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Research Article

Optimized Processing Techniques for Enhancing Fillet Yield from Low-Value Fish in Lamongan, East Java, Indonesia

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Abstract: Fishing activities often yield a bycatch of low-value fish (trash fish) that typically remains underutilized**.** This study aimed to identify the most effective preparation technique to improve the physical and chemical quality of fillets from three types of trash fish: Orangefin ponyfish (*Leiognathus bindus*), chacunda gizzard shad (*Anodontostoma chacunda*), and sardine (*Sardinella fimbriata*). A Randomized Block Design (RBD) was used with two factors: fish species and preparation technique, including mechanical treatment, blanching, immersion in 1% acetic acid, and immersion in 1% papain enzyme. Data were statistically analyzed and presented descriptively in tables and graphs. The results showed that treatment with 1% papain enzyme yielded the highest fillet yield (47.5% to 63.2%) and the shortest processing time. Additionally, the enzymatic treatment produced fillets with favorable chemical characteristics: moisture content (77.46% to 80.13%), protein (7.39% to 9.29%), fat (8.01% to 9.49%), and ash (1.55% to 2.83%). This study demonstrates that enzymatic preparation is effective in enhancing both the efficiency and quality of trash fish fillets, potentially increasing the added value of fishermen's catches.

Keywords: Chemical Composition, Enzymatic Processing, Fillet Yield, Papain Enzyme, Trash Fish.

1. INTRODUCTION

The marine fisheries sector plays a strategic role in supporting economic development and food security in Indonesia. With the second longest coastline in the world and high marine biodiversity, Indonesia holds enormous potential in marine resources [1, 2]. One of the key regions for the development of this sector is Lamongan Regency, East Java, which has been designated as a minapolitan area due to its significant contributions to fish production and processing [3, 4]. In practice, fishing operations yield not only high-value commercial species but also a significant bycatch of small, mixed, and low-quality fish, collectively termed low-value or "trash fish". These fish are often considered waste or by-products and remain underutilized. They are generally used as raw materials for animal feed, salted fish, or even discarded, potentially causing environmental issues such as pollution and foul odors during peak fishing seasons [5, 6]. However, low-value fish actually possess considerable

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potential as food ingredients if processed using appropriate technologies.

Several studies have shown that trash fish can be converted into value-added products such as biodiesel, biogas, food products, and other functional materials [7-12]. One promising form of food product diversification from low-value fish is fish fillet. Several species of trash fish found in Lamongan, such as orangefin ponyfish (*Leiognathus bindus* Valenciennes, 1835), chacunda gizzard shad (*Anodontostoma chacunda* Hamilton, 1822), and sardine (*Sardinella fimbriata* Valenciennes, 1847), are known to have high protein content and suitable characteristics for fillet production [13, 14]. The total marine capture fishery production in Lamongan reaches approximately 74818000 kg, with low-value fish contributing around 3880 kg (0.005%). The market prices are IDR 2000 kg^{-1} for orangefin ponyfish, IDR 3 125 kg^{-1} for chacunda gizzard shad, and IDR 2 300 kg^{-1} for sardine [15].

However, the production of fillets from low-value fish faces several technical challenges. The small size of the fish, varying body shapes, and complex bone and muscle structures complicate the process of separating meat from skin and bones. Both manual and mechanical techniques currently employed are often inefficient, time-consuming, and may compromise the quality of the resulting fillets [16-18]. Therefore, optimized processing techniques are needed to improve the efficiency of the filleting process, reduce processing time, and maintain the physico-chemical quality of the final product.

Various approaches have begun to be explored, including blanching, chemical treatments, and the use of proteolytic enzymes to accelerate the separation of muscle tissue and enhance fillet yields [19]. In this context, the main research question addressed in this study is: What is the most effective and efficient processing technique to improve fillet yield from low-value fish in Lamongan? This study aims to evaluate and optimize various processing methods for trash fish in order to obtain high fillet yields, with shorter processing times and good quality. Ultimately, this effort is expected to enhance the added value of fishery products and support the sustainable management of marine resources in Lamongan Regency, East Java, Indonesia.

2. MATERIALS AND METHODS

2.1. Material

The primary raw materials consisted of fresh trash fish, including orangefin ponyfish, chacunda gizzard shad, and sardine, with an average individual weight ranging from 50 g to 100 g. These fish were obtained from the fish auction site in Lamongan regency, East Java, Indonesia. After purchase, the fish were stored in a refrigerator and transported to the Agroindustrial Technology Laboratory, Faculty of Agricultural Technology, University of Jember, using a cooler box for approximately ±5 h. Upon arrival at the laboratory, the fish were stored in a freezer at –18 °C until analysis. Prior to use, the fish were thawed at room temperature, thoroughly washed, eviscerated, and uniformly chopped using a mincing machine. The additional materials used in the filleting preparation process included unripe papaya fruit (as a source of papain enzyme, a proteolytic enzyme derived from papaya- *Carica papaya* L.), lime juice, cooking oil, and chemical solutions such as acetic acid (CH_3COOH). Other analytical-grade chemicals used for laboratory analysis included TCA, NaOH, H_2SO_4, selenium, 3% boric acid, SDS (0.1% and 2%), and petroleum benzene [20].

2.2. The Preparation of Trash Fish Fillets

The fillet preparation procedure began with a deodorization step by adding lime juice equivalent to 15% (v w^{-1}) of the fish's weight for 10 min. After being thoroughly cleaned, the fish were prepared using four different preparation techniques: (i) Mechanical Method, based on the method by Fu *et al*. [21]: The fish were manually filleted using a knife from the tail to the head after being descaled and eviscerated. The process was conducted on fresh fish and immediately followed by chilling after filleting; (ii) Blanching Method, adapted from Nguyen *et al*. [22]: Cleaned fish were boiled at 100 °C for 5 min. After cooling to room temperature, the fish were manually filleted and stored in a freezer; (iii) Chemical Method, based on Moniharapon *et al*. [23]: The fish were soaked in acetic acid solutions at varying concentrations (0.25%, 0.5%, 0.75%, and 1%) for 30 min, followed by filleting and weighing of the resulting fillet; iv) Enzymatic Method, adapted from Ma *et al*. [24]: The fish were soaked in a papain enzyme solution

(a proteolytic enzyme derived from unripe papaya) at the same concentration levels as the chemical treatment (0.25%, 0.5%, 0.75%, and 1%) for 30 min prior to filleting. All preparation techniques were conducted under identical conditions. The fillet yield from each method was weighed and stored in a freezer for further analysis. Flowchart for the trash fish preparation process is presented in Figure 1.

2.3. Chemical Properties

The analyses conducted on the fillet products included: i) Fillet yield and flesh color, which followed the method by Urgessa *et al*. [25]; ii) Moisture, fat, ash, and crude protein content, which were analyzed based on standard AOAC procedures as described by Gukowsky *et al*. [26]; iii) Carbohydrate analysis was not performed, as carbohydrates are not considered a primary component in the chemical composition of fish, in accordance with findings by Eden and Rumambarsari [27], Elliott [28], Caulton and Bursell [29], and Weatherley and Gill [30].

2.4. Data Analysis

Quantitative data from the analyses were processed using Microsoft Excel for initial tabulation and visualization. Statistical analysis was performed using one-way ANOVA with the DSAASTAT software. If significant differences were found ($P \leq 0.05$), the analysis was followed by Duncan's Multiple Range Test (DMRT) to determine differences between treatments [31].

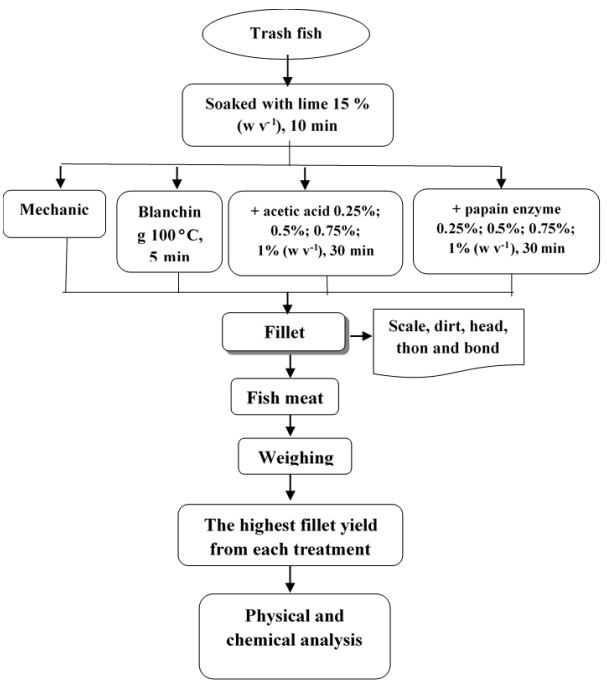

Fig. 1. Flowchart of trash fish preparation technique.

3. RESULTS AND DISCUSSION

3.1. Process Time

The analysis of variance showed a significant interaction between fish species and preparation techniques on fillet processing time ($P < 0.05$). Further analysis using Tukey's HSD test revealed significant differences among each treatment combination, as presented in Table 1.

Table 1 demonstrates that morphological differences among fish species affect the duration of the fillet process. Chacunda gizzard-shad has a relatively flatter and softer body shape, allowing faster separation of flesh from the bones. In contrast, orangefin ponyfish and sardine required longer processing times, with orangefin ponyfish consistently showing the longest fillet duration, likely due to its denser bone structure and firmer flesh texture. The 1% acetic acid treatment resulted in the shortest fillet times across all fish species, indicating its effectiveness in softening connective tissues by breaking peptide bonds, thereby facilitating easier flesh separation. Papain enzyme (proteolytic enzymes from papaya) exhibited a similar effect, particularly at concentrations of 0.25% and 1%, which statistically yielded significantly shorter fillet times compared to mechanical and blanching treatments. Papain acts by degrading myofibrillar proteins and connective tissues, aiding the filleting process [32]. Conversely, mechanical and blanching techniques tended to require longer fillet times, especially in fish with tougher or smaller body morphologies. This suggests that chemical or enzymatic pretreatments are more effective in reducing processing time for trash fish fillet production.

3.2. Yield

The analysis of variance revealed a significant interaction ($P < 0.05$) between fish species and preparation technique on fillet yield. The average fillet yields of the three types of trash fish under various treatments are presented in Figure 2.

Table 1. Average fillet processing time of three types of low-value fish in different preparation techniques (minutes).

Sr. No.	Treatment	Chacunda gizzard-shad	Orrangefin ponyfish	Sardine
1	Mechanics	2:52 g	6:49 a	6:23 b
2	Blanching	2:19 h	3:25 d	4:59 c
3	Acetic acid 0.25%	2:11 i	3:22 de	2:09 i
4	Acetic acid 0.50%	2:10 i	3:08 f	2:05 ij
5	Acetic acid 0.75%	2:57 g	3:06 f	1:58 k
6	Acetic acid 1%	2:00 ij	2:57 g	1:55 k
7	Enzym papain 0.25%	1:55 k	3:03 f	2:16 hi
8	Enzym papain 0.50%	1:52 k	3:09 f	3:22 de
9	Enzym papain 0.75%	1:55 l	3:20 de	3:08 f
10	Enzym papain 1%	1:53 k	3:10 f	2:11 i

Description: Different letters in the same row/column indicate significant differences (DMRT), $P < 0.05$).

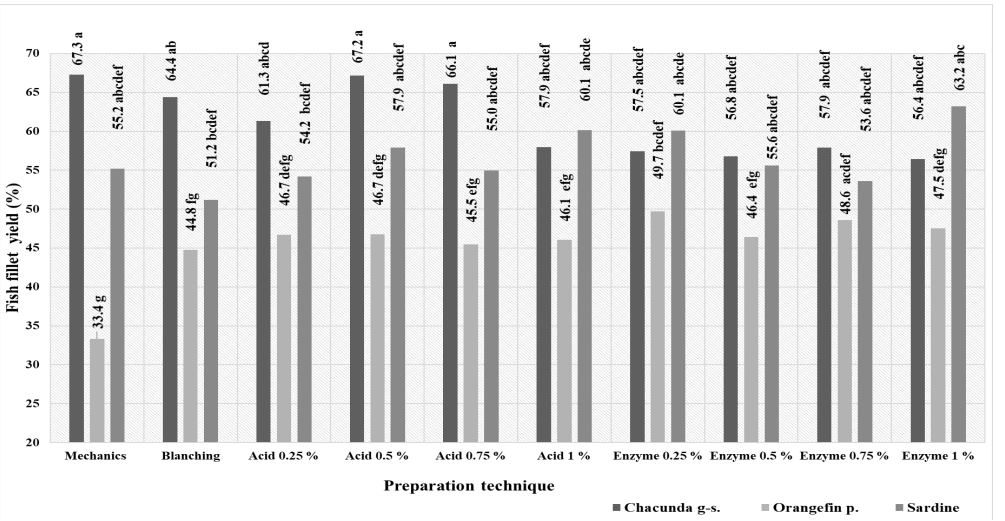

Fig. 2. Average fillet yield of three types of low-value fish in various preparation techniques (%).
Description: Different letters in the same row/column indicate significant differences (DMRT), $P < 0.05$).

The data presented in Figure 2 indicate that the mechanical filleting technique produced the highest yield significantly in chacunda gizzard-shad (67.3%). This is attributed to the fusiform and elongated body morphology of chacunda gizzard-shad, which has relatively thick flesh, making it easier to cut using mechanical methods. However, for sardine, the best result was achieved with 1% papain enzyme treatment, yielding the highest value (63.2%) and significantly differing from most other treatments. Similarly, in orangefin ponyfish, the 0.25% enzyme treatment produced the highest yield (49.7%), indicating the effectiveness of papain in softening connective tissues and facilitating the separation of flesh from bones. The enzyme papain can tenderize muscle tissue, simplifying the fillet process and increasing yield.

The general trend shows that increasing the concentration of papain enzyme tends to improve fillet yield, especially in fish with more complex muscle and connective tissue structures, such as sardine and orangefin ponyfish. This mechanism is supported by the proteolytic activity of papain, which breaks down myofibrillar proteins and connective tissues, resulting in softer flesh that is easier to fillet [33]. Previous studies have also supported these findings. For instance, papain has proven effective in increasing gelatin yield from bovine hides [33]. This supports previous findings by Zhang *et al*. [34], who reported a 20% increase in tenderization yield using papain on low-grade meat, and also on crocodile (*Crocodylidae* Cuvier) meat [35]. Although mechanical filleting produced the highest yield in chacunda gizzard-shad, this

approach is not optimal for small and flat-bodied fish like orangefin ponyfish. Therefore, enzyme- or acid-based treatments should be tailored to the specific morphology of the fish. Based on the best average yield for each technique and fish species, the general treatment recommendations are as follows: i) 1% papain enzyme treatment: consistently delivers high yield, especially for sardine and orangefin ponyfish, making it the most effective overall treatment for various types of trash fish; ii) 0.5% to 0.75% acetic acid treatment: yields relatively high results in chacunda gizzard-shad, though less effective than enzymes in other fish species; iii) Mechanical filleting: optimal for thick-fleshed fish such as chacunda gizzard-shad, but less suitable for smaller fish. This study extends current understanding by demonstrating that enzyme-based methods are not only effective in high-value fish but also improve processing of underutilized species, thus supporting sustainable fisheries.

3.3. Lightness

Lightness (L*) indicates the brightness level of the fillet color, where a value of 0 represents black and 100 represents white. The lightness values of trash fish fillets across different preparation techniques ranged from 18.83 to 25.01 (Figure 3). Based on the analysis of variance, a significant interaction was found between fish species and preparation technique ($P < 0.05$), indicating that the effect of one factor depends on the other.

The fillet with the highest lightness value was from chacunda gizzard-shad treated with 1% enzyme solution (25.01), which was significantly different from the mechanical treatment of the same species (21.52). The lowest lightness value was recorded in sardine under mechanical treatment (18.83). The 1% acetic acid treatment resulted in lightness values above 20 for all fish species, indicating that this method tends to preserve brightness [36].

