dates cultivated in south of Iran is small in size and delicious in taste with moisture content less than 15%. It has an extended shelf life (18 months) under normal room temperature. Size of Lulu dates is 1.5-2 cm and weight is 3-5 grams. Fruit colour is dark brown at tamar (Figure 10(d)).

9.5. Zahidi

Zahidi is one of the important commercial cultivars of date palm, cultivated largely in Iraq. It has light brown skin at tamar. Dates fall into a semi-dry date category with medium size (3-4 cm). Moisture content of dates is about 12% at tamar stage (Figure 10(e)).

9.6. Shishi

Shishi dates are brown coloured, oval shaped, with thick skin and somewhat dry texture. Shishi is a distinct reddish-brown coloured variety, caramel-like flavour and soft and chewy texture (Figure 10(f)).

9.7. Khalas

Khalas is oblong, oval-shaped fruit widely cultivated in Saudi Arabia, Persian Gulf and United Arab Emirates. It covers large area in Oman and is considered as original cultivar in the region. Fruit size of Khalas is 4.4 cm x 3.0 cm. Khalas is expensive date variety with brown skin colour (Figure 10(g)).

9.8. Muzafati

Mazafati is generally grown in Iran Bam, Ji-roft, Kahnuj in Kerman province, Saravan, Nikshahr, Haji Abad. Muzafati dates are soft, fleshy, sweet, medium sized (2.5–4.5 cm). Moisture content of dates at tamar stage is 32%-35%. Harvest time vary with varying location of orchards (Figure 10(h)).

9.9. Zaghlool

Zaghloul dates are basically grown in Egypt and also cultivated in India. Cultivation of date palm in Egypt was carried out 4000 BCE after Pakistan and eastern Arabia. Zaghloul dates cultivated largely in district Kutch, India on the west coast beside southern border of Pakistan. Size of Zaghlool dates is about 7 cm long, with red fruit colour at khalal stage (Figure 10(i)).

9.10. Siwi

Siwi dates are native to the Egyptian Oasis from which this delicious fruit derives its name. The Siwi date is renowned for its creamy texture and golden brown color (Figure 10(j)).

9.11. Deglet Nour

Deglet Nour is a famous date cultivar whose name originates from Arabic *daqlatu (a)n-nūr*, literally, called “date palm of light”, “heavenly date” from oldest Arabic daqal, a type of date palm cultivar originated in Tolga oasis, Algeria (Figure 10(k)).

9.12. Sagai

Sagai date cultivar originated in Arabian Peninsula and also propagated in Saudi Arabia. Sagai dates are famous for their two-toned colors. Tip of dates is golden and dry, while remaining part of fruit is soft and brown (Figure 10(l)).

9.13. Sukkary

Sukkary is a famous Saudi Arabian date cultivar. Fruit skin is light yellow or golden brown with fruit size 4.0 cm long and 3.0 cm in diameter. Sukkary dates are soft and extremely sweet (Figure 10(m)).

9.14. Safawi

Safawi dates are a special variety of dates mainly grown in Saudi Arabia, Al-Madinah region. Safawi dates are a soft, semi-dried date variety characteristically identified by their particular

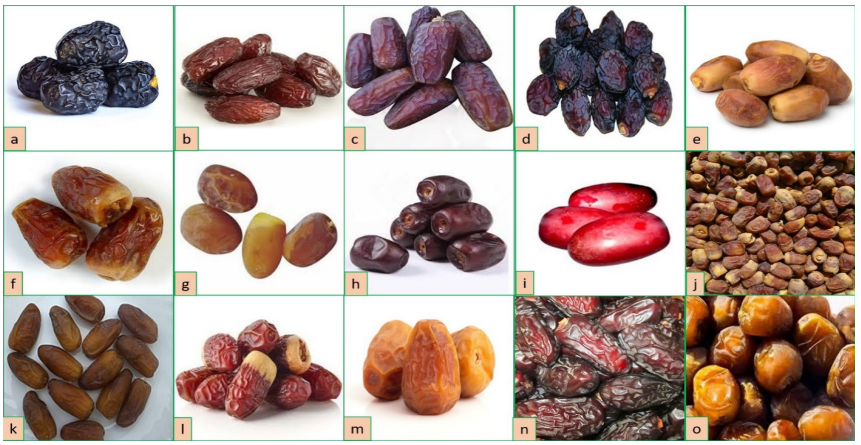

Fig. 10. International commercial varieties of dates: (a) Ajwa, (b) Medjool, (c) Mabroom, (d) Lulu, (e) Zahidi, (f) Shishi (g) Khalas, (h) Muzafati, (i) Zaghlool, (j) Siwi, (k) Deglet Nour, (l) Sagai, (m) Sukkary, (n) Safawi, and (o) Barhi.

dark brown colour at tamar stage, their length, and medium-size (Figure 10(n)).

9.15. Barhi

Barhi dates are a rare variety of dates native to Iraq, and belongs to the soft date type. It is very sweet in taste, crispy, and is consumed mainly at khalal stage. Barhi date palms were introduced from Basra, Iraq in 1913. Meaning of the name Barhi is possibly associated with the hot summer winds "bahr" at Basra. Size of the Barhi dates is 2-4 cm long and 2-3 cm in diameter. Barhi has earned the name in the international markets, where it is sold at high prices. Moisture content of Barhi dates at Khalal stage is about 60% (Figure 10(o)).

10. DISEASES IN DATE PALM (SUDDEN DECLINE DISEASE AND RED PALM WEEVIL)

Date palm is threatened by the sudden decline disease (bayoud like disease) and the Red Palm Weevil [55-59].

10.1. Sudden Decline Disease

Bayoud Arabic "Abiadh" meaning white, refers to the whitening of fronds of infected date palm. Bayoud was reported initially in 1870 in Morocco. Disease had already affected several date palm orchards in 1940, and after a century, disease had infected all Moroccan date palm orchards and western and central Algerian Sahara [60, 61]. In decline disease whole the crown of the mature date palm tree dried within 2-3 months (Figure 10(a-b)) depending on severity of disease in different areas. Decline disease was started few decades before in the area. Maitlo *et al*. [62] identified the pathogen and possible remedy to cure the disease. Identified pathogen was *Fusarium solani* which could be cured with a systemic fungicide Bavistin-DF (Carbendazim). The recommended dose of fungicide to control the disease is 3 g l^{-1}. Fungicide should be applied on the tree base and crown as needed to ensure that the fungicide completely reaches to the infected roots and base of fronds in the crown [62]. Initially, disease symptoms appear on the leaves starting from one side of the frond gradually spread to other side in this way whole the crown of the tree fall down in 2-3 months. Drying of fronds showed similar symptoms of Bayoud disease caused by *Fusarium oxysporum* in Morocco and Algeria [60, 63]. Decline disease destroyed many orchards and dispersed trees in district Khairpur [64]. Infected trees are increasing day by day. In some areas disease severely affected huge number of date palm trees. Recently, sudden decline disease is a big threat to current date palm cultivation generally in Khairpur (Figure 11(a-b)). Additionally, it restricts the extension of new cultivation in the area. In district Khairpur, after heavy rain and flood during the year 2022, it is expected that more than 0.1 million adult date palm trees have been destroyed with decline disease and number of infected trees is increasing day by day. Currently, it is recommended that diseased trees should thoroughly cut down and burned. On the other side un-infected trees should be treated with systemic fungicides as per recommendations of available literature. Previous studies observed

that decline disease is soil borne pathogen which infecting roots first causing blockage of xylem vessels which definitely stop transport of water and nutrients to aerial parts of the tree. Current disease situation is also result of the standing of the rain and flood water in the fields of date palm orchards caused rotting of the roots which is major cause of the spread of the disease in the area during floods in 2022.

10.2. Red Palm Weevil (*Rhynchophorus ferrugineus*).

Red Palm Weevil (RPW) is insect which eat soft tissues inside the trunk [65]. Generally, it enters in the trunk after removal of offshoots around the tree, if the injured side of the trunk are not sprayed with insecticide or not covered with mud [66]. In some cases, RPW enters in the trunk during growth of soft roots around the trunk [67, 69]. Slowly, RPW enter in the trunk and make the permanent big holes if tree is not cured or sometime trees fall down. Treatment with fumigating tablets Phosphotoxin (Aluminum phosphide 55%) is generally carried out. Tablets are applied in the holes, the insects are going through the holes inside the trunk, covered with a piece of plastic and then with mud. The treatments should be repeated until RPW is completely destroyed. Aluminum phosphide reacts with atmospheric moisture to liberate phosphine gas which kills the RPW [15]. International methods to control RPW include plant quarantine (transport of infected date palm should avoided), cultural (avoid cuts and injuries), mechanical (burn immediately all infected tissues), trapping (destroying the Weevil by trapping), biological and chemical (chemical control of RPW is very effective and applicable. RPW complete its life cycle during attack on the tree. Growth stages of RPW include egg (Figure 11(c)), larva (Figure 11(d)) and adult with wings (Figure 11(e)). Generally, insects cause harm to the tree at larva stage (Figure 11(d)), while at adult stage, RPW comes out to the tree trunk and fly to attack other trees and for reproduction. Adult Red Palm Weevil go inside the fibrous sheath covering around the trunk, and lays eggs which grow into feather less larva, and go inside the trunk for food; therefore, the control at initial stage is important to save the tree from further damage to the tree.

11. CONCLUSIONS AND RECOMMENDATIONS

This review study conclude that Pre- and Post-harvest practices are still need to be improved in the area. There is no applicable method to save the trees from monsoon rain and only fruits of early date varieties harvested before onset of monsoon rains can be saved. Different date factories exporting dates to other countries rely on the quality of dates prepared by the farmers which need to be improved according to international standards. Selection of new varieties of date palm through seed propagation is difficult and time consuming. Field evaluation of exotic cultivars showed that soil and climate of district Khairpur are suitable for cultivation of commercially important date varieties. Micropropagation is an alternative way and is an applicable procedure to multiply elite and rare date varieties for the cultivation in the area. Elite cultivars of date palm (Dedhi, Kashuwari, Gulistan, Samany and Barhi) have

Fig. 11. Adult trees of date palm infected with Sudden Decline Disease after heavy rains (a-b). Different growth stages of Red Palm Weevil, (c) egg, (d) larva, and (e) adult.

been successfully multiplied through tissue culture technique which must be extended to commercial level. Currently, date palm in Sindh is suffering from post-flood and rain effects which is big threat to date palm cultivation. It is recommended that fungicides should be applied in the fields which may reduce the disease spread, and the completely dried trees should be cut down and burned. Currently, in Pakistan, is a need for post-harvest management, to cultivate commercial varieties growing in the area and to import offshoots of commercially important cultivars of date palm, which will improve the varietal structure in the area.

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13. CONFLICT OF INTEREST

Authors declare that they have no any conflict of interest.

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Research Article

Hexavalent Chromium Detoxification and Bioremediation by *Bacillus sp.* from Tannery Effluents

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Abstract: This study examined the Cr(VI) absorption mechanism in indigenous Cr(VI)-tolerant bacterial strains. Five potential chromium-resistant bacterial strains were isolated indigenously from tannery effluent later identified as *Bacillus* sp. using phenotypic and genotypic techniques. In nutrient-rich media, Nutrient Agar, the concentration of Cr was analyzed for maximum tolerance (100 - 1500 µg/ml), and found that the strains showed growth even at 1500 µg/ml. Diphenylcarbazide (DPC) assay was performed to analyze the ability of chromium-reducing bacteria to reduce Cr under various conditions, such as pH, temperature, Cr(VI) content, incubation time, and inhibitors such as antibiotics and heavy metals (Ag, Ni, Zn, Mn and Co). In pilot research, *Bacillus licheniformis* (YAK4) and *Bacillus endophyticus* (YAK7), removed up to 95% Cr(VI) from tannery wastewater in 8 days. The obtained microbial-cleansed water was used afterward in a pot experiment to grow *Vigna radiata* and proved to be useful for the growth of plants. Capacitive heavy metal tolerance and Cr(VI) reduction potentials makes *Bacillus licheniformis* and *Bacillus endophyticus* an ideal option for decontaminating a Cr(VI) contaminated environment.

Keywords: Hexavalent Chromium Reduction, *Bacillus sp*., Tannery Effluent, Chromate Bioremediation, Chromate Resistant Bacteria (CRB), *Vigna radiata*.

1. INTRODUCTION

Chromium is a prevalent man-made pollutant found in soil, groundwater, and surface water. It is utilized in diverse industrial processes, such as metallurgy, chemical production, refractories, tanneries, and wood processing, and many more [1]. Common examples of heavy metals are cadmium, lead, chromium, copper, and extremely poisonous nickel which are found in industrial effluents [2]. These anthropogenic chemicals contaminate the soil, groundwater, sediments, and surface waterways also, including ecological and biological systems. Chromium plays an essential role in the growth of many organisms; however, it is poisonous, carcinogenic, and teratogenic at high concentrations [3]. The US EPA has listed chromium as a priority contaminant [4]. The effluents released by industries possessed Cr(III) and Cr(VI) ranges from ten to hundreds of mg/l. While the existence of nine valence states ranging from +2 to +6, hence, two distinct forms of chromium, namely Cr(III) and Cr(VI), have considerable importance [5]. Since the chromate anion shares structural similarities with SO_4^{2-} and is extremely soluble, it may pass the cellular permeability barrier and enter via sulphate transport channels [6]. The hexavalent form of chromium has a relatively high solubility in water and is thus reported to be a cause of lung cancer, kidney damage, ulcer, and many more [5]. The World Health Organization has category one carcinogen status for Cr(VI) (WHO). The drinking water guideline sets the maximum permitted chromium content in drinking water at 50 ug/l [5]. There exists an imminent need to implement rigorous environmental restrictions aimed at restricting the discharge of Cr(VI) into the environment, with the objective of limiting the

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adverse impacts of Cr(VI) on human health. [7]. Advanced treatment technologies like as reverse osmosis, ion exchange, membrane filtration, electrocoagulation, and electrodialysis have demonstrated efficacy in the removal of Cr(VI). However, they are costly and generate wastes that must be processed and disposed of later. [8, 9]. Hence, bioremediation is developing as a potential approach for removing Cr(VI) from industrial effluents. Several types of fungi and bacteria have been found to remove chromium [10]. Biological remediation techniques use bacteria that can tolerate Cr(VI) and provide cost-effective and environment friendly ways to remove Cr(VI) from contaminated areas [11]. Numerous researchers have investigated the capability of chromium-reducing bacteria to convert Cr(VI) into less hazardous forms. Microorganisms can tolerate the metals in their particular environment. These systems operate inside and outside of cells, lowering the amount of bioavailable metal present in the cell's surroundings [12]. Another protective method involves the expression of exporter proteins, which serve to regulate the metal concentration within the cell to a tolerable level. [13]. The enzymes synthesized by bacteria are diverse kinds and produced by different methods. For instance, intracellular or external membrane-bound reductases are metabolized by bacteria during the direct remediation process [14]. However, oxidants and reductants are generated by indirect mechanisms [15].

Serious health concerns have been raised by chromium emissions that are anthropogenic. According to Lauwerys *et al*. [16], Cr(VI) infusion in the human food chain can cause a number of physiological dysfunctions in humans, including irritability, respiratory infections, and certain allergies. Industrial effluents containing Cr(VI) infiltrate into the soil through the water bodies. As a result, it eventually makes its way into animals' food chains [16]. According to the United States Environmental Protection Agency, Cr(VI) is one of 17 substances that are dangerous to human health, and its allowable range in drinking water has been set at 0.05 mg/l [17]. Bacteria that are capable of tolerating Cr(VI) are utilized in biological remediation techniques and offer cost-effective and environmentally benign solutions for Cr(VI) detoxification from contaminated sites [18]. Thus, the objectives of this study were to (i) separate and identify new Cr(VI)-reducing bacteria from Cr(VI)-contaminated water, (ii) analyze the chromium reduction performance of bacteria, (iii) analyze the ability of bacterial species to degrade hexavalent chromium in the presence of inhibitory salts, heavy metals, and antibiotics, and (iv) determine the hexavalent chromium reduction in industrial wastewater.

2. MATERIALS AND METHODS

2.1. Isolation of Bacterial Strains and Culture Conditions

Five bacterial strains were isolated from the chromium-contaminated tannery effluent and identified based on their morphological characteristics, biochemical tests, physiological, and 16S RNA sequencing. These strains were analyzed for physicochemical properties and were streaked on nutrient agar medium (0.5 g Beef extract, 2.5 g Peptone, 2.5g NaCl, 1 g Yeast extract, and 7.5 g Agar into 500 ml distilled water). They were further checked for their minimum inhibitory concentration (MIC) at different concentrations of potassium dichromate ($K_2Cr_2O_7$) ranging from 100 µg/ml to 1500 µg/ml. All strains were incubated at 37 ℃ for 24 hrs. (pH 7). The results were noted, and strains were stored for a week at 4 ℃ for further testing.

2.2. Physiological Characterization

2.2.1. Temperature influence on bacterial growth

The optimal growth temperature for the isolated strains was identified by subjecting them to varying temperature conditions, i.e., 28 °C, 37 °C, and 42 ℃ and incubating them for 24 hrs. The growth characteristics of the strains were examined under both chromate (Cr(VI)) stress conditions alongside standard conditions (without stress), and the optical density was measured at a wavelength of 540 nm. Optical density (OD) indicates the amount of bacteria present in a suspension. The density of bacteria in colony-forming units (CFU) is measured through a spectrophotometer.

2.2.2. pH influence on bacterial growth

Five flasks of nutrient broth for this experiment were prepared and maintained at five different pH levels: 3, 5, 7, 9, and 11. Afterward, 50 µl of each

strain was added to tubes and incubated for 24 hrs. at 37 ℃. To check the bacterial growth, optical density (OD) was measured at 540 nm.

2.2.3. Metals influence on bacterial growth

Various metal resistance profiles, including Ag ($AgNO_3$), Zn ($ZnSO_4$), Mn ($MnSO_4$), Ni ($NiSO_4$), and Co ($CoCl_2$) were determined. For this objective, stock solutions of various metals were prepared by inoculating 1g of metal into 10 ml of distilled water. The nutrient broth was individually poured into tubes. For testing the resistivity of strains, two different metal concentrations: 100 µl/10 ml of broth and 400 µl/10 ml of broth were employed. The findings were evaluated after 24 and 48 hrs. at a temperature of 37 °C.

2.3. Chromium ($K_2Cr_2O_7$) Reduction Experiments

2.3.1. Chromium content determination

All the chromium reduction experiments were carried out in chromium-reducing broth (Tryptone 10 g, Yeast extract 5 g, NaCl 5 g, Citric acid 1 g, and Na_2HPO_4 6.9 g) and under aerobic conditions [19]. Hence, to measure the chromium reduction potential, the medium was supplemented with $K_2Cr_2O_7$ at a 200 µg/ml concentration. An individual test tube was filled with the bacterial culture that was prepared in nutrient broth beforehand. Each tube has 50 µl of bacterial culture, 10 ml of medium, and Cr(VI) as the stated concentration. Subsequently, the tubes were individually subjected to incubation for a duration of four days at a temperature of 37 °C. One milliliter of the bacterial culture was obtained after 24, 48, and 72 hrs. and centrifuged for five minutes at 12000 rpm, centrifugation was employed to get the bacterial pellet. Diphenylcarbazide (DPC) spectrophotometric technique was used to track Cr(VI) reduction [20]. In this experimental procedure, a volume of 100 µl of the supernatant was transferred from the Eppendorf tube to a test tube containing 10 ml of distilled water. The test tube was then filled with 1 ml of 0.5% of Diphenylcarbazide (0.5 g of 1,5-diphenylcarbazide, 100 ml of acetone) and a few drops of H_2SO_4. The tube was allowed to remain at room temperature for the next 15 to 20 minutes or until the color becomes purple [21]. At a wavelength of 540 nanometers, the optical density in the solution was measured. Based on the results obtained from the calibration curve, $K_2Cr_2O_7$ reduction was carried out. The percentage of chromium reduction was determined by calculating the final Cr(VI) concentration found in the medium using a standard curve that includes both the treatment and the control [20].

$$Cr(VI) reduction\ (\%) = \frac{C^i - C^f}{C^i} \times 100$$

where,
C^i = Initial $K_2Cr_2O_7$ concentration
C^f = Final $K_2Cr_2O_7$ concentration

2.3.2. Chromium reduction and effect of pH

In test tubes, chromium reduction media was prepared. We used five test tubes for each strain, with pH values of 3, 5, 7, 9, and 11. We added a 250 µg/ml supplement to the medium before administering the strains. The tubes were then kept in an incubator at 37 °C. At 540 nm, the optical density was measured after 24 and 48 hours, respectively, and a graph was drawn on the calibration curve to determine the amount of chromium present.

2.3.3. Chromium reduction and effect of temperature

In test tubes containing chromium reduction broth and 250 µg/ml $K_2Cr_2O_7$, we performed the experiment at three different temperatures, 28 °C, 37 °C, and 48 °C. In light of the findings of the earlier tests, the pH was settled (at optimum pH). The strains were added into the tubes and incubated for 24 and 48 hours. Following that, the optical density at 540 nm was measured to estimate the chromium concentration, and a graph was drawn in accordance with it.

2.3.4. Synergistic effect of pH and temperature on chromate reduction

The impact of Cr(VI) addition was examined in this study, coupled with the synergistic effect of varying temperature and pH for each strain. For this, chromate reducing broth was prepared by maintaining various pH levels (3, 5, 7, 9, and 11). The strains were incubated at different temperatures of 30 ℃, 37 ℃, and 48 ℃. $K_2Cr_2O_7$ was added to the medium at two distinct concentrations: 200 µg/ml and 400 µg/ ml for 24 and 48 hours, respectively.

A negative control was also placed alongside the other strains. The DPC test was then performed for a large number of samples in a microtiter plate. To bring the volume of the solution to 200 µl, 160 µl of water, 4 µl of bacterial inoculum, 20 µl of DPC, and about 2 µl of H_2SO_4 were added. Microtiter plate was allowed to rest for 15 - 20 minutes. At 540 nm, the optical density was measured. This experiment allowed us to determine the hexavalent chromium reduction by bacterial strains at optimum pH as well as temperature and analyze the effect synergistically.

2.4. Antibiotic Disk Diffusion Assay

Heavy metal resistance genes and antibiotic resistance genes are frequently found together on plasmids and can be utilized for further genetic manipulation studies of the existing resistance characteristic traits [22]. This experiment provides information about the presence of antibiotic resistance traits in bacterial strains. Muller Hilton agar was prepared, and 4 antibiotics (Erythromycin (15 µg), Neomycin (30 µg), Tetracycline (10 µg), and Ampicillin (5 µg) of above stated concentrations were taken and administered for each strain. After the 18-24 hours' incubation, a measuring scale was used to measure the inhibition zones for each antibiotic disc and mentioned in mm [23]. Afterward, the noted zones were compared with CLSI, 2020.

2.5. Determination of Chromium Reduction in Tannery Effluent

For this experiment, 250 ml of nutrient broth was inoculated with 50 µl of each strain; the OD was taken and then incubated for 7 days. After measuring the optical density of the broth, we added 50 ml of the broth from each flask into 500 ml of tannery water. We let it incubate for 10 days on the shaker at room temperature. The control was also placed alongside and the samples were extracted at regular intervals, specifically at 2, 4, 6, 8, and 10 days of incubation to monitor the chromium reduction by measuring the optical density. After 10 days of incubation, the final readings were taken and found considerable chromium reduction with the help of the Diphenylcarbazide method [24].

2.6. Microbial Treated Wastewater and Pot Experiment

Microbial-treated wastewater was used to cultivate plants. For this, we prepared small pots, and mung beans (*Vigna radiata*) were sown in each pot. Tap water was used for the control pot, while treated wastewater (5 ml) was given to the experimental plants. Seeds were allowed to grow for 7 days to determine the effect of chromium metal on plant growth. The seed germination and other changes were observed.

3. RESULTS

3.1. Bacterial Strains and Culture Conditions

Strains were extracted from sewage water at a tannery, and after morphological analysis and gram staining, we found that they were all gram-positive rods and positive for spore stain (Table 1). All strains showed positive growth till 1500 µg/ml of $K_2Cr_2O_7$. The strains were characterized and identified by 16S rRNA sequencing. Blast was performed on obtained sequence for each strain and accession number was assigned to them accordingly. These strains were identified as: YAK-1 *Bacillus foraminis*, YAK-2 *Bacillus thuringiensis*, YAK-4 *Bacillus licheniformis*, YAK-7 and YAM *Bacillus endophyticus*.

3.2. Comparative Analysis of pH and Temperature of Bacterial Isolates

To analyze the bacterial growth at varying pH, temperature, and time, the media was inoculated

Table 1. Biochemical analysis of the strains.

Isolates	Oxidase test	Starch hydrolysis	Indole test	VP	MR	Urease
YAK 1	+	+	-	-	+	-
YAK 2	-	+	-	+	-	-
YAK 4	+	+	-	+	+	-
YAK 7	-	-	-	-	+	-
YAM	+	-	-	-	+	-

with fresh cultures of the isolates. Five tubes from each set were incubated at three different temperatures: 28 °C, 37 °C, and 48 °C. At 37°C, all strains exhibited good growth, with YAK4 and YAM showing exponential growth, achieving OD values of 1.4 and 1.9, respectively (Figure 1A). Regarding different pH levels (3, 5, 7, 9, and 11), all strains demonstrated steady growth at pH 7. However, YAK4 performed particularly well at pH 5 and 9, with OD values of 1.2 and 1.09, respectively. In contrast, YAK1 and YAK2 showed optimal growth at pH 9, with OD values of 1.02 and 1.08 (Figure 1B). After 72 hours of incubation, all strains exhibited significant growth, with YAK7 and YAM displaying the best growth, with OD values of 2.0 and 1.76. In conclusion, the optimal conditions for growth were found to be 37°C and pH 7, with the highest growth observed after 72 hours (Figure 1C).

3.3. Heavy Metals Resistance Profile

In addition to chromium, five other heavy metals were tested for and employed for isolated strains. To test the strains' tolerance to metals, the strains were exposed to two distinct metal concentrations: 100 µg/ml and 400 µg/ml. The findings after 24 and 48 hours are shown in Figure 2. Optical density for each strain was observed at 540 nm. The results at a concentration of 100 µg/ml showed that YAK4 performed well against $ZnSO_4$, while YAK7 performed well against $MnSO_4$ after 24 hours, with optical densities of 0.519 for YAK4 and 0.521 for YAK7. YAK4 showed good performance against $ZnSO_4$, the OD was 0.491 after the time period of 48 hours, similarly, YAK2 showed significant growth against $MnSO_4$ with OD 0.495 as compared to its performance at 24 hours (Figure 2A). Comparatively, at 400 µg/ml conc. the observed value of OD was 0.294 at which YAK2 worked well under the influence of $AgNO_3$ and OD was 0.371 for YAK7 at which it exhibited effective growth against $MnSO_4$ after 24 hours of incubation. However, only YAK7 demonstrated good growth against $ZnSO_4$ after the incubation of 48 hours (Figure 2B).

3.4. Chromium ($K_2Cr_2O_7$) Reduction Experiments with Bacterial Strains

As the chromium content of the medium is decreased by the bacterial strains, a DPC test was performed to determine its concentration. While

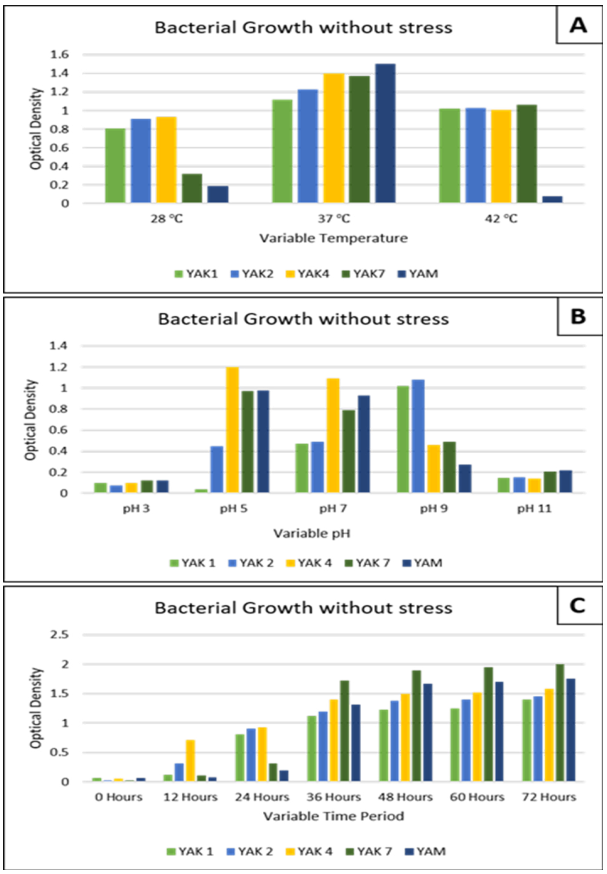

Fig. 1. Bacterial growth at variable temperatures (A), variable pH (B), and at different time intervals of 0 to 72 hrs (C).

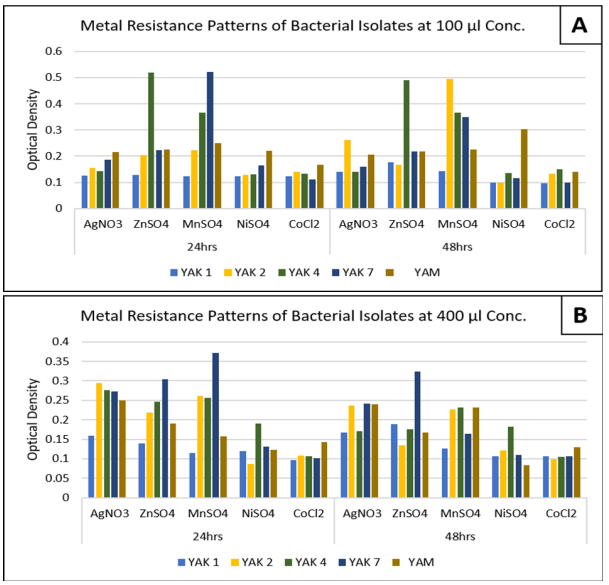

Fig. 2. Bacterial growth analysis and determining the heavy metal tolerance with 100 µg/ml of metal (A) and 400 µg/ml of metal at the time interval of 24 hours and 48 hours (B).

the concentration of $K_2Cr_2O_7$ was kept at 200 µg/ml with incubation durations of 24 and 48 hours, respectively, to check for reduction (Figure 3). All the bacterial strains performed well in reduction experiment, however, YAK7 and YAM reduced chromium concentration significantly well while the temperature was kept 37 ℃ at pH 7.