The variation in lightness values indicates that both treatment type and fish species influence the visual quality of the fillet, particularly color. The 1% enzymatic treatment on chacunda gizzard-shad yielded the highest brightness value, possibly due to proteolytic enzyme action softening the surface tissue and breaking down proteins, which enhances light reflectance. In contrast, mechanical treatment of sardine fillets resulted in the lowest brightness value, likely due to physical damage, oxidation, or release of endogenous pigments during cutting and washing. Acetic acid soaking produced relatively stable and brighter results. Acetic acid is known for its antimicrobial and antienzymatic effects, inhibiting the decomposition of histidine into histamine [37-41], and lowering pH, which may slow down color changes caused by oxidation. This may explain why the acid treatments maintained lightness values above 20 across all fish types. The highest lightness value (25.01) was observed in chacunda gizzard-shad fillets treated with 1%

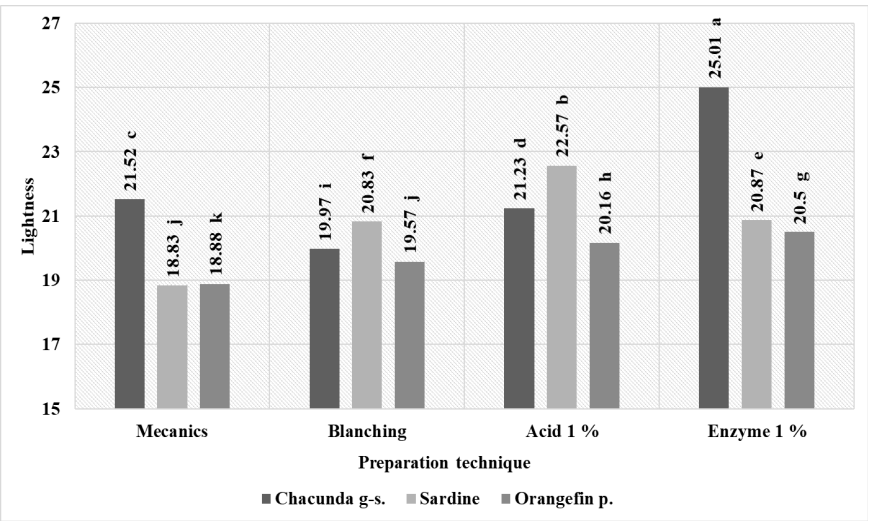

Fig. 3. Average brightness value of fillets of three types of low-value fish in various preparation techniques (scale: 0 to 100).

Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

enzyme immersion. This value is relatively low compared to fresh tilapia (*Oreochromis niloticus* Linnaeus, 1758) fillets (40.85 ± 0.32) [42], which indicates that trash fish fillets have a darker color intensity, possibly due to higher muscle pigment concentrations or post-capture stress. Factors such as myoglobin content, fish age, habitat, and post-harvest handling methods also influence the lightness value of fish flesh.

3.4. Water Content

The moisture content of fish fillets in this study ranged from 72.86% to 80.13%. Results from the two-way ANOVA showed that the preparation technique had a significant effect on moisture content ($P < 0.05$, whereas fish species had no significant effect, and there was no significant interaction between the two factors. This indicates that differences in moisture content were more influenced by the preparation methods than by the type of fish.

Figure 4 shows that the 1% enzyme treatment on sardines resulted in the highest moisture content (80.13% ± 0.36%), which was not significantly different from the acid treatment (78.94% ± 0.29%) and mechanical treatment (77.75% ± 0.31%), but was significantly different from the blanching treatment (76.57% ± 0.25%). Although the moisture content range among treatments was relatively narrow, the differences were statistically significant, indicating the effect of treatment on water retention in the fillet meat. High moisture content in fillet products has a direct impact on shelf life and susceptibility to microbial spoilage. The highest moisture content was observed in the 1% enzyme treatment, likely due to the enzyme's ability to break down connective tissue between muscle fibers, thereby enhancing the tissue's water-holding capacity. Previous studies have shown that proteolytic enzymes such as papain can increase water retention by breaking down the structure of myofibrillar proteins, allowing water to be more effectively trapped within the muscle matrix [43, 44]. In contrast, the blanching treatment resulted in the lowest moisture content. The rapid heating during blanching causes protein coagulation, leading to a loss of free water from the muscle tissue [45, 46]. This process also deactivates endogenous enzymes that help maintain tissue structure, further facilitating water loss during treatment.

The moisture content range in this study (72.86% to 80.13%) is comparable to the moisture content of several other fish species. For example, fillets of Baltic cod (*Gadus morhua* Linnaeus, 1758) contain about 79.5% to 79.7% moisture [47], while crescent grunter (*Terapon jarbua* Fabricius ex Forsskål in Niebuhr, 1775) fish from Pakistan have a moisture content of 73.22% [48]. In contrast, Atlantic salmon (*Salmo salar* Linnaeus, 1758) is reported to have a lower moisture content of 61.8% to 63.9% [49], likely due to its higher fat content and denser muscle structure. Other factors that influence moisture content in fish include species, body size, age, physiological status, habitat, and diet [50, 51]. However, in this study, fish species did not

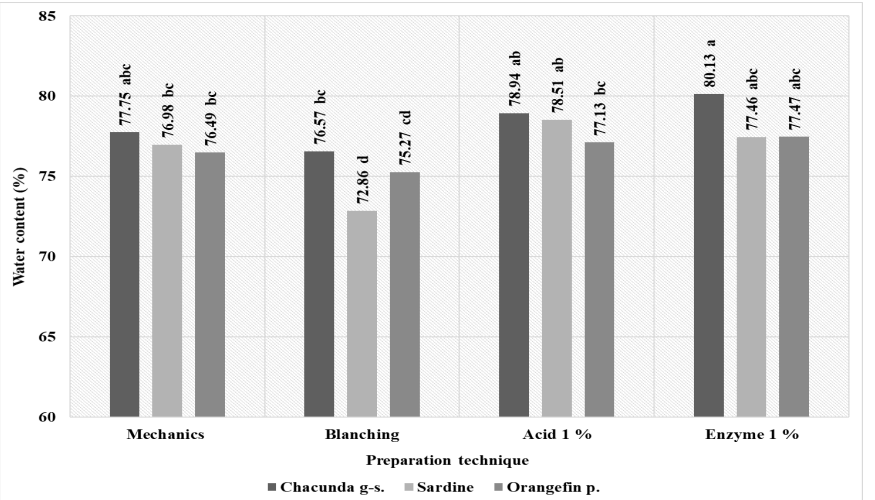

Fig. 4. Average water content of fillets of three types of low-value fish in various preparation techniques (%). Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

significantly affect moisture content, reinforcing that post-harvest treatment (preparation technique) is the dominant factor. Besides being a nutritional parameter, moisture content also determines the physical properties, texture, and shelf life of fillet products. Foods with high moisture content tend to deteriorate more easily due to microbial growth and chemical reactions [52]. Therefore, processing strategies that reduce moisture content (such as blanching) may be considered to improve product stability, though their impact on other attributes like texture and color should also be taken into account.

3.5. Protein Content

The protein content of fillets from the three types of low-value fish studied ranged from 7.39% to 10.59%. Two-way ANOVA results showed that both fish species and preparation techniques had a significant effect ($P < 0.05$) on protein content. However, there was no significant interaction between fish species and preparation techniques ($P > 0.05$), indicating that the effects of each factor were independent.

Figure 5 shows the effect of preparation techniques on the average protein content. The blanching treatment yielded the highest protein content (9.55 % ± 0.21%), which was not significantly different from the mechanical treatment (9.39 % ± 0.18% , but significantly higher than the 1% enzymatic treatment (8.31 % ± 0.14%), which had the lowest protein content. Figure 6 shows the effect of fish species on fillet protein content. Fillets from the orangefin ponyfish showed the highest protein content (9.55 % ± 0.19%), but this was not significantly different from the chacunda gizzard-shad (9.39% ± 0.17%). In contrast, the protein content of sardine (8.46 % ± 0.15%) was significantly different from the other two species. The differences in protein content among preparation techniques can be explained by the thermal and biochemical mechanisms occurring during the treatments. Although blanching involves heating, it actually helps preserve higher protein content [53]. This may be due to the blanching process inactivating proteolytic enzymes that could break down proteins, as well as inhibiting oxidation and nutrient degradation [54]. Additionally, blanching can improve color stability and antioxidant properties, which also contribute to the overall quality. [55, 56].

On the other hand, treatment with 1% papain enzyme significantly reduced protein content. This is consistent with literature stating that proteolytic enzymes like papain hydrolyze peptide bonds, producing smaller protein molecules (oligopeptides or free amino acids), some of which dissolve into the soaking medium and are lost from the fillet meat [57, 58]. As a result, the measured protein content appears lower due to the loss of these soluble fractions. In terms of fish species, chacunda gizzard-shad exhibited the highest protein content, likely due to its muscle tissue composition and habitat. This species tends to have denser muscle mass and higher motor activity, which generally correlates with higher protein levels [59]. The lower protein content observed in sardines may be attributed to their smaller body size and higher fat content, which can reduce the proportion of protein in the tissue. However, the protein content of fillets

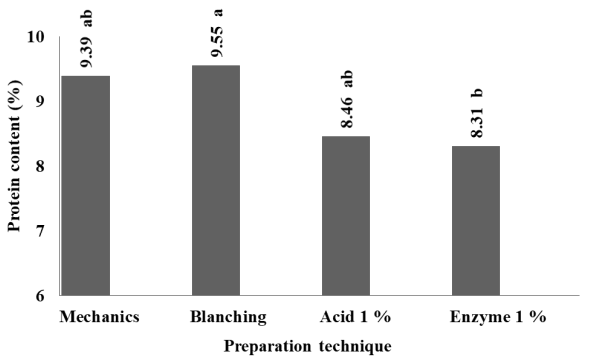

Fig. 5. Average protein content of fillets in various preparation techniques (%).
Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

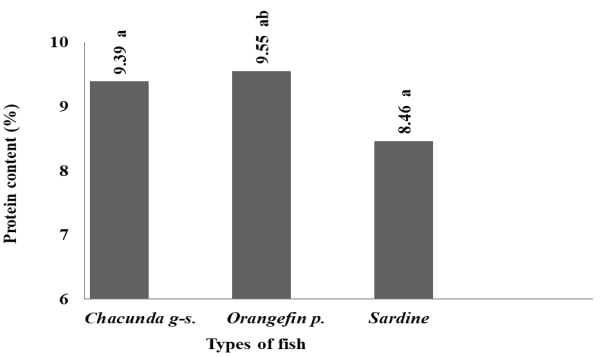

Fig. 6. Average protein content of fillets of three types of low-value fish (%).
Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

in this study remains relatively low compared to values reported for fresh species, such as orangefin ponyfish in India (12.4%) [60]. This discrepancy may be due to the condition of the raw material (trash fish), freshness status, or post-harvest handling methods. Protein tends to degrade more rapidly in fish of lower initial quality, which affects the final yield [61].

3.6. Fat Content

The fat content of the treated Trash fish fillets ranged from 5.87% to 9.49% (Figure 7), with the highest value found in orangefin ponyfish treated with 1% papain enzyme (a proteolytic enzyme derived from papaya) (9.49%), and the lowest value observed in the blanching treatment (5.87%).

The results of the two-way analysis of variance showed that the preparation technique had a significant effect on the fat content of the fillet ($P < 0.05$). Fish species and the interaction between fish species and preparation technique had no significant effect on fat content ($P > 0.05$). Thus, differences in fat content are more influenced by the preparation method than by the fish species used. The 1% papain enzyme treatment resulted in the highest fat content, likely due to the softening of muscle tissue caused by protein hydrolysis, which facilitates lipid extraction during testing. This aligns with the study by Imaizumi *et al*. [62], which stated that structural changes in tissue due to biochemical or thermal treatments can affect the release of nutrients, including lipids. Conversely, the blanching treatment produced the lowest fat content (5.87%). This may be due to partial dissolution of lipid content into hot water during the boiling process. A reduction in fat content due to blanching was also observed in boiled salmon fillets, which were reported to have lower fat content compared to other processing methods [63].

Overall, the fat content observed in this study was higher than that of Indian orangefin ponyfish (3.58%) as reported by Jeyasanta and Patterson [64], which may be influenced by differences in local species, environment, or post-catch handling methods. Fat content is also affected by moisture content, where fish with lower moisture levels tend to show higher percentages of crude fat [65]. A similar study on fish species in Iraq also showed fat content variation ranging from 0.97% to 6.46% [66], confirming that fish fat content can be strongly influenced by species and environment. Although high fat content can enhance the nutritional and caloric value of a product, it is not desirable for products such as surimi, since fat can interfere with gel formation [67]. Therefore, blanching can be recommended for gel-based industries, while enzymatic treatments are more suitable for high-energy products such as baby food or specialized diet products.

3.7. Ash Content

The ash content of fillets from the three types of low-value fish ranged from 1.55% to 3.19% (Figure 8). The results of the two-way analysis of variance showed that neither fish species, preparation technique, nor the interaction between them had a

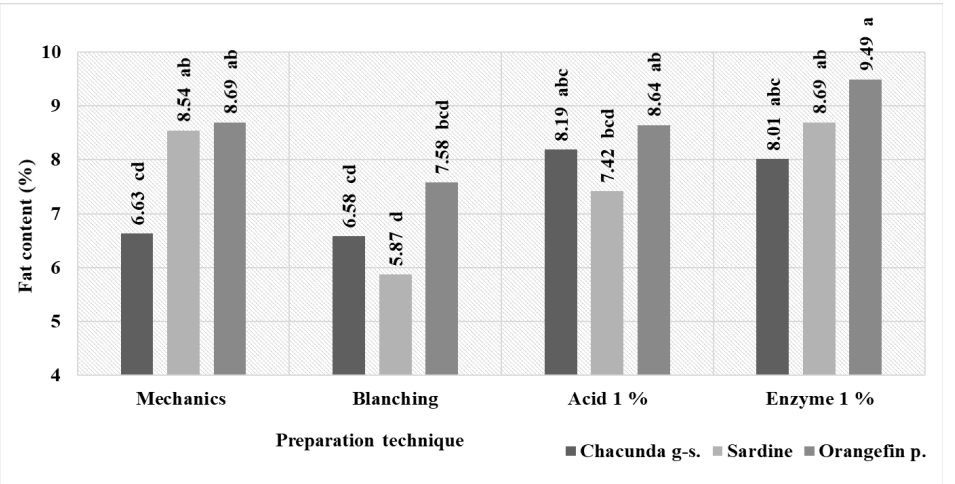

Fig. 7. Average fat content of fillets of three types of low-value fish in various preparation techniques (%).
Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

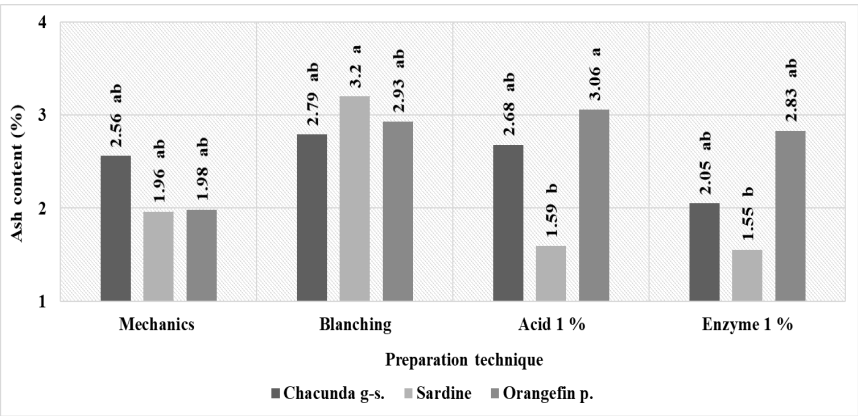

Fig. 8. Average ash content of fillets of three types of low-value fish in various preparation techniques.
(%) Description: Different letters in the same row/column indicate significant differences (DMRT, $P < 0.05$).

significant effect on the ash content of the fillets. However, descriptively, the lowest ash content was recorded in *orangefin ponyfish* treated with 1% papain enzyme (a proteolytic enzyme derived from papaya) (1.55 % ± 0.08%), followed by the 1% acetic acid treatment (1.59 % ± 0.09%). Meanwhile, the highest ash content was observed in the blanching treatment applied to *orangefin ponyfish* (3.19 % ± 0.11%) and *chacunda gizzard-shad* (2.85 % ± 0.10%).