3.5. Analyzing pH, Temperature, and Time in Comparison with $K_2Cr_2O_7$

$K_2Cr_2O_7$ was added as stress and examined the growth of bacteria at various pH levels, temperatures, and time intervals. Two amounts of Cr(VI) to the media—200 µg/ml and 500 µg/ml were employed. Chromate-reducing medium was used in test tubes and varied the values of 3, 5, 7, 9, and 11 for pH. The tubes were incubated at varying temperatures of 28 °C, 37 °C, and 48 °C. We observed bacterial growth after 24 and 48 hours. At 540 nm, the optical density 0.74 was determined and found that YAM exhibited optimal performance at 28 °C, while at OD 0.685, YAK7 performed best at 48 °C, with chromate concentration set at 200 µg/ml for a 24-hour incubation period (Figure 4A). At a concentration of 500 µg/ml, at 37 ℃ all strains showed nominal growth over a period of 24 hours

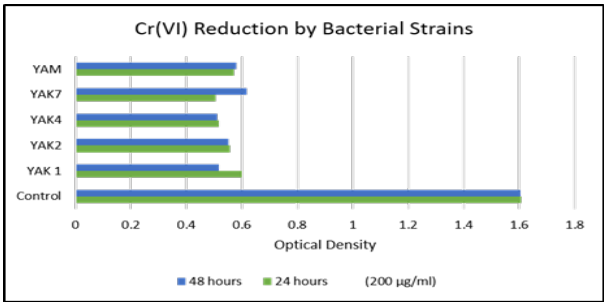

Fig. 3. Reduction of Cr(VI) at 200 µg/ml after 24 hours and 48 hours.

while YAK4 with OD value 0.744 demonstrated exponential growth at 28 °C after 48 hours (Figure 4B). The comparison of bacterial growth at variable pH revealed that at 24 hours YAK1 performed well at pH 5 while YAM showed exponential growth at pH 9 with a concentration of 200 µg/ml and optical density was 0.08 and 0.096 after 48 hours (Figure 5A). In comparison, the OD was 0.285 at which YAK7 demonstrated optimal performance at pH 9 when exposed to a concentration of 500 µg/ml after 48 hours of incubation (Figure 5B).

3.6. Chromium Content Determination

The concentration of $K_2Cr_2O_7$ in the media was determined by Diphenylcarbazide Assay (DPC). As the bacterial strains reduced the chromium content, a DPC assay was employed to assess rest of the chromium present in the media. The concentrations of chromium were 200 µg/ml and 500 µg/ml. The temperature differences (30 °C, 37 °C, and 48 °C) and pH levels (3, 5, 7,9, and 11) were used by providing 24 and 48-hour incubation periods, respectively. The outcomes revealed that with OD value 0.948 YAK1 exhibited the best reduction at pH 5 and 37 °C, with the measured reduction potential being 51%. For the strains YAK2 and YAM, the optical density observed was 0.953 and 0.94 at which the reduction was 54% and 60%, respectively (Figure 6).

On the other hand, the ideal pH was found to be pH 9 at 30 °C after 48 hours. With OD 1.749 YAK7 at pH 9 performed well while the temp was 30 °C, and the reduction potential was 78% (Figure 7A). However, at 37 °C, both YAK7 and YAM exhibited good reduction showed OD value 2.30 and 2.28 at pH 7, with potentials of 77% and 76%,

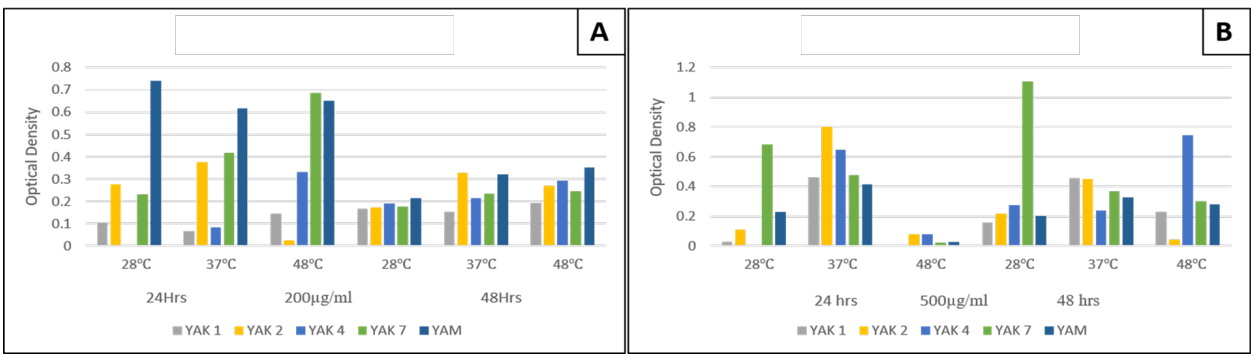

Fig. 4. Comparison of bacterial growth at various temperatures in the presence of different Cr(VI) conc. 200 µg/ml (A) 500 µg/ml (B).

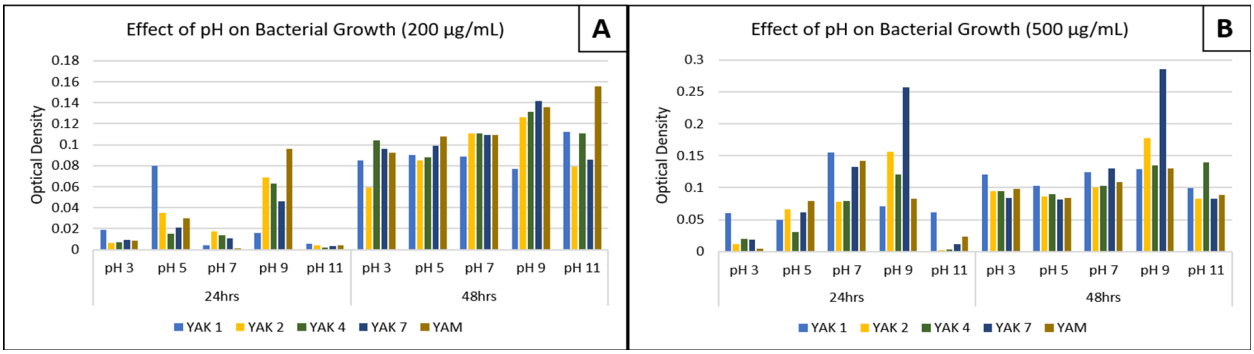

Fig. 5. Comparison of bacterial growth at different pH in the presence of variable Cr(VI) concentrations, 200 µg/ml (A) 500 µg/ml (B).

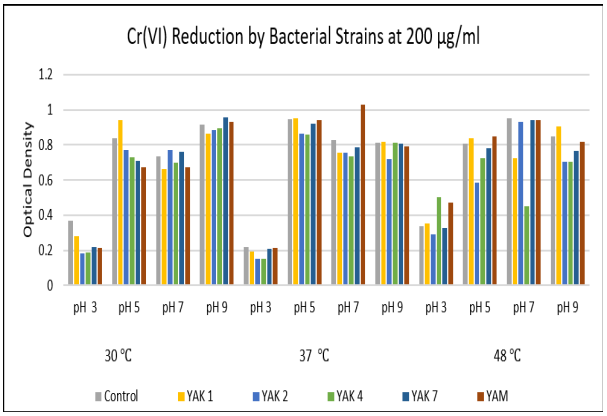

Fig. 6. Cr(VI) Reduction by Bacterial Strains at 200 µg/ml under variable pH at 30 °C, 37 °C, and 48 °C over a 24-hour time period.

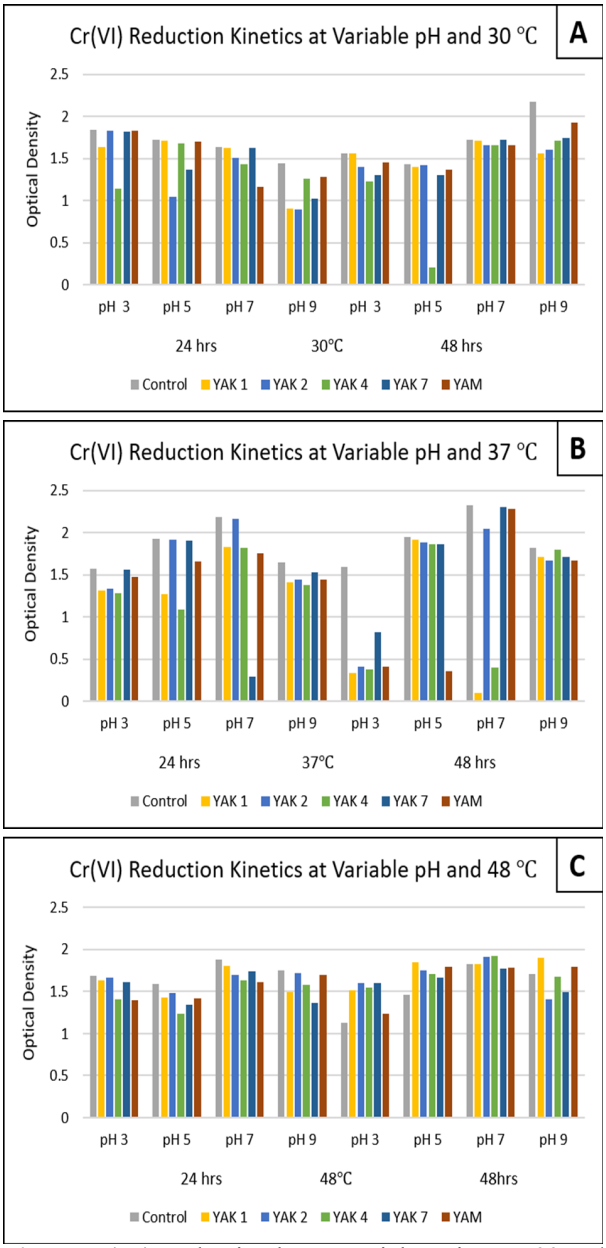

Fig. 7. Cr(VI) Reduction by Bacterial Strains at 500 µg/ml under variable pH at 30 °C (A), 37 °C (B), and 48 °C (C) with 24- and 48-hour time intervals.

respectively (Figure 7B). However, YAK4 with OD value 1.923 performed great reduction while the pH was 7 at 48 °C, and the reduction potential was 70% (Figure 7C).

3.7. Antibiotic Susceptibility Testing

Four antibiotic discs that were deemed to be more efficient against *Bacillus* species were taken. Muller Hilton agar it was used. Each plate comprised four discs (Figure 8). The zone of inhibitions was seen after the incubation period of 20 to 24 hours. After measurement, it was interpreted that all the strains were sensitive towards neomycin and tetracycline, however, resistance towards ampicillin (Table 2).

3.8. Bioremediation Experiment of Hexavalent Chromium

The efficiency of chromium-reducing strains was determined by supplementing the bacterial inoculated broth into the tannery wastewater. After

Fig. 8. Antibiotic disk diffusion testing.

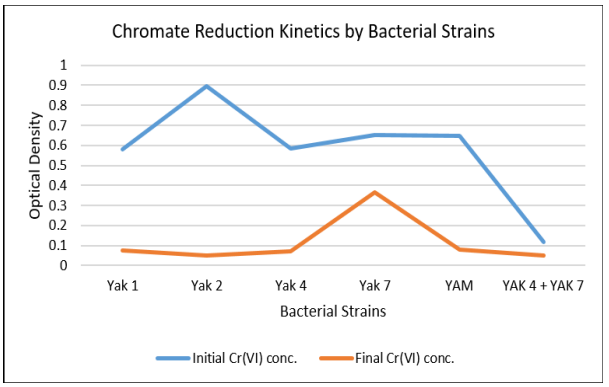

Fig. 9. Chromate reduction by chromium-reducing strains before incubation and 10 days after room-temperature incubation.

Table 2. Antibiotic testing.

Antibiotics	Strains				
	YAK 1	YAK 2	YAK 4	YAK 7	YAM
Erythromycin	R	I	S	I	I
Neomycin	S	S	S	S	S
Tetracycline	S	S	S	S	S
Ampicillin	R	R	R	R	R

Key: R = resistant, I = intermediate, S = sensitive

the incubation of 10 days, the chromium reduction was measured by performing a Diphenylcarbazide assay, and a graph was plotted based on the results (Figure 9). YAK4, YAK7, and YAM reduced chromate up to 87%, 67%, and 88% respectively.

3.9. Effect of Treated Wastewater on *Vigna Radiata*

The germination of *Vigna radiata* seeds was tested by using microbial-treated wastewater. Considerable growth of *Vigna radiata* was seen as compared to untreated wastewater. It was observed that after bacterial treatment, the toxicity of the original wastewater significantly decreased along with a decrease in the noxious harmful Cr(VI). After two days of exposure to the microbial-treated water, the germination of the young seedlings was successfully observed (Figure 10A). The transition to the seedling stage (post-germination) occurred after six days of continued watering with the treated solution. To assess the effects of the microbial treatment on seed growth, a control group was maintained, where the mung bean seeds were watered with standard tap water. This allowed for a comparative analysis of seedling development under different conditions (Figure 10B).

4. DISCUSSION

In this era of industrialization, the persistence and inability to degrade heavy metals, bioremediation is a major study topic worldwide [25]. In addition to other chemical agents, some heavy metals also contribute to contemporary concerns. Some metals, such as chromium, copper, cadmium, lead, selenium, and nickel, are natural poison reservoirs and negatively impact biological and ecological ecosystems [26].

The major industrial areas in Punjab are Kasur and Faisalabad, which are home to a number of significant enterprises that have helped the state's economy but have also aggressively polluted the region's otherwise beautiful environment. Due to the inability of heavy metals produced in large quantities by industries to degrade, their discharge into the biosphere has both an immediate and a cumulative impact [27, 28]. These heavy metals are byproducts of several industrial operations, such as leather tanning, wood preservation, and pulp manufacturing. They are discharged into the environment in the form of effluents, and their concentrations vary [25]. Due to their limited solubility in biota and persistent nature as a pollutant, heavy metals pose a significant threat to both human and environmental health. Several of the above-stated heavy metals are categorized as carcinogens and mutagens [29].

Chromium is one of the hazardous heavy

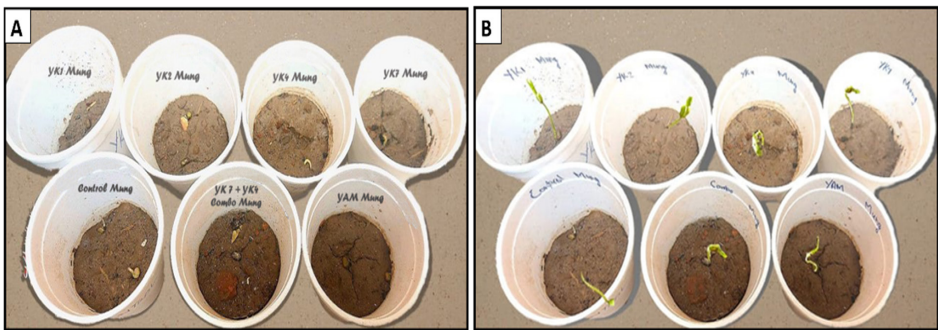

Fig. 10. *Vigna radiata* seed germination after 2 days (A), and *Vigna radiata* growth after 6 days of giving microbial-treated wastewater (B).

metals typically present in industrial effluent. It is also regarded as a necessary micronutrient for the growth of plants and many other species. Yet, when present in larger concentrations, chromium serves as a carcinogen and teratogen [30]. The chromium content in industrial effluents varies depending on the concentration utilized, ranging from 10th to 100th of mg/l in the form of Cr(III) or Cr(VI). Additionally, due to their stability, Cr(III) and Cr(VI)are more significant in the environment [31].

Cr(VI) is one of the primary causes of lung cancer, renal damage, chromate ulcer, and perforation of the nasal septum, according to earlier findings [32]. In addition, it is hazardous to species such as plants and animals that eat water contaminated with chromium. In contrast, the trivalent form of chromium is less harmful and is used by several species as a micronutrient. It is 100 times less hazardous than Cr(VI) in nature and also insoluble in water [33].

A total of 10 isolates that exhibited resistance to chromium were obtained from industrial sites in Faisalabad that had been chronically polluted with chromate. Among these isolates, 5 strains belonging to the *Bacillus* genus were selected for subsequent analysis. Several researchers have already identified and employed *Bacillus* isolates that exhibit resistance to chromate [27, 34-36]. Initial studies indicate that the strains were most likely *Bacillus sp*., since all the bacterial isolates were gram-positive rods capable of producing spores and exhibiting small, spherical, mucoid, irregularly shaped colonies. In a previous study various *Bacillus* species have shown strong chromium tolerance up to > 2500 mg/ml [37].

The bacterial strains were screened for their optimal pH and temperature by giving varying $K_2Cr_2O_7$ concentration, and we discovered that pH 9 was the best pH for growth at 30 ℃ at 200 µg/ml. After 24 hours of incubation at 48 °C, we observed that pH 7 was effective, while pH 5 was the ideal pH at 37 °C. Tan *et al*. [38] discovered that the pH in the range 6 and 9 was ideal for *Bacillus* species. These results are inline in the present study; however, a slight deviation from the results might be brought on by environmental circumstances. The ideal pH was found to be pH 9 at both temperatures, i.e., 30 °C and 37 °C, when we examined the findings at the concentration of 500 µg/ml. It supports the previous findings that the ideal pH 7 was determined at 48 °C [38, 39].

Due to the abundance of naturally occurring heavy metals in the biosphere, bacteria have resisted them [40]. In a study by Faisal *et al*. [26] explained how some bacterial strains might reduce heavy metals, including Cu, Ag, Se, Co, Cr, Zn, Mn, and Pb. We also assessed the growth potential of our isolated isolates by testing them against various heavy metals and discovered that the strains were effective against $AgNO_3$, $MnSO_4$, and $ZnSO_4$ at 100 µg/ml and 400 µg/ml concentrations. When the growth was assessed after 48 hours, 72 hours, and 168 hours, it was observed that the two strains, YAK4 and YAK7, performed exceptionally well at both concentrations. The current study's findings are consistent with those of [41, 42]. Chromium-resistant strains can also withstand additional heavy metals [33, 43].

Since wastewater lacks the proper nutrients for microbial growth and contains toxic compounds, it works as a particularly unfavorable environment for the growth of non-native bacteria. In earlier

research, there has been a lot of attention given to the evaluation of chromate reduction in lysogeny broth (LB) broth media [34, 44]; however, few studies have performed the bio reduction trials in actual industrial effluents. In this study original tannery effluent was used to perform the bioremediation studies at pilot scale level and examine the strain's ability in nutrient medium. The previously identified bacterial strains exhibit the ability to effectively remove Cr(VI) ions from the initial tannery wastewater. These isolates are therefore suitable to use in bioremediation experiments under real-world conditions. The chromate bio reduction potential of *Staphylococcus capitis* and *Bacillus sp*. JDM-2-1 was also investigated by Zahoor and Rehman [45] in the industrial wastewater. They discovered that both species had the ability to reduce Cr(VI) to Cr(III) by 81% and 85%, respectively. In chromium reduction media, the ability of the isolates was evaluated. After running a DPC test, it was determined that YAK1 exhibited the best reduction at pH 5 and 37 °C, with a reduction potential of 51%. At 30 °C, the ideal pH for the strains YAK2 and YAM was pH 9, with a decrease of 54% and 56%, respectively. For YAK4, pH 7 performed well at 48 °C, and the reduction potential was 76%; for YAK7, pH 9 performed well at 30 °C, and the reduction potential was 78%.

5. CONCLUSIONS

Based on the overall findings, it was determined that the strain YAK4 was identified as *Bacillus licheniformis*, whereas both YAK7 and YAM were identified as *Bacillus endophyticus*. These strains outperformed all others and were able to remove chromium by 75% to 80% when the temperature is 30 °C to 37 °C and pH between 7 and 9. Enhanced culture conditions can improve the reduction potential. We also observed that YAK4 and YAK7 performed well during the pilot scale study (*Vigna radiate* pot experiment). Hence, it was concluded that these strains have a high potential to reduce carcinogenic hexavalent chromium and may be employed in bioremediation applications because of their improved stability, high reduction potential, and ease of reusing.

6. ACKNOWLEDGMENT

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7. CONFLICT OF INTEREST

There are no conflicts of interest declared by the authors.

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Research Article

Evaluating the Bacterial Contamination in Used Cosmetic Products: A Potential Threat to Consumer's Health

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Abstract: Cosmetic products tend to be prone to microbial contamination as they contain growth factors, essential minerals, organic and inorganic compounds which provide favourable conditions for microbial growth. The aim of the present study is to evaluate the bacterial contamination in used cosmetic products at Hyderabad. A total of 22 samples of used cosmetic products belonging to Mascaras and Eyeliners were collected from beauty salons and homes, which were either used by a single person or used in sharing with other family members. The isolation and identification of contaminating bacteria was carried out on the basis of cultural, morphological, and biochemical characteristics. Out of 22 cosmetic samples 72.73% (n = 16) demonstrated bacterial growth, while 27.27% (n = 6) samples had no bacterial growth. A total of n = 41 bacterial isolates were recovered from used cosmetic samples. The majority of bacteria belonged to Gram-positive 82.93% (n = 34) while 7.32% (n = 03) were Gram-negative bacteria. Moreover, 9.76% (n = 04) samples showed the growth of mixed cultures. Bacteriological profiling revealed that *Bacillus* spp. were dominant with 63.41% (n = 26), followed by *Micrococcus* spp. 7.32% (n = 03), Coagulase-negative Staphylococci 7.32% (n = 03), *Staphylococcus aureus* 4.88% (n = 02), while *Proteus mirabilis* 2.44% (n = 01), *Citrobacter* spp. 2.44% (n = 01), *E. coli* 2.44% (n = 01). Our results have shown that, the shared cosmetics whether used at homes or in salons were more prone to bacterial contamination than non-shared home-based single users, suggesting that sharing increases their susceptibility to bacterial contamination, which can spread bacteria and cause skin infections.

Keywords: Bacterial Contamination, Cosmetics, Eye Liners, Mascara, Salon.

1. INTRODUCTION

Cosmetic products are being used worldwide because physical appearance is really vital for us these days [1]. A few active ingredients, stabilizers, additives, mineral pigments, colors, and fragrances are found in cosmetic items. Certain compound in cosmetics have the potential to negatively impact human health and aggravate long-term medical conditions [2]. It has been reported that these chemicals have the potential to enter the body through the skin, food, or inhaling [3]. In general, cosmetics fall into three categories: make-up (lipstick, eye makeup, etc.), rinse-off (shower gel, shampoo, toothpaste, liquid soap, etc.), and leave-on (facial cream, hand cream, antiperspirant, sunscreen, etc.) [4]. Conversely, there are three types for eye cosmetics: Eyeshadow, mascara, and eyeliner, these are applied to the eyes to accentuate their attractiveness [5]. Mascara has higher than allowable levels of bacteria than other goods. Furthermore, prolonged use of these items might constitute a health risk to humans, hence it has been suggested that the quality of these products be regulated and the consumers be warned to exercise caution while using inexpensive products [6]. The majority of females acquire eye infections each year from using mascara and eyeliner, which can result in temporary or permanent blindness [6]. The pigmented eye makeup items are called eyeshadows [7]. Furthermore, applying makeup on the eyelid can contaminate the container of the cosmetic product and increase the risk of eye infection and allergic response (redness and irritation) [8].

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After in-use contamination, microbial growth is supported by the high proportion of water content in cosmetics [9]. Long-term cosmetic product usage habits have an impact on human health as well as the state of the skin and hair. Because their usage habits have a major influence on the other effects of the cosmetic product as well as the incidence or non-occurrence of adverse reactions [10]. Cosmetics are susceptible to skin microbiota upon application, and there is also a possibility of minor contamination during production. Previously published literature has demonstrated the presence of many bacterial isolates including *S. aureus, S. epidermidis, Salmonella* spp., and *E. coli* in makeup items such as face powders, mascaras, and eyeliners. Additionally, a correlation between *S. aureus* and ailments including impetigo and conjunctivitis has been demonstrated [11]. When using personal care and cosmetic products, consumers should select items that won't be harmful to their health and aim to form good habits [12]. Cosmetic items provide an ideal environment for the growth of harmful bacteria in beauty salons. This might pose a risk to the health of the customer. Health risks can vary according to the services provided, the tools, makeup, and applicators utilized. Additionally, sharing equipment and cosmetics can lead to skin and eye infections, particularly in salons where sharing is prevalent [13]. The present study aimed to explore the bacterial contamination in the used cosmetic products from salon and home based usage at Hyderabad, Sindh. Two main categories of cosmetic products mascaras and eyeliners were included for bacteriological analysis.

2. MATERIALS AND METHODS

2.1. Collection of Used Cosmetic Products

The present study was carried out at Hyderabad city, Pakistan, to assess the bacterial contamination in used cosmetic products. A total of 22 used cosmetic product samples of different brands were collected from the different beauty salons, homes and used testers of these products displayed at vendors shops. The cosmetic products belonged to two main categories, which were further subdivided into three sub-categories based on the usage patterns of consumers such as individually used or used in sharing with others (Table 1). Each sample of the used cosmetic product was collected from consumers at salon or homes. The surfaces of all samples were cleansed with an aqueous mixture of 70% ethanol (v/v) prior to transportation to the research laboratory at the Institute of Microbiology, University of Sindh, Jamshoro in a sealed bag. All samples were then processed for bacteriological analysis according to FDA's Bacteriological Analytical Manual: Microbiological Methods for Cosmetics [14].

2.2. Processing of Used Cosmetic Samples

The cosmetic samples were processed at room temperature and neither incubated nor frozen before or after analysis, unless otherwise stated. All samples were processed aseptically in a laminar flow safety cabinet and labelled properly with the appropriate code according to the category and subcategory as mentioned elsewhere. All samples were initially examined for the physical characteristics including consistency (normal/thick/dried), smell (pungent/normal), wether date of expiry present/not present, and the name of manufacturer. For culturing the samples, different diagnostic media were used that included MacConkey's Agar, Blood Agar, Nutrient Agar and Mannitol Salt Agar.

2.3. Enrichment of Cosmetic Samples

With the help of sterile swabs, appropriate amounts (1 gm) of each of the samples of mascara and

Table 1. Category and subcategory-wise samples of cosmetic products.

Categories of samples	Sub-categories				
	Home-based (Single user / Non-shared) (*n* =)	Home-based (Multiple user/ Shared) (*n* =)	Salon-based (Multiple user/ Shared) (*n* =)	Total (*n* =)	Grand Total (*n* =)
Mascaras	4	5	5	14	22
Eyeliners	2	3	3	8	

eyeliners were transferred to sterilized and labeled test tubes containing 5 ml sterile nutrient broth. The broth tubes inoculated with cosmetic samples were then incubated at 37 °C for 24 hours. The next day, all tubes were observed for bacterial growth (turbidity). Samples that showed no turbidity/ visible growth were further incubated at 37 °C for 48 to 72 hours before labelling them as non-contaminated products.

2.4. Isolation and Identification of Bacteria from Used Cosmetic Products

2.4.1. Spread plate method for isolation of pure culture of bacteria

All cosmetic samples yielding positive growth by showing the turbidity in enrichment broth culture were serially diluted by preparing up to 10^{-3} dilutions [15]. Different volumes such as 50 µl, 100 µl and 150 µl inoculum were spread in duplicate on nutrient agar and Mannitol Salt Agar (MSA) plates using sterilized glass spreader for obtaining discrete bacterial colonies. After absorption of the inoculum, plates were incubated at 37 °C for 24 to 48 hours. On the following day, morphologically distinct and isolated colonies were selected and streaked on fresh nutrient agar plates, MSA and MacConkey's Agar plates to obtain pure cultures of each of the distinct colonies [16].

2.4.2. Morphological and biochemical characterization of isolates

Bacterial identification was carried out using conventional microbiological methods including, morphological characteristics by Gram staining and microscopic analysis. Cultivation on different diagnostic and selective media, and biochemical characterization using catalase test, coagulase test, patterns of hemolysis on blood agar, Citrate utilization test, TSI, and Sulfide Indole Motility (SIM) medium (Oxoid, UK) were also performed, according to Chessbrough [17].

3. RESULTS

3.1. Bacteriological Analysis of Cosmetic Products

Bacteriological analysis of the used cosmetic products demonstrated that out of 22 samples 72.73% (n = 16) were positive for bacterial growth thus considered as contaminated, while 27.27% (n = 06) samples were negative for bacterial growth. Overall, n = 41 bacterial isolates were isolated from the used cosmetic samples. The majority of bacterial isolates belonged to Gram-positive 82.93% (n = 34), whereas 7.32% (n = 03) showed the growth of Gram-negative bacteria, and 9.76% (n = 04) yeilded growth of mixed culture.

3.2. Distribution of Bacterial Isolates of Mascara and Eyeliner Cosmetic Samples

Overall distribution of isolated bacterial strains demonstrated that among Gram-positive bacteria *Bacillus* spp. 63.41% (n = 26), were highly prevalent followed by *Micrococcus* spp. 7.32% (n = 03), CoNS 7.32% (n = 03) and *S. aureus* 4.88% (n = 02), whereas Gram-negative rods accounted 7.32% (n = 03), which included *E. coli* (n = 01), *Proteus mirabilis* (n = 01), and *Citrobacter* spp. (n = 01). The remaining 9.76% (n = 04) samples showed the growth of mixed cultures (Figure 1).

3.3. Analysis of Hemolytic Activity in Bacteria Isolated from Cosmetic Samples

In order to determine hemolysis potential, all bacterial isolates recovered from used cosmetic products were cultured on blood agar to assess their potential for hemolysis of RBCs (Figure 2). As shown in Table 2, the majority of isolates (53.66%) were β-hemolytic, while 26.83% were α-hemolytic, and 19.51% of the bacterial isolates displayed

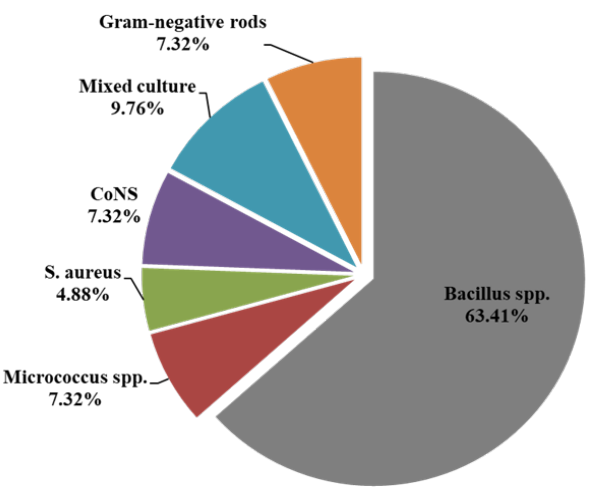

Fig. 1. Pie chart showing the overall distribution of bacteria isolated from used cosmetic products.