The ash content of the fish fillets in this study ranged from 1.55% to 3.19%, which is within the typical range for tropical fish products, and there were no significant statistical differences between treatments. This value is quite comparable to the ash content of Indian mackerel (*Rastrelliger kanagurta* Cuvier, 1816) at 1.75% [68], but lower than that of fresh Tilapia at 4.80% [69] and *Scomberoides commersonnianus* Lacépède, 1801, from Pakistan (3.58%) [70]. The blanching treatment tended to increase the ash content of the fillets. This is likely due to the relatively higher concentration of minerals remaining after water and dissolved substances are lost during the heat treatment. Conversely, the use of acetic acid and papain enzyme appeared to reduce the ash content, which may be linked to tissue softening and the possible dissolution of some mineral components during the soaking process [71]. Additionally, ash content is influenced by biological and environmental factors of the fish, including diet, age, and habitat. The mineral composition that forms the ash is correlated with the physiological and ecological characteristics of the fish, as explained in the study by Qubay *et al*. [72], which noted that fish from different environments show significant variations in mineral content and other inorganic elements. Thus, although not statistically significant, the trend in this data suggests [73, 74] that enzymatic and acid treatments can be used as alternatives to produce fillets with lower ash content, which is particularly relevant for processing industries such as surimi or gel products that require low mineral content.

4. CONCLUSIONS

This study shows that the enzymatic preparation technique using 1% papain is the most effective method for achieving the highest fillet yield and the fastest processing time for all three types of trash fish, namely: chacunda gizzard-shad by 56.4%, orangefin ponyfish by 47.5%, and sardine by 63.2%. This technique also produces stable chemical composition results, water content (77.46% to 80.13%), protein levels (7.39% to 9.29%), fat content (8.01% to 9.49%), and ash levels (1.55% to 2.83%). A novel finding from this study is that the enzymatic treatment consistently produces fillets with superior physical and chemical quality compared to mechanical, blanching, and acid soaking techniques. Therefore, the enzymatic technique is recommended as the optimal preparation method to enhance the added value and processing efficiency of trash fish.

5. ACKNOWLEDGEMENT

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6. ETHICAL STATEMENT

This study did not involve any human participants or live animal testing. All fish samples used in the experiment were obtained from local fishermen as part of regular fishing activities, and the handling procedures followed standard post-harvest practices without causing unnecessary harm. Therefore, ethical approval was not required for this research.

7. CONFLICT OF INTEREST

The authors state that they have no conflicts of interest.

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Research Article

Field Performance of Eight Commercial Date Palm Cultivars of Balochistan Grown under Agro-Climatic Conditions of District Khairpur, Pakistan

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Abstract: Field performance of eight commercial date palm cultivars of Balochistan was carried out under agro-climatic conditions of district Khairpur, Pakistan. Five years old offshoots of different date palm cultivars brought from Turbat and Panjgoor, Balochistan, and were cultivated in the Research Orchard of Date Palm Research Institute, Shah Abdul Latif University, Khairpur. In this study, vegetative, flowering, bunch and fruit physical characteristics were studied after ten years of plantation in the field. Fruits of different cultivars were collected at three distinct fruit growth stages (kimri, khalal and rutab) for morphological characterization (fruit colour, fruit length, fruit diameter, fruit weight, seed length, seed diameter, seed weight). Results of vegetative characteristics revealed that higher leaf/frond length (187.3 cm), pinnae number (208.6) and spine number (26.6) was observed in cv. Begum Jangi. Flowering characteristics showed that highest number of spathes (14.3) and number of strings per spathe (88.6) were noted in cv. Begum Jangi. Highest bunch length (44.6 cm) and bunch weight (15.4 kg) was observed in cv. Muzawati. Highest fruit length (4.3 cm, 5.3 cm and 5.6 cm) was recorded in cv. Aab-e-dandan at kimri, khalal and rutab stages respectively. Highest fruit weight (22.5 g) was observed in cv. Muzawati at kimri stage, 23.3 g in cv. Shakri at khalal stage and 24.0 g in cv. Muzawati at rutab stage. Highest seed length (2.41 cm) was noted in cv. Gogna at kimri stage, 2.97 cm at khalal and 3.0 cm at rutab stages were observed in cv. Aab-e-dandan. Higher pulp/fruit ratio (PFR) (94.6%) was observed in cv. Muzawati at khalal stage, whereas at rutab stage higher pulp/fruit ratio (94.4%) was recorded in cv. Halini at edible rutab stage. Findings of the current study revealed that soil and climate of district Khairpur were suitable for the cultivation of exotic commercial date palm cultivars in the area.

Keywords: Cultivars, Orchard, Kimri, Pinnae, Spine, Muzawati, Climate, Cultivation.

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is horticulturally important fruit crops belongs to the family Arecaceae is diploid (2n = 36) and dioecious; cultivated in the tropical and sub-tropical areas of the world [1, 2]. Pakistan is ranked at 6th position in dates export and production [3, 4]. Generally, the date palm is propagated via naturally occurring offshoots and seeds; however, the date palm cannot be propagated through seeds due to heterozygosity, therefore, elite date cultivars are propagated via offshoots for the production of true-to-type fruits; whereas the seed propagated date palms always produce fruits of inferior quality, and mainly indistinguishable to the mother tree [5]. In Pakistan dates occupy third position after citrus and mango in production and cultivated area [6]. Khairpur in Sindh and Turbat and Panjgur in Balochistan are the major dates producing and exporting areas in Pakistan. Khairpur is called biodiversity centre of the date palm enriched with 300 commercial and non-commercial varieties [4]. Commercially important varieties grown in district Khairpur are Aseel, Dhakki, Dedhi, Otakin, Kurh, Gajar, Karbalain, Khurmo, Fasli and Kashuwari. Several studies

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were conducted in different countries on the field performance of different date palm varieties for the evaluation of fruit quality [7-11]. Dates are oblong, berry with fibrous endocarp and fleshy mesocarp which constitutes 85-90% weight of the total fruit [12, 13]. Major constituents of dates are vitamins, minerals, sugar and several other compounds used as traditional medicine [14, 15]. Growth of dates is based on five stages, i.e., hababouk and kimri are early growth stages characterized with green colour, non-edible, and contain high tannins; next to the kimri is the khalal stage during which a particular colour of the fruit (red or yellow) is appeared, depending on the variety type; after khalal is the rutab stage (half ripened fruit), and final stage is tamar (fruit fully ripened) either on the tree in dry date cultivars or dried under the sun after harvesting the late khalal stage dates [16, 17]. Several studies have been conducted on vegetative, flowering and fruit evaluation of date palm in different regions of the world [17, 18-23]. Markhand *et al*. [4] characterized 85 date palm varieties; the study was based on the fruit size, fruit shape, fruit colour, perianth colour and size, micropyle position, fruit type (soft, dry or semi-dry) and edible stage (khalal, rutab or tamar). Abul-Soad *et al*. [24] carried out field evaluation (vegetative, flowering and fruit characteristics) of three Saudi Arabian date palm cultivars (Ajwa, Safawi and Ruthana) cultivated in Khairpur, Pakistan. Commercially important cultivars of date palm in district Khairpur are Aseel, Dhakki, Otakin, Kurh, Dedhi and Kashuwari, and are threatened by pests (Red Palm Weevil) and diseases (sudden decline disease and diplodia). Climate of district Panjgur is warm in summer with a maximum 45 °C, the annual rainfall is 25 mm, and the soil is sandy loam [25]. The sandy loam soil and shortage of water reduces the yield of the dates per tree in Turbat and Panjgur. In district Khairpur, the soil is clay loam, and temperature ranges from 45 to 50 °C during the summer (June and July). Cultivation of commercially important exotic date palm cultivars in district Khairpur is need of the time, which will boost existing varietal structure of date palm in the area. This study was conducted on field performance of eight commercial date palm cultivars originally belong to Balochistan, Pakistan to introduce in the district Khairpur. The study will be helpful to the date palm farmers in propagating elite date cultivars of Balochistan, exhibiting better adaptability in the area, in addition to the existing cultivars. The objectives of the current study were also extended to check field performance of eight commercial date palm cultivars of Balochistan grown under agro-climatic conditions of district Khairpur, Pakistan.

2. MATERIALS AND METHODS

2.1. Vegetative Characteristics

Vegetative characteristics were based on: trunk circumference, leaf length (cm), pinnae number, pinnae length (cm), leaf width, pinnae width, spine number, spine length (cm), spine area length (cm), measured with a measuring tape [26].

2.2. Flower Characteristics

Flower characteristics were taken as: date of spathes emergence (first and last), date of spathes pollination (first and last), number of spathes per tree, spathe length, number of strings per spathe, length of strings.

2.3. Bunch Characteristics

Bunch characteristics were taken as: bunch number per tree, bunch length, number of strings per bunch, string length, number of fruits per string (kimri stage), number of fruits per string at harvest (late khalal stage), harvest date, bunch weight and yield per palm [26].

2.4. Fruit Physical Characteristics

Fruits were obtained from ten years old trees at different stages, i.e., kimri, khalal and rutab cultivated in Research Orchard of Date Palm Research Institute, Shah Abdul Latif University, Khairpur (latitude 27.490418˚ N, longitude 68.761593˚ E). Average annual rainfall of Khairpur is 87.6 mm (3.45 in.) and temperature is 50 ˚C during July. Fruit physical characteristics were taken at kimri, khalal and rutab as fruit length, fruit width, fruit weight, fruit/ pulp ratio, seed length, seed width, seed weight. Vernier Caliper was used to measure the length and diameter of fruits.
Pulp/fruit ratio (PFR) was recorded (in %) by using the following formula:

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

Seed weight (SW) was calculated using the following formula:

$$SW = FW - PW$$

FW and *PW* are fruit weight and pulp weight respectively [26].

2.5. Data Analysis

Data collection was based on selection of three replicates for each treatment regarding vegetative, flower and bunch characteristics, whereas, ten randomly picked fruits were selected for each fruit growth stage for data analysis. The data were analyzed as two-way-ANOVA followed by LSD (≤ 0.05) as described by Steel *et al*. [27].

3. RESULTS

3.1. Vegetative Characteristics

In this study, data were obtained with two-way-ANOVA, exhibited the significant effect of cultivar (0.002), treatment (0.000) and combined effect of cultivar and treatment (0.000). Data presented in Table 1 show that higher tree circumference (192 cm) was noted in cv. Aab-e-dandan; whereas, lowest tree circumference (103 cm) was observed in cv. Gogna. Highest leaf/frond length (380.7 cm) was noted in cv. Begum Jangi; whereas, lowest leaf/frond length (264.5 cm) was recorded in cv. Halini. Number of pinnae (208.6) was higher in cv. Begum Jangi and lowest pinnae number (151.6) was found in cv. Halini. Highest pinnae length (41.39 cm) was noted in cv. Muzawati; whereas, lowest pinnae length (30.0 cm) was found in cv. Pashna. Higher leaf width (80.6 cm) was observed in cv. Aab-e-dandan and lowest leaf width (55 cm) was noted in cv. Pashna. Highest pinnae width (2.70 cm) was observed in cv. Kooznabad and lowest pinnae width (1.8 cm) was recorded in cv. Aab-e-dandan. Number of spines (26.6) was higher in cv. Begum Jangi and lower number of spines (17.0) was noted in cv. Pashna. Highest spine length (13.33 cm) was observed in cv. Aab-e-dandan and lowest spine length (8.40 cm) was observed in cv. Halini. Spine area length was significantly higher in cv. Gogna (107.3 cm), and lower in cv. Halini (52.1 cm).

3.2. Flowering Characteristics

Data presented in Table 2 show that first spathe emergence was noted in cv. Shakri on 11th February; whereas, in cv. Halini first spathe emergence was observed on 12th March. Earlier pollination was carried out in cv. Aab-e-dandan on 20th February, and late pollination was done in cv. Halini on 21st March. Last spathe emergence was found in cv. Aab-e-dandan on 23rd February and in cv. Halini last spathe emergence was noted on 21st March. Last spathe pollination was carried out earlier in cv. Shakri on 27th February, and in cv. Halini pollination was done on 29th March. Highest number of spathes (15) was recorded in cv. Aab-e-dandan; whereas, lowest number of spathes (11.67) was observed in cv. Shakri. Highest length of spathe (76.77 cm) was noted in cv. Halini and lowest spathe length (48.66 cm) was observed in cv. Muzawati. Significantly highest number of spikelets per spathe (88.66) was recorded in cv. Begum Jangi and lowest number

Table 1. Vegetative characteristics of different date palm cultivars of Balochistan grown in district Khairpur, Pakistan.

Cultivars	Trunk circumference (cm)	Leaf length (cm)	Pinnae number	Pinnae length (cm)	Leaf width (cm)	Pinnae width (cm)	Spine number	Spine length (cm)	Spine area length (cm)
Aab-e-dandan	192 ± 0.5^{a}	342.3 ± 1.1^{d}	194 ± 0.5^{b}	40.2 ± 1.3^{b}	80.6 ± 0.4^{a}	1.8 ± 0.5^{c}	19 ± 1.1^{bc}	13.3 ± 0.5^{a}	77 ± 1.2^{ab}
Begum Jangi	187.3 ± 1.1^{b}	380.7 ± 0.8^{a}	208.6 ± 1.2^{a}	36.2 ± 1.2^{d}	78.5 ± 1.3^{b}	2.0 ± 0.6^{bc}	26.6 ± 1.3^{a}	11.4 ± 1.5^{bc}	101.3 ± 1.5^{b}
Gogna	103 ± 0.72^{g}	293.3 ± 1.2^{f}	181.3 ± 0.6^{bc}	34 ± 0.8^{de}	64.0 ± 0.4^{c}	2.5 ± 1.3^{ab}	25.0 ± 0.6^{b}	11.6 ± 1.5^{bc}	107.3 ± 0.6^{a}
Halini	161.6 ± 0.6^{e}	264.5 ± 0.6^{d}	151.6 ± 0.7^{d}	31.4 ± 0.5^{e}	67.5 ± 0.6^{bc}	2.4 ± 1.2^{bc}	18 ± 0.5^{de}	8.4 ± 0.6^{d}	52.1 ± 1.2^{b}
Kooznabad	185.1 ± 0.7^{c}	328.3 ± 0.5^{b}	177.6 ± 0.6^{d}	38.8 ± 1.5^{c}	71.9 ± 0.2^{bc}	2.70 ± 1.3^{a}	20 ± 1.5^{d}	9.8 ± 0.7^{bc}	81.0 ± 1.5^{c}
Muzawati	176 ± 1.2^{d}	353.4 ± 1.3^{c}	179.6 ± 1.2^{c}	41.3 ± 2.2^{a}	78.3 ± 1.2^{b}	2.6 ± 1.4^{ab}	21.6 ± 0.4^{c}	11.9 ± 0.6^{bc}	71.6 ± 1.5^{d}
Pashna	160.3 ± 0.7^{e}	330.3 ± 0.7^{c}	180.6 ± 0.5^{bc}	30 ± 0.7^{ef}	55 ± 0.8^{d}	2.0 ± 0.5^{b}	17.0 ± 1.1^{c}	12.0 ± 0.5^{b}	83.3 ± 0.5^{c}
Shakri	158.6 ± 0.3^{f}	341.3 ± 1.2^{d}	196.6 ± 0.4^{b}	38.9 ± 0.4^{c}	71.6 ± 1.5^{bc}	1.8 ± 1.4^{d}	25.3 ± 1.4^{b}	10.8 ± 1.3^{c}	104.6 ± 0.5^{b}
LSD (0.05)	0.000**	0.000**	0.000**	0.000**	0.000**	0.02**	0.000**	0.05*	0.000**

Variability: Cultivar = 0.002, Treatment = 0.000, CV x Treatment = 0.000.
Values followed by the same letter are not significantly different at $p < 0.05$.

Table 2. Flowering characteristics of different date palm cultivars of Balochistan grown in district Khairpur, Pakistan.

Cultivars	Date of 1st spathes emergence	Date of 1st spathes pollination	Date of Last spathes emergence	Date of last spathes pollination	Number of spathes	Spathes length (cm)	Number of spikelets per spathes	spikelet length (cm)
Aab-e-dandan	14-02-2012	20-02-2012	23-02-2012	03-03-2012	11.0 ± 0.8^{c}	66.5 ± 0.5^{b}	43.6 ± 1.1^{cd}	41.3 ± 1.2^{b}
Begum Jangi	02-03-2012	08-03-2012	11-03-2012	17-03-2012	14.3 ± 0.5^{a}	62.7±1.2^{c}	88.6±0.6^{a}	43.1±0.4^{a}
Gogna	17-02-2012	24-02-2012	25-02-2012	04-03-2012	13.0 ± 1.5^{b}	51.6 ± 0.5^{d}	47.2 ± 1.1^{c}	27.1 ± 1.4^{de}
Halini	12-03-2012	21-03-2012	21-03-2012	29-03-2012	10.3 ± 1.5^{d}	76.7 ± 0.5^{a}	34.6 ± 1.4^{d}	26.5 ± 1.3^{e}
Kooznabad	20-02-2012	27-02-2012	02-03-2012	08-03-2012	12.0 ± 1.5^{c}	62.3 ± 1.6^{c}	30.8 ± 1.3^{e}	28.8 ± 0.5^{de}
Muzawati	06-03-2012	13-03-2012	15-03-2012	23-03-2012	13.0 ± 1.6^{b}	48.6 ± 0.5^{f}	53.2 ± 0.6^{b}	30.1 ± 0.6^{d}
Pashna	26-02-2012	05-03-2012	07-03-2012	15-03-2012	11.6 ± 0.6^{c}	52.5 ± 0.8^{e}	21.3 ± 1.8^{f}	20.9 ± 1.5^{de}
Shakri	11-02-2012	21-02-2012	20-02-2012	27-02-2012	10.6 ± 1.5^{d}	55.3 ± 1.6^{e}	51.8 ± 0.6^{b}	35.6 ± 0.4^{c}
LSD (0.05)					0.005**	0.000**	0.000**	0.000**

Variability: Cultivar = 0.006, Treatment = 0.002, CV x Treatment = 0.000.
Values followed by the same letter are not significantly different at $p < 0.05$.

of spikelets (21.33) was noted in cv. Pashna. Significantly highest length of spikelets (43.11 cm) was observed in cv. Begum Jangi and lowest length of spikelets (20.55 cm) was recorded in cv. Halini.

3.3. Bunch Characteristics

Data presented in Table 3 show that highest bunch number (av. 14.3) was observed in cv. Begum Jangi followed by cv. Muzawati (av. 13.0) and Gogna (av. 13.0); whereas, lowest bunch number (av. 10.6) was observed in cv. Shakri followed by cv. Halini (av. 10.3). Highest bunch length (44.6 cm) was recorded in cv. Muzawati followed by Begum Jangi (43.0 cm) and Shakri (43.0 cm); whereas, lowest bunch length (38.8 cm) was noted in cv. Aab-e-dandan. Number of strands per bunch (65) were higher in cv. Begum Jangi followed by cv. Muzawati (51.5) and cv. Shakri (51.7); whereas, lowest number of strings (36.5) was found in cv. Halini. Highest strand length (42.4 cm) was noted in cv. Aab-e-dandan, and lowest strand length (33.1 cm) was observed in cv. Pashna. Significantly highest number of fruits per strand (35.7) was recorded in cv. Muzawati followed by cv. Pashna (31.7); whereas, lowest number of fruits per strand (27.8) was noted in cv. Aab-e-dandan. Significantly higher number of fruits per strand at harvest time (26.2) was observed in cv. Pashna, and lowest number of fruits per strand at harvest time (21.8) was observed in cv. Aab-e-dandan. Earlier harvest was done in cv. Aab-e-dandan on 3rd July; whereas, late harvest was done in cv. Halini on 17th September. Highest bunch weight (15.4 kg) was found in cv. Muzawati followed by cv. Pashna (13.6 kg); whereas, lowest bunch weight (10.7 kg) was noted in cv. Aab-e-dandan. Significantly higher total yield per tree (150.3 kg) was observed in cv. Muzawati; whereas, lowest yield per tree (97 kg) was noted in cv. Shakri.

3.4. Fruit Physical Characteristics at Different Fruit Growth Stages (kimri, khalal and rutab)

Data presented in Table 4 show fruit physical characteristics recorded at three different growth stages (kimri, khalal, and rutab). Fruits of Aab-e-dandan (Figure (1a)), Begum Jangi (Figure (1b)), Gogna (Figure (1c)), Halini (Figure (1d)), Kooznabad (Figure (1e)), Muzawati (Figure (1f)), Pashna (Figure (1g)) and Shakri (Figure (1h)) were green at kimri stage, and remained inedible due to occurrence of high amount of tannins. The fruits of Aab-e-dandan (Figure (2a)), Begum Jangi (Figure (2b)), Gogna (Figure (2c)), Halini (Figure (2d)), Kooznabad (Figure (2e)), Muzawati (Figure (2f)), Pashna (Figure (2g)) and Shakri (Figure (2h)) at khalal stage acquired a particular colour and sweet taste. The fruits of Aab-e-dandan (Figure (3a)), Begum Jangi (Figure (3b)), Gogna (Figure (3c)), Halini (Figure (3d)), Kooznabad (Figure (3e)), Muzawati (Figure (3f)), Pashna (Figure (3g)) and Shakri (Figure (3h)) also acquire sweet taste due to increase in sugar content at rutab stage; whereas, half of the fruit remain yellow or red reflecting the particular fruit colour. Results presented in Table 4 indicate that highest fruit length (4.33, 5.30 and 5.63 cm) at kimri, khalal and rutab stages respectively was noted in cv. Aab-e-dandan, and lowest fruit length (2.7 cm in cv. Halini at kimri stage, 4.3 cm

Table 3. Bunch characteristics of different date palm cultivars of Balochistan grown in district Khairpur, Pakistan.

Cultivars	Number of bunches	Bunch length (cm)	Strands per bunch	Strand length (cm)	Total fruits per strand	Total fruits/ strand at harvest	Bunch weight (kg)	Total yield (kg/Palm)	Date of harvest
Aab-e-dandan	11.0 ± 0.7^{c}	38.8 ± 1.1^{c}	43.1 ± 0.7^{c}	42.4 ± 0.6^{a}	27.8 ± 0.8^{c}	21.8 ± 0.4^{c}	10.7 ± 0.6^{c}	100 ± 0.7^{c}	03-07-2012
Begum Jangi	14.3 ± 1.2^{a}	43.0 ± 1.3^{b}	65.0 ± 0.3^{a}	36.3 ± 0.7^{c}	29.2 ± 0.5^{d}	24.1 ± 1.4^{b}	12.7 ± 1.5^{c}	137 ± 0.5^{b}	11-09-2012
Gogna	13.0 ± 0.5^{b}	40.3 ± 1.5^{c}	41.6 ± 1.4^{c}	34.8 ± 1.3^{d}	28.7 ± 1.4^{d}	22.5 ± 0.5^{b}	11.7 ± 1.2^{d}	120 ± 1.3^{d}	07-07-2012
Halini	10.3 ± 0.6^{d}	39.6 ± 0.5^{d}	36.5 ± 1.3^{d}	35.5 ± 0.5^{e}	30.0 ± 1.5^{c}	23.8 ± 1.3^{d}	12.3 ± 1.6^{c}	99.1 ± 1.2^{f}	17-09-2012
Kooznabad	12.0 ± 0.7^{c}	42.5 ± 0.6^{c}	37.5 ± 1.2^{d}	36.8 ± 1.3^{c}	31.3 ± 1.3^{b}	23.2 ± 1.5^{c}	11.4 ± 0.6^{d}	120.8 ± 0.7^{d}	17-08-2012
Muzawati	13.0 ± 0.8^{b}	44.6 ± 0.8^{a}	51.5 ± 0.6^{b}	39.2 ± 0.4^{b}	35.7 ± 1.4^{a}	25.2 ± 1.6^{b}	15.4 ± 0.5^{a}	150.3 ± 0.5^{a}	14-09-2012
Pashna	11.6 ± 0.6^{c}	41.0 ± 0.7^{c}	37.3 ± 0.7^{c}	33.1 ± 1.2^{f}	31.7 ± 1.1^{b}	26.2 ± 0.7^{a}	13.6 ± 0.4^{b}	130 ± 1.5^{c}	14-08-2012
Shakri	10.6 ± 0.7^{d}	43.0 ± 0.8^{b}	51.7 ± 1.2^{b}	36.9 ± 1.3^{c}	29.8 ± 1.5^{c}	22.3 ± 1.5^{e}	11.2 ± 0.5^{d}	97.0 ± 1.5^{f}	06-07-2012
LSD (0.05)	0.000**	0.000**	0.000**	0.000**	0.000**	0.001**	0.003**	0.000**	

Variability: Cultivar = 0.004, Treatment = 0.001, CV x Treatment = 0.000.
Values followed by the same letter are not significantly different at $p < 0.05$.

and 4.8 cm in cv. Kooznabad at khalal and rutab stages respectively) was observed. Significantly highest fruit width (2.5 cm in cv. Shakri at kimri stage, 2.90 cm in cv. Halini at khalal stage and 3.1 cm in cv. Halini at rutab stage) was observed; whereas, lowest fruit width (2.0 cm) was recorded in cv. Begum Jangi at kimri stage, 2.12 cm in cv. Pashna at khalal stage, 2.6 cm in cv. Gogna at rutab stage. Highest fruit weight (22.5 g) was noted in cv. Muzawati, 23.3 g in cv. Shakri at khalal stage and 23.7 g in cv. Shakri at rutab stage; whereas, lowest fruit weight (9.8 g, 11.3 g and 11.9 g) was observed in cv. Begum Jangi at kimri, khalal and rutab stages respectively. Highest seed length (2.7 cm was noted in cv. Shakri at kimri stage), 2.8 cm in cv. Pashna at khalal stage and 2.9 cm in cv. Pashna at rutab stage; whereas, lowest seed length (2.0 cm) was recorded in cv. Muzawati at kimri stage, 2.17 cm in cv. Halini at khalal stage and 2.4 cm in cv. Halini at rutab stage. Seed weight (2.0 g, 2.4 g and 2.6 g) was higher in cv. Shakri at kimri, khalal and rutab stages respectively; whereas, lowest seed weight (1.0 g) was noted in cultivars Begum Jangi and Halini at kimri stage, 1.1 g was observed in cv. Kooznabad at khalal stage and 1.50 g was noted in cv. Muzawati at rutab stage. Highest seed width (0.9 cm) was found in cv. Gogna at kimri stage, 0.9 cm in cv. Aab-e-dandan at khalal stage and 1.20 cm in cv. Pashna at rutab stage; whereas, lowest seed width (0.56 cm) was observed in cv. Aab-e-dandan at kimri stage, 0.8 cm in cv. Kooznabad at khalal stage and 0.9 cm in cv. Kooznabad at rutab stage. Significantly highest flesh weight (21.6 g, 22.0 g and 22.5 g) was noted in cv. Muzawati at kimri, khalal and rutab

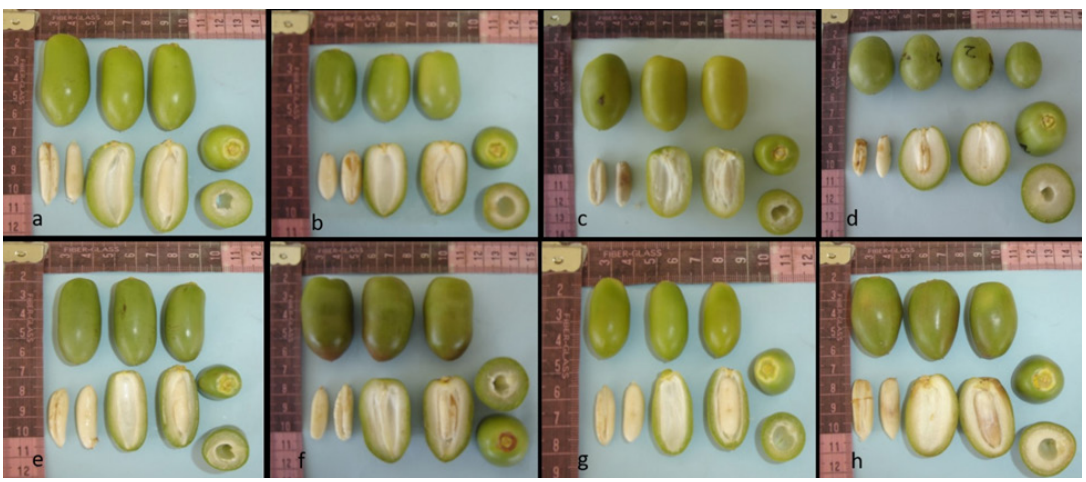

Fig. 1. Fruit of different date varieties at kimri stage, (a) Aab-e-dandan, (b) Begum Jangi, (c) Gogna, (d) Halini, (e) Kooznabad, (f) Muzawati, (g) Pashna, (h) Shakri.

Fig. 2. Fruit of different date varieties at khalal stage, (a) Aab-e-dandan, (b) Begum Jangi, (c) Gogna, (d) Halini, (e) Kooznabad, (f) Muzawati, (g) Pashna, (h) Shakri.

Fig. 3. Fruit of different date varieties at Rutab stage, (a) Aab-e-dandan, (b) Begum Jangi, (c) Gogna, (d) Halini, (e) Kooznabad, (f) Muzawati, (g) Pashna, (h) Shakri.

stages respectively; whereas, lowest flesh weight (8.8 g, 9.5 g and 9.9 g) was recorded in cv. Begum Jangi at kimri, khalal and rutab stages respectively. Higher pulp fruit ratio (94.9%) was noted in cv. Halini at kimri stage followed by cv. Aab-e-dandan (92.2%); whereas, higher pulp fruit ratio (94.6%) was observed in cv. Muzawati at khalal stage followed by cv. Halini (91.4%). Significantly highest pulp fruit ratio (95.4%) was observed in cv. Halini at rutab stage followed by cv. Muzawati (93.7%). Lowest pulp fruit ratio (89.0%) was observed in Pashna at kimri stage followed by cv. Begum Jangi (89.8%). Whereas, significantly lower pulp fruit ratio (84.4% and 83.5%) was recorded in the fruits of cv. Begum Jangi at khalal and rutab stages of respectively.

4. DISCUSSION

Vij *et al*. [19] conducted study on different date palm cultivars (Hillawi, Shamran, Khadrawi, Deglet Noor, Medjool, Barhee, Zahidi, Dayri, Khalasa, Hayani, Thoory and Itima) regarding vegetative, flowering and fruit physical characteristics. El-Alwani and El-Ammari [28] studied morphological characteristics (trunk diameter, leaf length, width of leaf base, blade length, spine area length, spine number, pinnae length, pinnae width and pinnae number). Different studies [29-37] observed the variations in growth parameters in different date palm cultivars, cultivated under different environmental conditions, i.e., fruit setting, yield, fruit physical and chemical characteristics. Aslam *et al*. [38] conducted study on physio-chemical properties of dates grown in Turbat, Balochistan; the data was recorded in cultivar Begum Jangi, i.e., fruit length (3.1 cm), fruit weight (6.87 g), fruit width (1.9 cm), seed length (1.8 cm) and seed weight (0.7 g). Similarly, the data were recorded in cultivar Halini, i.e., fruit length (2.9 cm), fruit

Table 4. Fruit physical characteristics of different date palm cultivars at kimri, khalal and rutab stages.