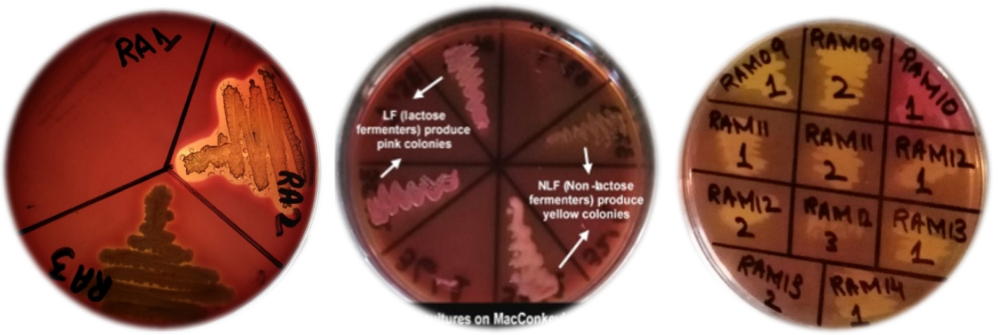

Fig. 2. Pure cultures and hemolytic analysis of bacterial isolates of cosmetic products.

Table 2. Analysis of haemolytic activity of isolated bacteria.

Bacteria	α- hemolytic % (n=)	β- hemolytic % (*n*=)	γ- hemolytic % (n=)
Bacillus spp.	24.39 (10)	36.59 (15)	2.44 (01)
Micrococcus spp.	0 (0)	0 (0)	7.32 (03)
S. aureus	0 (0)	4.88 (02)	0 (0)
CoNS	0 (0)	0 (0)	7.32 (03)
Mixed culture	0 (0)	7.32 (03)	2.44 (01)
Gram-negative rods	2.44 (01)	4.88 (02)	0 (0)
Total	26.83 (11)	53.66 (22)	19.51 (08)

γ-hemolysis. The majority of *Bacillus* spp. (36.59%) were β-hemolytic, whereas all *S. aureus* displayed β-hemolysis on blood agar plates. Among the mixed culture (7.32%) were β-hemolytic, and 2.44% were γ- hemolytic in nature. The hemolytic profiling of Gram-negative bacteria demonstrated that (4.88%) were β-hemolytic and 2.44% were α-hemolytic. On ther otherhand both α-hemolytic and β-hemolytic activity was absent in CoNS and *Micrococcus* spp., thus were considered as γ- hemolytic bacteria.

3.4. Sub-category wise Bacterial Load Analysis of Eyeliner Samples

Eight Eyeliner samples demonstrated the growth of only Gram-positive bacteria as no Gram-negative bacteria was recovered out of *n* = 13 positive samples. Based on usage patterns of eye liner samples, data demonstrate that eyeliner samples 37.5% collected from beauty salons were found contaminated, whereas 12.5% of the home-based multiple user samples yielded bacterial growth and 25% failed to show any bacterial growth. Moreover, all home-based single user (non-shared) samples (25%) were negative for bacterial growth (Table 3). Two types of bacteria belonging to *Bacillus* spp. (*n* = 11) and *S. aureus* (*n* = 02) were recovered from eyeliner cosmetic products.

3.5. Sub-category wise Bacterial Load Analysis of Mascara Samples

Out of 14 mascara samples, 85.71% (*n* = 12) were found contaminated and 14.29% (*n* = 2) were negative for bacterial growth. Based on usage patterns, the samples were categorized as single user

Table 3. Sub-category-wise distribution and bacterial contamination in eyeliner samples.

Sub-categories	Total sample % (n=)	Contaminated samples % (n=)	Non-contaminated samples % (n=)
Salon Products (shared)	37.5 (03)	37.5 (03)	0 (0)
Homebased single user (non-shared)	25 (02)	0 (0)	25 (02)
Homebased multiple user (Shared)	37.5 (03)	12.5 (01)	25 (02)
Total	**100 (08)**	**50 (04)**	**50 (04)**

and shared at either home or beauty salons. Among the beauty salons and home-based multiple users samples 35.71% (*n* = 5/5) and 14.29% (*n* = 2/4) were found contaminated, respectively; whereas 14.29% (*n* = 2/4) of the home-based signle usage samples did not yield any bacterial growth (Table 4). The mascara sample's bacterial load analysis demonstrated contamination of seven different types of bacteria, including *Bacillus* spp. (*n* = 15), followed by *Micrococcus* spp. (*n* = 03) and CoNS (*n* = 03). While the Gram-negative rods were the least prevalent bacteria in Mascara samples with one isolate of each of the *E. coli*, *Proteus mirabilis.* and *Citrobacter* spp. and mixed culture growth (*n* = 04).

4. DISCUSSION

The present study reports the bacterial contamination in different used cosmetic products, that were further sub-divided based on the usage patterns by the consumers. Although, a number of research studies have been carried out on the consequences of consumer behavior, use, and inadequate preservation from different countries, there are a few studies on bacterial contamination of used cosmetic products published from Pakistan, particularily from Sindh province [18, 19]. Despite the fact that the cosmetic products are prepared under strictly controlled conditions, which inhibit the growth and proliferation of microorganism during usage and increase their shelf life, depending upon the effectiveness of the preservatives [20]. The current study has therefore evaluated the bacterial contamination in used cosmetic products at Hyderabad, Sindh. The data of current study showed that the makeup items yeilded bacterial growth in the used products, indicating contamination from the customers during the usage of the product. Our finding are supported by published studies that have reported the contamination in used cosmetic produtcs [21, 22]. The current study has demonstrated that majority of cosmetic samples were contaminated with either one or more than one bacterial isolates. Gram-positive bacteria were highly prevalent in used cosmetic products as compared to Gram-negative bacteria. Gram-positive isolates included *S. aureus*, CoNS, *Bacillus* spp. and *Micrococcus* spp., while Gram-negative bacteria consisted *Proteus mirabilis*, *Citrobacter* spp*.,* and *E. coli*. The major concern is the presence of bacterial pathogens including *E. coli*, *Proteus mirabilis, Citrobacter* spp., and *S. aureus* in used cosmetics products [21].

Used eye cosmetic samples of mascara demonstrated immense bacterial diversity yielding both Gram-positive and Gram-negative bacteria. The presence of pathogenic bacteria such as *E. coli*, *Citrobacter* spp., and *Proteus* spp. in the mascara raises concern about the safety and health issues of the consumers. Our results are in agreement with previous studies demonstrating presence of *Salmonella, Citrobactor and Klebsiella* spp. in cosmetic products [22]. High bacterial diversity in mascara samples may possibly be attributed to the aqueous formulation of mascara and its high potential of interacting with bacteria originating from the eyelashes surfaces and environmental exposure [23, 24]. Moreover, eye cosmetics, specifically mascaras have been demonstrated to be associated with ocular infections in consumers using contaminated products [25, 26]. Presence of potentially pathogenic bacteria may pose serious threat of infections among consumers as these products are applied on skin near to eyes and mouth area [21]. Moreover, using testers at beauty counters or stores, sharing makeup at home, and applying cosmetics in salons may also expose the customers to contamination and skin infections. It has been noted that the testers are not routinely cleaned and are also exposed to the outside air as

Table 4. Sub-category-wise bacterial load analysis of mascara samples.

Sub-categories	Total sample % (n=)	Contaminated samples % (n=)	Non-contaminated samples % (n=)
Salon Products (shared)	35.71 (05)	35.71 (05)	0 (0)
Homebased single user (non-shared)	28.57 (04)	14.29 (02)	14.29 (02)
Homebased multiple user (Shared)	35.71 (05)	35.71 (05)	0 (0)
Total	**100% (14)**	**85.71 (12)**	**14.29 (02)**

well as the consumers may touch and test them, these shared cosmetics in salons and homes are often highly contaminated [22].

Previously published studies have demonstrated the presence of *Bacillus* spp., *Staphylococcus* spp., and *E. coli* in the cosmetic samples. *Staphylococcus* spp. have been shown to cause skin infections including acne. Although *Bacillus* spp. are known to be the transient skin microflora, some of its species have been reported to cause necrotizing cellulitis of skin and severe eye infections [27]. *Staphylococcus* spp. are commensal organisms found on the skin; however, published studies have reported the clinical significance of *S. aureus* in conjunctivitis [28]. In the current study, Staphylococcal spp. were recovered from both mascara and eyeliner cosmetic products. Our results are in agreement with previously published studies in which they have shown the presence of *Staphylococcus* spp. in cosmetic products especially mascara, eyeshadow and lip-gloss [29, 30].

It has been reported that most of the cosmetic products expire within 3-12 months [21], suggesting that except the consumers having medical issues, the cosmetic product would not be the source of infection until their expiry date [21]. However, the frequent contamination of used cosmetic products may result due to using them beyond the expiry date or not stored properly under hygienic conditions. Moreover, when such expired cosmetic products containing the preservatives are applied on the skin, they may alter the skin microflora [31]. The skin microflora of every individual tend to be diverse and in some cases may be harmful for another individual, thus, sharing the same cosmetic product between many people poses a high risk of contamination and spread of skin infections. In conclusion, the high prevalence of bacterial contamination in used cosmetic products pose serious threats to consumers health especially when applied near eyes and mouth area [32] and warrants the need of conducting a comprehensive investigation to ensure better user compliance to mitigate the risk of infectious disease in consumers.

5. CONCLUSIONS

The present study investigated the bacterial contamination of used cosmetic products being used by individuals and in sharing at homes and salons. High level of bacterial contamination has been observed in shared cosmetic products as compared to those used by single consumers. Contamination of pathogenic bacteria in the used cosmetic products may pose serious threat of infections as well as other serious issues to consumer's health.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Antimicrobial Finish for Cotton/polyester from Natural Bio-extracts

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Abstract: This study develops and applies sustainable antimicrobial finishes derived from *Azadirachta indica* (Neem), *Butea monosperma,* and *Litchi chinensis* leaf extracts to 50/50 cotton/polyester blend fabrics. The antimicrobial efficacy of these finishes was evaluated, revealing a 100% reduction in microbial growth after 22 hours and six days. Before and after applying antimicrobial finish FTIR, SEM and fabric aesthetic properties were checked. The antimicrobial finish was applied by the pad dry cure method and finish was fixed by using of polyurethane binder. In case of aesthetic properties (stiffness, smoothness appearance) antimicrobial finish had positive effect on 50/50 cotton/polyester. The treated fabrics also exhibited significant increases in stiffness ($p < 0.001$, $\eta^2 = 0.85$). Additionally, the smoothness appearance of treated fabrics was assessed, revealing a slight decrease in smoothness appearance ratings as compared to untreated controls group, although this decrease was not statistically significant ($p = 0.29$). Fourier Transform Infrared (FTIR) spectroscopy revealed changes in functional groups, indicating successful finish application. Scanning Electron Microscopy (SEM) micrographs displayed surface modifications and filament breakage. The results were analyzed through ANOVA. The fabric properties were checked by using AATCC standard test methods. These eco-friendly finishes offer promising alternatives to synthetic antimicrobials, enhancing textile sustainability and consumer safety. The findings of this study contribute to the development of sustainable and environmentally friendly textile finishes.

Keywords: Antimicrobial, Natural Extracts, Aesthetic Properties *A. indica*, *L. chinensis*, *B. monosperma*, Smoothness and Surface Appearance.

1. INTRODUCTION

Regular polymers with a plant-based (vegetable) origin have a position with materials. People have been using it for hundreds of years with the sole purpose of insuring (bodies) and covering (temperature, dust, daylight, wind, and so on). Additionally, clothing has played a vital role in human existence and has become essential with the advancement of trend-setting technologies. Among specialised materials, clinical materials are a very promising field that is directly related to human prosperity and well-being. It includes resources used by wards, paramedical staff, specialists, attendants, and pre- and post-employable tasks [1, 2]. There are bacterial or other harmful germs everywhere in our surroundings that can harm or benefit humans. The epidermis, nasal passages, stomach, and other areas of the human body are home to a variety of bacterial species [3, 4]. Antimicrobial additions to materials can protect the human body from harmful germs. Microorganisms are too small for the human unaided eye to see. Microbes, growths, diseases, and green growth are all considered microorganisms [5]. Neem or *Azadirachta indica*, is widely distributed over the Indian subcontinent and belongs to the *Meliaceae* (Mahogony) family. It is one of the plants with the most elaborate naturally occurring mixes. Because of its antibacterial and healing qualities, neem has been used in traditional Indian medicine for hundreds of years. Neem has been demonstrated to be less toxic to warm-blooded animals, such as humans. Neem subordinates have been used in

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toothpastes, cosmetics, toiletries, and home-grown medications due to the plant's exceptional qualities [6, 7]. *B. monosperma* leaves have shown useful in treating diabetes, glycosuria, and skin conditions. It has demonstrated impressive results in eliminating tapeworms and roundworms. The antibacterial activity of *B. monosperma* has been investigated by El-Shafei *et al*. [8]. *Litchi chinensis* (Lychee) is an evergreen, soapberry tree within the *Sapindaceae* family. The results of the litchi organic product, for example, blossoms, pericarp, and seeds have shown anti-oxidative properties [9-11].

The textile industry's reliance on synthetic antimicrobial agent's poses environmental and health risks. Developing sustainable, eco-friendly alternatives that maintain fabric aesthetic properties is crucial. This study aims to create effective, plant-based antimicrobial finishes balancing efficacy, aesthetics, and environmental sustainability. Existing literature highlights the potential of plant-based antimicrobial agents as eco-friendly alternatives to synthetic chemicals. However, limited studies have investigated the application of these agents on cotton/polyester blend fabrics. By investigating the effects of *A. indica, B. monosperma,* and *L. chinensis* extracts on fabric properties, this research contributes to the development of sustainable textile finishes. The primary objective of this study is to develop and evaluate sustainable antimicrobial finishes derived from natural plant extracts, specifically *Azadirachta indica* (Neem), *Blepharis monosperma,* and *Litchi chinensis*, for application on 50/50 cotton/polyester blend fabrics. This research aims to address the growing concern of antimicrobial resistance and environmental pollution caused by synthetic antimicrobial agents in the textile industry.

2. MATERIALS AND METHODS

In this study antimicrobial finish was extracted from three plants leaves, i.e., *A. indica, B. monosperma* and *L. chinensis* and applied on 50/50 cotton/polyester, 50% concentration solution was used. The fabric samples were cut, treated with antimicrobial finish and then tested to govern their effectiveness as antimicrobial fabrics. Antimicrobial agents were extracted from leaves of *A. indica*, *B. monosperma* and *L. chinensis*. Extractions of antimicrobial agents from plants were carried out in laboratory of Botany Department, Government College University, Lahore. Antimicrobial finish was applied in National Textile University (NTU) Faisalabad. Binder was used to improve the durability of finish. Antimicrobial testing was carried out in Centre of Excellence in Molecular Biology (CEMB). To check the presence of antimicrobial finish on fabrics pre-test post-test, FTIR test was conducted at the Institute of Chemistry, University of the Punjab, SEM test was conducted in The Centre for Solid State Physics, University of the Punjab, Lahore. Fabric properties were rechecked after applying antimicrobial finish whether it affected the fabric properties or not. This part of experiment was conducted in National Textile University, Faisalabad.

2.1. Sample Preparation

Samples of fabrics 50/50 cotton/polyester were taken from fabric trader of Faisalabad. Its quality was authenticated by the study co-supervisor at NTU Faisalabad. Sample size was five yards which depended upon the checking fabric properties and tests. Unfinished fabric was taken and these were bleached at NTU. Untreated fabric was taken as control group and the presence of microorganisms was checked in CEMB. The fabric consists on plain weave with 217 GSM.

2.2. Fabric Preparation

After purchasing the fabric 50/50 cotton/polyester was first de-sized. In de-sizing enzyme Bactasal HTN was used in ratio of 1 g/l. The pH was 5-6 and temperature was 60-70 °C. The cotton/polyester fabric was dipped in solution for 45 minutes. After de-sizing, scouring was done by using NaOH 4 g/l and wetting agent 2 g/l, detergent was used in the ratio of 1 g/l at 90 °C, temperature was maintained during the process. The cotton/polyester fabric was treated for 1 hour. In bleaching of cotton/polyester took H_2O_2 5 g/l, NaOH (pH 10-10.5) 2 g/l, stabilizer 2 g/l and requesting agent 2 g/l. The temperature of the process was 90 °C. The fabric was treated in this solution for one hour.

2.3. Development of Finish

The leaves of *A. indica* (Neem), *B. monosperma* and *L. chinensis* were collected from the botanical garden of Government College University, Lahore. The plant leaves of *A. indica*, *B. monosperma* and

L. chinensis were identified and authenticated. The leaves were washed and then dried for two months under shadow. These were grinded by using a stainless-steel grinder into very fine powder. This powder was stored in air tight, high density poly ethylene vessels before extraction. The weights of dry powder of leaves *A. indica* (Neem), *B. monosperma* and *L. chinensis* were 500 g each. The ratio of grinded leaves and distilled water was 100 g/250 ml. This process was repeated for *B. monosperma* and *L. chinensis*. This soaked material was left for 7 days and stirred it twice a day. After that it was filtered by using muslin cloth then filtered again by using Whatman filter paper. The filtered extracts of *A. indica (*Neem), *B. monosperma* and *L. chinensis* were concentrated by a rotary film evaporator.

2.4. Application of Finish

200 milliliters of *A. indica* (neem) leaf extract, 50 milliliters of polyurethane binder, and 150 milliliters of distilled water were combined to create the diluted concentration of finish. For *B. monosperma* and *L. chinensis,* the same ratio was applied. The pad dry cure machine at NTU was used to apply the antimicrobial finish. The pad dry cure machine (method) was used for two minutes of drying and three minutes of curing at 120 °C and 150 °C, respectively.

2.5. Washing

To confirm that the fabric samples had been cleaned, eco-friendly laundry was performed at home using AATCC test method 135-2003. The following tools were used:

- A washing machine that operates automatically.
- A tumble dryer that operates automatically.
- Features for line and drip drying, the 1993 AATCC standard reference detergent was use.

2.6. Antimicrobial Test

The bacteria under investigation were those discovered during the experiment. Microbes, both Gram-positive and Gram-negative, were examined. These bacteria were studied in CEMB under normal conditions. For textile testing, the usual conditions are 20 ± 2 °C and 65 ± 2% relative humidity.

2.7. Aesthetic Properties

2.7.1. Stiffness

For the stiffness test, the Shirley stiffness tester was used. A fabric strip was cut that was a 6 × 1-inch sample on Shirley stiffness tester underneath the template. Both were slowly pushed forward. The fabric strip was dropped over the edge of the stiffness tester platform by the movement of the template. The sample was continued until the tip of sample as saw in mirror cut both index lines. The bending length noted from the scale. Each sample was tested four times at each end and reading was taken by strip turned over. Mean value both warp and weft were calculated.

2.7.2. Smoothness appearance

The 1993 AATCC Standard Reference technique was applied for the surface appearance test using an automated washing machine. The three-dimensional replicas depicted in Figure 1 were compared to the treated samples. The surface appearance as an aesthetic property was checked by using AATCC Technical Manual /2004 TM 124-2001 203 test method. The surface appearance was measured by evaluating the smoothness appearance of surface of plane fabric sample. Three samples were cut from fabric 15 inches square. Surface appearance was checked after five home laundries. After machine washing the fabric samples were evaluated by using standard replica in standard. The fabric samples were observed by three trained observers by using standard replicas and rate independently. In room all lights turned off, the overhead bright light was the only way of light source. The trained observer

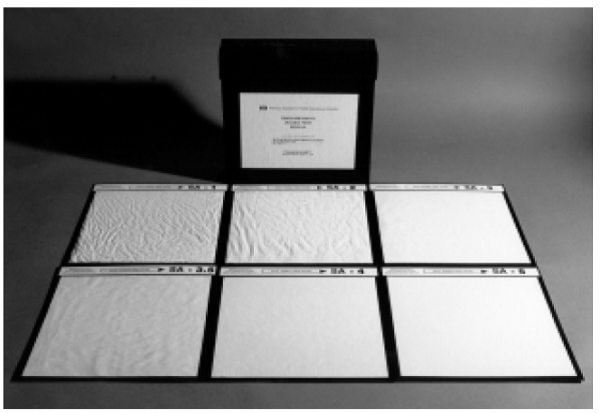

Fig. 1. AATCC 3-D smoothness appearance replicas.

was standing four feet away from the board on which testing samples and standard replicas were mounted on each side of testing sample to enable comparative evaluation. Then assign the number which present on most nearly match the finished appearance. The detail of smoothness appearance replicas is as under.

- SA-4 Replica. Smooth, finished appearance.
- SA-3 Replica. Mussed, no pressed appearance.
- SA-2 Replica. Rumpled, obviously wrinkled appearance.
- SA-1 Replica. Crumpled, creased and severely wrinkled appearance.

2.8. Sustainability in Home Laundry

Cut the sample from fabric in standard testing atmosphere. Samples were placed on the flat surface. Automatic washing machine did laundry by the following steps as washing, rinsing, and drying. In washing, automatic washing machine weights the fabric samples. According to sample size water level was selected. The temperature for washing and rinsing was less than 29 °C. Add 1993 AATCC standard reference detergent by the ratio of 1 g/l. Add fabric samples to washing machine, set the washer cycle and time. After that rinsed and dried the samples then line dry the samples. In line dry, hung each sample in vertical direction by clipping it two corners. The fabric samples were dried at room temperature, below 26 °C.

2.9. Scanning Electron Microscopy (SEM)

Sample Preparation: Treated and untreated fabric samples were cut into 1 cm × 1 cm pieces and mounted on aluminium stubs using double-sided adhesive tape.

- Coating: Samples were sputter-coated with a thin layer of gold (10 nm) to enhance conductivity and prevent charging.
- Microscopy: A scanning electron microscope (SEM) (Model: JEOL JSM-6610LV) was used to examine fabric surfaces.
- Operating Conditions: Accelerating voltage: 15 kV; working distance: 10 mm; magnification: 500X and 1000X.
- Image Analysis: SEM images were analysed using Image software to assess surface modifications and filament breakage.

2.10. Fourier Transform Infrared (FTIR)

Sample Preparation: Treated and untreated fabric samples were cut into small pieces and mixed with potassium bromide (KBr) powder.

- Pellet Formation: The mixture was pressed into a transparent pellet using a hydraulic press.
- Spectroscopy: A Fourier Transform Infrared (FTIR) spectrometer (Model: Shimadzu IRTracer-100) was used to analyse fabric chemical composition.
- Operating Conditions: Scan range: 4000-400 cm^{-1}; resolution: 4 cm^{-1}; number of scans: 32.
- Spectral Analysis: FTIR spectra were analyzed using Shimadzu IR Solution software to identify functional groups and peak shifts.

3. RESULTS

3.1. Effect on Aesthetic Property

The results of aesthetic properties (stiffness, smoothness appearance) on treated and untreated cotton/polyester fabric with *A. indica, B. monosperma* and *L. chinensis* are presented in Table 1. Table 1 presents the results of Pillai's test, which indicates a significant difference ($p = 0.000$) in the effect of antimicrobial finishes on the stiffness (warp, weft) and smoothness appearance of cotton/polyester fabric. The effect size was large ($\eta^2 = 0.995$). To further investigate the differences, ANOVA was applied to compare the effects of *A. indica*, *B. monosperma*, *L. chinensis*, and the control group on stiffness (warp, weft) and smoothness appearance. The F-test results show a significant difference ($p = 0.001$) in the effect of antimicrobial finishes on stiffness (warp) of cotton/polyester fabric, with a large effect size ($\eta^2 = 0.85$).

Table 1. Effect of antimicrobial finish on stiffness and smoothness appearance of cotton/polyester fabric.

	Plants		
	F	**P**	**η^2**
Multivariate	72.32	0.000	0.995
Univariate			
Stiffness Wrap (cm)	151.19	0.001	0.85
Stiffness weft (cm)	2.4	0.143	0.47
Stiffness warp+ weft (cm)	9.941	0.001	0.651
Smoothness Appearance	1.50	0.29	0.36

However, no significant differences were found in the effects of antimicrobial finishes on stiffness (weft) and smoothness appearance. Additionally, the F-test results indicate a significant difference in the effect of antimicrobial finishes on combined stiffness (warp + weft) of cotton/polyester fabric, with a large effect size ($\eta^2 = 0.651$).

Table 2 shows that *A. indica and B. monosperma* and *L. chinensis* plant leaves extracts antimicrobial finish have effect on stiffness warp of cotton/polyester fabric as compared to control group. One-way ANOVA showed that the difference in antimicrobial finish between control group (M = 3.84, SD = 0.31), the first experimental group *A. indica* (M = 4.65, SD = 0.43), second experimental group *B. monosperma* (M = 4.54, SD = 0.32) and third experimental group *L. chinensis* (M = 4.38, SD = 0.20) were statistically significant (F = 151.19, p = 0.001, $\eta^2 = 0.85$). Results revealed that control group scored significantly lower than the experimental groups. However, the three experimental groups' *A. indica*, *B. monosperma* and *L. chinensis* antimicrobial finish significantly affects the stiffness warp. The significant difference between control group and the first, second and third (*A. indica*, *B. monosperma*, *L. chinensis*) experimental group is also evident from the big difference in the mean values and remarkable difference in standard deviation (control = 0.31, *A. indica* = 0.43, *B. monosperma* = 0.32, and *L. chinensis* = 0.20). When we compare the stiffness (warp) of cotton/polyester fabric treated with antimicrobial finishes from different plant extracts (*A. indica*, *B. monosperma*, and *L. chinensis*) to the control group. The results show that all treated groups have significantly higher stiffness (warp) values than the control group. Table 2 shows that *A. indica*, *B. monosperma* and *L. chinensis* plant leaves extracts antimicrobial finish have effect on stiffness (warp + weft) of cotton/polyester fabric as compared to control group. One-way ANOVA showed that the difference in antimicrobial finish between control group (M = 3.54, SD = 0.24), the first experimental group *A. indica* (M = 4.00, SD = 0.37), second experimental group *B. monosperma* (M = 4.01, SD = 0.27) and third experimental group *L. chinensis* (M = 3.97, SD = 0.35) were statistically significant (F = 9.94.19, p = 0.001, $\eta^2 = 0.651$). Results revealed that control group scored significantly lower than the experimental groups. However, the three experimental groups' *A. indica*, *B. monosperma* and *L. chinensis* antimicrobial finish significantly affects the stiffness warp. The significant difference between control group and the first, second and third (*A. indica*, *B. monosperma*, *L. chinensis*) experimental group is also evident from the big difference in the mean values and remarkable difference in standard deviation (control = 0.24, *A. indica* = 0.37, *B. monosperma* = 0.27, *L. chinensis* = 0.35). The reason was that antimicrobial finish made a coating on treated fabric which increases the stiffness of antimicrobial treated fabric as compare to untreated fabric. So, this finish significantly affects the aesthetic properties (stiffness, smoothness appearance) of cotton/polyester fabric.

The results of microorganisms' reduction on treated and untreated cotton/polyester fabric with *A. indica, B. monosperma* and *L. chinensis* are presented in Table 3. It is observed that after 22 hours there were no microorganisms' growth shown while after six days microorganisms growth has shown on untreated fabric. The treated fabrics show 100% reduction on all samples after 22 hours, after six days there was no microorganisms shown treated fabric while five microorganisms shown on untreated cotton/polyester sample.

The cotton-polyester blend fabric IR spectrum given in Figure 2 shows that a broad peak at 1730

Table 2. Effect of antimicrobial finish on stiffness of cotton/polyester fabric.

	Control Group		***A. indica***		***B. monosperma***		***L. chinensis***	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Stiffness warp (cm)	3.84	0.31	4.65	0.43	4.54	0.32	4.38	0.20
Stiffness weft (cm)	3.24	0.18	3.28	0.33	3.48	0.23	3.56	0.53
Stiffness warp + weft (cm)	3.54	0.24	4.00	0.37	4.01	0.27	3.97	0.35
Smoothness Appearance (SA Replica)	3.50	0.50	3.00	0.00	3.00	0.50	3.00	0.00

Table 3. Quantitative analysis test results of treated and untreated cotton/polyester sample.

	Untreated	*A. indica*	*B. monosperma*	*L. chinensis*	Reduction %
Readings after 22 hours					
1st reading	0	0	0	0	100%
2nd reading	0	0	0	0	100%
3rd reading	0	0	0	0	100%
Readings after 6 days					
1st reading	2	0	0	0	100%
2nd reading	1	0	0	0	100%
3rd reading	2	0	0	0	100%

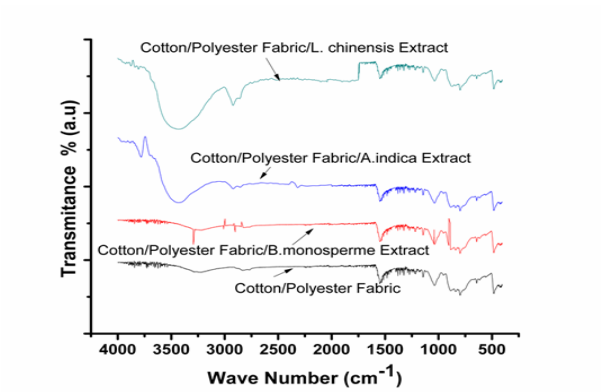

Fig. 2. FTIR Spectra of untreated vs treated cotton/polyester blend fabrics.

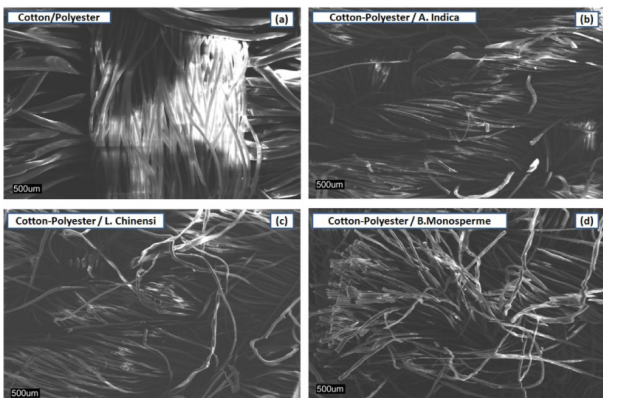

Fig. 3. SEM micrographs of untreated and treated cotton-polyester fabric.

cm^{-1} is characteristic of carbonyl stretching of unsaturated ester. The width of the peak reduced and the peak value has been shifted to higher wave number, that is, 1750 cm^{-1}. A small peak in the region between 800 and 850 cm^{-1} can be accounted for out-of-plane bending of aromatic ring system. The peak at 1250 cm^{-1} and 1300 cm^{-1} may be due to C-O stretching of the polymer back bone. An intense peak at 2350–2360 cm^{-1} can be attributed to methylene C–H stretching [14]. The small peak close to 3000 cm^{-1} can be correlated to C-H stretching of aromatic ring. An interesting feature in the above-discussed spectrum was that an additional sharp small peak observed at around 3600 cm^{-1} corresponds to free -OH groups of cellulose components indicating that solvent treatment had increased the extent of amorphous region in the cotton component of the material. The observed small peaks between the regions 1110–1150 cm^{-1} were due to cellulosic component of the fiber materials.