Growth Stage	Cultivars	Fruit Length (cm)	Fruit Width (cm)	Fruit Weight (gm)	Seed Length (cm)	Seed Weight (gm)	Seed Width (cm)	Flesh Weight (gm)	Pulp Fruit Ratio (%)
Kimri	Aab-e-dandan	4.3 ± 1.2^{a}	2.2 ± 1.1^{bc}	13.8 ± 0.8^{c}	2.2 ± 0.5^{b}	1.0 ± 1.6^{c}	0.5 ± 0.5^{bc}	12.8 ± 0.5c	92.2 ± 1.4^{b}
	Begum Jangi	3.5 ± 0.6^{abc}	2.0 ± 1.2^{bc}	9.8 ± 0.4^{c}	2.1 ± 0.6^{b}	1.0 ± 1.4^{b}	0.6 ± 0.6^{c}	8.8 ± 0.6^{d}	89.8 ± 1.3^{d}
	Gogna	3.6 ± 1.3^{ab}	2.3 ± 1.4^{bc}	15.1 ± 1.2^{c}	2.4 ± 0.3^{b}	1.3 ± 1.2^{b}	0.9 ± 1.1^{a}	13.8 ± 0.4^{c}	91.3 ± 1.5^{c}
	Halini	2.7 ± 0.7^{c}	2.4 ± 0.5^{b}	19.7 ± 0.6^{b}	1.9 ± 0.4^{c}	1.0 ± 0.5^{c}	0.7 ± 1.4^{b}	18.7 ± 1.3^{b}	94.9 ± 0.7^{b}
	Kooznabad	3.5 ± 0.5^{abc}	2.2 ± 0.6^{bc}	10.2 ± 0.6^{d}	2.2 ± 0.4^{b}	0.9 ± 0.7^{d}	0.7 ± 0.5^{b}	9.3 ± 0.6^{d}	90.5 ± 0.5^{c}
	Muzawati	3.3 ± 0.4^{bc}	2.2 ± 0.4^{bc}	22.5 ± 1.2^{a}	2.0 ± 0.5^{d}	0.9 ± 1.5^{d}	0.6 ± 1.2^{c}	21.6 ± 1.5^{a}	95.9 ± 1.5^{a}
	Pashna	3.2 ± 0.5^{bc}	1.9 ± 1.1^{c}	13.7 ± 0.5^{c}	2.3 ± 1.2^{b}	1.5 ± 0.5^{ab}	0.6 ± 1.4^{c}	12.2 ± 1.3^{c}	89.0 ± 1.5^{d}
	Shakri	4.01 ± 0.6^{b}	2.5 ± 0.6^{a}	21.6 ± 0.4^{b}	2.7 ± 0.7^{a}	2.0 ± 0.3^{a}	0.8 ± 0.6^{b}	19.6 ± 1.5^{b}	90.7 ± 0.6^{c}
	LSD (0.05)	0.03**	0.05**	0.000***	0.002**	0.07^{ns}	0.08^{ns}	0.000**	0.042*
Khalal	Aab-e-dandan	5.3 ± 0.5^{a}	2.3 ± 0.8^{c}	14.6 ± 0.4^{bc}	2.9 ± 0.6^{a}	1.34 ± 0.6^{d}	0.9 ± 1.4^{b}	13.2 ± 1.7^{d}	90.8 ± 0.9^{c}
	Begum Jangi	4.6 ± 0.4^{b}	2.4 ± 1.3^{c}	11.3 ± 0.6^{d}	2.2 ± 1.7^{cd}	1.7 ± 0.5^{b}	0.8 ± 1.2^{b}	9.5 ± 0.9^{c}	84.4 ± 0.3^{d}
	Gogna	4.9 ± 0.6^{b}	2.4 ± 1.5^{c}	15.9 ± 1.3^{c}	2.5 ± 1.4^{bc}	1.5 ± 0.7^{c}	1.0 ± 1.5^{a}	14.3 ± 0.5^{d}	90.3 ± 0.5^{c}
	Halini	4.4 ± 0.5^{b}	2.9 ± 1.4^{b}	21.6 ± 0.5^{b}	2.1 ± 1.7^{d}	1.8 ± 1.3^{b}	0.9 ± 0.7^{c}	19.8 ± 0.3^{c}	91.4 ± 0.8^{b}
	Kooznabad	4.3 ± 1.5^{c}	2.4 ± 0.8^{c}	11.8 ± 0.4^{bc}	2.5 ± 1.5^{c}	1.1 ± 0.6^{d}	0.8 ± 0.6^{d}	10.6 ± 0.6^{f}	89.9 ± 0.7^{c}
	Muzawati	5.0 ± 1.2^{ab}	2.7 ± 0.5^{c}	23.3 ± 1.2^{a}	2.6 ± 0.6^{c}	1.2 ± 0.8^{c}	0.9 ± 0.3^{c}	22.0 ± 0.4^{a}	94.6 ± 0.6^{a}
	Pashna	4.3 ± 0.7^{c}	2.1 ± 0.8^{d}	14.9 ± 1.5^{bc}	2.8 ± 1.5^{b}	1.8 ± 1.1^{b}	0.9 ± 0.3^{c}	13.0 ± 1.1^{d}	86.8 ± 0.5^{d}
	Shakri	4.8 ± 1.3^{b}	2.4 ± 0.5^{a}	23.3 ± 0.7^{a}	2.8 ± 0.6^{b}	2.4 ± 1.5^{a}	1.0 ± 0.6^{b}	20.8 ± 1.4^{b}	89.3 ± 0.4^{c}
	LSD (0.05)	0.004**	0.07**	0.000**	0.005**	0.06^{ns}	0.07^{ns}	0.000**	0.051*
Rutab	Aab-e-dandan	5.6 ± 1.4^{a}	2.7 ± 0.8^{c}	15.5 ± 0.6^{d}	3.0 ± 0.8^{a}	1.9 ± 0.6^{bc}	1.0 ± 0.3^{c}	13.5 ± 0.5^{e}	87.4 ± 0.6^{c}
	Begum Jangi	4.8 ± 0.6^{d}	2.7 ± 0.5^{c}	11.9 ± 0.6^{f}	2.6 ± 1.1^{cd}	1.9 ± 0.8^{b}	0.9 ± 0.8^{d}	9.9 ± 0.6^{f}	83.5 ± 0.4^{e}
	Gogna	5.1 ± 1.5^{ab}	2.6 ± 0.6^{d}	16.7 ± 0.7^{c}	2.7 ± 1.5^{c}	1.8 ± 0.7^{c}	1.1 ± 0.5^{b}	14.8 ± 0.8^{d}	89.0 ± 1.5^{c}
	Halini	4.8 ± 0.6^{d}	3.1 ± 0.4^{a}	22.1 ± 0.6^{b}	2.4 ± 0.9^{d}	1.9 ± 0.8^{b}	1.0 ± 0.4^{b}	20.1 ± 1.1^{c}	95.4 ± 0.8^{a}
	Kooznabad	4.8 ± 1.5^{d}	2.8 ± 1.4^{c}	12.9 ± 1.3^{e}	2.7 ± 0.8^{c}	1.7 ± 1.5^{c}	0.9 ± 0.6^{c}	11.1 ± 1.5^{f}	86.5 ± 0.5^{c}
	Muzawati	5.5 ± 1.3^{b}	2.9 ± 0.6^{b}	24.0 ± 1.5^{a}	2.8 ± 1.5^{bc}	1.5 ± 1.6^{d}	1.0 ± 0.4^{b}	22.5 ± 1.1^{a}	93.7 ± 0.4^{b}
	Pashna	4.9 ± 0.5^{c}	2.8 ± 0.5^{b}	15.5 ± 0.5^{d}	2.9 ± 0.4^{b}	1.9 ± 0.8^{b}	1.2 ± 0.5^{a}	13.5 ± 1.2^{e}	87.4 ± 0.7^{d}
	Shakri	5.1 ± 0.5^{c}	2.8 ± 0.7^{b}	23.7 ± 1.3^{b}	2.9 ± 1.5^{b}	2.6 ± 0.4^{a}	1.1 ± 0.3^{b}	21.0 ± 0.5^{b}	88.9 ± 0.8^{c}
	LSD (0.05)	0.006**	0.05**	0.000**	0.05**	0.08^{ns}	0.06^{ns}	0.000**	0.041*

Values followed by the same letter are not significantly different at $p < 0.05$.

weight (8.57 g), fruit width (1.3 cm), seed length (1.8 cm) and seed weight (0.54 g). Further, the data recorded in cultivar Pashna, i.e., fruit length (2.8 cm), fruit weight (4 g), fruit width (1.5 cm), seed length (1.9 cm) and seed weight (0.64 g). On the contrary, the fruit length (4.8 cm) and fruit weight (11.9 g) was observed in cultivar Begum Jangi, whereas fruit length (4.8 cm) and fruit weight (22.1 g) was observed in cultivar Halini in district Khairpur at rutab stage. Dates at rutab stage contain high moisture content compared to tamar stage. Generally, the dates grown in district Khairpur showed excellent fruit size and weight in all studied cultivars of Balochistan. Soil of district Khairpur is different to the soil of Turbat and Panjgur, Balochistan. Sandy loam soil of these areas and water shortage reduces the bunch number and yield per tree in Turbat and Panjgur. In the present study such growth parameters were studied showed differences among different cultivars regarding vegetative, flowering and fruit physical characteristics, but simultaneously all growth parameters were normal and did not show any type abnormal growth. Morphological attributes of tree and fruits showed that soil (clay loam) and climatic conditions of district Khairpur

(Shah Abdul Latif University, Khairpur) were suitable for the growth of exotic date palm cultivars brought from Balochistan, Pakistan cultivated in district Khairpur, Pakistan. Temperature in district Khairpur fluctuate from 40 °C (during May) to 50-52 °C (June to July), which is acceptable range for growth and ripening of date palm fruit from kimri to rutab stage. Soil of Turbat and Panjgur is sandy loam and average temperature is 45-50 °C during July. Plants show variation in yield, vegetative traits and morphological properties of fruits and seeds in response to environmental changes [39, 40]. El-Sharabasy and Sherif [20] conducted field evaluation of three dry date palm cultivars (Sakkoti, Bertamoda and Gondila) regarding vegetative, fruit physical and chemical characteristics. Bacha *et al*. [41] studied fruit physical and chemical characteristics of four date palm cultivars (Seleg, Sakhi, Khudari and Nebut Seif) during three stages of fruit development (kimri, khalal and tamar). In the current study fruit physical characters were studied at three distinct fruit growth stages (kimri, khalal and rutab). Solangi *et al*. [17] conducted study on the physico-chemical attributes of three Saudi Arabian cultivars of the date palm i.e., Ajwa, Safawi and Ruthana, and observed that soil and climate of district Khairpur, Pakistan was suitable for the cultivation. Quality of the fruits is important character to obtain a maximum economic profit and create a better relationship between demand and supply. Kimri is the second fruit growth stage after hababouk (longest period of the fruit growth) characterized as green, inedible, and exhibits rapid growth. Al Udhaib [42] recorded the fruit length (27.5 mm) and fruit weight (5.8 g) at kimri stage. Weekly growth (90% at kimri stage) decreased to 20% at late kimri stage was observed by Tafti and Fooladi [43]. Abdul-Hamid *et al*. [44] and Solangi *et al*. [17] observed differences in fruit dimensions and length in cv. Ajwa i.e., fruit diameter (14.6 mm) and fruit length (26.4 mm). Khodabakhshian and Khojastehpour [45] recorded fruit measurements at kimri stage such as, length, width and thickness (34.4, 17.5, and 16.80 mm, respectively). Similarly, the current study described the fruit measurements from kimri (Figure 1) to rutab stage (Figure 3), showed differences in fruit colour, size and weight. After kimri stage, fruit gradually convert to edible khalal stage (third fruit growth stage), and is characterized with the development of a particular colour of the fruits (red or yellow) depending on the type of variety (Figure 2). Late khalal stage is the last fruit growth stage before conversion of fruits into rutab, and is characterized with highest fruit weight, length and dimension. Several studies [17, 46-47] observed that after kimri stage, fruit converts to the next stage, the khalal, which brings a change in the fruit colour i.e., green to yellow or red depend on the variety, with increase in fruit length and width, decrease in weekly growth and increase in sugars. Al-Jasass *et al*. [48] described different colours in Moroccan dates at khalal. Biglari *et al*. [49] stated that change in the colour of dates was generally due to genetic differences develop particular colour pigments at khalal stage. Different pigments (carotenoid, chlorophyll and anthocyanin) produce green, yellow and red colours in dates from kimri to khalal stage [50]. In the current study, colour of all fruits was green at kimri stage, whereas the colour of the fruits at khalal stage was red (Shakri and Muzawati) and yellow (Aab-e-dandan, Begum Jangi, Gogna, Halini, Kooznabad and Pashna). Fruit dimensions of the dates were higher at khalal stage [51]. Studies conducted on dates in Tunisia [52], Iran [53] and Pakistan [17, 24] reported higher fruit dimensions at khalal. Similarly in the present study higher fruit dimensions were noted at khalal stage. Edible rutab stage is obtained before harvesting during which the khalal stage dates convert into edible rutab stage, whereas, dates remain attached in the bunches on the tree. Half of the date fruit turn into soft brown or black from one side with development of sweet taste due to high concentration of sugar and is called rutab [17, 42] (Figure 3). Al-Shahib and Marshall [47] described that after harvest storage of khalal dates under low temperature can save from spoilage. Ahmed *et al*. [54] suggested that proper fruit harvest stage is rutab, which saves the fruit from ripening failure, however, fruit harvesting at early khalal stage consume more time in drying process. Khalal stage, dates contain high moisture content, therefore fruit contain more weight as compared to tamar stage dates which contain low weight due to significant decrease in moisture content [17, 55]. The size of date fruit reduces at rutab and tamar stage compared to kimri and khalal stages of fruit growth [45]. Tafti and Fooladi [43] observed difference in weight of the fruit at ripening stage in cv. Shamsaei. The current study described size of fruits increases rapidly from kimri to khalal, while decrease from rutab to tamar due to decrease in the moisture content.

5. CONCLUSIONS

Field evaluation of eight date palm cultivars of Balochistan exhibited normal vegetative, bunch and fruit morphology. Variations were observed among different cultivars regarding vegetative, flowering and fruit physical characteristics were cultivar dependent, but fruit size of all studied cultivars was excellent. Pollination time varies among different studied cultivars did not affect the quality of fruits. Highest yield was noted in cv. Muzawati at harvest time. Evaluation of fruit physical characteristics showed significant variations among fruits of different cultivars at different growth stages regarding colour, size and weight. Cultivar Aab-e-dandan showed higher fruit size at different growth stages. The obtained results may not comparable to the dates obtained from their original place, i.e., Turbat and Panjgur, Balochistan due to variation in climate and soil and water availability. The results obtained in the present study will support the selection of elite commercial date palm cultivars to cultivate and streamline the varietal structure of the date palm in the area.

6. CONFLICT OF INTEREST

Authors declare that they have no any conflict of interest.

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Research Article

Bacterial Etiology and Antibiotic Susceptibility Patterns in Urinary Tract Infection among Patients with Various Renal Conditions

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Abstract: Urinary tract infections (UTIs) are a common medical problem affecting a significant number of individuals around the world. The administration of UTIs in patients with renal abnormalities can be challenging, as these patients might require specific consideration and antibiotic regimens. The purpose of this study, which was carried out in the District Bahawalpur, was to investigate the prevalence, bacterial composition, and antimicrobial resistance pattern of UTIs in individuals with renal conditions, including renal calculi, renal cysts, and renal failure. In this descriptive cross-sectional study, 50 clinical samples from Bahawal Victoria Hospital, Bahawalpur, were subjected to microbiologic analysis using urine culture and drug susceptibility testing, and statistical analysis using descriptive statistics. Mean Patient Age was 28.52 ± 2.99, and the female to male ratio was 27:23. Among 50 patients 44% had right kidney stones, and 56% of patients faced UTIs for the first time. The most common pathogen was *Escherichia coli* (*E. coli*) (50%), which was followed by *Klebsiella spp* (20%), *Pseudomonas spp* (16%), *Morganella spp* (8%), and *Staphylococcus aureus* (6%). Azetronam was effective against some bacteria, including 4.0% of *Escherichia coli* and 8.0% of *Klebsiella spp*. Amoxicillin and Polymyxin were less effective than Augmentin and Amikacin, which were the most often used antibiotics. Our study demonstrates that antibiotic selection based on bacterial etiology is crucial for effective UTI treatment in patients with renal abnormalities. These findings improve patient outcomes and address the challenge of antibiotic resistance in patients with renal conditions.

Keywords: Antibiotic Susceptibility, Bacterial Etiology, Renal Conditions, Urinary Tract Infection.

1. INTRODUCTION

Urinary tract infections (UTIs) are a typical medical problem affecting a huge number of individuals around the world. However, people with renal conditions, like those with ongoing specific renal conditions (SRC) such as Bilateral kidney stone, right kidney stone, left kidney stone, bladder stone, renal cyst and renal failure, are at an increased risk of developing UTIs. These patients frequently have altered immune systems, which leads to intermittent or more extreme UTIs. Moreover, the utilization of immunosuppressive prescriptions in patients with SRC can additionally increase the risk of UTIs. The administration of UTIs in patients with renal abnormalities can be challenging, as these patients might require specific consideration and antibiotic regimens. Kidney stones influence many individuals, and up to 10% of all people will have a kidney stone in the course of their life [1]. Patients with renal diseases frequently had polymicrobial infections, with different microbes in their urine samples. Alternately, those with ordinary renal function had isolated bacterial infections, normally caused by *Escherichia coli* [2]. *Klebsiella pneumoniae is* the most prevalent intestinal pathogen, followed by *Pseudomonas aeruginosa* and several *Enterococci species* [3]. *E. coli* is the most prevalent uropathogen. However, in patients with specific renal conditions (SRC), *P. aeruginosa*, *Enterococcus faecalis*, and other non-*Enterobacteriaceae species* were more likely to cause infections [4]. The key component of

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standard UTI therapy is the use of antibiotics. The appropriate antibiotics should be chosen by considering the antibiotic susceptibility pattern of the causative isolate, infection type (community-acquired or hospital-acquired infection), the patient's conditions, including age, gender, history of allergy, underlying diseases, prior antibiotic consumption, taking other medications, history of prior UTIs, site of infection, and other factors [5]. Various antibiotics, including Amoxicillin, Ceftriaxone, Cephalexin, Ciprofloxacin, Fosfomycin, Levofloxacin, Nitrofurantoin, and Trimethoprim/sulfamethoxazole, treat urinary infections. Little has been accomplished despite the great efforts, and more study is needed to develop an antibiotic substitute for the treatment of UTIs [5]. In our study, the prevalence, bacterial composition, and antibiotic susceptibility patterns of UTIs in individuals with SRC were examined. UTIs are frequent infection that affects the genitourinary system and may be linked to numerous structural, metabolic, or functional problems of the kidneys. To create successful prevention measures and direct suitable treatment techniques, it is essential to understand the connection between SRC and the occurrence of UTIs. Additionally, investigating the bacterial etiology and patterns of antimicrobial resistance of UTIs in this particular patient population can offer insights into the selection of antimicrobial drugs for the best patient management. This study aims to enhance clinical decision-making by contributing to the understanding of UTIs in patients with SRC and to enhance clinical decision-making in the management of UTIs in these patients.

2. MATERIALS AND METHODS

2.1. Study Design

This study was conducted at the Bahawal Victoria Hospital (BVH), Bahawalpur, Pakistan, from July 2022 to January 2023. Urine samples were collected from the patients of the outdoor and indoor departments using aseptic techniques in a sterile container, which was then carried to the pathology section of Quaid-E-Azam Medical College (QMC), Bahawalpur, for further processing. This study was approved by the Ethical Review Committee of the Islamia University of Bahawalpur (letter No. IUB/ERC/20/2022). Specimens were collected using a straightforward random sampling technique in accordance with international safety regulations and biosafety standards, informed consent ensuring confidentiality and anonymizing data during analysis and reporting. Patient's clinical history including age, gender (male & Female), UTI history, hygiene as well as the presence of bilateral kidney stone, right kidney stone, left kidney stone, bladder stone, renal cyst, and renal failure was collected from the patient. Patients without above given conditions were excluded from the study samples. Contaminated specimens (with three or more pathogens exceeding 100,000 colonies/mL) were also excluded from analysis.

2.2. Data Collection

Fifty urine samples were collected from patients showing positive symptoms for UTIs in sterile conditions in the morning from the outdoor patient ward. The patient's identification and the date of collection were written on the sample container before the urine analysis process started. A sterile tube was used for culture after 0.5-1.0 mL had been thoroughly mixed and aseptically transferred. Before doing biochemical analysis with a dipstick or automatic reader, the sample's physical properties, i.e., color, clarity, and odor, were recorded. To achieve a clear supernatant for turbid or discolored materials, centrifugation was performed prior to testing. A refractometer was used to analyze the sediment's specific gravity, and at 100X and 400X magnifications, microscopic analysis revealed cellular components, casts, crystals, and bacteria. Chemical analysis was performed using 9 reagent "Chemistript", which measured nitrite, leukocyte esterase, pH, protein, glucose, ketones, Urobilinogen, and bilirubin semi-quantitatively. These strips could also detect hemoglobin, erythrocytes, and myoglobin.

2.3. Isolation of Uropathogens

While urine culture remained the preferred test, positive dipstick testing was considered specific for asymptomatic bacteriuria. A sterilized platinum wire loop (0.001 mL) was used to spread the urine sample on petri dishes in UV UV-irradiated culture hood to maintain a sterilized environment to avoid environmental contamination on LAMIL PLUS. The prepared media was poured into the Petri dishes before cooling down and solidifying. Urine samples were spread on MacConkey agar and blood agar (OXOID, UK). The culture dishes were incubated

at 37 °C for 18-24 hours. The catalase and oxidation tests were performed to detect oxidase and catalase-positive colonies.

2.4. Gram Staining

Samples were smeared on slides, each labeled with its sample number, forming colonies. A primary crystal violet stain was applied, followed by fixer and decolorizing steps each lasting 30 seconds. Safranin was then used for 30 seconds. Gram staining differentiates Gram-negative and Gram-positive bacteria [6].

2.5. Biochemical and Oxidation Test

Bacteria produce toxic superoxide and hydrogen peroxide (H_2O_2) during aerobic respiration, which can cause cell death. To counteract this, they generate superoxide dismutase for superoxide and catalase for H_2O_2. Gram-positive and gram-negative bacteria can be distinguished using the catalase test, which entails adding 3% H_2O_2 to a culture and watching for bubbles [7], [8]. A variety of sugars were added to carbohydrate fermentation broth, and fresh overnight cultures were inoculated. The color change of broth was monitored after 24 to 28 hours incubation [9]. Oxidase enzymes, which catalyze the production of H_2O or H_2O_2 during aerobic respiration, are detected by the oxidase test. Aerobic, facultative, and microaerophiles generally have oxidase activity, but obligatory anaerobes don't have oxidase activity. With the exception of Bacillaceae, the majority of Gram-positive species are oxidase negative, whereas the majority of Gram-negative organisms have oxidase activity [8].

2.6. Antimicrobial Susceptibility

After sterilizing the loop, colonies from Petri dishes were transferred to Muller-Hilton agar. Using a cotton swab stick, the sample was spread across the plate for 24 hours at 37 °C. Different types of antibiotics were used to test antibiotic sensitivity of identified bacteria, including Ceftazidim (CAZ) 30 µg, Levofloxin (LEO) 5 µg, Penicillin (P) 10 µg, Tetracycline (TGC) 30 µg, Cefoxitin (FOX) 30 µg, Erythromycin (E) 30 µg, clarithromycin (CLR) 15 µg, Moxifloxacin (MXF) 5 µg, Meropenem (MEM) 10 µg Teicoplanin (TEC) 30 µg, Imipenem (IMI) 10 µg, Sulphamethoxazole (SXT) 25 µg, Amox + Clav (AUG) 30 µg, Vancomycin (VA) 30µg, Cefotaxime (CTX) 30 µg, Fusidic acid (FD) 10 µg, linezolid (LNZ) 30 µg, Clindamycin (DA) 10 µg, and Tobramycin (TOB) 10 µg (Sensi-Discs™ Ceftazidime, CAZ-30). The Disc diffusion test was qualitative, which categorizes susceptibility derived by RIS category, including resistance, intermediate, and sensitive, following MIC standards [10]. Antibiotic discs for Gram-negative rods and Gram-positive cocci were placed on the agar, followed by 12-hour incubation. The resulting zones indicated bacterial sensitivity, intermediate, and resistance response. The zone size for each medication was interpreted using the standards established by the Clinical and Laboratory Standards Institute (CLSI) [11]. Results were recorded and used to recommend suitable treatment.

2.7. Statistical Analysis

Statistical analyses were conducted using software like SPSS V.27. Descriptive statistics, encompassing frequencies, percentages, means, and standard deviations, summarized the demographic and clinical traits of the study group. The prevalence of urinary tract infections (UTIs) in patients with renal stones and diabetes was determined by calculating case proportions within the sample. To gauge the correlation between factors and UTI occurrence, inferential tests were performed. Chi-square test examined categorical variables (e.g., gender, hygiene, previous operations). The antimicrobial resistance of uropathogens was assessed, noting proportions of resistance, intermediate, and sensitivity to antibiotics. The zones of inhibition around antibiotic discs were measured, and diameters were recorded. Comparing resistance patterns among bacterial species involved a chi-square test to ascertain statistical significance. P-values < 0.05 were considered significant.

3. RESULTS

3.1. Demographic and Clinical Characteristics of Study Participants

This study explored UTIs in individuals with renal conditions, providing insights into their prevalence, bacterial etiology, and antimicrobial resistance patterns. It involved 50 participants with renal stones, with a higher female-to-male ratio. A slightly higher percentage of patients had bladder stones than bilateral kidney stones, which

impact both kidneys. Only a very small fraction of subjects had kidney failure. Renal cysts were uncommon; however, left kidney stones were more prevalent. Right kidney stones were found in the largest group. Participants' mean age was 28.52 ± 2.99 years. A little over half of the participants reported experiencing their first UTI, and a lower percentage reported having a second episode. A comparable percentage reported a third episode, and only a tiny percentage experienced more than three UTI episodes (Table 1). Visually depicts UTI prevalence among participants with various renal conditions, aiding our understanding of how these conditions influence UTI rates. Chi-square tests showed no significant association between types of SRC and UTI frequency. Confidence interval with 95% confidence level is used to determine UTI frequencies in population based on the study samples.

3.2. Frequency Distribution of Urinary Tract Infections among Renal Condition Categories

The study explored UTI frequency distribution among SRC categories (Table 2). Notably, UTI frequencies significantly varied. Bilateral kidney stone, kidney failure, left kidney stone (LKS), and right kidney stone (RKS) spanned the 1st to "More" frequency categories, signifying broader UTI ranges. Conversely, bladder stones and renal cysts mostly appeared in the 1st category. Urinary tract infections were present in a significant percentage of patients with bilateral renal abnormalities at variable frequencies, suggesting persistent infections. Right kidney stones were present in a substantial number of cases, indicating a greater vulnerability to UTIs. The most prevalent kidney stones were on the left, suggesting a higher risk of infection. Urinary tract infections linked to renal cysts and bladder stones were very uncommon.

3.3. Severity and Clinical Presentation of Ultrasound Injuries in Renal Calculi, Cyst and Failure

This study investigated the severity and clinical presentation of UTIs in people with renal calculi, cysts, and failure. Despite the fact that several of the 37 patients lacked microbiological data, doctors

Table 1. Mean, standard deviation, and percentage of variables.

Variables		
Age (Mean ± SD, Median (IQR))	28.52 ± 2.99, 24.50(40)	
Gender Frequency		
Female	27(54%)	
Male	23(46%)	
Renal Abnormalities		
Mean ± SD	8.33 ± 8.641	
Bilateral Kidney stone	2(4%)	
Bladder Stone	3(6%)	
Kidney failure	6(12%)	
Left kidney stone	16(32%)	
Renal cyst	1(2%)	
Right kidney stone	22(44%)	
UTI Frequency		
Mean ± SD, Median (IQR)	1.68 ± 0.86, 1.00(1.00)	95% confidence interval
1st time	28(56%)	0.0097, 0.3203
2nd time	11(22%)	0.0063, 0.1537
3rd time	10(20%)	0.0048, 0.1452
More	1(2%)	-0.0448, 0.1298

Note: Demographic and Clinical Characteristics of Study Participants.

Table 2. Distribution of UTI frequencies among patients with various renal abnormalities.

Renal abnormalities	UTI frequency (%)				Total (%)
	1st	2nd	3rd	More	
Bilateral	4	0	0	0	4
Bladder Stone	6	0	0	0	6
Kidney failure	2	2	8	0	12
Left Kidney Stone	18	10	4	0	32
Renal cyst	0	2	0	0	2
Right Kidney Stone	26	8	8	2	44
P-value	0.224	0.263	0.285	0.306	

treated their symptoms. Notably, pus cell counts, indicating considerable inflammation or infection, while yeast presence, indicating compromised immunity. While epithelial cells, white and red blood cell casts, indicating hemorrhage, Inflammation, trauma, and neoplasia infection. The data distribution was revealed by percentiles, with the 25th percentile indicating that at least 25% of patients had values that were at or below 1.00. The 75th percentile showed that at least 75% of patients had values at or below 1.00 for several parameters, whereas medians showed that around 50% of patients had values between 1.00 and 2.00 (Table 3).

3.4. Antibiotic Sensitivity and Resistance

The antibiotic sensitivity and resistance among microorganisms. Azetronam and tazobactam had inconsistent efficacy, while Imipenem and cotrimoxazole showed high sensitivity to various bacteria. Cephalexin consistently demonstrated sensitivity, indicating its safety for treatment. Nitrofurantoin and ciprofloxacin exhibited clear resistance, underscoring the need for cautious use. Azetronam was effective against some bacteria, including 4.0% of *E. coli* and 8.0% of *Klebsiella species*. Imipenem generally showed sensitivity, especially against S. aureus. Cotrimoxazole displayed high sensitivity across all bacteria. Sensitivity rates varied for ciprofloxacin and nitrofurantoin. Notably, *S. aureus* was 100.0% sensitive to vancomycin, offering valuable insights into UTI antibiotic resistance and sensitivity patterns (Table 4). The p-value indicates Antibiotic susceptibility significance in contrast with bacterial etiology.

3.5. Bacterial Etiology and Antibiotic Resistance in UTI Patients with Renal Conditions

The study investigated UTI bacterial etiology and antibiotic resistance in patients with bilateral kidney stones, right kidney stones, left kidney stones, bladder stones, renal cyst and renal failure (Table 5). *Escherichia coli* (*E. coli*) was the most common pathogen (50%), followed by *Klebsiella spp* (20%), *Pseudomonas spp* (16%), *Morganella spp* (8%), and *Staphylococcus aureus* (6%). Antibiotic prescriptions varied based on the bacteria. Ciprofloxacin and Imipenem were common for *E. coli*. Moxifloxacin was preferred for *Klebsiella spp* and *Morganella spp*. *Pseudomonas spp* showed resistance to many antibiotics. Moxifloxacin was primary for *Staphylococcus aureus*.

Table 3. Mean and standard deviation for the urine analysis test.

		Yeast	Pus cell	WBC cast	RBC cast	Granule	Epithelial cell
Mean		1.16	1.81	1.30	1.16	1.03	1.51
Std. Deviation		0.374	0.397	0.463	0.374	0.164	0.507
Percentiles (IQR)	Q1 = 25	1.00	2.00	1.00	1.00	1.00	1.00
	Q2 = 50	1.00	2.00	1.00	1.00	1.00	2.00
	Q3 = 75	1.00	2.00	2.00	1.00	1.00	2.00

Note: Summary of urine analysis statistical analysis and percentiles for measured parameters.

Table 4. Percentage of antibiotic susceptibility.

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistant (%)	Bacterial Etiology (%)					p-value
				E. coli	*Klebsiella spp*	*Morganella spp*	*Pseudomonas spp*	*S. aureus*	
Azetronam	4.0	88	8	52	20	4	15	6	0.220
Imipenem	94	6	0	0	0	0	0	100	0.287
Tazobactam	44	56	0	42	25	10	10	10	0.265
Cotrimoxazole	6	88	6	47	9	18	9	6	0.241
Cephalexin	16	84	0	59	21	7	4	7	0.241
Ceftazidime	32	54	14	48	12	11	7	11	0.241
Amikacin	56	22	22	36	36	9	0	18	0.241
Sulbactam	38	58	4	46	20	10	13	10	0.241
Ceftriaxone	20	74	6	40	27	5	18	8	0.220
Ciprofloxacin	36	16	48	53	15	0	7	23	0.220
Levofloxacin	32	24	44	50	16	16	0	16	0.265
Cefepime	24	66	10	54	21	9	6	9	0.241
Sulphamethoxazole	4	92	4	52	21	8	15	2	0.220
Gentamycin	26	60	14	53	13	0	23	10	0.220
Amox+clav	28	44	28	54	27	0	9	9	0.241
Cefotaxime	18	62	20	41	22	12	16	6	0.220
Nitrofurantoin	44	48	8	33	20	12	25	8	0.220
Cephalexin	4	96	0	52	20	8	16	2	0.220
Fosfomycin	16	80	4	52	20	0	20	7	0.241
Vancomycin	4	94	2	53	21	8	17	0	0.220
Linezolid	4	96	0	52	20	8	16	2	0.220
Clindamycin	2	94	4	51	21	8	17	2	0.220
Teicoplanin	4	92	4	52	21	6	17	2	0.220
Azithromycin	0	96	4	50	20	8	16	4	0.220
Clarithromycin	2	94	4	53	21	8	14	2	0.220
Erythromycin	0	98	2	51	20	8	16	4	0.220
Moxifloxacin	2	94	4	51	21	8	17	4	0.220
Cefadroxil	2	88	10	54	15	6	15	6	0.265
Ampicillin	2	76	22	60	13	0	18	7	0.220
Cefixime	2	80	18	55	12	7	17	7	0.241
Total				50	20	8	16	6	

Note: Microbial profile of urine culture and antibiotic resistance/sensitivity patterns.

4. DISCUSSION

The study provides insights into UTIs in patients with renal conditions, specifically renal stones and diabetes. The gender distribution showed more females (54%) than males (46%), consistent with previous research [12-14]. Prevalence of Bilateral kidney stone, right kidney stone, left kidney stone, bladder stone and renal cyst among participants: bilateral kidney stones (4%), bladder stones (6%), kidney failure (12%), left kidney stones (32%), renal cysts (2%), and right kidney stones (44%). Sample characteristics and geographic factors can influence variation in prevalence rates [15-17]. These findings highlight the diverse renal conditions present in the study population, which may contribute to the susceptibility and recurrence of UTIs.