Figure 3 portrayed the outcome of treatment of extract on cotton/polyester fabric. Figure 3(a) is the SEM image of untreated cotton/polyester fabric. The fabric appears to have a smooth and compact structure with tightly packed fibers. The fibers are predominantly linear and parallel, suggesting minimal surface modification or additional treatment. This is likely the control sample, showing the baseline morphology of untreated Cotton/Polyester. Figure 3(b) shows the results of fabric that was treated with *A. indica*, these results indicate that the surface of the fabric shows increased roughness compared to the control. Fibers seem less aligned and have a scattered appearance, suggesting that *Azadirachta indica* (Neem) extract or treatment caused some disruption in the fiber arrangement. This change might indicate that the extract influenced the fabric's surface, potentially due to deposition or chemical interactions. Figure 3(c) shows SEM results of fabric treated with *L. chinensis* these results show that the morphology is marked by the presence of loosely aligned fibers with visible branching and irregular patterns. *Litchi chinensis* treatment seems to have further altered the fiber structure, potentially increasing porosity or roughness. This could indicate that the treatment affected the fiber bonds or introduced new surface

features, enhancing texture. Figure 3(d) shows that when fabric was treated with *B. monosperma* the fibers display the most disrupted and entangled appearance among the samples. The *Butea monosperma* treatment likely caused significant modifications, leading to a highly irregular surface structure. The increased roughness and random alignment suggest a stronger interaction between the treatment and the fabric, which may impact properties like absorption, texture, or durability. The treated cotton/polyester fabric shows presence of finish as compare to untreated fabric. The untreated cotton/polyester fabric exhibits a smooth surface, whereas the treated samples display increasing roughness. Moreover, the fiber arrangement in the untreated sample is characterized by parallel and compact fibers, whereas the treated samples show progressively more disordered and entangled structures. Notably, each treatment, namely *Azadirachta*, *Litchi*, and *Butea*, has distinct effects on the fabric, with *Butea monosperma* causing the most significant disruption. These structural changes can have a profound impact on the physical, chemical, and mechanical properties of the fabric, including breathability, durability, hydrophilicity, and antimicrobial activity, depending on the purpose of the treatments.

4. DISCUSSION

In this study the antimicrobial finish derived from plant extracts impacts fabric aesthetic properties due to physical and chemical changes. The deposition of extract particles onto the fabric surface alters its texture and smoothness, while interactions between extract compounds and fiber molecules affect stiffness and flexibility. Fourier Transform Infrared (FTIR) spectroscopy reveals corresponding material structure changes, including modifications to functional groups and hydrogen bonding. Specifically, shifts in carbonyl stretching (1730 cm^{-1}) indicate interactions between extract compounds and fiber ester groups, influencing stiffness, and changes in hydroxyl stretching (3600 cm^{-1}) suggest hydrogen bonding between extract compounds and fiber hydroxyl groups, affecting smoothness. These interactions and structural changes correlate with observed alterations in fabric aesthetic properties. Previous results show that F test indicate the significance difference of antimicrobial finish on stiffness property of cotton/polyester fabric [12, 13]. In current study it was observed, that in case of stiffness (warp + weft) F-test indicates that there was significance difference of antimicrobial finish on stiffness (warp + weft) on cotton/polyester fabric and the effect size was large ($\eta^2 = 0.651$). In previous studies it was indicated that antimicrobial finish did not significantly affect the smoothness of cotton/polyester fabric [14, 15]. While current study proves the same that there is no significance difference of antimicrobial finish on smoothness appearance of cotton/polyester fabric. Previous studies suggested that the three experimental groups' *A. indica*, *B. monosperma* and *L. chinensis* antimicrobial finish significantly affect the stiffness warp [16, 17]. The present study proves the same, that antimicrobial finish effects the warp stiffness of cotton/polyester fabric. In a previous study it was suggested that with the duration of 6 days the presence of microorganisms was noticed on untreated fabric [18], our results support these findings. Previous studies confirmed that the fabrics which were treated with *L. chinensis* [19-22] show 100% reduction on all three samples after 22 hours and after six days there was no colony of microorganisms was found, similar findings are observed in the present study.

5. CONCLUSIONS

This study successfully developed sustainable antimicrobial finishes from *A. indica, B. monosperma,* and *L. chinensis* leaf extracts for cotton/polyester fabrics. Results showed excellent antimicrobial efficacy, improved stiffness, and minimal impact on smoothness appearance. These eco-friendly finishes offer promising alternatives to synthetic antimicrobials, enhancing textile sustainability and consumer safety. Future research will focus on scalability, exploring new agents, and in-vivo assessments.

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Investigation of Paternally Inherited Allele Mutation at Short Tandem Repeat (STR) Locus D7S820 Leading to Parent-Child Mismatch

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Abstract: During paternity investigation with Identifiler™ set of autosomal Short Tandem Repeats (STRs), a genetic mismatch was observed with D7S820 between the disputed father and child. The genotype at this locus in the disputed father, mother and child was 10/10, 11/12 and 11/11, respectively. The combined paternity index and probability of paternity after including the mutation in the calculation were 7.6×10^7 and 0.9998, respectively. Both values supported the suspicious father as the biological father of the child. Further analysis of Y-STRs revealed matching of all the alleles of the child with that of the suspicious father. It suggested that the mismatch at the D7S820 locus might be a case of mutation. DNA sequencing of D7S820 PCR products of the child and both the parents helped in determining that the child inherited the expanded repeat of the paternal allele 10, which was transmitted as allele 11 in the child from the suspicious father.

Keywords: Paternity Index, Y-STRs, DNA Typing, Paternity Test, Forensic Science.

1. INTRODUCTION

The human genome is densely packed with repetitive DNA sequences. The length of the core repeat units, the number of adjacent repeat units, and/or the overall length of the repeat area are used to categorize these repeating sequences, which occur in various sizes. Short Tandem Repeats (STRs), often known as microsatellite markers, are DNA sections with brief repeat units typically 2–6 bp in length [1]. The benefits of STRs have been demonstrated, and this makes them particularly ideal for human identification. A population's variation in the number of repeat units leads to numerous alleles at the STR locus. An STR marker is transmitted from each parent, resulting in repeat sizes that can vary between the two alleles. Due to the significant variability in the number of repeats found in STR markers among individuals, these loci serve as effective tools for human identification [2]. STRs have become popular DNA markers because these can easily be amplified by polymerase chain reaction (PCR) without the problem of differential amplification and typed by gel electrophoresis. For the amplification of STRs used in human identity testing, multiplex PCR with fluorescently labeled primers has been an essential approach. Multiplex PCR is frequently identified as the bottleneck in the STR workflow of extraction, quantification, amplification, separation, and detection [3]. Polymorphism of STR markers has been utilized in a range of case studies, such as determining parentage and various forensic applications. STRs are classified into three types based on the structure of their repeat units: simple repeats, compound repeats, and complex repeats [2]. When compared to other genetic markers, STRs exhibit a significantly high mutation rate, which is viewed

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as a limiting factor for their application in forensic investigations [4-6]. Mutations occurring during meiosis can affect the interpretation of paternity tests. A meiotic mutation could produce inconsistent outcomes at a location [7-9]. A repeat unit could be inserted or deleted from the DNA strand. The gain or loss of repetitive units in alleles mainly caused by replication slippage at one or more loci results in an allelic mismatch in the questioned child and complicates the interpretation of the analytical results [10]. This type of allele mismatch, which diverges from the Mendelian inheritance pattern due to size differences from the parental allele, may influence the paternity or maternity of a child. According to recommendations from the Forensic Society, a single allele mismatch generally does not provide sufficient grounds for exclusion unless there are more than two mismatches [11]. However, this should be validated by analyzing additional markers to confirm an inclusion or exclusion determination [7].

A recent study demonstrated that the AmpFlSTR® Identifiler® Plus Kit™ was unable to amplify the child's allele at the D7S820 locus during standard paternity testing. This resulted in an inconsistency between the parent and child due to a single-step mutation [12]. The D7S820 STR loci display a wide range of allele numbers, making them particularly valuable for forensic investigations and paternity determinations [13].

In this study, we present a case related to a paternity test that revealed a paternal allele mismatch at the D7S820 locus in the child. We obtained DNA profiles for the child, the mother, and the alleged father, which confirmed the allele mismatch at this particular locus. Additionally, we analyzed Y-chromosome STR markers from both the child and the alleged father. The results showed identical DNA profiles for the two. Consequently, we conclude that the mismatch at the D7S820 locus likely indicates a mutation in the paternal allele, which has been inherited by the child.

2. MATERIALS AND METHODS

2.1. Sample Collection

To determine the paternity of a child with the suspected father, initially, six samples were collected at the Institute of Biomedical and Genetic Engineering (IBGE) from the suspected father, the child, and the child's mother. To validate the initial findings, an additional six samples were obtained from the same individuals after obtaining informed consent, resulting in a total of 12 samples. The samples included both blood and buccal swabs, with the blood samples being preserved in Vacutainer™ tubes containing Acid Citrate Dextrose (ACD).

2.2. DNA Extraction and DNA Profiling

The QIAamp® DNA Blood Mini Kit, produced by QIAGEN GmbH in Germany, was utilized for DNA extraction from blood samples, adhering strictly to the manufacturer's guidelines. A modified DNA isolation protocol was applied to extract DNA from buccal swabs using the same QIAamp® DNA Blood Mini Kit. The concentration of the extracted DNA was assessed using NanoDrop 2000/2000c Spectrophotometers from Thermo Scientific. Following the manufacturer's instructions, DNA samples were amplified using the AmpFl STR Identifiler™, AmpFl STR Profiler™, AmpFl STR Cofiler™, and AmpFl STR Y-filer™ kits from Applied Biosystems, Foster City, USA. The PCR amplified products from the AmpFl STR kits were separated via capillary electrophoresis and analyzed with appropriate internal size standards and allelic ladders on the ABI3130 Genetic Analyzer, employing Data Collection software. Allele sizing and genotyping were conducted using GeneMapper ID version 3.1 software [14].

2.3. Sequencing of D7S820 Alleles

DNA samples from the presumed father, mother, and child were amplified at the D7S820 gene using a specific primer pair from the AmpFlSTR Identifiler™ kit [15]. The resulting PCR product was purified with the AccuPrep® PCR Purification Kit from Pioneer, a Korean company, and subsequently sequenced using the BigDye Version 3.1 terminator-ready reaction kit, employing both forward and reverse D7S820 primers (Perkin Elmer, Foster City, USA). Samples were genotyped using the ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.4. Statistical Analysis

The Paternity Index (PI) is calculated using the formula PI = X/Y, where X represents the

likelihood that the alleged father could pass on the required allele, and Y represents the chance that a different male of the same ethnicity might transmit that allele. This measure indicates whether the suspect is the biological father of the child. Following the guidelines set forth by the Paternity Testing Commission of the International Society for Forensic Genetics, we also computed the Combined Paternity Index (CPI) and the Probability of Paternity (W) [16, 17]. The allelic frequency was also computed to calculate the CPI and W.

3. RESULTS AND DISCUSSION

A routine DNA paternity test was conducted and the samples were first analyzed using the Identifiler™ set of 16 STR markers. A mismatch was identified at the D7S820 locus between the child and the disputed father (Figure 1). At this locus, the disputed father is homozygous for allele10, the mother is heterozygous with alleles 11 and 12, and the child is homozygous for allele 11. The DNA profiling was repeated using the Identifiler™ set of STR markers, which again revealed that the D7S820 locus was the only site of mismatch (Table 1). Further analysis was performed using a 10 loci STR Profiler™ kit, which consistently confirmed the mismatch at the D7S820 locus alone, showing perfect matches at all other loci. If both tested individuals were the child's biological parents, the genotype of the child would be 10/11; however, only allele 11 was detected in the child's electropherogram (Figure 1). A child can be considered the biological offspring of the presumed father if all STR loci match during comparison. Conversely, a child is not the biological child of the suspected father if there are two or more STR loci that indicate exclusion. This situation suggests that the probability of paternity would fall below 90%, indicating a definitive lack of blood relation between the child and the suspected parent. Thus, it remains plausible that the alleged father is not the biological parent, or that a mutation may account for the discrepancies [18]. Mutations can occur at the STR loci, similar to any

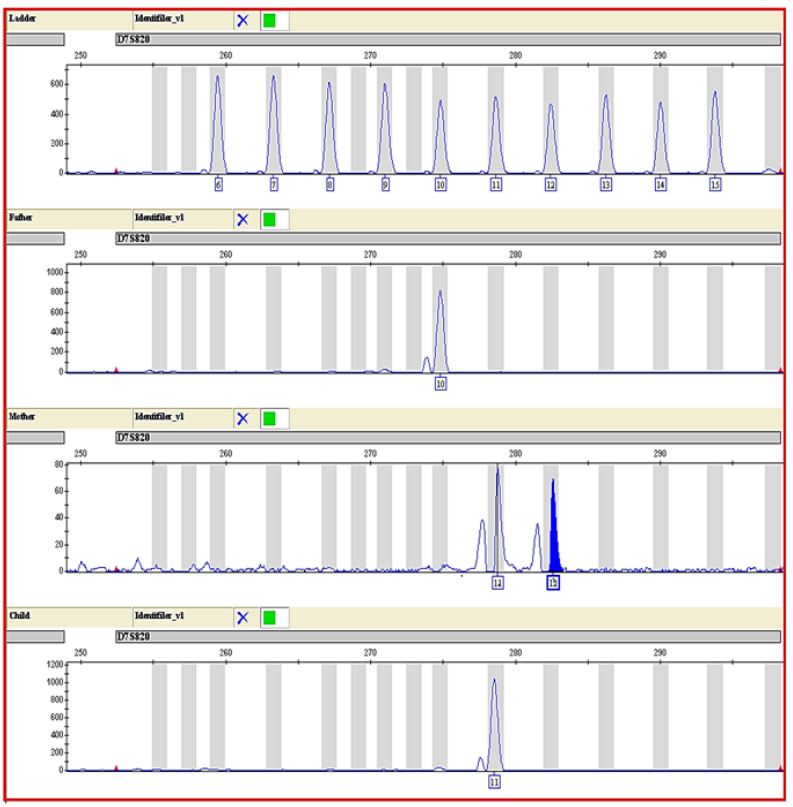

Fig. 1. Screenshots of genotype of the locus D7S820 of the Father, mother and child. Amplicons generated using Identifiler set of STR markers were analysed in ABI3130 Genetic Analyser and electropherograms of alleles were obtained using GeneMapper® ID Software v3.2. The genotyping results at D7S820 locus showing father homozygous for allele 10, mother heterozygous 11/12 and child homozygous for allele 11. Child sharing allele 11 only from his mother and father lack this allele in his profile at D7S820 locus.

Table 1. The DNA profiles of parents and child generated with 15 autosomal STR Identifiler™ markers. Only for the marker, D7S820, no match was observed between the suspected father's allele with the child (Highlighted as red).

STR Panel	Marker	Dye	Father		Mother		Child	
			Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Identifiler_v1	D8S1179	B	11	11	10	16	11	16
Identifiler_v1	D21S11	B	31.2	31.2	27	31.2	31.2	31.2
Identifiler_v1	**D7S820**	**B**	**10**	**10**	**11**	**12**	**11**	**11**
Identifiler_v1	CSF1PO	B	10	13	11	12	10	12
Identifiler_v1	D3S1358	G	16	17	16	17	16	17
Identifiler_v1	TH01	G	7	9	8	9.3	7	8
Identifiler_v1	D13S317	G	8	11	11	12	11	11
Identifiler_v1	D16S539	G	12	13	11	12	12	12
Identifiler_v1	D2S1338	G	19	20	18	25	19	25
Identifiler_v1	D19S433	Y	14	15	12	13	12	14
Identifiler_v1	vWA	Y	18	18	15	15	15	18
Identifiler_v1	TPOX	Y	8	8	8	11	8	11
Identifiler_v1	D18S51	Y	15	16	14	16	15	16
Identifiler_v1	AMEL	R	X	Y	X	X	X	Y
Identifiler_v1	D5S818	R	10	12	11	12	10	11
Identifiler_v1	FGA	R	24	24	21	23	23	24

region of DNA, and the STR alleles may change over time. Each STR locus currently possesses known alleles derived from prior individuals [19]. For instance, a study by Youngest *et al*. [20] found that all paternal fragments from the child were identical to those of the alleged father, except for one locus, CSF1PO, which exhibited a mutation. While mutations in the STR locus can lower the paternity index, it may still be concluded that the child is indeed the biological child of the alleged father [20]. Similarly, a mismatch was reported at the locus D13S317 in a study conducted by Singh *et al.* [21]. The estimated combined paternity index (CPI) and probability of paternity (W) for 15 loci after including mutation in the calculation were 7.6×10^7 and 0.9998. Our results indicate that there was a strong likelihood that the suspected father was also a biological father. The CPI and W values in this instance were so high that additional analysis was not necessary.

We conducted a further examination of the DNA from both the infant and the father, focusing on 16 Y chromosomal STR markers specific to the male lineage. The profiles revealed that all 16 Y chromosomal locations of the child matched those of the father (Table 2). It is important to note that the Y chromosome is passed down paternally without recombination, which raises the possibility that other males on the paternal side, such as the grandfather, uncles, and their male descendants, could share a similar Y-STR profile with the child. Nonetheless, this possibility was ruled out after thorough interviews with family members and the child's mother. The findings from the DNA profiling and additional evidence strongly indicate that the mismatch of paternal alleles at the D7S820 locus likely stems from a mutation event. Somatic mutations can occur at STR loci used in forensic analysis, and it is conceivable that the DNA profile derived from a buccal swab may differ from that obtained from a hair or blood sample. If such a mutation takes place early in the embryo's development, it is more likely to be uniformly present across all tissues. To further eliminate the possibility of somatic cell mutation, we also analyzed blood samples for the same set of autosomal and Y-STR markers. The results consistently aligned with those obtained from the buccal swab samples.

The alleles of the mother, child, and suspected father were directly sequenced to further characterize the D7S820 mutant allele. The suspicious father

Table 2. A comparison of Y-STR profiles between the alleged father and son reveals complete similarity at every analyzed locus.

STR Panel	Marker	Dye	Father		Child	
			Allele 1	Allele 2	Allele 1	Allele 2
Yfiler_v2	B_DYS456	B	15		15	
Yfiler_v2	B_DYS389I	B	14		14	
Yfiler_v2	B_DYS390	B	22		22	
Yfiler_v2	B_DYS389II	B	30		30	
Yfiler_v2	G_DYS458	G	15		15	
Yfiler_v2	G_DYS19	G	14		14	
Yfiler_v2	G_DYS385	G	14	18	14	18
Yfiler_v2	Y_DYS393	Y	14		14	
Yfiler_v2	Y_DYS391	Y	10		10	
Yfiler_v2	Y_DYS439	Y	10		10	
Yfiler_v2	Y_DYS635	Y	25		25	
Yfiler_v2	Y_DYS392	Y	10		10	
Yfiler_v2	R_Y_GATA_H4	R	12		12	
Yfiler_v2	R_DYS437	R	16		16	
Yfiler_v2	R_DYS438	R	11		11	
Yfiler_v2	R_DYS448	R	19		19	

only had 10 GATA repeats, the mother had 11 and 12 GATA repeats, and the child had 11 GATA repeats in homozygosity (Figure 2). Results show a repeated motif (GATA) mutation, which may have happened in the paternal germ cells. As was previously mentioned, STRs are generally subject to mutations, with replication slippage being the primary cause of these mutations [5, 9, 12, 22, 23]. The average autosomal STR mutation rate is thought to be less than 0.1%, and it would take about 1000 instances of parent-offspring allele transmission to detect one mutation in STR markers. The average mutation rate ranges from 0.0-0.7% in commonly used STR markers and the rate of mutation has been observed four times high in tetra-nucleotide repeats than the dinucleotide repeats [23]. The D7S820 STR marker is located on chromosome 7q21.11. The core repeat unit is GATA and the number of alleles observed at this locus ranges from allele number 5 to 16, with a mutation rate

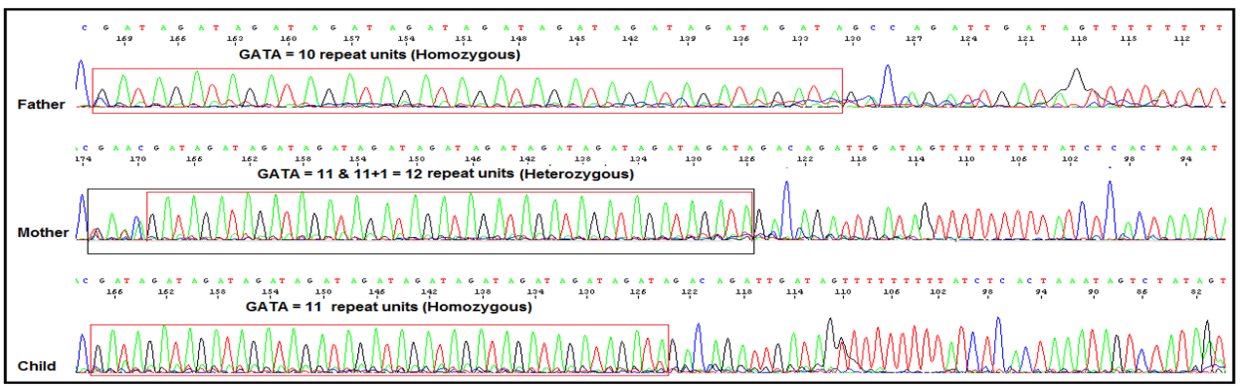

Fig. 2. DNA sequence electropherogram of the D7S820 locus. The top panel showing the DNA sequence obtained from father's DNA. The DNA sequencing results, indicating the presence of only 10 GATA repeats (allele 10) in father. The middle panel showing DNA sequence obtained from the Mother. The mother's first GATA repeats showing mixed peaks and rest of the 11 GATA repeats are homozygous indicating the DNA sequences of allele 12 and 11 respectively. The lower panel shows a DNA sequence of the child with 11 GATA repeat units (homozygous for allele 11). It indicates that the normal allele 11 of child is inherited from the mother and father's allele get mutated and loosed a repeat unit during gamete formation.

of 0.1%. In this particular case of paternity, there is a possibility of one mutation event for the allele mismatch at the D7S820 locus. The allele 10 of the father is expanded by a complete GATA repeat unit and transmitted to the child as allele 11.

Another explanation would be the presence of a null allele in the father or child. A null allele can be produced via a primer binding site mutation, which prevents the amplification of the original allele. An individual would type as a homozygote if they are heterozygous and have a primer binding site mutation for one of the alleles. Null alleles, which are brought on by mutations in the primer binding site, might cause discrepant DNA types at a certain locus when comparing DNA typing results from various kits. Although null alleles are uncommon, it's crucial to realise that they must be taken into account when interpreting prospective matches. Using an unlabeled primer pair from a separate kit, D7S820 was amplified and sequenced to further rule out the possibility of a null allele (Research Genetics MapPair kit, ver 8.0).

4. CONCLUSIONS

The study indicates a genetic mismatch involving the D7S820 locus between the child and the disputed father. Specifically, the alleged father is homozygous for allele 10, while the mother exhibits a heterozygous profile with alleles 11 and 12. In contrast, the child is homozygous for allele 11. This mismatch may be attributed to a potential mutation, as STRs are known to be susceptible to such genetic changes over time.

5. ETHICAL STATEMENT

The study was approved by the ethical committee of Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan, and Quaid-i-Azam University, Islamabad, Pakistan.

6. ACKNOWLEDGMENTS

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Research Article

Prevalence of Self-Medication and Assessment of its Consequences on Health among Female University Students in Islamabad, Pakistan

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Abstract: Self-medication (SM) is the drug attainment and consumption without consulting a doctor/physician against a particular disease. This cross-sectional study assessed SM and its consequences among the female students of the International Islamic University Islamabad (IIUI), Pakistan. A total of 180 female students from different academic departments were selected on the basis of stratified random sampling technique and the collection of data on SM was done with a pre designed questionnaire. Findings displayed that the prevalence of SM among the female students of IIUI, Islamabad, Pakistan was 32.22 to 55.6%. The information source of medicines in students was 48.9 % from doctors/physicians and 33.9% from the internet. The disease conditions for which SM was adapted include headache, flue, cough and cold in about 64.4% students and abdominal pain in 38.3% students. The most common medicines used for SM were pain killers for 49.4% students, vitamin supplements for 30.6% students and antibiotics for 16.7% students. About 46.1% students obtain medicines for SM from the medical stores and 33.3% obtain from hospitals pharmacy without doctors/physician's prescription. Majority were using medicines for seasonal (40%) or viral (21.7%) infections or during accidental cases (26.1%). Overall, 68.88% students confirmed that they experienced adverse effects on health after SM. 74.35% students felt some common side effects, 67.16% experienced drug/medicine resistance against other diseases and 58.06% experienced poor immune system due to SM. In this regard, medication through regular consultations with doctors/physicians is necessary and the execution of proper policies on advertising, marketing and selling of medicines with precautionary measures is recommended to avoid adverse effects on health.

Keywords: Self-medication (SM), Health Effect, Viral Infection, Antibiotics, Pain Killers.

1. INTRODUCTION

The desire of taking medicine for treatment with self-prescribed medicines remained a crucial aspect of life. Self-medication (SM) especially is very important in the maintenance of individual's health [1]. People have general perception that medicine should be taken during any disease or poor health conditions. Individuals who use self-prescribed medications are more concerned about their good health and disease treatment. People act differently to treat their symptoms of disease which is common among communities. SM practice is great drive in terms of self-care where the individuals undertake actions to improve their health and to prevent diseases after sickness or any physical or internal injury [1-8]. According to Alano *et al*. [9], SM is one of the elements of self-care, the way of taking medicines by individuals for the treatment of self-identified diseases or associated symptoms.

Hussain and Khanum [10] also explained SM as taking different types of medicines without the supervision of professional regarding the dosage, indication and course of treatment. Nevertheless, SM not essentially means the only use of modern medicines, yet it also includes the use of herbs and other natural remedies [11, 12]. During any disease

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condition, SM is the first option to adapt and hence is a common practice globally. The diseases are considered as minor, when they last for limited time and which are not life threatening. In some government bodies, SM reduces costs and assists health professionals to pay attention on some other very severe health conditions [9] and in the developing countries; various types of diseases are cured with SM practices. Some common health conditions for which individuals use self-prescribed medicines include flu, cold, headache, heart burn, constipation, minor skin problems and insect bites, etc., [13]. Though, SM is old practice and if it is employed appropriately, it limits the dependency on doctors. However, when abused, SM could cause delay in appropriate treatment and diagnosis of disease and may cause toxicity and drug interactions with serious side-effects [14, 15]. Some of the problems associated with SM include toxicity due to excess dose of medicine, inappropriate diagnoses, drug addiction, drug-drug interactions and various side effects in people. Other concerns associated with SM are increased pathogens resistance, health hazards and wastage of resources and money.

There are much SM anecdotal evidences available on inappropriate purchasing of medicines to treat particular disease condition, but very limited inquiries have quantified their consequences [13]. Pharmacists play a crucial role by assisting the individuals with pertinent information about medicines they use as SM. Advertising is another way to make individuals aware about the medicines used without prescription. It should always be friendly and encouraging for the individual to get advice from a pharmacist or physicians [16]. A lot of people are not aware about the drugs adverse effects which they use and also recommend to other individuals. Since this practice causes very serious problems to the individuals themselves and to those whom they suggest such medication. In this regard, the potential SM problems should be addressed to the individuals to reduce risks associated with SM. Antimicrobial resistance is a present global issue predominantly in some under develop countries where the use of antibiotics without doctor's prescription is common [14, 17]. As SM practice in educated student community is a substantial health problem [18], students are thought to be the role models in maintaining good health. Due to their vigorous part in cyberspace and media, students of the university could take more imperative part in this area [7, 19]. Also, the university students are thought to be very susceptible to SM because of their social interactions and their duties as future parents [19-22]. Despite SM being an issue of global concern, a very limited data on SM has been reported on those who belong to non-medical profession from Pakistan. Hence, the present study was conducted to uncover the information, approaches and practices towards SM among the female students of university. As there is very limited data available on this issue, this research helps in finding out the major factors that are responsible for SM and associated side effects among female university students from the Islamabad region of Pakistan.

2. MATERIALS AND METHODS

2.1. Study Design and Settings

This study was conducted to estimate the use of self-care medicine by female students at the International Islamic University, Islamabad Pakistan. The respondents were selected from different academic departments at the International Islamic University Islamabad, Pakistan female campus with the exclusion of teaching and nonteaching staff. The respondents were included based on their ability to read Urdu, being at least under the age of 18 and above and willingness to participate in the study. Those students who study pharmacy or medicine were not included in the study, since they are experts in SM with professional information about medication. Data from the selected respondents was collected through electronic questionnaire written in English. The questionnaire based on multiple parts was designed following previous studies [7]. The first part was based on the respondent's demographic information. The second part includes information on SM practice. The selected questions were about the information sources and various practices on SM. For some questions, the scaled measured contain two options by "Yes" and "No". Regarding SM, the variables assessed in this study includes: steps or measures taken by the respondent during sickness, distribution of the respondents regarding source of obtaining medicine, conditions or cases for self-prescribed medicines, types of self-prescribed medicines, disease symptoms regarding SM, information sources regarding SM and types of potential side effects experienced from SM.

2.2. Statistical Analysis

Computer analysis typically requires that people answer to questions or personal observation be converted into numbers. The obtained data was maintained in excel sheets and analyzed statistically with SPSS (SPSS, IBM Corporation, Version 26) and Chi-square test was performed to assess variables association [7].

2.3. Consent and Ethical Consideration

The survey for data collection was initiated with a brief explanation about the nature of the study to the respondents. The respondents were prior informed that their responses to the given questionnaire will be considered as consent to collect and process the data for research purpose.