This study's insights into UTI distribution among renal calculi, cysts, and failure categories offer valuable perspectives. Comparing them with other studies helps identify commonalities and disparities, shedding light on underlying factors. In participants with bilateral renal stones, 11.5% had recurring UTIs, aligning with prior research [18]. In this study, the highest number of UTI cases (50 cases) occurred in the age group of 1-9 years, indicating a higher prevalence among younger individuals. This aligns with previous research showing increased UTI incidence in pediatric populations [19]. Factors like anatomical differences, incomplete bladder emptying, and lower hygiene awareness contribute to the higher susceptibility in young children [20, 21]. As age increased, UTI frequency generally decreased, with the 10-19 and 20-29 age groups having 12 and 11 UTI cases, respectively. Age-related urinary tract changes, hormonal factors, and comorbidities likely contribute to this trend. In the 60-69, 70-79, and 80-89 age groups, UTI frequencies were even lower, with 6, 5, and 2 cases, respectively, consistent with previous studies noting reduced UTI incidence in the elderly [22]. Factors such as immune function changes, bladder dysfunction, and comorbidities may underlie the decreased susceptibility to UTIs in older individuals. In this study, UTI prevalence and severity were assessed using various parameters. Yeast presence in urine was relatively low (mean value: 1.16), aligning with previous studies reporting lower fungal UTIs compared to bacterial UTIs [23]. Mean values for WBC cast and RBC cast confirmed a mild to moderate presence of white and red blood cells, further supporting UTI occurrence [24].

Table 5 presents insights into microbial profiles of UTIs and antibiotic resistance/sensitivity patterns. Variations in sensitivity and resistance rates for each antibiotic-bacteria combination were observed. Azetronam showed 4.0% to 8.0% sensitivity against *E. coli* and *Klebsiella spp*., consistent with previous studies [24]. Beta-lactam drug imipenem demonstrated high overall sensitivity except for *S. aureus*, which displayed above 90% resistance, in line with its effectiveness against most bacteria but not MRSA strains [25] *S. aureus* develops resistance to β-lactam antibiotics through two main mechanisms: the production of β-lactamase enzymes, which render the antibiotic inactive, and the use of an alternative penicillin-binding protein (PBP2a), which has a low affinity for these antibiotics and allows the bacteria to survive despite their presence [26, 27]. It also highlights the role of BlaR1 receptor senses β-lactams and initiates β-lactamase production [28-30].

Table 5. Antibiotic prescriptions tailored to microbial profiles in study participants.

Antibiotics	**Bacterial etiology (%)**					**Total (%)**	**p-value**
	E. coli	***Klebsiella spp***	***Morganella spp***	***Pseudomonas spp***	***S. aureus***		
Amikacin	0	2	0	0	0	2	0.287
Amoxicillin	4	0	0	0	0	4	0.287
Augmentin	4	2	2	0	2	10	0.265
Ciprofloxacin	22	2	2	2	2	30	0.287
Imipenem	18	2	0	4	0	24	0.241
Moxifloxacin	2	10	4	6	0	22	0.220
Polymyxin	0	2	0	4	2	8	0.265

Cotrimoxazole showed high sensitivity rates (80.0% to 100.0%) for all bacteria, supporting its use as a first-line treatment for complicated UTIs [31]. However, resistance to antibiotics like ciprofloxacin and nitrofurantoin was evident, highlighting the global concern about increasing antibiotic resistance in UTIs [32]. Consistent with previous research, this study identified *E. coli* as the most common pathogen, accounting for 50% of cases [33] because *E. coli* uses a variety of CUP pili to attach to and invade urinary tract cells, particularly those in the bladder [34]. By establishing quiescent intracellular reservoirs (QIRs) and intracellular bacterial communities (IBCs) [35]. It evades the host's immune system through an aggressive immune response, particularly a strong inflammatory reaction involving lymphocytes causes significant damage to the urinary tract's protective lining (the mucosal uroepithelium) [36]. Ciprofloxacin is frequently prescribed for *E. coli* infections (22%), along with Imipenem (18%), which is effective against Gram-negative bacteria [37]. Proper antibiotic selection is crucial to effectively target this prevalent pathogen. *Klebsiella spp* exhibited varying susceptibility patterns. Moxifloxacin was the preferred choice in 10% of cases, supported by studies [38]. Less common antibiotics included Augmentin, Amikacin, Amoxicillin, and Polymyxin, suggesting the need for additional treatment options for diverse *Klebsiella spp*. infections. *Pseudomonas spp* infections displayed widespread drug resistance, with only 2% responding to the antibiotic. Imipenem and Polymyxin were used in 4% of cases, indicating the demand for broad-spectrum antibiotics against *Pseudomonas spp* [39].

The study offers new insight into healthcare that may affect clinical practice and existing treatment recommendations for urinary tract infections (UTIs) in individuals with SRC. The continued use of cotrimoxazole and imipenem as first-line therapies for complex UTIs and highlighting the alarming resistance patterns, especially for ciprofloxacin and nitrofurantoin, and the proven efficacy of these medications against different bacterial strains. Furthermore, the particular patterns of susceptibility in Pseudomonas and Klebsiella species emphasize the need for individualized antibiotic treatment based on regional resistance profiles especially in patients with SRC to improve patient's outcome and treatment effectiveness.

5. CONCLUSIONS

Our research focused on UTIs in patients with renal calculi, cyst and failure. The key data indicate that *E. coli* is the most prevalent pathogen. For a successful course of treatment, specific antibiotic selection based on bacterial etiology was essential. For *E. coli* infections, Ciprofloxacin and Imipenem are routinely recommended, whereas Moxifloxacin was effective against *Klebsiella spp*. Antibiotic resistance seen in *Pseudomonas species*. Due to different resistance patterns, culture, and sensitivity testing is crucial for assisting treatment decisions. Antibiotic resistance must be combatted with cautious prescribing techniques, particularly for *Pseudomonas species*. These findings improve patient outcomes and address the challenge of antibiotic resistance in UTIs with renal conditions.

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7. ETHICAL STATEMENT AND PARTICIPATION CONSENT

The study has been approved by the Ethical Review Committee of "The Islamia University of Bahawalpur, Pakistan" under the letter number IUB/ERC/20/2022. Written informed consent was obtained from all participants before their inclusion in the study.

8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Analysis of the Physicochemical Characteristics of the Soil in the Malakand District, Khyber Pakhtunkhwa, Pakistan

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Abstract: The aim of the present study was to assess the physicochemical characteristics of the soil of 20 selected sites in the district of Malakand, Pakistan. The study showed that the soil texture of District Malakand was predominantly sandy loam (50%) to silty loam (25%) and loamy sand (25%). The pH value of the soil ranged from 6.7 to 8.2. The electrical conductivity of soil samples ranged from 0.201 dS/m to 0.683 dS/m. The organic matter content has a range of 0.70% to 2.27%. In the macronutrients, the Nitrogen content was found in the range of 300 ppm to 1500 ppm, Potassium from 28 ppm to 190 ppm, Phosphorus from 6.2 ppm to 14.4 ppm, Calcium element from 2.18 ppm to 7.75 ppm, Magnesium from 3.24 ppm to 6.09 ppm and Sodium from 12.21 ppm to 18.29 ppm. Similarly, microelements such as Zinc ranged from 1.37 ppm to 2.22 ppm and Manganese from 0.53 ppm to 1.63 ppm. The results showed a significant Positive Pearson's correlation coefficient, with the highest correlation were observed between Pb-Na (r = 0.803), followed by P-N (r = 0.759) and N-EC (r = 0.677); whereas, a significant negative Pearson's correlation coefficient were found for Ca-K (r = -0.579), Mg-K (r = -0.467) and Ni-P (r = -0.454). The probability values show a significant correlation ($p < 0.01$* and $p < 0.05$**) between pH and Ec (0.023**), Ca-K (0.003*), N-Mg (0.041**), K-Mg (0.018**), Zn-Ca (0.038**), and Zn-Mg (0.046**). This study provides valuable insights into the physicochemical characteristics of soil in Malakand Distrcit, contributing to a better understanding of soil health and its implications for agriculture and environmental sustainability.

Keywords: Soil Texture, Soil Organic Matter, Micronutrients, Macronutrients, Malakand.

1. INTRODUCTION

Soil contamination with heavy metals is a significant environmental and health issue in Malakand district, Khyber Pakhtunkhwa, Pakistan due to industrial activities and agricultural practices. The lack of comprehensive data on soil quality in the region hinders effective policy making and mitigation strategies, potentially threatning agricultural productivity, environmental sustainability and human health. The economic prosperity and happiness of a country are reliant on its natural properties and resources. If a country is to continue as a successful and prosperous unit, that country needs to have a widespread and perfect account of all its main resources [1]. Among all the resources, the actual capital and the ultimate asset of a country is its soil. For the production of more and good quality crops, the soil must have all essential nutrients in a balanced quantity. The insufficient

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quantity of nutrients in the soil will produce serious diseases in plants. The nutrients in the soil are grouped into two categories such as macro and micronutrients. Macronutrients are required by plants in greater quantity and include N, P, K, S, Ca, and Mg while micronutrients are required by plants in lesser quantity and include Fe, Cu, Zn, B, Mo, Cl and Mn [2, 3]. These macronutrients and micronutrients are required by plants for various physiological functions in their bodies. Nitrogen element enhances the growth and development of living tissues in the plant body whereas Phosphorus element is needed for the development of seeds and fruits, cell division and stimulation of root initiation and development. Potassium element enlarges the size of grains or seeds and develops the quality of fruits. In addition, Potassium may also activate about 60 enzymes in the plant body [4].

Soil has an important and crucial role in achieving the aims and objectives of sustainable development goals (SDGs) [5]. According to an estimate about 50% - 70% of overall soils are deteriorated or polluted due to extensive human-induced soil erosion which has eventually resulted in food security issues [6]. Moreover, it is believed that only 11% of the total land surface all over the world in arable land types is supposed to be available to keep up with the increasing demand for 50% farming products to nourish about 9.5 billion individuals by 2050 [7]. As a result, it is necessary and crucial to know and understand soil, its genesis, development, properties and behavior to support land use preferences and choices that may affect the ecological health and sustainable produce of the soil [8, 9]. Soils and their properties are substantially affected by geographic and topographical dynamics such as temperature, gravity, water, vegetation, pressure differences, wind, chemical interaction, topography and living organisms [10]. Diverse characteristics of soil like moisture content, permeability, porosity, temperature, depth, constancy, nutrient substances, etc., can significantly impact the nature of flora growing upon it [11]. The soil has a dynamic zone made up of minerals (parent rocks), organic matter (debris from animals and plants), soil water, and soil air [12].

The soil-plant association is significant as both are dependent on each other and the surrounding environment. The sustenance, water, minerals and nutrients required by plants to grow and survive are provided by soil whereas, the formation and improvement of soil are interlinked with plants [13]. The utility of soil for maintaining and sustaining human, plant, and animal activities, including farming, is influenced by the quality of the soil [14]. The quality of soil can be measured by a set of parameters including physical, chemical and biological properties of soil. An appropriate parameter should have a solid correlation with the specific soil function, which can be reproducible and economical to evaluate [15].

The physicochemical properties of soil determine the health of particular ecosystems [16-18]. Deforestation, overgrazing, and other human activities for agricultural and farming purposes have consistently and progressively diminished vegetation cover, leading to embraces for soil erosion, particularly in the mountainous regions [19]. Additionally, it may result in waterlogging that can cause nutrients to leak out of the soil depriving the soil of some essential nutrients [20]. Consequently, regular monitoring of the physicochemical characteristics of soil is essential to ensuring the sustainability of the environment and ecosystem. The physicochemical characteristics of soil in Malakand district vary significantly due to factors such as location, land use, and irrigation practices. In order to determine the soil fertility and production, the objective of this research was to assess and evaluate the physicochemical characteristics of the soil in the study region district of Malakand, Pakistan.

2. MATERIALS AND METHODS

2.1. Research Area

The present research was conducted in the district of Malakand, Khyber Pakhtunkhwa, Pakistan. The study area is located at 34° 35′ North latitude and 71° 57′ East longitude (Figure 1). It has a lush green valley of Malakand bounded by mountains. The area has sandy-loamy soil with sufficient moisture content which is a peculiar feature of the area. The mean annual rainfall recorded ranged between 600 to 650 mm. The area has diverse climatic conditions because the winters are cooler and the summers are warmer. Malakand district has historic significance and it has a rich floristic composition. The local people mostly get their livelihood from farming and

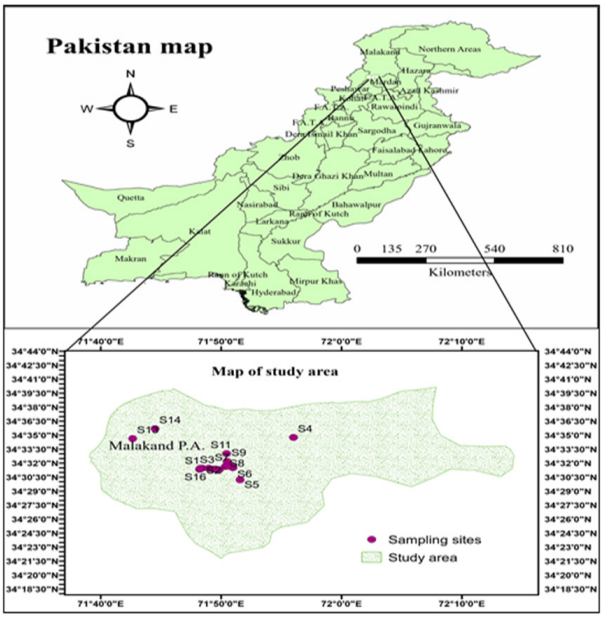

Fig. 1. Map of the study area.

livestock rearing. The majority of the people speak the Pashto language whereas, a few Gujjar families speak Gujari language [21].

2.2. Sample Collection

Twenty soil samples were randomly taken at a depth of 0-20 cm from the selected sites. The collected soil samples were retained in polythene bags and were properly labeled then dried and made fine powder with the sieve of 2 mm mesh for further analysis. The unbroken soil samples were collected using the core drill and wrapped instantly in air-tight bags and properly sealed with candle wax to prevent loss of moisture [3, 22].

2.3. Physicochemical Analysis of the Soil Samples

The soil samples were analyzed physicochemically at the Pakistan Tobacco Board, soil research laboratory, Khan Ghari Mardan, Khyber Pakhtunkhwa, Pakistan.

2.3.1. Determination of soil texture

The soil texture of the collected soil samples was determined by using the hydrometer method [23].

2.3.2. Determination of soil pH

An electrode pH meter (PCE-228) was used to determine the soil samples' pH in a 5:1 water-to-soil solution [24].

2.3.3. Determination of soil electrical conductivity (EC)

An electrical conductivity meter was used to measure the electrical conductivity of soil samples.

2.3.4. Determination of soil organic matter

In order to calculate the organic matter content of soil samples, the organic carbon contents of the soils were first calculated by using the Walkley and Black technique [25] and then multiplied by 1.724 [26].

2.3.5. Determination of total Nitrogen and Phosphorus in the soil

Micro-Kjeldhal digestion distillation procedures [27] and electro-photometer methods [28] were used to calculate the total content of nitrogen and phosphorus.

2.3.6 Elemental analysis of soil sample

For the examination of various elements present in soil samples, the AB-DTPA (Ammonium Bicarbonate-diethylenetriaminepentaacetic Acid) method was used [29]. An inductively coupled plasma (ICP) Spectrophotometer can be used to use the AB- DTPA soil test more effectively [30].

2.4. Statistical Analysis of Collected Data

The collected data were analyzed for basic descriptive statistical analysis through Microsoft Excel (Version 2016) and Origin (2019). The Pearson correlation coefficient of the data was also determined to study the inter-relationship among the different parameters of soil.