3. RESULTS

3.1. Demographic Status of Respondents

Figure 1 (a-e) depicts the socio-economic status of the respondents including age, monthly family income, expenditure on drugs/medicines, nationality and living place of the respondents. The respondent's age is one of the significant variables in social research which is associated with person's behavior and attitude at different stages of life. Regarding the age, 117 (65.0%) respondents fell under 21 to 25 years and 50 (27.77%) respondents were under the age group of 18-20 years. The remaining 13 (7.2%) respondents were under the age group of 26-29 years (Figure 1a). The data further showed gross monthly family income of the respondents where maximum 63 (35.0%) respondents showed their family income up to 41,000 to 50,000 PKR, 49 (27.22%) respondents showed their family income above 50,000 PKR, 43 respondents showed income up to 31,000 to 40,000 PKR and 25 respondents revealed 20,000 to 30,000 PKR family income respectively (Figure 1b). Expenditure of the respondents on medicines/ drugs showed maximum 82 (45.6%) respondent's expenditure was up to 1100 to 20,000 PKR, 42 (23.3%) respondent's medicines/drugs expenditure was in range between 500 to 1000 PKR, 36 (20%) respondent's medicines/drugs expenditure was 20,000 to 30,000 PKR and 20 (11.11%) respondent's medicines/drugs expenditure was 31,000 to 40,000 PKR (Figure 1c). The data further indicated 137 (76.1%) respondents were Pakistani nationals while 43 (23.9%) respondents were from other countries (Figure 1d). Regarding the living place of respondents, 92 (51.1%) were living in

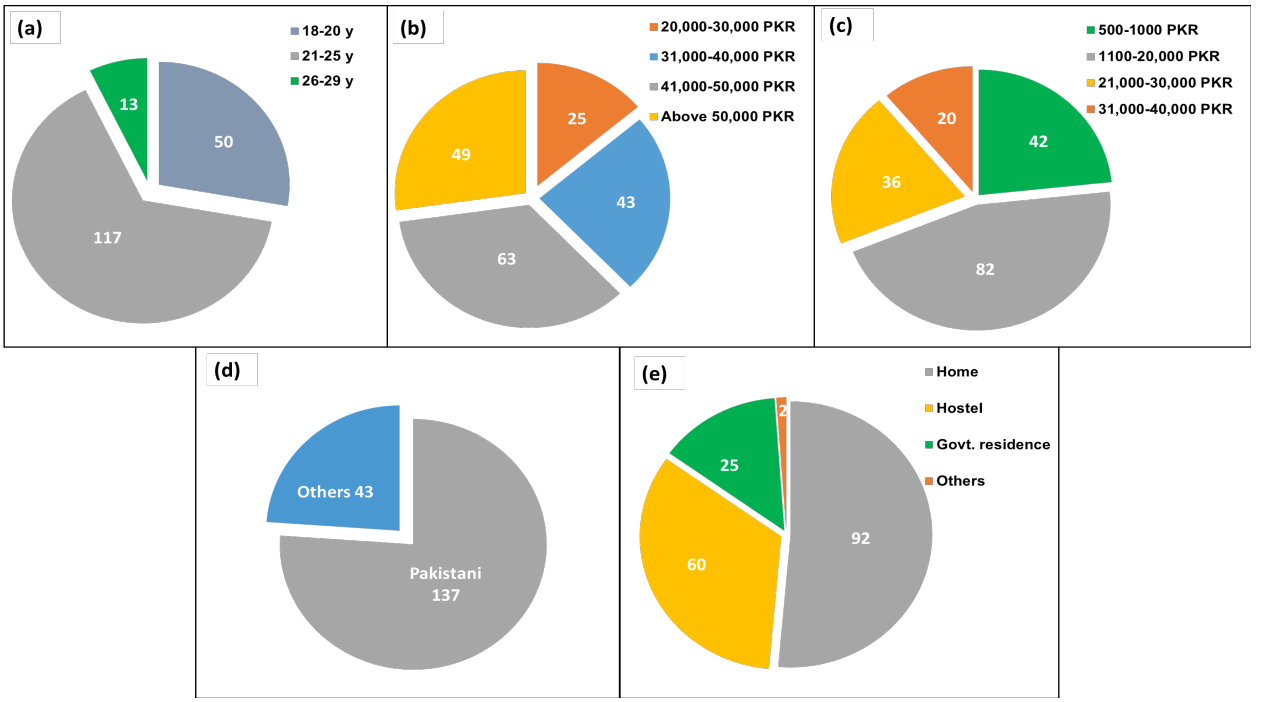

Fig. 1. Respondents' distribution on the basis of socio-economic status (n = 180), (a) distribution on the basis of age, (b) monthly family income, (c) expenditure of respondents on drugs/medicines, (d) nationality of the respondents, and (e) living place of respondents.

ancestral homes, 60 (33.3%) were living in hostels, 25 (13.9%) were living in government residence and 2 (1.1%) respondents marked others as their place of living (Figure 1e).

Figure 2 (a-c) provides information about respondents' socio-demographic status including marital status, family type and ethnicity. Out of 180 respondents, 99 (55.0%) were living as nuclear family, 51 (28.33%) as joint family, 16 (8.88%) as single parent and 14 (7.77%) as extended family (Figure 2a). Data further showed marital status of respondents, where 145 (80.55%) were single, while 25 (13.88%) were married (Figure 2b). The respondents were further asked to mention their ethnicity where 67 (37.2%) were Punjabi, 49 (27.2%) were Balochi, 36 (20.0%) were Pashtun and 28 (15.6%) were Sindhi (Figure 2c).

Figure 3 (a-d) shows details about the religion, area of residence, academic departments and condition of drug consumption of the respondents. Out of 180 respondents, 137 (76.1%) were Muslims (Islam), 32 (18%) were Christians (Christianity) and remaining were from other religions (Hindu and others) (Figure 3a), and about 63 (35.0%) respondents were from rural and 117 (65.0%) were from urban areas of the country (Figure 3b). Regarding the academic departments of the respondents, 38 (21.1%) respondents were from Psychology, 37 (21.6%) from Sociology, 36 (20.0%) from Education, 23 (13%) from Mass and Media Communication, 26 (14.4%) from Arts and Architecture and 20 (11.1%) were from the History department of the University (Figure 3c). Data on conditions of medicine/drug consumption showed that 99 (55.0%) respondents consumed medicines due to chronic diseases such as liver, kidney and cardiac problems, while 81 (45.0%) respondents consumed medicines due to some other health problems (Figure 3d).

3.2. Respondents' Knowledge About Self-Medication

Table 1 depicts the details of measures or steps taken by the respondent during disease condition. Almost 100 (55.6%) respondents sometimes prefer, 58 (32.2%) respondents always prefer, while 22 (12.2%) never preferred SM when they are sick. Almost 120 (66.7%) respondents sometimes consulted, 36 (20.0%) respondents always consulted and 24 (13.3%) respondents never consulted a doctor/physician when they are sick. 89 (49.4%) respondents sometimes ignore the sickness, 48 (26.7%) respondents always ignored and 43 (23.9%) respondents never ignored the feeling of sickness.

Table 2 shows the details of distribution of the respondents regarding the sources of obtaining medicines for self-care. 96 (53.3%) of

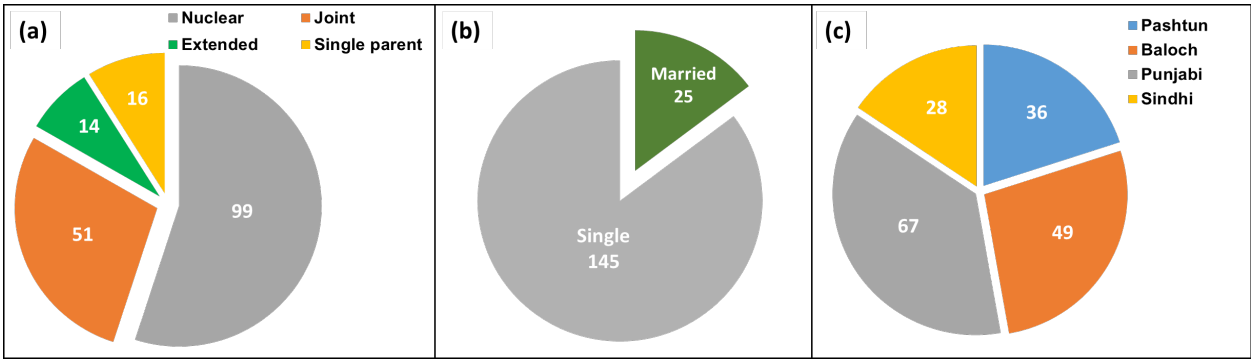

Fig. 2. Respondents' distribution on the basis of socio-demographic status (n = 180), (a) family type, (b) marital status, and (c) ethnicity of respondents.

Table 1. Distribution of the measures or steps taken by the respondents (n = 180) during sickness.

S/No.	Measures/Steps	Always	Sometime	Never/Not at all
1	Self–medication	58 (32.22%)	100 (55.6%)	22 (12.2%)
2	Consult a doctor/physician	36 (20.0%)	120 (66.7%)	24 (13.3%)
3	Ignore the feeling of sickness	48 (26.7%)	89 (49.4%)	43 (23.9%)

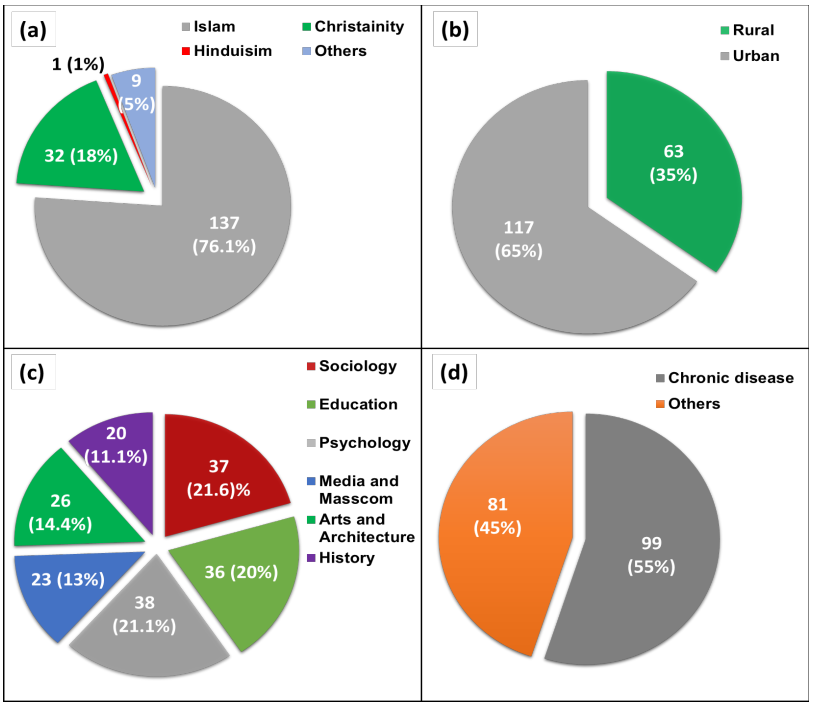

Fig. 3. Respondents' distribution (n = 180) on the basis of (a) religion, (b) area of residence, (c) academic departments, and (d) condition of the drug consumption.

the respondents obtained medicines sometimes from hospital pharmacy section while 60 (33.3%) respondents always obtained medicines from hospital pharmacy section. 76 (42.2%) sometimes obtained medicines from relatives while 24 (13.3%) always obtained medicine from friends or relatives. 83 (46.1%) respondents always obtained medicines from medical stores while 78 (43.3%) sometime obtained medicines from medical stores. 93 (51.7%) respondents obtained medicines sometime from friends or family. The 87 (48.3%) respondents sometime obtained medicines from previous hospital visit while 26 (14.4%) respondents always obtain medicines from previous hospital visits.

Table 3 shows details of conditions or cases due to which the respondents prefer SM. 72 (40.0%) respondents always preferred, 88 (48.9%) sometimes preferred and 20 (11.1%) never preferred SM in case of seasonal infections. 73 (40.6%)

Table 2. Distribution of the respondents (n = 180) regarding the source of obtaining medicine.

S/No.	Sources of medicine	Always	Sometime	Never/Not at all
1	Hospital pharmacy	60 (33.3%)	96 (53.3%)	24 (13.3%)
2	Relatives	24 (13.3%)	76 (42.2%)	80 (44.4%)
3	Medical stores	83 (46.1%)	78 (43.3%)	19 (10.6%)
4	Use left over from previous hospital visit	26 (14.4%)	87 (48.3%)	67 (36.7%)
5	Obtain drug/medicine from friends or family	25 (13.9%)	93 (51.7%)	62 (34.4%)

Table 3. Distribution of the respondents (n = 180) regarding condition or cases for self-medication.

S/No.	Conditions for SM	Always	Sometime	Never/Not at all
1	In case of seasonal infections	72 (40.0%)	88 (48.9%)	20 (11.1%)
2	In case of viral diseases	39 (21.7%)	73 (40.6%)	68 (37.8%)
3	In case of emergency or accidents	47 (26.1%)	54 (30.0%)	79 (43.9%)

sometimes preferred, 39 (21.7%) always preferred and 68 (37.8%) never preferred SM in case of viral diseases. 47 (26.1%) respondents always preferred SM in case of emergency or accidents while 54 (30.0%) preferred sometimes and 79 (43.9%) never preferred SM in case of emergency or accidents.

The details of types of medicines preferred by the respondents in SM are given in Table 4 where 89 (49.4%) respondents always used medicines, 66 (36.7%) sometimes use while 25 (13.9%) never used pain killers. 97 (53.9%) respondents sometimes use antibiotics, 30 (16.7%) always use and 53 (29.4%) never used antibiotics. 83 (46.1%) respondents sometimes use vitamins, 55 (30.6%) always use and 42 (23.3%) respondents never used vitamins as medicine for self-care. Some respondents used antacid (5% always, 23.3% sometimes, 71.7% never), anti-malarial (8.3% always, 25% sometimes, 71.7% never) and oral rehydration salts (13.3% always, 35% sometimes and 51.7% never) as type of medications in SM. The details of disease symptoms for which the respondents adapt medication are given in Table 5. Around 116 (64.4%) respondents pointed headache and 116 (64.4%) pointed cough and cold as disease symptoms due to which they prefer SM. 62 (34.4%) respondents pointed allergy as major cause, 69 (38.3%) respondents pointed abdominal pain or diarrhea and 58 (32.2%) claimed inability to sleep/ insomnia as symptoms due to which they prefer SM.

Table 6 provides data about the information sources of SM. About 88 (48.9%) respondents to great extent consulted, 72 (40.0%) respondents to some extent consulted and 20 (11.1%) never consulted the doctors/physicians to get information about SM. 19 (10.6%) respondents consulted nurses to great extent, 74 (41.1%) consulted to some extent and 87 (41.1%) never consulted nurses to get information on SM. About 61 (33.9%) respondents used internet as a source of information to a great extent, 75 (41.7%) to some extent and 44 (24.4%) never used internet as a source of information on SM. 52 (28.9%) respondents got information from relatives or friends to great extent, 82 (45.6%) to some extent and 46 (25.6%) never got information from relatives or friends on SM. 47 (26.1%) consulted pharmacists for information on SM to great extent, 72 (40.0%) to some extent and 61 (33.9%) never consulted a pharmacist for information on SM. Moreover, 35 (19.4%) respondents consulted ordinary newspapers and magazines to great extent, 55 (30.6%) to some extent and 90 (50%) never consulted ordinary newspapers and magazines for information on SM. 35 (19.4%) used leaflets to great extent, 65 (36.1%) to some extent and 80 (44.4%) never used leaflets for information on SM. Around 27 (15.0%) respondents used medical books to great extent, 73 (40.6%) to some extent and 80 (44.4%) never used medical books for information on SM.

Table 4. Distribution of the respondents (n = 180) regarding types of medication.

S/No.	Types of medication	Always	Sometime	Never/ not at all
1	Pain killers	89 (49.4%)	66 (36.7%)	25 (13.9%)
2	Antibiotics	30 (16.7%)	97 (53.9%)	53 (29.4%)
3	Anti-malarials	15 (8.3%)	45 (25.0%)	120 (71.70%)
4	Antacids	9 (5.0%)	42 (23.3%)	129 (71.7%)
5	Oral rehydration salts	24 (13.3%)	63 (35.0%)	93 (51.7%)
6	Vitamins	55 (30.6%)	83 (46.1%)	42 (23.3 %)

Table 5. Distribution of the respondents (n = 180) based on disease symptoms responsible for self-medication.

S/No.	Disease symptoms	Agree	Undecided	Disagree
1	Headache	116 (64.4%)	38 (21.1%)	26 (14.4%)
2	Flu, cough and cold	116 (64.4%)	39 (21.7%)	24 (13.3%)
3	Inability to sleep/Insomnia	58 (32.2%)	50 (27.8%)	72 (40.0%)
4	Allergy	62 (34.4%)	52 (28.9%)	66 (36.7%)
5	Abdominal pain or diarrhea	69 (38.3%)	39 (21.7%)	72 (40.0%)

Table 6. Distribution of the respondents (n = 180) regarding information sources of self-medication.

S/No.	Information sources of self-medication	To great extent	To some extent	Never/Not at all
1	Doctors/ Physicians	88 (48.9%)	72 (40.0%)	20 (11.1%)
2	Nurses	19 (10.6%)	74 (41.1%)	87 (41.1%)
3	Internet	61 (33.9%)	75 (41.7%)	44 (24.4%)
4	Relatives or friends	52 (28.9%)	82 (45.6%)	46 (25.6%)
5	Pharmacists	47 (26.1%)	72 (40.0%)	61 (33.9%)
6	Ordinary newspaper or magazines	35 (19.4%)	55 (30.6%)	90 (50.0%)
7	Leaflets concerning medicines	35 (19.4%)	65 (36.1%)	80 (44.4%)
8	Medical books	27 (15.0%)	73 (40.6%)	80 (44.4%)

Figure 4 shows details about the contribution of advertisement of medicines in SM. 63 (35.1%) respondents confirmed that advertisement of medicines is very helpful in SM while 82 (46.1%) showed neutral responses. Only 34 (18.9%) respondents disagreed regarding the contribution of advertisement of medicines in SM.

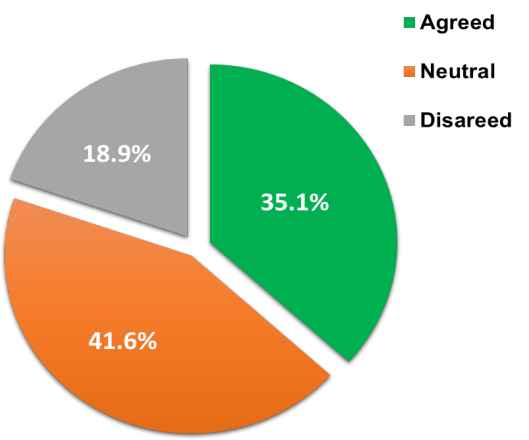

Figure 4. Perception of respondents (n = 180) about advertisement of medicines contributing in SM.

Table 7 shows a close relationship between types of potential adverse effects and awareness of the respondents about medicines. 58 (74.35%) respondents confirmed that they experienced some common side effects, 14 (17.94%) were undecided and 6 (7.69%) respondents did not agree about it. In response of any resistance to drugs/medicines, 45 (67.16%) respondents agreed that SM causes resistance to drugs/medicines and makes certain drugs ineffective, 12 (17.91%) were undecided, and 10 (14.92%) disagreed. When asked about the effects of SM on immune system, 18 (58.06%) agreed that SM causes poor immune system. The 10 (32.25%) respondents were undecided about it, while 3 (9.67%) disagreed about the effects of SM on immune system. Overall for the types of potential adverse effects, among 180 respondents, 124 (68.88%) respondents faced different adverse side effects, 36 (20.0%) were not sure about it and 19 (10.55%) respondents never faced side effects after SM.

Table 7. Awareness about medicines and type of potential adverse effects experienced by the respondents (n = 180) after SM.

Type of potential adverse effects	Awareness about medicine				
	Agree	Undecided	Disagree	Any other	Total
Common side effects	58 (74.35%)	14 (17.94%)	6 (7.69%)	0 (0.0%)	78 (100.0%)
Resistance to drugs/medicines	45 (67.16%)	12 (17.91%)	10 (14.92%)	0 (0.0%)	67 (100.0%)
Poor immune system	18 (58.06%)	10 (32.25%)	3 (9.67%)	0 (0.0%)	31 (100.0%)
Any other	3 (75.0%)	0 (0.0%)	0 (0.0%)	1 (25.0%)	4 (100.0%)
Total	124 (68.88%)	36 (20.0%)	19 (10.55%)	1 (0.55%)	180 (100.0%)
Chi-square: 50.619		DF:19		Significance level: .0000	

4. DISCUSSION

In this study, data regarding steps/measures taken by the respondents during sickness/illness showed that majority of respondents primarily ignored the feeling and did not take any step or measure, while few respondents consulted doctors/physician and few opted SM to treat the disease. Based on the findings of this study that the prevalence of SM practice among female students of the university in Islamabad Pakistan was 32.22% (always practice SM) to 55.6% (sometimes practice SM). A lot of studies have previously reported the prevalence of SM among individuals from different regions of the world [3-7, 19, 23, 24-34]. It has been found that SM in the developing countries is a common practice with prevalence ranging from 25.6 to 73.6%. It is also associated with a positive perception of the country's healthcare system [33]; that's why its prevalence is high in the developing countries [35]. University students are also among the groups having high rate of SM [20, 22, 36]. In Nepal, the reported self-medication rate was 59% in the six-month period preceding while in India, the rate of SM was 31% with extensive variation. For example, in the South Indian coastal areas, 71% prevalence of SM was reported [26]. Study from Saudi Arabia indicated that the SM prevalence among the public was 59% [1] and other inquiries on SM from Saudi Arabia recognized lower prevalence as reported 52.8% in Dammam and higher prevalence of 93.1% in Majmaah and 75.2% in Qassim [5, 6, 34]. From Iran, 44.8% prevalence of SM has been reported among students [7].

Some studies have shown 18 and 87% rate of SM in Iran [37, 38], 86.4% in Brazil [29] and 55% in Egypt [27]. In the Northern Ireland and UK, 41.5% people were using medicines without a prescription of doctor [32]. 27% people in Spain were suffering from pain due to self-medication practices [23]. The high prevalence of SM is a serious issue for the decision and policy-makers in the health sector [32]. It shows that SM practice is very common across health, gender, culture, race, occupation and social status or any other socio-demographic state.

In this study, when asked about the source of obtaining medicines for SM, about 46.1% of the respondents stated that they obtained/purchased medicines for SM from the medical stores without the prescription of doctor/physician. 33.3% from the hospital pharmacy, 14.4 % from the previous treatments or leftovers and 13.9% obtained medicines from friends or family. Parallel outcomes were reported from Ethiopia and Saudi Arabia where the respondents obtained medicines from the previous treatments [1, 3].

Regarding the disease category experienced by the respondent, it was found that majority of the respondent's always choose SM when they got seasonal (40.0%) or viral (21.7%) infections or when there was any emergency /accident (26.1%). In other studies, SM was found to be significantly associated with having a chronic illness as described from Addis Ababa, Serbia and Riyadh [1, 3, 4, 25]. When asked about disease symptoms that lead to SM, data from the respondents here showed that headache (64.4%), cough, flu, common cold (64.4%), abdominal pain (38.3%), allergies (34.4%) and inability to sleep/insomnia (32.2%) were the most common symptoms for which the respondents agreed in practicing SM. Correspondingly, same symptoms have been reported as common in previous inquiries for SM [1, 39, 40]. Gupta *et al*. [41] also reported headache and fever as the most common symptoms treated with SM.

When asked the respondents about the information sources for SM, they revealed that doctors/physicians (48.9%), internet (33.9%), friends and relatives (28.9%) and pharmacists (26.1%) were the highly consulted sources to get information about SM. According to the World Health Organization, around half of the drugs distributed, prescribed and sold are those which are not justified clinically worldwide [42] and people still use those medicines. A study from Iran also showed in about 40% of students, the social networks and internet were the information sources on SM [7].

Another source of information the respondents revealed was through advertisement of medicines that provide information on SM. In this study, 35.1% of the respondents were agreed about contribution of advertisement in the provision of information on SM while 46.1% of the respondents showed neutral responses and 18.9% were disagreed. Previous studies have reported that the use of advertisements and medical applications for the dissemination of knowledge about SM could be supportive in the management of many chronic diseases [1, 43, 44]. In addition, the sources of information on medicines

including the family, social media and internet can affects the knowledge and the SM practice among consumers [45-46].

Regarding the opinion about side effects, dangers or risks associated with SM, the majority of respondents in this study were aware that SM causes infections and may damage different body organs with the initiation of adverse reactions. Previous inquiries from Riyadh and Dammam Saudi Arabia suggested that the level of knowledge about SM may vary among consumers and there was no surety of safety in SM for many of the respondents [5, 47].

When asked about type of medicine used for SM, majority of respondents confirmed that 49.4% used pain killers, 30.6% used vitamins and 16.7% used antibiotics as types of medicine in SM. A study in Iran reported about 47% of students, using antibiotics as self-prescribed medicine. About 33% of the students widely used two common medicines in SM like the sedatives and cold remedies [7]. Additionally, analgesics and antibiotics are also used frequently for SM in most studies carried out in Finland, Iran, Saudi Arabia, Spain and Turkey [36, 48, 49]. Another study from Iran reported the use of antibiotic as SM among the 53.3% nursing students [50].

When asked about potential adverse effects experienced after SM, majority of the respondents (74.35%) confirmed the appearance of some common side effects after SM. 67.16% experienced drug resistance against other diseases and 58.06% respondent's experienced poor immune system due to SM. Overall for the types of potential adverse effects, 68.88% respondents experienced different adverse side effects, 20.0% were undecided and 10.55% respondents never experienced side effects after SM.

Similarly, previous findings have shown adverse effects of SM including misdiagnosis, long-term use of drugs, ingesting toxic drug doses and drug interactions and associated complications [2, 17]. These adverse effects could worsen the health condition of patient and may leave the disease untreated. Also in many studies, the trend of higher drug usage among the individuals with higher education level as compared to the individuals with low education level have been reported [51, 52]. It has been advocated that the trend of using medicines is influenced by various factors where some are related to the demographic attributes of individual like gender, age, education level, financial status and occupation, etc., and some are related to the knowledge of individuals about medicines. It is believed that SM among patients is the first point where the patients face early disease symptoms which leads to severe side effects like bacterial resistance, drug dependency, intoxication and complicated disease symptoms [14, 17]. Through prompt necessary actions, these side effects could be minimized for safer public health [53].

5. CONCLUSIONS

The prevalence of self-medication (SM) practice is high and common among the female university students of Islamabad, Pakistan. The use of pain killers, antibiotics and vitamins as self-prescribed medicine among the students was higher during seasonal and viral infections including headache, flue, cough, cold and diarrhea or during an emergency or accident. The majority of self-medicating students experienced numerous side effects due to limited knowledge about the risks and side effects associated with medicines. In parallel with the growing SM trend, there is a dire need of spreading knowledge to familiarize the female students about the uses of medicines in consultation with doctors/physicians. Further, some proper policies should be implemented on the advertising and selling of medications with precautionary measures to prevent adverse effects of SM on individual health.

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7. CONFLICT OF INTEREST

The authors declare no competing or potential conflict of interest.

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Research Article

A Morphometric Study of Epidermal Appendages in Commonly Existed Angiosperms in Faisalabad, Pakistan

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Abstract: Epidermal appendages are mono-cellular or multicellular, root hairs, and trichomes that grow on an epidermis. Commonly found members of angiospermic families were selected focusing on their presence or absence, position, and types of surface appendages (colliculate, hairy, wart and papil). Trichomes types (simple hairs, tubercle-based hair minerals) concrete, secretory hairs, vesicular hairs, moniliform hairs, and dendritic hairs were also considered. The epidermal appendages were studied in thirty species of angiospermic families collected from Botanical Garden University of Agriculture Faisalabad, Pakistan, by using light microscope. The data was then subjected to ANOVA and cluster analysis to investigate relationship among different angiosperm species. The Maximum cell length/width on abaxial epidermis is recorded in *Strelitzia reginae Regel & Koch* and *Terminalia bellirica* (Gaertn.) Roxb, which is closely related to the *Alpinia allughas* Retz., and *Crinum asiaticum* L.. Minimum cell length on upper epidermis is recorded in *Ficus lyrata* which is closely related to the *Colocasia esculentum.* Maximum number of stomata is observed in *Kigela africana.* Minimum number of stomata is recorded in *Terminalia bellirica* and *Ficus lyrata*. Maximum number of stomata on adaxial epidermis is recorded in *Pentas lanceolata.* Minimum number of stomata is recorded in *Ravenala medaghas cariensis*. On adaxial surface, the maximum number of trichomes is recorded in *Terminallia bellirica* and *Kigela Africana*, and the minimum number is recorded in *Campsis rodicum.* The present study emphasizes the distinctive and similar features of those epidermal appendages. These observations will provide as a basis for subsequent research on the physiology and ethnobotany of the chosen species.

Keywords: Plants, Families, Leaves, Root Hairs, Stomata, Trichome.

1. INTRODUCTION

Flowering plants, also called angiosperms, are recognized members of numerous plant families worldwide (total 400-500) with around 4 million species. These plants, constituting at least 95% of all vascular plant species, significantly impact the human world and its survival. Taxonomy plays a crucial role in categorizing and distinguishing plant species by organizing them according to morphological, epidermal, and phytochemical traits [1]. Within plant taxonomy, diverse plant species are designated and described based on genus and species. Various taxonomists utilize epidermal features to recognize plants belonging to specific genera and families. In the field of plant taxonomy, attributes like trichome, stoma characteristics, and anticlinal cell wall patterns in epidermal cells are employed for identification. [2-5]. Epidermal traits encompass epidermal cells, stomata, and epidermal inserts. These attributes play a crucial role in the classification of taxa within different plant families

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[6]. Epidermal analysis mainly involves the classification of stoma type and trichome variation [7]. Trichomes and hydathodes represent additional characteristics that can serve as effective taxonomic instruments in the epidermis and other plant organs. Numerous systematic approaches have employed them for both systematic and classification objectives [8-12].

A specific trichome species frequently serves as a distinguishing feature for species, genera, and entire plant families. Trichome morphology is a key aspect in characterizing the epidermal properties within the taxonomy of the Combretaceae family. Trichomes, as a taxonomic trait, hold significance across various plant groups, ranging from specific to familial levels. Different feather types are specified to highlight distinct sub-genera and sections within the *Jatropha* L. genus [13]. The characteristics of the leaf epidermis are becoming more and more important in solving the current taxonomic disturbances [14, 15].

In the realm of leaf epidermis, anatomical features like stomata, trichomes, and other markers have demonstrated their potential taxonomic significance as valuable anatomical tools [16, 17]. In leaves of dicotyledonous plants, stomata are dispersed, while in monocotyledonous leaves, they are arranged in parallel rows. The quantity of stomata on leaf surfaces varies considerably among distinct plant species, but typically, the lower epidermis of the leaf tends to possess a greater number of stomata compared to the upper surface [18]. Plant stoma patterns and distribution were found to be very useful as diagnostic tools in plant taxonomy and systematic [19]. Trichomes are found on the surfaces of plant organs, serving as outer extensions of living cells that interface initially with the external environment surrounding the plant.

The scientific literature proposes various functions of trichomes, including safeguarding against herbivores and small chewing insects, minimizing transpirational water loss, aiding gaseous exchange in humid conditions, and attracting pollinators [20-22]. The aim of this study was to explore the diversity, taxonomic traits, types, positioning, patterns, and significance of epidermal appendages observed in different families of angiosperm plants.

2. MATERIALS AND METHODS

2.1. Collection and Preservation of Leave Samples

Frequent visits of botanical gardens were carried out in university of agriculture, Faisalabad, to collect leaves specimens of some angiosperm families. In total 30 species of angiosperm plants were considered (Table 1). The leave samples were preserved by labeling them. The leave samples were preserved in anatomical jars, dipping them in 70% methanol solution. The observations and measurements were taken many times in order to ensure accuracy.

2.2. Anatomical Studies

The preserved samples as well as fresh leaves were used for anatomical features studies. For the study of different anatomical features light microscope was used. Magnifier 10X was used for observation of epidermal peels of leave specimens. The epidermis of the leaves specimens was peeled-off with the help of scalpel razor blade as its method has proved to be effective and accurate for peeling-off the large number of specimens. The debris on leave was removed by brush. To peel off the abaxial surface the leaf must be placed on the tile with its adaxial surface facing upwards. The adaxial surface was scrapped until the abaxial surface appeared. The epidermal peels were dipped in water for few seconds so as to clear the surface from chloroplasts (traces of greenish material). And then peels were washed with water. The abaxial and adaxial epidermis was removed along with the mesophyll cells by using scalpel blade, until only the epidermis of the leaf remained on the tile. The epidermal peels of scrapped surfaces were placed on the clean glass slides also putting few drops of water and cover the peels with cover slips. The following anatomical Characteristics of both the abaxial and adaxial epidermis were studied during investigation:

- Epidermal cell Length and width
- Stomatal number
- Trichome number & length

2.2.1. Cell length

To measure the length of cells in the abaxial and adaxial epidermis of leaves, fresh leaf samples are collected and the epidermal layers are carefully

Table 1. Trichome analysis from the study of epidermal appendages in some angiosperm families.

Sr. No.	Species	Common name	Family	Trichome type abaxial/adaxial
1	*Hemarocallis fulva* L.	Orange day-lilly	Asphodelaceae	Absent/absent
2	*Pentas lanceolata* (Forssk.) Defiers	Egyptian star cluster	Rubiaceae	Absent/Absent
3	*Chlorophytum comosum* (Thunb.) Jaques.	Spider plant	Asparagaceae	Absent/Present
4	*Alpinia allughas* Retzius.	Black galangal	Zingiberaceae	Absent/Absent
5	*Ravenala medaghas* Sonn.	Traveler tree, Traveler palm	Strelitzaceae	Absent/Absent
6	*Strelitzia reginae* Banks.	Crane flower, Bird of Paradise	Strelitzaceae	Absent/Present
7	*Gardenia floribunda* L.	Cape jasmine	Rubiaceae	Present/T-shaped
8	*Rhondelatia adorata* Jacq.	Cleveland	Rubiaceae	Absent/ Unicellular
9	*Campsis rodicum* (L.) Seem. ex-Bureau.	Trumpet vine	Egnoniaceae	Absent/ Hair clusters
10	*Campsis grandiflora* (Thunb.) K. Schum.	Chinensis trumpet vine	Egnoniaceae	Absent/Absent
11	*Cana indica* L.	Wild cana lilly	Cannaceae	Absent/Absent
12	*Colocasia esculentum* (L) Schott.	Elephant ears	Araceae	Absent/Present
13	*Cricum asiaticum* Linn.	Giant Cricum asiaticum	Amaryllidaceae	Present/Present
14	*Barlaria cristata* L.	Philippine violet	Acanthaceae	Absent/Absent
15	*Terminallia bellirica* (Gaertn.) Roxb.	Bastard myrobalan	Combretaceae	Unicellular/Unicellular
16	*Morus leavigata* Wall.	Mulberries	Moraceae	Absent/Absent
17	*Schizinthus terebunthifolus* Raddi.	Brazilian pepper tree	Anacardaceae	Present /T-shaped
18	*Litchi chinensis* Sonn.	Lychees	Sapindaceae	Absent/Absent
19	*Kigela Africana* (Lam.) Benth.	Sausage	Bignoniaceae	Absent/Unicellular
20	*Tamarindis indica* L.	Tamarind	Fabaceae	Short branched/present
21	*Artocarpous lakucha* Roxb.	Monkey fruit	Moraceae	Present/present
22	*Erthrina herbaca* L.	Cherokee bean	Phylanthaceae	Short-branched/ dendritic
23	*Hematoxylon campechianum* L.	Logwood tree	Fabaceae	Absent/Absent
24	*Diospyrose embryopteris* (Desr.) Kostel.	Butter fruit	Enabaceae	absent /Hair-clusters
25	*Annona squamosal* L.	Sugar apple	Annonaceae	Absent/ Absent
26	*Lawsonia innermis* L.	Henna	Lythraceae	Absent/Absent
27	*Ficus netalensis*Hochst.	Banyan	Moraceae	Absent/Absent
28	*Ficus lyrata* Warb.	Fiddle leaf fig	Moraceae	Absent/Short branches
29	*Ficus bengalensis* L.	Natal fig matuba	Moraceae	Absent/Absent
30	*Ficus elastic* (Roxb.) Hornem.	Rubber fig	Moraceae	Absent/Absent

peeled off. The epidermal strips are cleared using a 10% NaOH solution or a commercial clearing agent, then stained with 0.1% toluidine blue to enhance cell visibility. The stained samples are mounted on glass slides and observed under a light microscope at 40x magnification. Digital images are captured and analyzed using image analysis which is calibrated with a micrometer scale for accurate measurements. A statistically significant number of cells are measured from multiple fields of view on both the abaxial and adaxial surfaces. The average cell length and variability are then determined through statistical analysis [23, 24].

2.2.2. Stomatal number and length

To identify and calculate the number of stomata, fresh leaf samples are collected and cleared using a 10% NaOH solution, then neutralized in 5% acetic acid. The cleared samples are stained with 0.1% toluidine blue to highlight the stomatal structures. The stained leaf samples are mounted on glass slides and observed under a light microscope at 40x or higher magnification. Images are captured using a digital camera attached to the microscope. Stomatal density is calculated by counting the number of stomata in a defined area (e.g., 0.1 mm^2) and averaging the counts from multiple fields of view, expressed as stomata per square millimeter. Stomatal size is measured using the software's measurement tool, and statistical analysis is performed to determine the average size [25].

2.2.3. Trichome number and length

Fresh leaf samples were collected carefully. Using a stereomicroscope or light microscope at 10x to 40x magnification, trichomes were observed and counted in a defined area (e.g., 1 cm^2) on both the abaxial and adaxial surfaces. The length of individual trichomes was measured using image analysis software or a calibrated eyepiece. Recent studies have utilized similar methods for accurate trichome analysis [26, 27].

2.3. Statistical Analysis

The data on stomatal, trichome number and length were analyzed using ANOVA to identify significant differences among angiosperm species. The cluster analysis was performed to explore relationships and groupings based on trichome characteristics. The data was then subjected to multivariate (PCA and cluster) analysis to investigate the relationship between different angiosperm species samples collected from new and old botanical gardens of the university of agriculture, Faisalabad, Pakistan.

3. RESULTS

3.1. Comparative Leaf Epidermal Studies

3.1.1. Abaxial epidermal cell length/ width

Maximum cell length/width on abaxial epidermis is recorded in *Strelitzia reginae* and *Terminalia bellirica* which is closely related to the *Alpinia allughas, Crinum asiaticum*. Minimum cell length on upper epidermis is recorded in *Ficus lyrata* which is closely related to the *Colocasia esculentum* (L). Schott (Figure 1(a)).

3.1.2. Adaxial epidermal cell length/width

Maximum cell width is recorded in *Lawsonia innermis* and *Ficus netalensis* L., and while other shows little variations in cell width. Minimum cell width on abaxial epidermis is recorded in *Gardenia floribunda* L. and *Ficus lyrata* Warb (Figure 1(b)).

3.1.3. Abaxial epidermal stomatal length

Maximum stomatal length is recorded in *Gardenia floribunda, Campsis rodicum.* Minimum stomatal length is recorded in *Terminelia bellirica* and *Ficus lyrata*.

3.1.4. Adaxial epidermal stomata length

Maximum stomatal length is recorded in *Ravenala medaghas cariensis* and *Litchi chinensis.* Minimum stomatal length is recorded in *Diospyros embryopteris* and *Rhondolatia adorata.*

3.1.5. Abaxial number of stomata

Maximum number of stomata is observed in *Kigela africana.* Minimum number of stomata is recorded in *Terminalia bellirica* and *Ficus lyrata* (Figure 2(a)).

3.1.6. Adaxial number of stomata

Maximum number of stomata on adaxial epidermis

is recorded in *Pentas lanceolata.* Minimum number of stomata is recorded in *Ravenala medaghas cariensis* (Figure 2(b)).

3.1.7. Abaxial trichome length

Maximum length of trichome on abaxial epidermis is recorded in *Tamarindis indica* and *Terminallia bellirica*. Minimum length is observed in *Gardenia floribunda* (Figure 3(a)).

3.1.8. Adaxial trichome length

Maximum trichome length is recorded in *Tamarindis indica* and *Colocasia esculentum*. Minimum trichome length is recorded in *Chlorophytum comosum* and *Gardenia floribunda* (Figure 3(b)).

3.1.9. Abaxial number of trichomes

Maximum number of trichomes is recorded in *Terminallia bellirica and Schizinthus terebunthifolus* and while minimum number is recorded in *Gardenia floribunda* (Figure 4(a)).

3.1.10. Adaxial number of trichomes

Maximum number of trichomes is recorded *Terminallia bellirica and Kigela africana.* Minimum number is recorded in *Campsis rodicum* (Figure 4(b)).

Dendogram is clustered into two main groups first group contains 15 species and second group contains 15 species. Both groups are further subclustered in two more groups. In first group *Tamarindis indica and Erthrina herbaca shows more similarity as they are close to cluster.* In second group *Hemamatoxylon campechianum and Ficus netalensis* more similarity as they are close to each other.

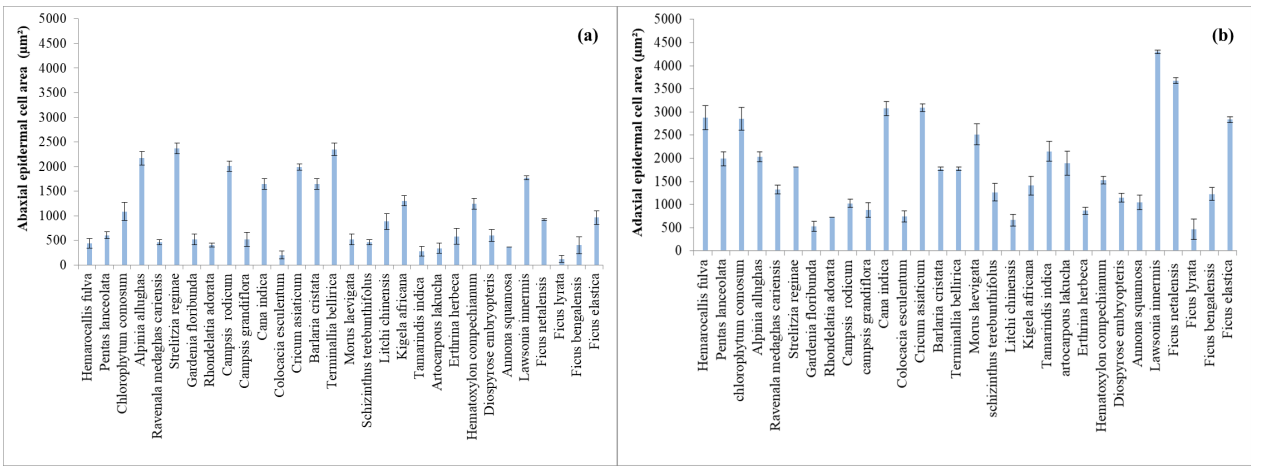

Fig. 1. (a) Abaxial Epidermal cell areas (µm²) from the study of epidermal appendages in some angiosperm families and **(b)** Adaxial epidermal cell areas (µm²) from the study of epidermal appendages in some angiosperm families.

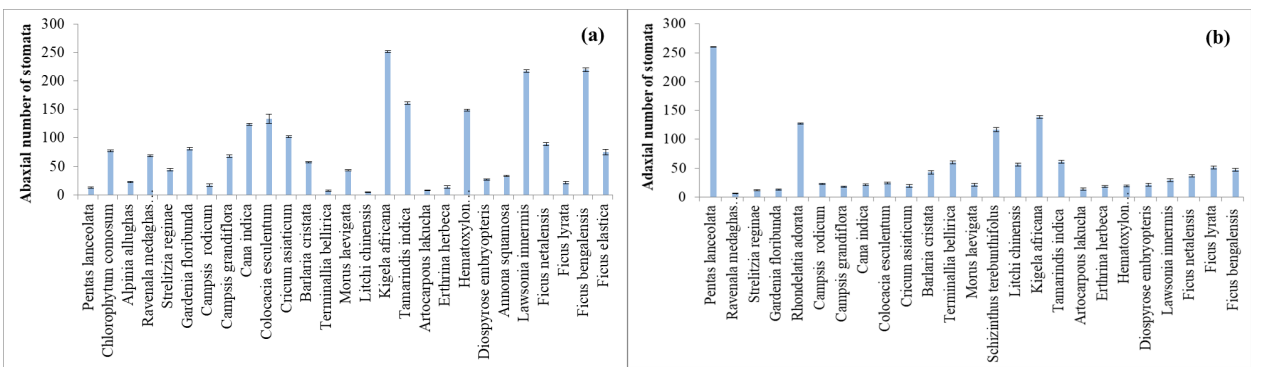

Fig. 2. (a) Abaxial stomata number from the study of epidermal appendages in some angiosperm families and **(b)** Adaxial stomata number from the study of epidermal appendages in some angiosperm families.

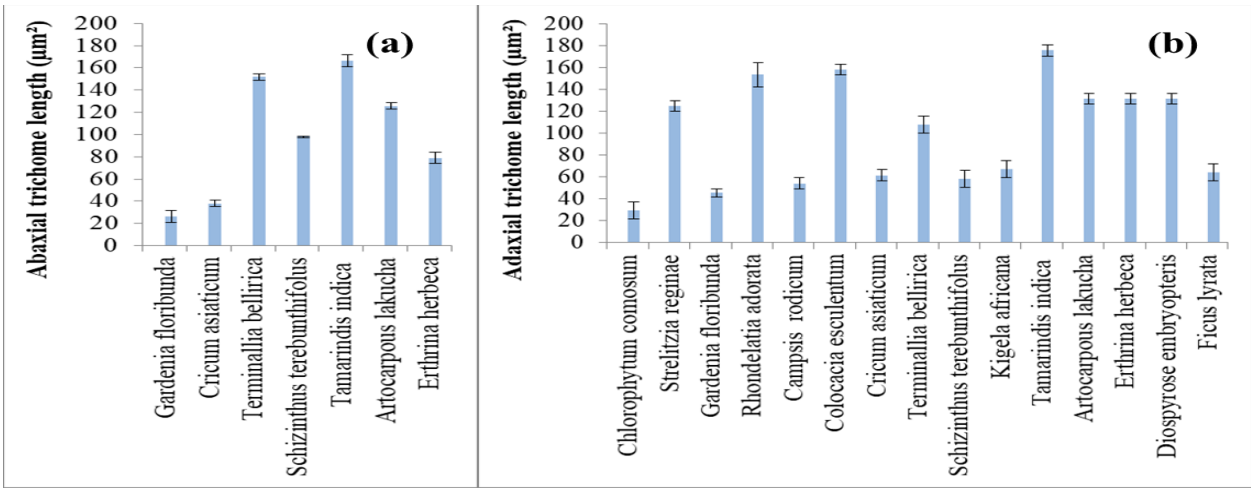

Fig. 3. (a) Abaxial trichome lengths (µm²) from the study of epidermal appendages in some angiosperm families and **(b)** Adaxial trichome lengths (µm²) from the study of epidermal appendages in some angiosperm families.

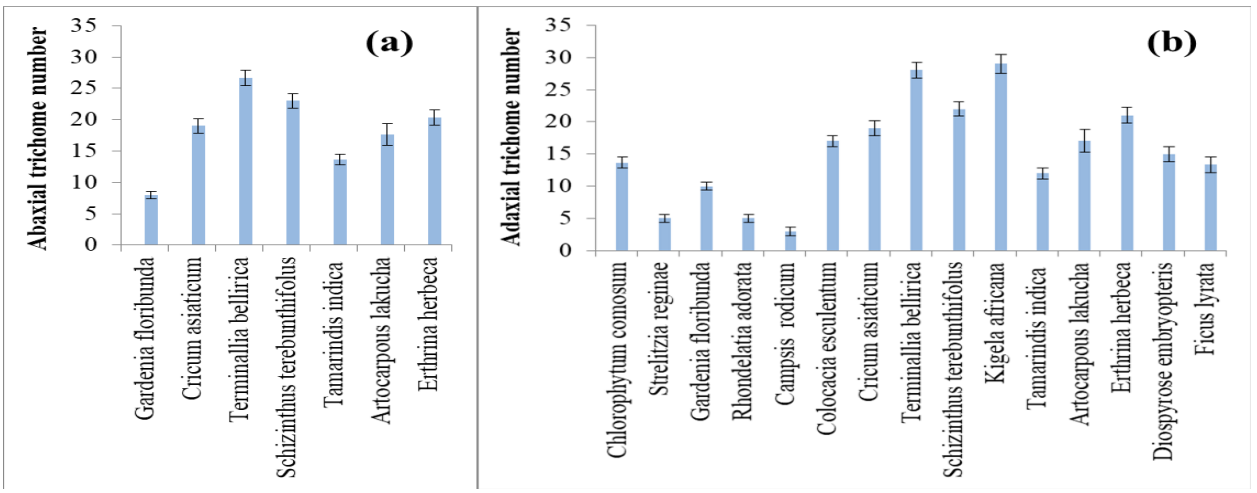

Fig. 4. (a) Abaxial trichome number from the study of epidermal appendages in some angiosperm families and **(b)** Adaxial trichome number from the study of epidermal appendages in some angiosperm families.

4. DISCUSSION

The ANOVA results in Table 2, demonstrated significant differences in trichome characteristics across the studied parameters. For abaxial trichome length (µm²), the species factor was highly significant (F = 11.71), with a mean square (MS) of 5451.60, indicating substantial interspecies variation. The standard error (SE = 12.46) reflects reliable measurements. Similarly, for adaxial trichome length (µm²), the species effect was pronounced (F = 21.85, MS = 8327.08, SE = 11.27), highlighting its dependence. For abaxial trichome number, the species factor was non-significant (F = 0.0003), suggesting uniformity across species. In contrast, adaxial trichome number showed significant variation among species (F = 4.56), indicating a potential role in species differentiation or adaptive mechanisms. The larger SE values associated with trichome numbers may reflect higher variability in environmental influence or sampling differences. These findings suggest that trichome length is a more consistent trait compared to trichome number, which may be influenced by external factors. Studies on the epidermal surfaces revealed a number of important micro-morphological characters and revealed interesting specific differences that are important for the identification of these characters. There are many changes in the smooth and glabrata species. For example, the highest and lowest stomatal length/ width (L/W) ratios were found among species. Taxonomic studies of epidermal appendages of angiosperm families' species revealed interesting

Table 2. Analysis of variance (ANOVA) abaxial and adaxial trichome length and number respectively.

Sr. no.	ANOVA	SOV	DF	SS	MS	F-ratio	SE
1.	Abaxial trichome length (µm²)	Species	29	158096.5088	5451.603752	11.71024378	12.45714574
		Error	60	27932.4864	465.54144	--	--
		Total	89	186028.9952	--	--	--
2.	Adaxial trichome length (µm²)	Species	29	241485.5534	8327.08805	21.84841472	11.27135129
		Error	60	22867.8048	381.13008	--	--
		Total	89	264353.3582	--	--	--
3.	Abaxial trichome number	Species	29	1868.377778	0.626682667	0.000289327	722
		Error	60	2226	2166	--	--
		Total	89	357.6222222	--	--	--
4.	Adaxial trichome number	Species	29	8016.233333	1.292920007	4.55692	9457.555556
		Error	60	28432.66667	28372.66667	--	--
		Total	89	36448.9	--	--	--

SOV: Source of variable, **DF:** Degree of freedom**, SS:** Sum of square, **MS:** Means of square**, F-ratio:** F- ratio, **SE:** Standard error

significant results on observing the epidermis of the leaves and comparative leaf studies by comparing the characters of leaf surfaces of the angiosperm plant species (see Table 1). Epidermis of leaf cells having different length/width ratios most of the specie's epidermis lack stomata or less density of stomata and trichomes. Herewith micro-hairs were often present but most probably on the leaf surface not on observing epidermis of the leaf. Thirty species were observed here with from *Hemarocallus fulva* to *Ficus elastica*. Epidermal glands are important in relative research in angiosperms arising from epidermal cells [28]. Glandular characters were observed on studying the leaf surfaces of angiosperm plant species. Clearly found glands on the surfaces of leaf *Chlorophytum comosum* showed clear glands on the adaxial surface of leaf but on adaxial and abaxial surface clear glands were observed in *Gardenia floribunda.* Trichomes can vary widely in families and smaller plant groups and even in the same plant.

On the other hand, trichomes within a group of plants sometimes have significant uniformity. Plant hair types have been used successfully in the classification of genera and even in some families and in the identification of specific hybrids [29]. Observational and comparative studies of leaf surfaces of angiosperm families were observed and trichomes were present on the leaf surface. Unicellular, multicellular, hair-clusters, y-shaped, t-shaped, dendrites, with short branches, and gland-headed leaf epidermal features are sometimes influenced by environmental conditions [30, 31]. There is substantial evidence for their overall genetic control [32-34]. As such, they have been employed for plant species discrimination at various taxonomic ranks [35]. *Ficus lyrata* sword is bifacial and hypostomatic. The upper epidermal (adaxial epidermis layer) cells are iso-diametric to the lower rectangle according to the shape and are covered with a visible soft flat cuticle. This is followed by a sub epidermal cell layer - hypodermis - followed by lithocysts (12 m length and 12 m width) formed by large cells found here and giant epidermal cells protruding into the mesophyll. The function of these facialized cells is unknown. Inside the lithocysts, long solitary systolites (which accumulate calcium carbonate) are found suspended from a left arm (attached to the top of the lithocysts) [36]. On the other hand, *Ficus lyrata* is fiddle leaf fig belongs to Moraceae family. The present study gives the Abaxial and adaxial surfaces with epidermal appendages that unicellular, short

branched trichomes appears on the adaxial surface. The comparative study showed the distinguished characters of leaf of *Ficus lyrata*. The leaf blade has elliptical shape, which means distribution or regulation of a vascular system, such as in the wing or leaf of an insect. Vascular patterns in insect wings are often used to identify and distinguish species. The venation pattern is observed as secondary veins branched towards margins. The leaf margins are lobed. Apex of the leaf is emarginated and base is obtuse. On the abaxial surface there are young and permanent old trichome bases consisting of one or three surrounding cells with one or less circular rings in the middle and *M. oppositifolius* var. pubescens; however, trichome bases were recorded only on the adaxial surface of the genus. The hair base as a character has been shown to be taxonomically useful in distinguishing these characters from *Mallotus* [37]. On both the surfaces of leaf epidermis and leaf area trichomes were observed *Terminallia bellirica* and *Tamarindis indica* have the maximum number of trichomes and the minimum number is recorded in *Crinum asiaticum*. On adaxial surface the maximum number of trichome is recorded. *Terminallia bellirica* and *Schizinthus terebunthifolus, Kigela africana*. The minimum number is recorded in *Campsis rodicum*.

5. CONCLUSIONS

The present study provides detailed insights into the epidermal characteristics crucial for taxonomy and physiological understanding. Analysis revealed significant variations in epidermal traits such as cell length, stomatal density, and types of epidermal appendages like trichomes. Species-specific distinctions were observed, such as *Pentas lanceolata* showing maximum abaxial cell length and *Ficus elastica* exhibiting minimum upper epidermal cell length. *Strelitzia reginae* displayed the lowest stomatal count on the abaxial surface, contrasting with species like *Crinum asiaticum* and *Morus leavigata*, which showed higher stomatal densities. ANOVA and cluster analysis provided statistical validation of these distinctions, highlighting relationships among species based on their epidermal features. These findings contribute foundational knowledge for future studies in plant taxonomy, physiology, and ethnobotany, emphasizing the importance of epidermal characteristics in plant classification and ecological adaptation.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Bioinformatics Analysis of a 4bp Homozygous Deletion Mutation of EDAR Gene Identified as an Important Cause of Hypohidrotic Ectodermal Dysplasia in Pakistan

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Abstract: Hypohidrotic Ectodermal Dysplasia (HED) is a rare congenital disorder characterized by reduced hair, the absence of sweat glands, dental anomalies, and craniofacial malformations. This condition can be inherited through X-linked, autosomal recessive, or autosomal dominant modes of inheritance. Mutations in four specific genes (EDAR, EDA, WNT10A, and EDARADD) are known to cause HED. In this study for the identification of pathogenic mutations in a consanguineous Pakistani family with the autosomal recessive form of HED, microsatellite markers were utilized to genotype the known loci associated with the disease. The condition in this family was mapped to the EDAR gene locus located on chromosome 2q11-q13. Upon screening the EDAR gene, a novel homozygous 4bp deletion (718delAAGA) in exon 8 was identified, which segregated with the disease phenotype. This 4bp deletion in the EDAR gene results in a frameshift and early termination of translation, producing a truncated protein of 245 amino acids instead of the normal length of 539 amino acids. Various bioinformatics tools were employed to analyze the pathogenic mutation linked to a significant number of HED cases in Pakistan. I-TASSER was used to model the protein structure, CASTp facilitated the identification of various pockets, and STiTCH 3.1 determined EDA to be the ligand for EDAR. Docking analysis were conducted for both the mutant and wild-type EDAR proteins with EDA, revealing notable differences in the interaction sites between the docked complexes of the normal and mutant forms of EDAR with the ligand EDA. These analyses provided insights into the protein's structural features, active sites, interactions, and the overall impact of the mutation.

Keywords: HED, EDAR, Congenital Disorder, Ectodermal Dysplasia, Homozygous Deletion.

1. INTRODUCTION

The incidence of Ectodermal Dysplasia (ED), a rare congenital disorder affecting at least two or more ectodermal tissues (skin, teeth, hair, sweat glands, and nails), is approximately seven instances per 10,000 people [1]. The most prevalent kind of ED in humans, known as Hypohidrotic Ectodermal Dysplasia (HED), is characterized by faulty tooth, sweat gland, and hair development [2]. Most sufferers of HED have fewer or ineffective sweat glands, which reduces their capacity to sweat. The body regulates its temperature in part through sweat, which cools the body as it evaporates off the skin. In hot conditions, an inability to sweat can result in hyperthermia, which can have life-threatening health effects. HED is a Mendelian disorder and can be inherited as autosomal recessive, autosomal dominant and X-linked recessive trait. Mutations of four genes, namely Ectodysplasin A (EDA); Ectodysplasin A receptor (EDAR); ectodysplasin A receptor-associated death domain (EDARADD)

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and WNT10A are responsible for causing HED. The four genes mentioned account for approximately 90% of the HED cases [3, 4]. A recurrent missense mutation (p.W434R) in exon 12 of EDAR gene has been identified in two consanguineous Kashmiri families [5]. The presence of a mutation at position 398 in the EDAR gene leads to a significant decrease in its binding capacity to EDARADD, resulting in the development of HED [6].

Here, in this study, we ascertained a consanguineous family demonstrating autosomal recessive inheritance of HED. By genetic linkage analysis with microsatellite markers for the known HED loci, it was mapped to EDAR locus on chromosome 2q11-q13. The NF-kB activating member of the tumor necrosis factor receptor family is encoded by the EDAR gene. The transmembrane receptor for the soluble ligand ectodysplasin A is the encoded protein. It is a component of a signaling pathway that is crucial for interactions between the ectoderm and mesoderm, two embryonic cell layers, and is necessary for skin formation before birth. The connections between the embryonic cell layers are crucial for the development of various ectoderm-derived features, including as the skin, hair, nails, teeth, and sweat glands [7-9]. The mutation screening of the EDAR gene revealed a homozygous 4bp deletion (718delAAGA) associated with the disease in our HED family. This deletion mutation induces a frameshift that leads to premature termination of the protein product. Consequently, the truncated protein is composed of 245 amino acids, compared to the normal 539 amino acids. This specific deletion has also been documented in other Pakistani families, resulting in a similar HED phenotype, and appears to be a significant contributor to HED in Pakistan [10]. Given its importance as one of the common causes of recessively inherited HED, this deletion mutation has been characterized using various bioinformatics tools to establish the relationship between protein structure and function, as well as to conduct active site analysis, interaction studies, and protein-protein docking analysis.

2. MATERIALS AND METHODS

2.1. Sample Collection

A consanguineous Pakistani family (Figure 1A) from the rural Sindh province (specifically Sukkur city) was selected for this study due to their diagnosis of autosomal recessive HED. This family had not previously been included in any studies. Ethical approval was obtained from the Institutional Review Board, and informed consent was secured from the patients, family members, and control individuals before sample collection. An experienced dermatologist conducted clinical examinations of the affected individuals at a local hospital. All affected subjects exhibited the hallmark clinical signs and symptoms of HED, including thin, dry, light-colored, and brittle skin,

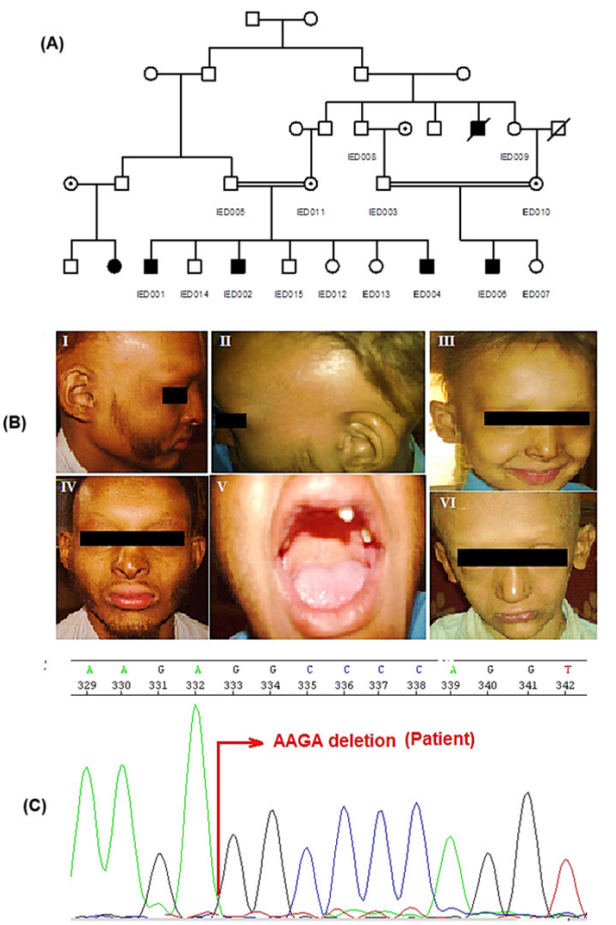

Fig. 1. (A) The pedigree of a Pakistani family affected by the autosomal recessive form of HED is depicted. Affected males and females are represented by filled squares and circles, respectively, while unaffected individuals are shown with open symbols. The study included individuals with Lab I.D. numbers, and double lines between symbols indicate consanguineous marriages. (B) Clinical observations of HED are displayed. Patients (I-IV & VI) exhibit characteristics such as fine scalp hair, absent eyebrows and eyelashes, a flattened nose, prominent lips, hyperpigmentation, and periorbital wrinkling. One of the affected family members (V) shows permanent conical teeth. (C) An electropherogram of a patient illustrates a homozygous 4bp deletion (del718AAGA) in exon 8 of EDAR gene.

a limited number of deformed teeth, decreased sweating capacity, periorbital wrinkles, and hyperpigmentation. Furthermore, the patients presented facial characteristics such as a prominent forehead, thick lips, and a flattened nasal bridge (Figure 1B). Family members seldom enter into consanguineous relationships, as they rarely marry outside the family.

2.2. Extraction of Genomic DNA

Approximately 5cc venous blood samples were obtained from the affected and normal family members. Genomic DNA was isolated from peripheral blood following a standard organic method of DNA extraction from whole blood [11]. DNA was quantified by spectrophotometry, by measuring absorbance of optical density at 260 nm and dilution of 40ng/μL for each sample was prepared for PCR amplification.

2.3. Genotyping

The HED disease phenotype in family was tested for linkage by using microsatellite markers tightly linked the known loci (Table 1). The genomic DNA from each available family member was amplified through PCR in a 25 μL reaction volume. The PCR reaction mixture consisted of 40 ng genomic DNA, 200 mM of each dNTP, 20 pmol of each primer, 1X PCR buffer and 1 U of Taq DNA polymerase. The PCR amplification was conducted for 35 cycles using the following conditions: initial denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. A final extension step at 72°C for 7 minutes was performed using a thermal cycler 9600. The amplified PCR products were separated and visualized on an 8% non-denaturing polyacrylamide gel under UV-illumination, and the genotypes were assigned and recorded.

2.4. Mutation Screening of EDAR Gene

Primers were designed from intronic sequences of the EDAR gene to facilitate PCR amplification of all protein-coding exons (2–12) and exon/intron splice junctions. Genomic DNA was used as the template in a 30 litres reaction volume to screen for mutations. The ACCuPrep® PCR purification kit (Bioneer corporation, Seoul, Korea) was used to purify the PCR products, and the BigDye ver 3.1 Cycle Sequencing Kit was used to sequence them in the Applied Biosystems 3130 Genetic Analyzer (Life technologies, USA).

2.5. Bioinformatics Analysis

2.5.1. Sequence retrieval

The EDAR gene consists of 9 exons, and it encodes a protein with 539 amino acids. The gene's sequence was retrieved from the OMIM database using the MIM number 604095.

2.5.2. Structure modeling

An online technique, PSIPRED, was used to estimate the secondary structure of the EDAR protein McGuffin *et al*. [12]. The threading technique through I-Tasser [13, 14] was used to construct three-dimensional (3D) structures. Using RAMPAGE server [15], protein 3D models (wild and mutant) were assessed.

Table 1. Known Loci for ectodermal dysplasia.

Locus I.D. No.	Gene	Chromosomal location	Reported markers in the region	Markers available and analyzed
ED1	EDA	Xq12-q13	DXS7159	DXS7132, DXS6800
ED2	EDAR	2q11-q13	D2S1890 D2S2954 D2S1888	D2S436, D2S410
ED3	EDARDD	1q42.2-q43	D1S2680 D1S2850 D1S2678	D1S235, D1S547, D1S1609
ED4	WNT10A	18q22.1-18q22.3	D18S857-D18S815	D18S858 ATA7D07, ATA82B02

2.5.3. Pocket identification

Protein pockets were detected using Computed Atlas of Surface Topography of proteins (CASTp) [16].

2.5.4. Protein-protein docking

Using the STITCH3.1 database [17], the framework of protein interactions was examined. Using the PatchDock server [18, 19], protein-protein docking analysis was performed. After being collected, the first 10 docked complexes were sent to the FireDock server for improvement [20, 21]. Version 5.0 of the ViewerLite programme was used to display docked protein and ligand complexes. After the docking process, the interactions and 2D representations of protein-ligand complexes were analyzed using LIGPLOT [22].

3. RESULTS AND DISCUSSION

3.1. Genotyping and Mutation Screening

Genotyping using microsatellite markers for the known loci showed linkage of the family to EDAR gene locus on chromosome 2q11-q13 (Figure 1A). On subsequent sequencing and mutation screening in 12 exons and splice site junctions of EDAR gene revealed a homozygous 4bp deletion (718delAAGA) mutation in exon 8 associated with the disease in our family (Figure 1B). The EDAR mutation, specifically 718delAAGA, was found to be inherited recessively in all affected family members. However, in the obligate carriers within the family, the mutation was present in a heterozygous state. The disease-association of the mutation was further confirmed by analyzing the ethnically matched 100 control samples for 4bp deletion of EDAR. None of the control sample had this change.

In individuals with HED, the EDAR gene has been found to include over 20 different mutations. While deletion mutations of the EDAR gene have been described, the majority of these variants impact only a single amino acid residue within the receptor protein. A small number of EDAR mutations lead to the creation of an atypical ectodysplasin A receptor. These genetic alterations can disrupt the signaling pathways essential for the development of ectodermal structures such as hair follicles and sweat glands. This class of mutation results in the autosomal dominant form of HED, where each cell possesses one copy of the mutated gene. Conversely, additional mutations in the EDAR gene can completely inhibit the production of ectodysplasin A receptor protein. This inactivity of the receptor prevents the initiation of vital chemical signals necessary for ectoderm-mesoderm interactions and the proper development of ectodermal structures. When such mutations affect both copies of the EDAR gene in a cell, it leads to an autosomal recessive form of HED. A related study also identified the same mutation in the EDAR gene [23], but our research included an extensive analysis using various bioinformatics tools to characterize the differing outcomes of such mutations on the disease manifestation associated with a deletion mutation identified in Pakistani families.

3.2. Structure Prediction and Evaluation

Based on the PSIPRED results for protein secondary structure prediction (Figure 2), it is anticipated that the standard EDAR structure comprises 10 helices, 4 strands, and 14 coils. The number of helixes and coils in a mutant EDAR are altered and change to 2 and 7, respectively. There are still the same amount of strands. The size of the protein is impacted by frame shift mutation, which also affects the amount of structural characteristics.

I-Tasser was utilised to estimate the 3D structure of the normal and mutant EDAR using a threading technique. Helixes, beta sheets, and coils can be seen in the formations in Figure 3. Remains in favoured, allowed, and outlier regions have been identified by evaluation of these projected

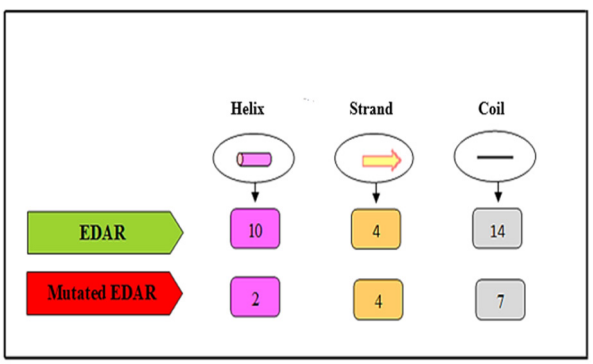

Fig. 2. A comparison of the PSIPRED results for the normal and mutated EDAR reveals the predicted number of structural features for both structures.

structures. For normal and mutant EDAR, the favoured, allowed, and outlier residue percentages are 334 (74.9%), 82 (18.4%), 30 (6.7%), and 161 (66.3%), 61 (25.1%), and 21 (8.6%), respectively. For both predicted structures, it is evident that a significant proportion of residues falls within the favored region, indicating the reliability of the predicted structures. Second, the difference in the amount of residues in favoured, allowed, and outlier regions demonstrates the difference between the two structures (wild and mutant). Protein function as well as size, structure, and conformation are all impacted by mutations, which results in a sick condition.

3.3. Analysis of Active Sites

The active site in proteins is typically a hydrophobic region with side chain atoms. For the purpose of creating drugs and predicting 3D structures, it is useful to identify compounds that bind to target proteins. Additionally, since these pockets contain particular amino acids, their prediction is helpful for mutational research. Using CASTp, a total of 59 pockets for normal EDAR and 20 for mutant EDAR have been located. Mutation effected structure of protein and its active sites. The mutation causes the changes in the size, structure, conformation, active sites of the protein. All these changes ultimately affect the normal gene function.

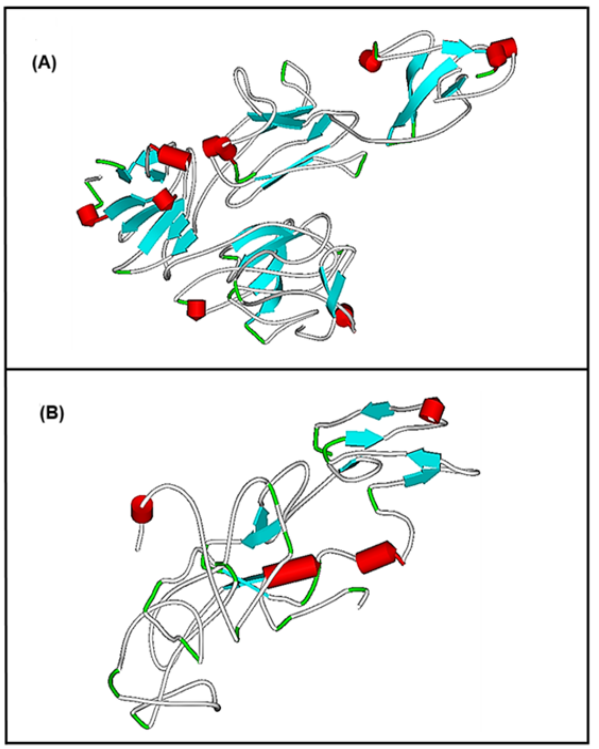

Fig. 3. The 3D structure prediction of both (A) Human EDAR and the (B) Mutated EDAR was performed using I-Tasser. The structures were displayed in a Schematic display style through ViewerLite v5.0.

3.4. Protein-Protein Docking

The EDAR functional partners were predicted using the Stitch3.1 server. Figure 4 displays predicted functional partners for EDAR gene. The protein that exhibited the highest interaction score (0.998) with EDAR was selected for further analysis and considered as the protein ligand, identified as EDA.EDA plays a role in epithelial-mesenchymal signalling during the development of ectodermal organs. To explore the docking procedure as well as the impact of mutation, this protein ligand was docked with both the normal and mutant structures of EDAR. The docking complex of the EDAR receptor and EDA ligand is shown in Figure 5(A), while the docking complex of the mutant EDAR receptor and EDA ligand is shown in Figure 5(B).

The EDAR wild type structure is altered by mutation, becoming a mutant structure that is smaller than usual. Change in the structure also affects the conformation, which affects the protein's interaction sites (i.e. active sites). The receptor protein's active site is where ligands engage and bind. The ligand interaction also alters when a wild type structure is altered because the active site changes. Table 2 presents the docking results, highlighting the receptor/ligand residues engaged in the interaction of both normal and mutant forms of EDAR with the ligand EDA. The docking contact involves certain residues on both the ligand and the receptor.

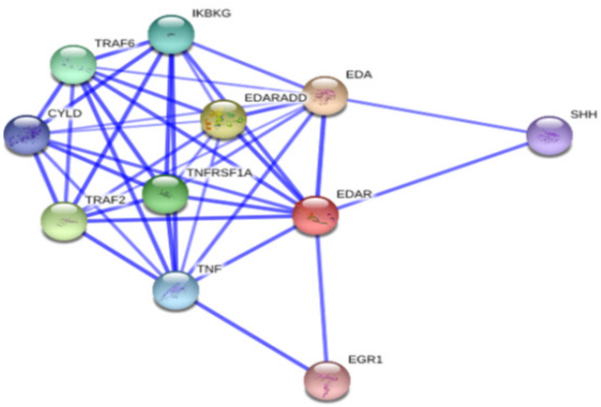

Fig. 4. Using the STITCH3.1 server, the predicted functional partners of EDAR were identified. Among these partners, EDA showed the highest interaction score with EDAR, which was 0.998.

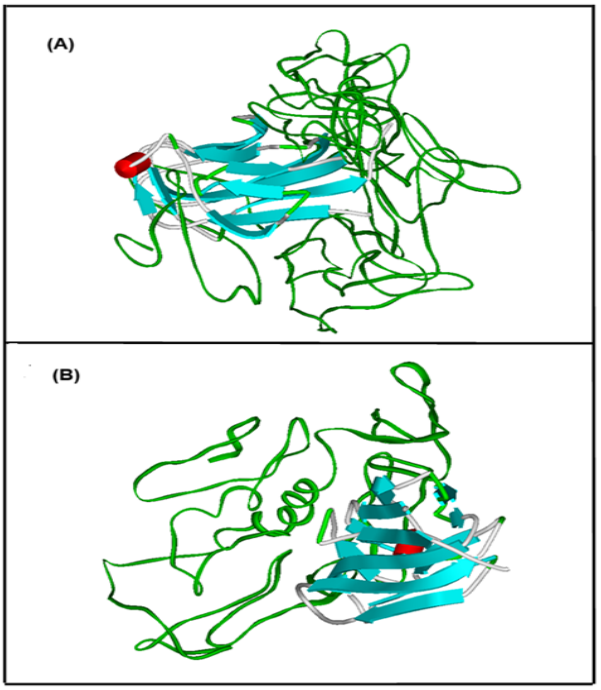

Fig. 5. Visualization of Docking Results using ViewerLite: (A) EDAR with EDR, (B) Mutated EDAR with EDR. Receptor: Green Color, Solid Ribbon display style, Ligand: Color by secondary type and Schematic display style.

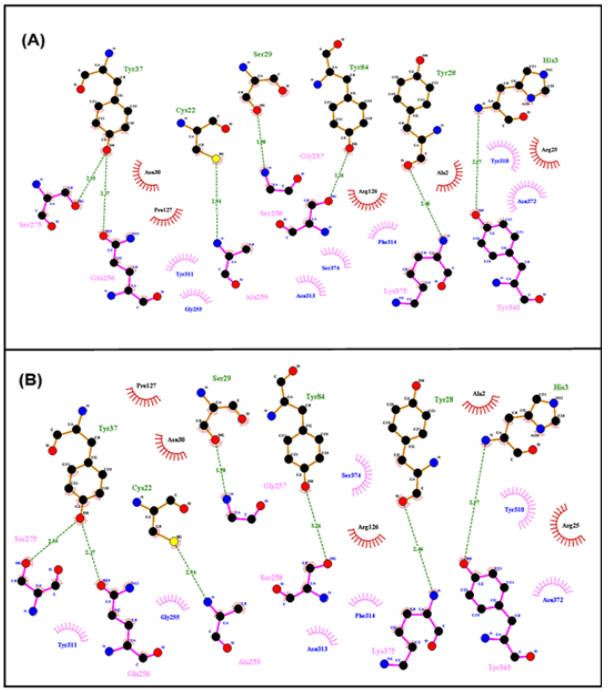

Fig. 6. Dimplot Results for Docking Interactions; a) Normal EDAR with EDA, b) Mutated EDAR with EDA. Ligand Residues Involved in Hydrophobic Interactions are shown in blue color and represented by pink spoked arcs ( ). Receptor Residues Involved in Hydrophobic Interactions are shown in black color and represented by brick red spoked arcs ( ). Green dotted lines (.....) show Hydrogen Bonding. Receptor residues involved in H-Bonding are shown in Olive Green Color. Ligand residues involved in H-Bonding are shown in pink color.

Non-covalent interactions, such as hydrogen bonds and hydrophobic interactions, play a crucial role in facilitating the attachment of ligand/receptor residues. The docking analysis revealed the specific residues of both the receptor and the ligand that participated in hydrogen bonding and hydrophobic interactions, as listed in Table 2. The results indicate significant differences in the interaction locations between the docked complexes of normal and mutant EDAR with the ligand EDA. The amino acids involved in these interactions at the binding site of the receptor protein provide valuable insights into the disparities in the interaction sites. Figure 6

Table 2: Receptor (EDAR) and ligand (EDA) residues involved in interactions.

Receptor-ligand	Hydrogen bond interactions		Hydrophobic interactions		Figure
	Ligand residues	Receptor residues	Ligand residues	Receptor residues	
EDAR-EDA	Gln256, ly257, Ser258, la259, Ser275, yr343, Lys375	His3,Cys22, Tyr28, Ser29, Tyr37, Tyr84	Gly255, Tyr310, Tyr311, Asn313, Phe314, Asn372, Ser374	Ala2, Arg25, Arg126, Pro127, Asn30	5(A)
Mutated EDAR-EDA	Gln256, Gly257, Ser258, Ala259, Ser275, Tyr343, Lys375	His3, Cys22, Tyr28, Ser29, Tyr37, Tyr84,	Gly255, Tyr310, Tyr311, Asn313, Phe314, Asn372, Ser374,	Ala2, Arg25, Asn30, Arg126, Pro127,	5(B)

presents the dimplot data, illustrating the docking interactions between EDAR (normal and mutant) and the ligand EDA. It also assesses the differences between the docking interactions of the normal/ wild type structure of EDAR and its mutant form with EDA ligand in our docking results. Protein size, structure, conformation, and interaction sites have all undergone significant change as a result of mutation. The protein's function is significantly altered as a result of these changes in structure, confirmation, and interaction site, which results in the disease state.

4. CONCLUSIONS

Structure prediction, interaction, and docking analyses of wild-type and mutant EDAR revealed significant structural and functional alterations resulting from a 4-bp homozygous deletion. PSIPRED results indicated a decrease in the number of helices (from 10 to 2) and coils (from 14 to 7) in the mutant EDAR. I-Tasser and RAMPAGE analyses confirmed variations in the three-dimensional structures, highlighting changes in residues in favored, allowed, and outlier regions. CASTp analysis demonstrated a significant reduction in active pockets (from 59 in normal EDAR to 20 in mutant EDAR), suggesting disrupted active sites. Docking studies involving the ligand EDA revealed altered interaction sites alongside hydrogen bonding and hydrophobic interaction changes. These structural and functional modifications compromise protein activity, contributing to disease conditions associated with the mutation.

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6. CONFLICT OF INTEREST

Authors declare no conflict of interest.

7. ETHICAL STATEMENT

The study was approved by the ethical committee of Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan, and Quaid-i-Azam University, Islamabad, Pakistan.

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Research Article

Biomass Carbon Sequestration Potential of Conifers in Relation to Tree Structural Traits and Anthropogenic Disturbance Stimuli in Kashmir Himalaya

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Abstract: It is essential to quantify the amount of carbon stored in the biomass of forest species to determine the potential for mitigating climate change through forest management. This study aimed to estimate the biomass carbon stock (BCS) of coniferous tree species in 16 temperate (TFs) and 4 subalpine forests (SFs) in the state of Azad Jammu and Kashmir (AJK). BCS was calculated for individual trees using allometric equations. The total BCS was 66.5 ± 6 .8 Mg ha^{-1}, with 42.4 ± 7.3 Mg ha^{-1} (63.7%) in TFs and 24.2 ± 4.1 Mg ha^{-1} (36.3%) in SFs. The dominant species, *Pinus wallichiana* A.B. Jacks. and *Picea smithiana* (Wall.) Boiss., had corresponding BCS totals of 22.4 ± 4.6 (33.7%) and 21.7 ± 4.8 Mg ha^{-1} (21.7%), respectively. *Abies pindrow* Royle had a BCS of 14.1 ± 3.8 Mg ha^{-1} (21.2%), while the lowest value of 8.3 ± 1.3 Mg ha^{-1} (15.5%) was found in *Cedrus deodara* (Roxb. ex D. Don) G. Don. TFs showed healthier structural attributes, with higher tree diameter at breast height (DBH) (155.7 ± 8.2 cm) and density (157.1 ± 4.2 trees ha^{-1}) compared to SFs, which had lower DBH (131 ± 7.4 cm) and density (113.9 ± 7.7 trees ha^{-1}). The forests in this region are facing significant deforestation, with 154.0 ± 6.4 stumps ha^{-1} in temperate forests and 48.8 ± 2.8 stumps ha^{-1} in subalpine forests. Statistical analysis revealed a significant correlation between BCS and tree girth, height, and total stem density. This study highlights the allocation trends of BCS among keystone species in a climate-sensitive region and emphasizes the need for forest conservation in the context of climate change.

Keywords: Biomass, Carbon, Conifers, Himalayas, Kashmir, Forest, Regeneration.

1. INTRODUCTION

Carbon dioxide (CO_2) is the most significant anthropogenic greenhouse gas (GHG) contributing to climate change, predominantly emitted through the combustion of fossil fuels, industrial processes, and deforestation.[1]. Climate change has altered the natural structural and functional ecology of the forest ecosystems in the Himalayan region where a radical transformation is observed in natural species composition, forest regeneration and phenological patterns [2, 3]. The outbreak of invasive species, increased risk of species extinction and forest carbon losses are also attributed to climate change [4, 5]. Western Himalayan forests are generally classified into coniferous and broad-leaved forests [6, 7] and provide significant ecological, economic, and aesthetic services including edible seeds, essential oils, resins, flavours and vital medicinal compounds [8]. Coniferous forests are dominant regional carbon sinks and sustain significant biomass and carbon stocks as compared to broad-leaved forests [9].

Ecological and physiological variations among the forest species are attributed to the altitudinal gradient correlated with climatic and non-climatic factors [10, 11]. These diverse ecosystems maintain differential CO_2 sequestration potentials depending on the site temperature, solar

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radiation, precipitation, atmospheric pressure, wind velocity, slope aspect, nutrient availability, growth stage and disturbance regimes [12]. Deforestation for timber, fuelwood and raw materials in these delicate ecosystems causes a remarkable loss of tree cover, biomass and natural carbon stock [13].

Forest conservation for ecosystem balance and carbon management through rehabilitation is of great importance as natural forests play a significant role in mitigating climate change by atmospheric CO_2 sequestration and biomass production [8, 14]. Large trees make a higher amount of biomass and hence they capture more CO_2 in their woody portions as compared to the lower strata. Forest tree species, being a vital sink for ambient CO_2, retain about 50% carbon in their total standing biomass [15].

Analysis of the forest structure and composition is a prerequisite to assessing carbon content in the forest biomass. The species-wise analysis is still deficient and the accurate quantification of carbon in dominant tree species of the Himalayan forests region is required to evaluate climate change mitigation potential [11]. This study is intended to quantify the tree BCS in the tree species belonging to the Pinaceae family in temperate forests (TFs) and subalpine forests (SFs) of the Kashmir Himalayan region. It also aimed to find the relationship between carbon stocks and structural traits (tree density and size) and provide baseline data from the relatively less explored regions for forest conservation, carbon management and policy decisions.

2. MATERIALS AND METHODS

2.1. Study Area

The present study was carried out in the western Himalayan TFs and SFs of Azad Jammu and Kashmir (AJK), Pakistan (Figure 1). The study region is situated between Longitude 73° - 75° and Latitude 33° - 36° (Table 1) having an area of 13,297 km^2, enriched with unique phytodivesity and a greater species indigenousness. TFs and SFs in this region are symbolized with widespread growth of conifers including *Abies pindrow, Cedrus deodara*, *Picea smithiana*, and *Pinus wallichiana* [16, 17].

The area is characterized by mild summers (June to August) with an average temperature of 10-15 °C whereas the temperature falls below 0 °C in the winter season (November-May). The annual rainfall remains about 1500 mm with extreme in the monsoon season (July to August) in some regions whereas the entire study area accepts heavy snowfall during the winter season (November to April). Topographically, the area is steep and mountainous with carved valleys covered with vegetation. Soils are loamy and highly susceptible to erosion due to deforestation, overgrazing, and heavy precipitation [18, 19].

2.2. Sampling Techniques

Field surveys were conducted in the study area during spring 2019-20. Primary data was collected

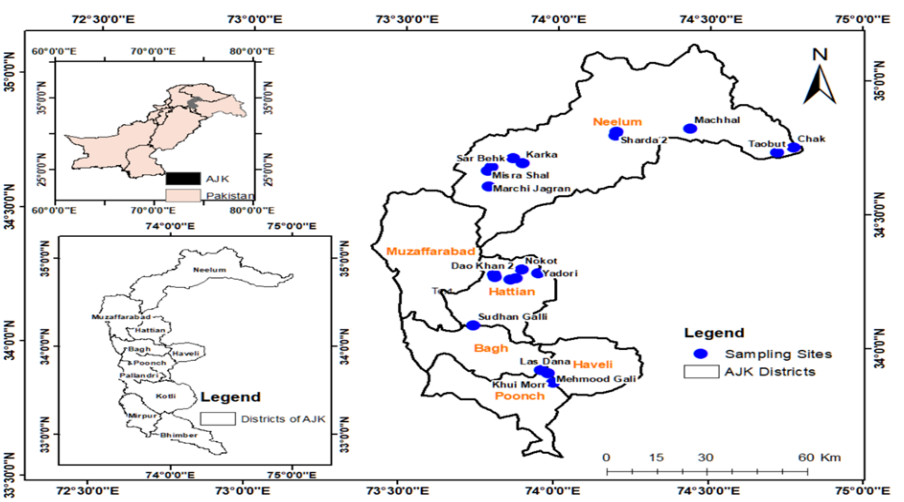

Fig. 1. Map of the study area indicating sampling sites in the temperate and subalpine coniferous forests.

Table 1. Location and characteristics of the studied coniferous forests.

Site No.	Site name	District	Vegetation zone	Location		
				N	E	Elevation (m)
1	Marchi Jagran	Neelum	Temperate	34° 35.061	73° 46.537	1930
2	Sharda 1	Neelum	Temperate	34° 47.886	74° 11.411	1950
3	Machhal	Neelum	Temperate	34° 48.762	74° 25.778	2060
4	Nokot	Hattian	Temperate	34° 17.338	73° 53.337	2071
5	Sharda 2	Neelum	Temperate	34º47.336	74°11.398	2100
6	Sudhan Galli	Bagh	Temperate	34° 04.498	73° 44.184	2185
7	Mehmood Gali	Poonch	Temperate	33º 52.100	73º 59.570	2225
8	Taobut	Neelum	Temperate	34° 43.579	74° 43.210	2286
9	Khui Morr	Haveli	Temperate	33º 54.230	73º 58.531	2375
10	Misra Shal	Neelum	Temperate	34° 39.312	73° 46.404	2458
11	Karka	Neelum	Temperate	34° 41.205	73° 52.841	2480
12	Chak	Neelum	Temperate	34° 45.050	74° 46.311	2502
13	Las Dana	Bagh	Temperate	33º 55.050	73º 57.290	2525
14	Sar Behk	Neelum	Temperate	34° 40.221	73° 46.767	2680
15	Dao Khan 1	Hattian	Temperate	34° 15.488	73° 48.186	2685
16	Dao Khan 2	Hattian	Temperate	34° 16.172	73° 27.15	2700
17	Barthwar Galli Lower	Hattian	Subalpine	34° 15.29	73° 51.821	2825
18	Barthwar Galli Top	Hattian	Subalpine	34°15.130	73° 51.232	2923
19	Bichhkarla Doga	Neelum	Subalpine	34° 41.837	73° 50.892	2984
20	Yadori	Hattian	Subalpine	34° 16.420	73° 56.530	3165

at 20 sites comprising 16 TFs and 4 SFs and in AJK (Table 1). Geographical attributes of the study sites including latitude, longitude and altitude were recorded using a GPS device. The forest sites were classified based on the intensity of anthropogenic disturbances, which included steepness of the terrain, soil erosion, and grazing pressure. The sites were categorized into three disturbance classes: low, moderate, and high. For low disturbance sites (Class 1), the terrain was relatively flat or had a gentle slope, with minimal soil erosion and limited grazing impact. Sites classified as moderate disturbance (Class 2) had moderate slopes, which contributed to some degree of soil erosion, and grazing intensity was more noticeable, affecting vegetation structure. High disturbance sites (Class 3) were characterized by steep slopes, which led to significant soil erosion, and high grazing intensity, resulting in overgrazing and noticeable degradation of vegetation. Deforestation intensity was quantified by counting the number of stumps within each plot. Similarly, seedlings count was used to describe forest regeneration potential. A total of ten square plots of 400 m^2 (20 × 20 m) were established at each forest site for data collection through the stratified random sampling method. Tree diameter at breast height (DBH ≥10 cm) and height (H) values were measured using standard protocols with the help of a conventional measuring tape and digital laser rangefinder respectively [20].

2.3. Forest Biomass and Carbon Stock Assessment

Aboveground tree biomass (AGTB) in living trees including stems, branches, twigs, and leaves was computed after calculation of the growing stock volume density (GSVD) using allometric models [21, 22]. Individual AGTB values were obtained by multiplying GSVD (m^3 ha^{-1}) with the applicable biomass expansion factor (BEF Mg/m^3). The BEF's of *Pinus wallichiana* were considered as 1.68 (for GSVD < 10 m^3 ha^{-1}), 0.95 (for GSVD = 10 – 100 m^3 ha^{-1}) and 0.81 (for GSVD > 100 m^3 ha^{-1}). For other

coniferous trees with GSVD > 200 m^3 ha^{-1}, a BEF value of 1 was used whereas in the case of GSVD ≤ 160 m^3 ha^{-1}, a recommended BEF equation was used [23, 24]. Belowground tree biomass (BGB) of roots in tree species was estimated using recommended equation [25]. AGTB and BGB collectively made total tree biomass (TTB) in all living biomass components [8]. Biomass carbon stock (BCS) was computed by using biomass to carbon conversion factor of 0.50 applied to the obtained biomass value in each tree species [15, 26].

2.4. Data analysis

Multivariate analysis of BCS versus forest structural attributes and disturbance stimuli was carried out through Principal Component Analysis (PCA) using R (v4.4.2) software [27]. Generalized linear models (GLMs) with normal distribution and reciprocal function were applied for bivariate analysis for the calculated dataset. To express the similarities and statistical variations among the BCS pools, overall correlation trends among the biomass carbon stock, forest structural attributes and disturbance stimuli were presented in illustrative and numerical forms. The statistical analysis was performed using Past (v.5.0.2) software [28].

3. RESULTS

3.1. Biomass Carbon Distribution

BCS was computed in four coniferous tree species across the study region. Total BCS was computed as 66.5 ± 6.8 Mg ha^{-1}, from which 50.0 ± 7.1 Mg ha^{-1}C was recorded in the AGTB whereas BGB was recorded as having a total of 16.6 ± 4.6 Mg ha^{-1}C. TFs produced higher carbon content which was quantified as 42.4 ± 7.3 Mg ha^{-1} with respective BCS values of 31.9 ± 4.6 and 10.5 ± 2.5 Mg ha^{-1} in AGTB and BGB portions. SFs ecosystem showed comparatively lower BCS which was totaled as 24.2 ± 4.1 Mg ha^{-1}. Corresponding Carbon stock values in the AGTB and BGB portions in the SFs were recorded as 18.1 ± 4.3 and 6.1 ± 2.1 Mg ha^{-1} (Table 2).

Pinus wallichiana and *Picea smithiana* were perceived as codominant species in the region with respective total BCS values of 22.4 ± 4.6 and 21.7 ± 4.8 Mg ha^{-1} followed by *Abies pindrow* (14.1 ± 3.8 Mg ha^{-1}C). *Cedrus deodara* exhibited the lowest total BCS (8.3 ± 1.3 Mg ha^{-1}) in the studies region. Total BCS in the AGTB components in *Pinus wallichiana* and *Picea smithiana* was 17 ±

Table 2. Above and belowground biomass and carbon stock in the temperate and subalpine coniferous forests.

Forest Type	Species	Aboveground tree biomass (Mg ha^{-1})	Belowground biomass (Mg ha^{-1})	Total tree biomass (Mg ha^{-1})	Aboveground tree biomass carbon (Mg ha^{-1})	Belowground biomass carbon (Mg ha^{-1})	Total tree biomass carbon (Mg ha^{-1})
Temperate	*Abies pindrow*	13.4 ± 3.5	4.5 ± 1.4	17.9 ± 4.3	6.7 ± 1.3	2.3 ± 0.8	8.9 ± 2.4
	Cedrus deodara	12.4 ± 3.7	4.2 ± 1.5	16.6 ± 4.6	6.2 ± 1.8	2.1 ± 0.6	8.3 ± 2.1
	Picea smithiana	20.6 ± 4.2	6.6 ± 1.2	27.2 ± 5.4	10.3 ± 3.7	3.3 ± 0.3	13.6 ± 3.6
	Pinus wallichiana	17.5 ± 4.1	5.6 ± 1.6	23.1 ± 4.3	8.8 ± 2.6	2.8 ± 0.4	11.6 ± 2.5
	Sub total	**63.8 ± 7.4**	**20.9 ± 3.5**	**84.7 ± 11.7**	**31.9 ± 4.6**	**10.5 ± 2.5**	**42.4 ± 7.3**
Subalpine	*Abies pindrow*	7.6 ± 2.3	2.8 ± 0.8	10.4 ± 2.3	3.8 ± 0.6	1.4 ± 0.4	5.2 ± 1.7
	Cedrus deodara	0.0	0.0	0.0	0.0	0.0	0.0
	Picea smithiana	12.1 ± 3.2	4.1 ± 1.1	16.2 ± 4.1	6.1 ± 1.3	2.0 ± 0.3	8.1 ± 1.4
	Pinus wallichiana	16.4 ± 3.1	5.4 ± 1.3	21.8 ± 3.2	8.2 ± 2.4	2.7 ± 0.3	10.9 ± 2.6
	Sub total	**36.1 ± 5.4**	**12.2 ± 3.3**	**48.3 ± 5.4**	**18.1 ± 4.3**	**6.1 ± 2.1**	**24.2 ± 4.1**
BOTH	*Abies pindrow*	21.0 ± 4.8	7.3 ± 2.4	28.2 ± 3.3	10.5 ± 2.1	3.6 ± 1.3	14.1 ± 3.8
	Cedrus deodara	12.4 ± 1.4	4.2 ± 1.1	16.6 ± 3.2	6.2 ± 1.8	2.1 ± 0.4	8.3 ± 1.3
	Picea smithiana	32.7 ± 5.1	10.7 ± 3.2	43.4 ± 7.4	16.4 ± 2.3	5.3 ± 1.5	21.7 ± 4.8
	Pinus wallichiana	33.9 ± 6.4	11.0 ± 3.1	44.9 ± 6.3	17.0 ± 4.1	5.5 ± 1.4	22.4 ± 4.6
TOTAL		**99.9 ± 8.4**	**33.1 ± 4.3**	**133.1 ± 11.7**	**50.0 ± 7.1**	**16.6 ± 4.6**	**66.5 ± 6.8**

4.1 and 16.4 ± 2.3 Mg ha^{-1} individually while *Abies pindrow* and *Cedrus deodara* exhibited 10.5 ± 2.1 and 6.2 ± 1.8 Mg ha^{-1}C separately. BGB carbon content varied between the maximum of 5.5 ± 1.4 Mg ha^{-1} in *Pinus wallichiana* to the minimum of 2.1 ± 0.4 Mg ha^{-1} in *Cedrus deodara* (Table 2).

In the TFs region, *Picea smithiana* was the most noteworthy carbon sequestering species with 13.6 ± 3.6 Mg ha^{-1} BCS followed by *Pinus wallichiana* (11.6 ± 2.5 Mg ha^{-1}), *Abies pindrow* (8.9 ± 2.4 Mg ha^{-1}) and *Cedrus deodara* (8.3 ± 2.1 Mg ha^{-1}). SFs revealed that *Pinus wallichiana* made the maximum total BCS (10.9 ± 2.6 Mg ha^{-1}) in these ecosystems whereas *Picea smithiana* (8.1 ± 1.4 Mg ha^{-1}) and *Abies pindrow* (5.2 ± 1.7 Mg ha^{-1}) were successive species (Table 2).

Considering site-specific BCS production in TFs, Sudhan Galli yielded the maximum BCS as 176.6 ± 8.4 Mg ha^{-1} followed by Sharda 2 (161.2 ± 6.5 Mg ha^{-1}) and Nokot, (100.1 ± 5.7 Mg ha^{-1}) whereas Marchi Jagran TF was recorded as having the minimum site-specific BCS as 65.6 ± 4.7 Mg ha^{-1}. Among the SFs, Yadori produced the highest amount of site-specific BCS as 130.2 ± 8.4 Mg ha^{-1} while Lower Barthwar Galli yielded the lowest total of 58.8 ± 4.6 Mg ha^{-1}C.

3.2. Forest Structural Attributes

The average DBH value in coniferous trees was recorded as 143.3 ± 6.5 cm, relatively high (155.7 ± 8.2 cm) in the SFs and low (131 ± 6.4 cm) in the TFs region. Similarly, the average tree height was noted as 27.8 ± 3.1 m with a higher value (28.1 ± 3.8 m) in the TFs and a lower value (27.4 ± 3.2 m) in the SFs region. The individual maximum tree girth (172.1 ± 7.5 cm) and height (35.51 ± 5.3 m) were recorded in *Pinus wallichiana* whereas *Cedrus deodara* showed the minimum DBH (68.14 ± 4.8 cm) and height (12.83 ± 1.8 cm) values (Table 3). Sudhan Galli, Sharda 2, Yadori, Nokot, Machhal, Chak, Dao Khan 1, Taobut and Misra Shal showed an average DBH range of 150-250 cm. All these forests and some other sites (Las Dana, Misra Shal, Sar Behk, Mehmood Gali, Dao Khan 2, Khui Morr, Sharda 1, Karka and Marchi Jagran) presented tree height range of 20-48 m. Barthwar Galli Lower, Bichhkarla Doga and Barthwar Galli Top showed minimum average tree DBH (> 100 cm) and height (> 15 m).

The total average tree density in the study region was 135.5 ± 6.4 trees ha^{-1}. A higher density value of 157.1 ± 4.2 trees ha^{-1} was recorded in the TFs ecosystem with *Pinus wallichiana* (240.9 ± 8.8

Table 3. Forest structural attributes of studied temperate and subalpine coniferous forests.

Structural attributes	Forest type	*Abies pindrow*	*Cedrus deodara*	*Picea smithiana*	*Pinus wallichiana*	Total
Tree DBH (cm)	Temperate	157.4 ± 9.4	136.3 ± 7.5	176.8 ± 6.6	152.2 ± 8.3	155.7 ± 8.2
	Subalpine	143.8 ± 8.3	0	188.2 ± 11.1	192.0 ± 10.3	131.0 ± 7.4
	Average	**150.58 ± 9.3**	**68.14 ± 4.8**	**182.49 ± 9.7**	**172.1 ± 7.5**	**143.3 ± 6.5**
Tree height (m)	Temperate	27.8 ± 2.1	25.7 ± 3.2	29.7 ± 4.1	29.2 ± 4.5	28.1 ± 3.8
	Subalpine	30.0 ± 3.7	0	38.0 ± 3.2	41.8 ± 3.7	27.4 ± 3.2
	Average	**28.89 ± 4.7**	**12.83 ± 1.8**	**33.87 ± 3.3**	**35.51 ± 5.3**	**27.8 ± 3.1**
Tree density (trees ha^{-1})	Temperate	107.3 ± 7.4	123.8 ± 8.3	156.5 ± 6.1	240.9 ± 8.8	157.1 ± 4.2
	Subalpine	120.0 ± 5.3	0.0	130.0 ± 6.5	205.6 ± 8.5	113.9 ± 7.7
	Average	**113.7 ± 8.3**	**61.9 ± 3.5**	**143.3 ± 6.1**	**223.3 ± 11.3**	**135.5 ± 6.4**
Forest regeneration (seedlings ha^{-1})	Temperate	80.0 ± 6.1	100.0 ± 6.3	21.0 ± 2.1	161.0 ± 8.3	90.5 ± 5.8
	Subalpine	150.0 ± 4.7	0.0	40.0 ± 5.1	10.0 ± 0.4	50.0 ± 4.1
	Average	**115.0 ± 6.2**	**50.0 ± 4.8**	**30.5 ± 3.6**	**85.5 ± 7.2**	**70.3 ± 4.6**
Deforestation intensity (stumps ha^{-1})	Temperate	158.0 ± 7.8	134.0 ± 6.4	117.0 ± 5.1	207.0 ± 8.2	154.0 ± 6.4
	Subalpine	73.0 ± 5.1	0.0	65.0 ± 4.3	57.0 ± 4.2	48.8 ± 2.8
	Average	**115.5 ± 5.0**	**67.0 ± 4.7**	**91.0 ± 4.7**	**132.0 ± 5.6**	**101.4 ± 4.4**

trees ha^{-1}) as the most abundant species. The total tree density in SFs was 113.9 ± 7.7 ha^{-1} where the minimum density of 120 ± 5.3 ha^{-1} was chronicled in *Abies pindrow* (Table 3). Some sites including Sudhan Galli, Yadori, Sharda 2, Machhal, Chak, Nokot, Taobut and Misra Shal exhibited improved growth parameters and higher tree density (980 to 600 stems ha^{-1}) while Barthwar Galli Top yielded the lowest density of 79.2 ± 4 trees ha^{-1}.

Deforestation, as a threat to the sustainability of the forest ecosystem, was recorded as 101.4 ± 4.4 stumps ha^{-1}, ranging from 154 ± 6.4 in TFs to 48.8 ± 2.8 stumps ha^{-1} in the SFs. In the TFs region, the discrete deforestation rate in *Pinus wallichiana* (207 ± 8.2 stumps ha^{-1}) was the highest followed by *Abies pindrow* (158 ± 7.8 ha^{-1}), *Cedrus deodara* (134 ± 6.4 ha^{-1}) and *Picea smithiana* (117 ± 5.1 ha^{-1}). Inversely, *Pinus wallichiana* in the SFs region showed a low deforestation count (57 ± 4.2 stumps ha^{-1}) but logging rates of *Abies pindrow* (73 ± 5.1 ha^{-1}) and *Picea smithiana* (65 ± 4.3 ha^{-1}) were relatively higher (Table 3). The maximum deforestation was recorded at Barthwar Galli Lower (1666.6 ± 9.7 stems ha^{-1}) followed by Barthwar Galli Top (933.3 ± 6.8 stems ha^{-1}), Bichhkarla Doga (666.6 ± 5.7 stems ha^{-1}) and Marchi Jagran (512.5 ± 5.1 stems ha^{-1}) whereas deforestation rate at Sudhan Galli was the minimum (104.2 ± 3 stems ha^{-1}).

The average seedlings count in both forest types was 70.3 ± 4.6 ha^{-1}, which was higher (90.5 ± 5.8 ha^{-1}) in TFs and lower (50 ± 4.1 ha^{-1}) in SFs. *Abies pindrow* showed the highest regeneration potential with an average of 115 ± 6.2 seedlings ha^{-1} and particularly 150 ± 4.7 seedlings ha^{-1} in SFs. In the TFs region, *Pinus wallichiana* also showed a noteworthy regeneration capability at the rate of 161 ± 8.3 seedlings ha^{-1} but it reduced to 10 ± 0.4 ha^{-1} in the SFs (Table 3). Karka, Dao Khan 1, Mehmood Gali, and Bichhkarla Doga forests were found to have healthier regeneration rates (1167-588 seedlings ha^{-1}) whereas a reduced regeneration was noted at Sharda 2, Dao Khan 2, and Misra Shal (50-21 seedlings ha^{-1}).

Multivariate analysis (PCA) revealed a significant relationship between total tree carbon content and structural attributes. PCA distinguished species based on structural attributes including tree DBH, height, density, regeneration and deforestation (Figure 2(a)). Site-wise multivariate correlation analysis also revealed the same fact that carbon stock is influenced by forest growth stage, disturbance stimuli and altitude (Figure 2(b)). Bivariate linear models explained the statistical relationships of BCS with forest structural traits and various anthropogenic disturbance stimuli (Table 4).

4. DISCUSSION

Forest BCS management is one of the key approaches to minimize the challenging impacts of climate change, following the Kyoto Protocol [1, 29]. Sustainable forest management by improving forest health and plantation is regarded as a significant tool for improved atmospheric CO_2

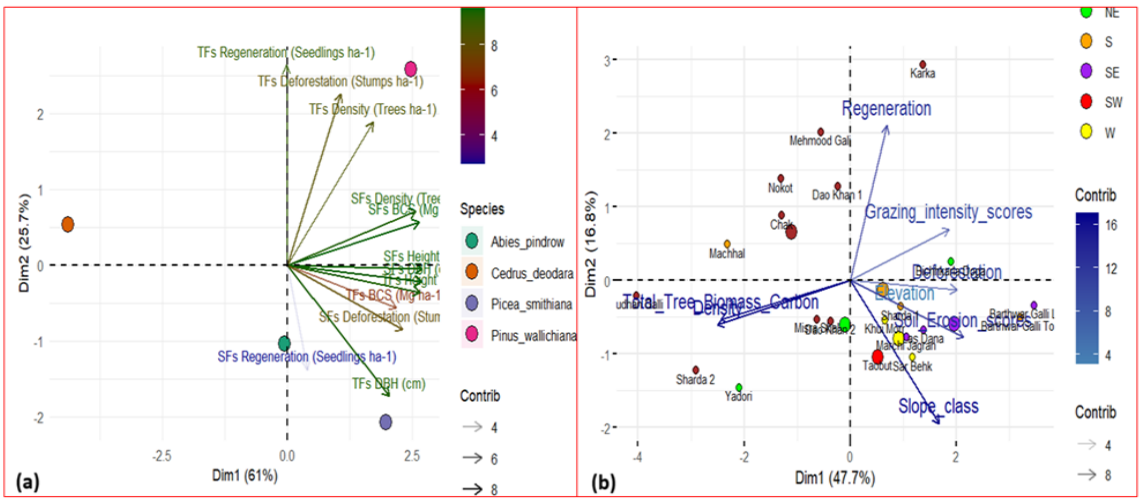

Fig. 2. PCA expression of correlation between (a) species-wise BCS and (b) site-wise BCS with forest structural attributes and disturbance stimuli.

Table 4. Supplementary table of GLM depicting statistical associations of BCS with forest structural attributes and disturbance stimuli.

GLM-Carbon Stock VS		Dispersion phi (estimated)	Slope a		Intercept b		Log likelihood	G:	
			Value	Std. error. a	Value	Std. error. b		Value	p (slope = 0)
Species	DBH	961.8	-0.000346	0.00019	0.0132	0.004008	-1	6.3875	0.01149
	H	32.905	-0.001914	0.000968	0.07062	0.020567	-1	7.7704	0.00531
	Density	1247.8	-0.00065	0.000357	0.01967	0.007789	-1	8.949	0.00278
Sites	DBH	358.89	-3.57E-05	3.36E-06	0.01001	0.000468	-9	88.511	5.06E-21
	H	26.592	-0.000195	2.52E-05	0.05354	0.003563	-9	49.049	2.50E-12
	Density	28557	-1.10E-05	2.11E-06	0.00285	0.00031	-9	24.646	6.89E-07
	Regeneration	91140	3.03E-05	3.52E-05	0.00094	0.002397	-9	1.3072	0.2529
	Deforestation	73877	-0.034698	0.01024	8.4626	0.61561	-9	16.226	5.62E-05
	Altitude	126480	4.71E-07	4.34E-07	0.00037	3.80E-05	-9	1.2288	0.26765
	Slope	0.67211	0.0033508	0.001717	0.18663	0.12195	-9	4.6155	0.03169
	Erosion	0.67211	0.0033508	0.001717	0.18663	0.12195	-9	4.6155	0.03169
	Grazing	0.35495	0.0034115	0.001235	0.17872	0.087256	-9	13.554	0.00023

sequestration. Assessing BCS in local carbon pools not only supports the policy decisions to mitigate climate change but reveals the challenges and offers sustainable forestry options and management approaches like species selection for reforestation and afforestation at the regional scale. Therefore, a higher volume of BCS is expected in well-managed forest ecosystems [30].

Several studies in AJK state have reported carbon counts in various terrestrial carbon pools at small landscapes with insights into factors affecting the carbon sequestration potential [31-35]. The current study focused on a cluster of four keystone coniferous tree species, widely distributed across Himalayan TFs and SFs ecosystems with a diverse range of structural and geographic considerations, growth and yield aptitudes, disturbance regimes, rejuvenation capacities and management options. It was observed that BCS was generally supported by growth parameters and variant ecological circumstances at specific forest sites whereas similar factors abandoned BCS production in other species [29].

Many regional studies reported comparatively higher BCS at different locations depending on geographical characteristics, ecological conditions, species composition, climate and forest sampling strategies [31, 35-40]. The decreased BCS presented by this study is attributed to the carbon exhaust and susceptibility of delicate Himalayan TFs and SFs ecosystems due to the combination of climatic changes, environmental circumstances and larger anthropogenic pressure that decreases forest carbon stocks and limit the livelihood options for local communities [41].

This study investigated that *Picea smithiana* and *Pinus wallichiana* yielded greater BCS totalities and contributed 33.8% and 32.6% respectively in the total tree carbon count as both species were recorded as having the greater DBH and tree height. Similarly, *Abies pindrow* yielded an intermediary BCS count (21.2%) with a medium tree size while *Cedrus deodara* exhibited the lowest BCS (12.4%) with a reduced tree size. Bivariate correlation analysis through GLM showed that besides various other factors (allometric models, tree density, wood-specific gravity, growing stock volume and site climatic conditions etc.), BCS is exclusively dependent on tree size and significantly associated with overall growth conditions as individual tree biomass remains directly proportional to tree DBH (Figure 3(a)) and height (Figure 3(b)). BCS in coniferous trees in relation to tree size emphasized that high altitude coniferous forests in are required to be conserved for carbon management as tree size in these forests is abridged as compared to some other forests in the Himalayan region [42-45].

The diversity of tree growth determinants comprising texture and structure of local soils, moisture content, nutrients availability, light duration, and quality, inter and intra-specific competition and climatic limitations may decline the tree growth and carbon accumulation potential [46]. Another important factor that directly affects BCS is overall tree density. Low density with sparse and disturbed forests produces low BCS [47]. The current study explicated the fact that the cumulative BCS in TFs was higher and was significantly supported by the larger stem density count (Figure 3(c)).

The literature review rationalized that low BCS count is attributed to lower tree density as compared to some other Himalayan forests [35, 48-52]. Species-specific trends in the BCS followed the site-specific trends where BCS was found to be dependent on individual tree DBH (Figure 4(a)), height (Figure 4(b)) and density (Figure 4(c)) in all four coniferous trees across the study area. TFs and SFs in the region are currently facing high deforestation intensity. Low tree density in the TFs and SFs has resulted from heavy deforestation, timber and fuelwood extraction, intensive grazing and browsing, soil loss through erosion and compaction, agriculture and infrastructure development as well as unsustainable use of forest resources [31]. The well-understood and expected associations between BCS and disturbance stimuli emphasize the importance of substantial forest management to achieve a reasonable carbon sequestration capacity. It demonstrates that forest conservation is a key mitigation tool against climate change [14].

Numerous anthropogenic stimuli in the region including increased rates of deforestation and cattle grazing as well as reduced rates of forest natural rehabilitation put a negative impact on the forest carbon sequestration potential. It was analyzed that deforestation rates were mostly higher in the forests with a low BCS count (Figure 5(a)). Similarly, forest sites with higher grazing intensity yielded dwindled total BCS values (Figure 5(b)). As a result of tree removal and cattle grazing, forest regeneration potential may decline and therefore BCS reduction takes place (Figure 5(c)). Tree species with higher density (i.e., *Pinus wallichiana*) were predetermined for deforestation even at the premature growth stage due to ease of access and species abundance. Forest

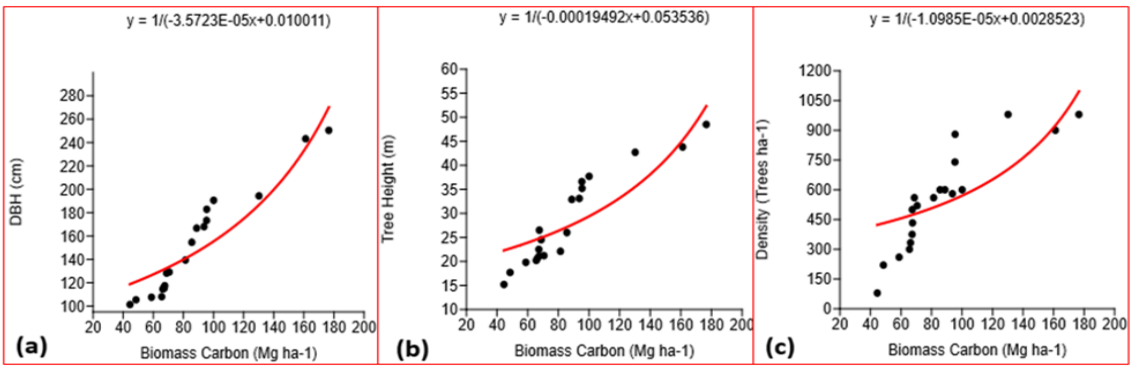

Fig. 3. GLM based correlation of site-wise BCS with average (a) tree DBH, (b) tree height, and (c) tree density.

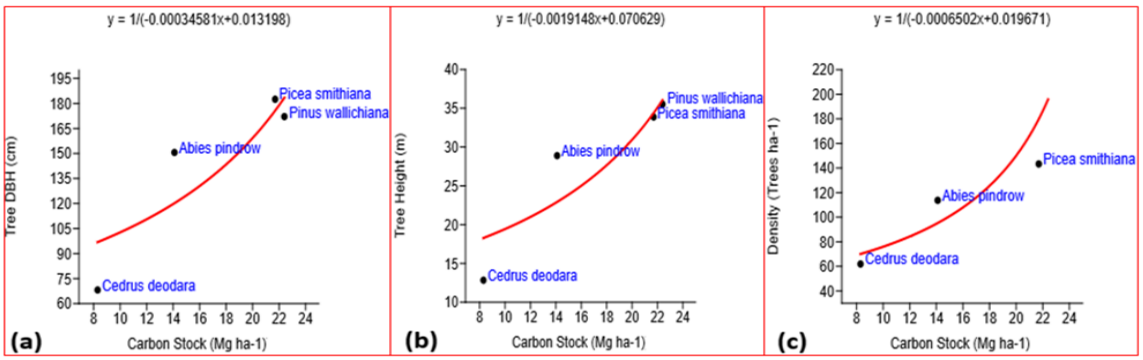

Fig. 4. GLM based correlation of species-wise BCS with average (a) tree DBH, (b) tree height, and (c) tree density.

degradation takes place essentially due to seasonal migration to access animal grazing supplies, trampling and unapproachability to alternate fuel and shelter resources which eventually decrease the productivity and natural biomass carbon capture [1, 13].

A remarkable verdict is that the forest ecosystem is fairly reviving without any dedicated conservation effort even with increased deforestation intensity. The overall recovering correlation was noticed between deforestation and natural regeneration rates at the studied forest sites. Although natural regeneration in *Pinus wallichiana* in the TFs and *Abies pindrow* in SFs is supporting the species endurance, all these keystone species need a comprehensive implementation of a focused conservation plan for an improved rate of carbon sequestration potential, species regeneration and forest cover [8, 9, 14].

Forest site features, especially altitude is an important factor influencing vegetation growth and BCS production [11]. This study was carried out in a broader altitudinal range (1930 to 3165 m above sea level). GLM conveyed a decrease in the total BCS along with increasing altitude. Although some sites at higher elevations showed a handsome amount of BCS but the overall relationship between the altitude and forest BCS remained negative mostly as an effect of climatic variations coupled with the decrease in the total tree DBH, tree density and deforestation (Figure 6(a)). Moreover, harsh climatic conditions at higher elevations in the Himalayan coniferous TFs and SFs are reported to suppress the growth and development of forests species, decrease species diversity and ultimately reduce the overall carbon sequestration potential of the biomass carbon pools [3, 11, 12]. Bivariate analysis through GLM disclosed that BCS production declined along the intensifying site slope (Figure 6(b)). Analogous trends were shown between the forest BCS values and soil erosion intensity (Figure 6(c)).

Besides the forest structural attributes and multiplicity of anthropogenic pressure regimes, topographical features including varying steepness and soil erosion intensity also influence the forest BCS production [39, 53]. It was perceived that grassroots reliance on the forest resources for livelihood is causing forest and soil degradation in

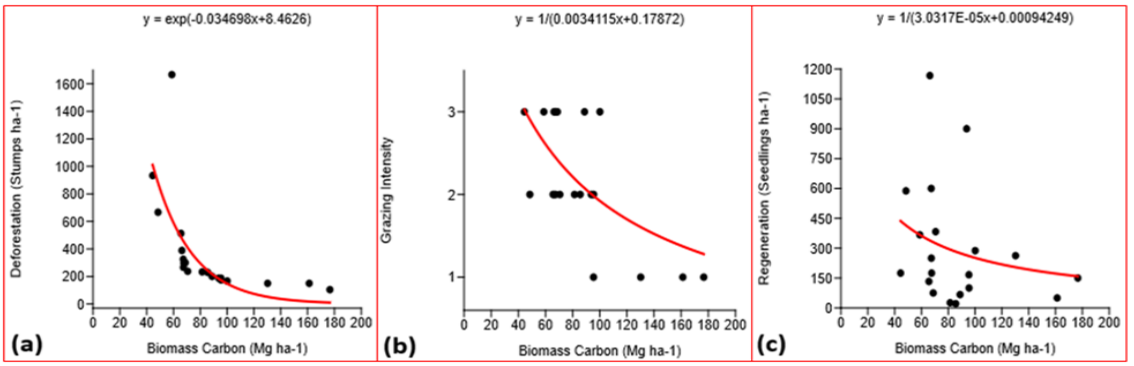

Fig. 5. GLM based correlation of site-wise BCS with average (a) deforestation rate, (b) grazing intensity, and (c) regeneration potential.

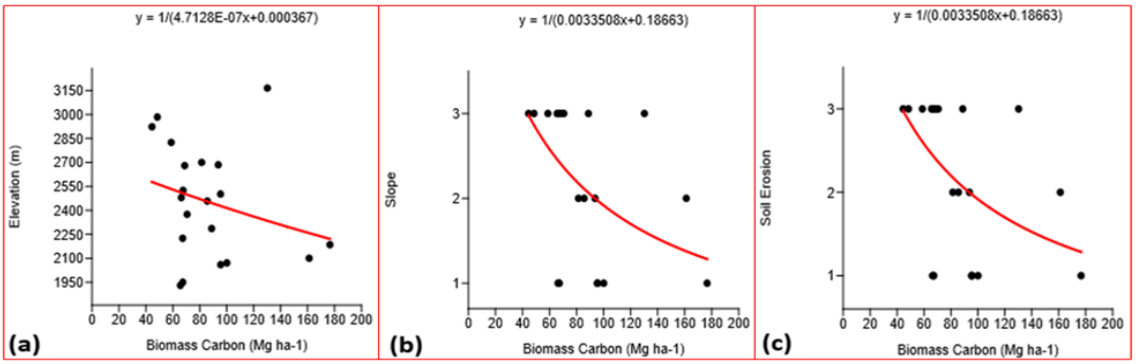

Fig. 6. GLM-based correlation of site-wise BCS with average (a) site elevation, (b) slope class, and (c) soil erosion.

the Himalayan region and which in turn local BCS stocks. Conservation and management of forests integrated with public policies and communal involvement, provision of alternate fuel, fodder and timber resources to the people living around these forests can dynamically recover carbon sequestration and climate change mitigation potential [54-56].

Forest carbon sequestration potential varies depending on forest type and management practices, with community-driven forest management emerging as an effective strategy to boost carbon storage while supporting local livelihoods. The socio-economic dimensions of forest conservation for carbon sequestration in the Himalayan region are crucial for both ecological health and community well-being [8, 41]. In the Western Himalayas, temperate forests play a critical role in climate change mitigation due to their significant carbon storage capacity. However, factors like topography and climate, including low temperatures and limited water availability, can hinder carbon sequestration by affecting tree growth and biomass accumulation [8, 10, 16]. Moreover, forest degradation, rather than area loss alone, represents a major threat to carbon stocks, highlighting the need for sustainable management practices [57] .

5. CONCLUSIONS

This research focused on the quantification of BCS in keystone tree species grown in Western Himalayan coniferous TFs and SFs of the Kashmir region using standard protocols. It was concluded that BCS is markedly depleted and vitally dependent upon the growth of forest species, maturity stage and tree density. *Picea smithiana* and *Pinus wallichiana* are dominant CO_2 sequestering species having larger DBH, height and density scores. Forest BCS showed a decrease along an altitudinal gradient as severe climates limit tree growth rates. Currently, anthropogenic drivers of deforestation and forest degradation including settlements, agriculture, wood fuels, seasonal migration and intensive grazing are major threats to existing carbon stocks in Himalayan coniferous forests. TFs ecosystem holds much significant importance to achieving a sustainable climate change mitigation potential as they can sequester an adequate amount of atmospheric CO_2 subjected to forest conservation. Although Himalayan coniferous forests are naturally regenerating, it is necessary to implement a precise conservation plan intended for GHG management using indigenous natural forest resources. Besides adding numbers to the national and regional forest carbon inventory, this document recommends an accurate estimation of carbon stocks in all other forest species and soil. Improved forest cover and accurate species-wise estimations of carbon stocks can reinforce the national economy through Reducing Emission from Deforestation and Forest Degradation ($REDD^+$) initiatives of the United Nations Framework Convention on Climate Change (UNFCCC).

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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d. Reports

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