3. RESULTS AND DISCUSSION

3.1. Physicochemical Analysis of the Soil

3.1.1. Physical characteristics

Physical properties of the selected plant samples across the study area showed that sand particles were found in the range from 20.18% to 85.28%

with a mean value of 59.21%. The silt particles were in ranged from 10.35% to 74.60% with a mean value of 35.64%. The clay particles ranged from 3.23% to 7.11% with a mean value of 3.43%. The soil texture of the collected 20 samples showed that based on texture 50% soil samples tested were sandy loam, followed by textural class silty loam and loamy sand 25% each (Table 1). The soil of Kot, Maina, Hayankot, Salgro, Ghari Usmainkheil, Wazirabad, Kharkai, Mekhband, Aladand, Thana and Piran was sandy loam while the soil of Wartair, Musamena, Meherday, Sakhakot and Kopar was found silty loam (Figure 2). The textural distribution of soil reveals that the soil of Agra and Selai Patai is loamy sand. The soil of Kot, Maina, Hayankot, Salgro, Ghari Usmainkheil, Wazirabad, Kharkai, Mekhband, Aladand, Thana and Piran was sandy loam while the soil of Wartair, Musamena, Meherday, Sakhakot and Kopar was found silty loam (Figure 2). The physical properties of soil play a significant role in the water-holding capacity, saturation of root zone, aeration and absorption of water by plants [31]. The development of soil aggregates mainly depends on soil texture. One of the basic alterations in the soil texture is the superficial layers and it is one of the key causes that controls water potential, organic matter binding cation exchange as well as other activities [32]. The results of the present study for textural class determination are in with the results of previous studies in the area [1, 3, 33].

3.2. Chemical Properties

Chemical properties of selected soils reveal that:

pH value: pH value ranged from 6.7 to 8.2 with a mean value of 7.6 indicating the slightly alkaline nature (Table 2). This is considered to be the best soil for plant growth, root absorption and nutrient uptake [34]. The study showed that the pH value was recorded highest in plain areas as compared to hilly areas which is might due to the presence of more organic matter in plain areas than in hilly areas.

Electrical conductivity (Ec): Electrical conductivity of the tested soil samples ranged from 0.21 dS/m

Table 1. Physical properties of soil of district Malakand, Pakistan.

S. No.	Locality name	Textural class	Sand (%)	Silt (%)	Clay (%)
1	Kopar	Silty Loam	21.25	74.6	4.15
2	Sakhakot	Silty Loam	20.35	76.42	3.23
3	Meherday	Silty Loam	21.47	74.37	4.16
4	Musamena	Silty Loam	20.18	76.27	3.55
5	Wartair	Silty Loam	20.44	74.27	5.29
6	Piran	Sandy Loam	70.56	23.12	6.32
7	Thana	Sandy Loam	67.24	26.4	5.36
8	Alladand	Sandy Loam	70.19	25.47	4.34
9	Totakan	Sandy Loam	69.43	24.15	6.42
10	Mekhband	Sandy Loam	70.55	25.23	4.22
11	Kharkai	Sandy Loam	68.26	24.63	7.11
12	Wazirabad	Sandy Loam	67.37	26.5	6.13
13	Ghari Usmani Khel	Sandy Loam	67.16	27.24	5.6
14	Salgaro	Sandy Loam	68.27	27.3	4.43
15	Haryankot	Sandy Loam	66.28	26.31	7.41
16	Maina	Loamy Sand	70.46	25.19	4.35
17	Kot	Loamy Sand	75.25	18.26	6.49
18	Selai Patay	Loamy Sand	80.14	14.48	5.38
19	Agraa	Loamy Sand	84.16	12.41	3.43
20	Khanorai	Loamy Sand	85.28	10.35	4.37

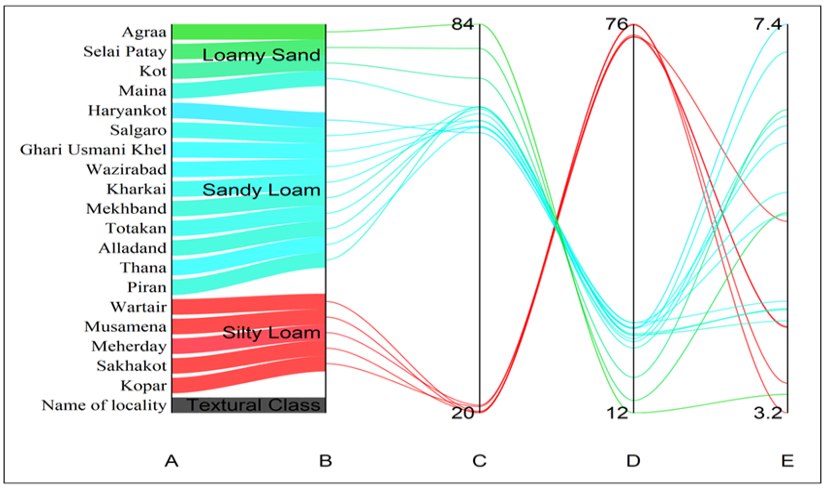

Fig. 2. Soil Texture classes distribution in different localities of District Malakand.

to 0.68 dS/m with a mean value of 0.43 dS/m. The electrical conductivity of the soil (EC) is a characteristic used to examine the salinity of the soil and is a crucial aspect in determining the quality of the soil. The results of the current analysis demonstrated that the soil's electrical conductivity (EC) is within a normal range. The sources of salts in the soil are mainly irrigation water, solubility of minerals, rise in water table and use of excessive fertilizers in the study area.

Organic matter: Organic matter content in the soil ranged from 0.70% to 2.27% with a mean value of 1.37%. Soil organic matter is rich in mineral substances and promotes soil fertility. It also has a role in soil texture and promoting water holding capacity of the soil. It also adds important minerals like nitrogen, phosphorus, sulphur, calcium, etc. to the soil and affects greatly to soil's physical and chemical properties [35].

Nitrogen content: Nitrogen content ranged from 300 ppm to 1500 ppm with a mean value of 735 ppm. The Phosphorus content ranged from 6.2 ppm to 14.4 ppm with a mean value of 9.46 ppm. A substantial amount of organic matter in the soil may be entrusted with the highest nitrogen and phosphorus content [36, 37].

Other macro elements: Other maceo elements reported from tested soil were K which ranged from 28 ppm to 190 ppm with a mean value of 62.20 ppm, Ca element ranged from 2.18 ppm to 7.75 ppm with a mean value of 5.62 ppm, Mg element ranged from 3.24 ppm to 6.09 ppm with a mean value of 4.74 ppm and Na element ranged

Table 2. Descriptive analysis of soil data by using statistical tools.

Descriptive analysis	**Chemical parameters of soil**										
	pH	EC (dS/m)	OM (%)	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Na (ppm)	Zn (ppm)	Mn (ppm)
Min	6.70	0.20	0.70	300	6.20	28.00	2.18	3.24	12.21	1.37	0.53
Max	8.20	0.68	2.27	1500	14.40	190.0	7.75	6.09	18.29	2.22	1.63
Mean	7.66	0.43	1.37	735	9.46	62.20	5.62	4.74	15.36	1.66	0.95
Median	7.80	0.40	1.38	650	9.20	50.10	5.90	4.79	15.49	1.61	0.90
SD	0.48	0.15	0.35	380.2	2.51	37.56	1.38	0.76	1.35	0.19	0.27
SE	0.11	0.03	0.08	85	0.56	8.40	0.31	0.17	0.30	0.04	0.06
Kurtosis	-0.04	-0.66	1.56	-0.66	-0.94	6.83	0.63	-0.32	1.32	2.45	1.14
Skewness	-0.10	0.26	0.76	0.69	0.42	2.38	-0.71	-0.03	-0.42	1.27	1.06

Key: Max-Maximum, Min-Minimum, SD-Standard Deviation, SE-Standard Error and ppm-Parts per Million.

from 12.21 ppm to 18.29 ppm with a mean value of 15.36 ppm. The relatively small values of these macro elements may be accredited to the loss of macro elements from the soil may be due to human activities like farming, harvesting, or climatic aspects leading to percolating that can speed up the movement and inertness of these elements [12, 34, 38]. The value of microelement Zinc ranged from 1.37 ppm to 2.22 ppm with a mean value of 1.66 ppm. The value of Manganese ranged from 0.53 ppm to 1.63 ppm with a mean value of 0.95 ppm. Low values of these microelements were observed in the soil samples, which is reflective of the low unevenness in the soil's geochemical attributes [39]. It was found that the microelement concentrations in the analyzed soil samples were within the recommended standard range for typical soils [40]. The results of the present research on the chemical characteristics of soil were comparable and associated with those of earlier investigations, with a few minor variations that could be driven by a number of geomorphological features of the region under investigation [41-44].

3.3. Pearson's Correlation Coefficients among the Soil Parameters

To investigate the correlations between these various chemical features in the soil, Pearson's correlation coefficients were calculated, as shown in Table 3. The results showed a significant Positive Pearson's correlation coefficient, with the highest correlation observed between Pb-Na (r = 0.803), followed by P-N (r = 0.759) and N-EC (r = 0.677); whereas, a significant negative Pearson's correlation coefficient were found for Ca-K (r = -0.579), Mg-K (r = -0.467) and Ni-P (r = -0.454). The correlation coefficients between 0.9 and 1.00 are considered to be very highly correlated, 0.7 and 0.9 are considered to be highly correlated, 0.5 to 0.70 are considered to be moderately correlated, 0.25 to 0.50 are considered to be a low correlation, and values less than 0.2 are considered to have a low correlation [45]. The findings of past investigations [46, 47] are consistent with and comparable to the results of Pearson's correlation coefficients among various chemical characteristics of soil.

3.4. Probability Values among the Soil Parameters

The Probability values of the chemical properties of soil showed that significant correlations existed at ($p < 0.01$* and $p < 0.05$**) between different chemical properties of soil such as pH and Ec (0.023**), Ca-K (0.003*), N-Mg (0.041**), K-Mg (0.018**), Zn-Ca (0.038**), Zn-Mg (0. 046**) as shown in table 4. The results of Probability values of the chemical properties of soil and significant values at ($p < 0.01$* and $p < 0.05$**) of the current study are parallel and in line with the results of previous studies [48-50].

Table 3. Pearson's correlation coefficient among the soil parameters.

Correlation	PH	EC	OM	N	P	K	Ca	Mg	Na	Zn	Ni	Cd	Cr	Pb	Mn
PH	1														
EC	-0.449	1													
OM	0.303	0.255	1												
N	-0.280	0.677	0.453	1											
P	-0.075	0.493	0.181	0.759	1										
K	0.178	0.251	0.422	0.408	0.250	1									
Ca	0.080	-0.296	-0.062	-0.368	-0.092	-0.579	1								
Mg	-0.188	-0.087	-0.330	-0.398	-0.252	-0.467	0.377	1							
Na	0.184	0.091	0.381	0.142	0.148	0.448	0.052	-0.100	1						
Zn	0.148	0.051	0.275	0.097	0.086	0.624	0.404	-0.385	0.460	1					
Ni	-0.284	-0.171	0.152	-0.218	-0.454	-0.156	0.007	0.253	0.272	-0.150	1				
Cd	0.147	0.286	0.388	-0.048	-0.133	0.583	0.312	-0.070	0.525	0.657	0.115	1			
Cr	0.043	-0.001	0.076	0.046	-0.059	-0.390	-0.446	-0.236	0.219	0.459	0.308	0.289	1		
Pb	0.433	0.140	0.618	0.157	0.163	0.527	-0.063	-0.263	0.803	0.448	0.258	0.573	-0.084	1	
Mn	0.172	-0.270	0.396	-0.244	-0.344	0.291	-0.096	-0.055	0.366	0.335	0.601	0.538	0.403	0.431	1

Key: EC stands for electrical conductivity, OM for organic matter.

Table 4. Probability among the soil parameters.

Probability	pH	EC	OM	N	P	K	Ca	Mg	Na	Zn	Ni	Cd	Cr	Pb	Mn
pH	1														
EC	**0.023****	1													
OM	0.903	0.861	1												
N	0.115	0.999	0.977	1											
P	0.375	0.986	0.777	0.999	1										
K	0.774	0.857	0.968	0.963	0.856	1									
Ca	0.632	0.101	0.396	0.067	0.348	**0.003***	1								
Mg	0.213	0.356	0.089	**0.041****	0.141	**0.018****	0.949	1							
Na	0.781	0.649	0.938	0.723	0.734	0.976	0.413	0.337	1						
Zn	0.733	0.585	0.879	0.658	0.640	0.998	**0.038****	**0.046**	0.979	1					
Ni	0.111	0.234	0.738	0.177	**0.021****	0.255	0.512	0.859	0.122	0.262	1				
Cd	0.732	0.889	0.954	0.418	0.286	0.996	0.089	0.383	0.991	0.999	0.686	1			
Cr	0.572	0.496	0.625	0.577	0.401	**0.044****	**0.024****	0.157	0.176	0.979	0.906	0.891	1		
PB	0.971	0.722	0.998	0.746	0.755	0.991	0.394	0.130	0.999	0.976	0.135	0.987	0.362	1	
Mn	0.763	0.124	0.958	0.149	0.068	0.893	0.34	0.408	0.943	0.925	0.997	0.992	0.961	0.971	1

Key: EC stands for electrical conductivity, OM for organic matter. Bold r-values are significant at **$p < 0.01$*** and **$p < 0.05$****.

4. CONCLUSIONS

The present research encompassed the physicochemical characteristics of the soil in District Malakand, Pakistan. Three types of soil make up the distinctive soil textural class: sandy loam, loamy sand, and silty loam. The pH levels in the sites selected were found to be slightly alkaline to neutral, indicating their compatibility with the growth of plants. The soil had good pH ranging between 6.7 to 8.2, significant organic matter content ranged from 0.70% to 2.27% and The electrical conductivity of soil samples ranged from 0.201 dS/m to 0.683 dS/m clearly pointed to the soil's fertility in the study area. In the macronutrients, the Nitrogen content was found in the range of 300 ppm to 1500 ppm, Potassium from 28 ppm to 190 ppm, Phosphorus from 6.2 ppm to 14.4 ppm, Ca element from 2.18 ppm to 7.75 ppm, Magnesium from 3.24 ppm to 6.09 ppm and Sodium from 12.21 ppm to 18.29 ppm. Similarly, microelements such as Zinc ranged from 1.37 ppm to 2.22 ppm and Manganese from 0.534 ppm to 1.634 ppm. The content of the micronutrients and macronutrients were found to be within the permissible range, indicating that it is suitable for planting of different forest species, especially the Pinus forests. The soil of the farmed land was found to be ideal for the cultivation of numerous fruit and vegetable species. Although soil erosion and deforestation were the two main threats that were identified to be damaging the natural physicochemical composition of the soil in the selected sites, they need to be conserved. The present study reveals that the soil in district Malakand has favourable physicochemical charateristics, including organic matter, optimal nitrogen level, suitable moisture content, and a pH range conducive to plant growth. Additionally, the soil contains sufficient amounts of macro and micronutrients, indicating a fertile and productive soil environment. Based on these findings, it is recommended that farmers can adopt sustainable agricultural practices, such as crop rotation and optimal fertilizer application, to maintain soil fertility and productivity.

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Integron Mediated Multiple Heavy Metal and Antibiotic Resistance in Plant Growth Promoting Epiphytic Bacteria

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Abstract: The present study was conducted to investigate the co-selection of antibiotic and heavy metal resistance in epiphytic bacteria isolated from *Withania somnifera, Ficus benghalensis, Olea europaea* and *Aloe vera*. Thirty epiphytic bacterial strains were isolated. Six isolated strains were selected and observed to have significant multiple heavy metals and antibiotics resistance. Single and synergistic effect of heavy metals and antibiotics constantly boosted the growth rate of selected bacterial isolates. Inoculation of these epiphytic bacteria caused increment in seedling, shoot and root length upto 58-70%, 25-37%, 87-125% respectively, while there was an increase in number of leaves upto 25-50% of *Triticum aestivum.* These epiphytic bacteria exhibited high extracellular antioxidant potential with rise in DPPH (2, 2-diphenyl 1-picrylhydrazyl) scavenging ability (33-59%) and phenols concentration (78-173 µg/ml). Phylogenetic analysis revealed 99-100% similarity of these bacterial strain AN1 *(Staphylococcus pasteuri)*, AN2 *(Microbacterium paraoxydans)*, BG4 *(Pseudomonas azotoformans)*, BG6 *(Staphylococcus haemolyticus)*, OL21 *(Staphylococcus haemolyticus)*, and AV2 (*Paenobacillus lactis)*. Sixty-six percent of these bacteria carried *IntI1* gene having similarity with *XerC* integrase/recombinases superfamily conserved domains. Our findings suggested that existence of *IntI1* gene in epiphytic bacterial genome helps in their survival under stress environment.

Keywords: Epiphytic Bacteria, Plant Growth Promotion, Antioxidant Activity, DPPH Activity, *IntI1* Gene.

1. INTRODUCTION

The phyllosphere, a distinctive ecological niche, undergoes significant perturbations in response to variations in environmental parameters, including temperature, UV exposure, and relative humidity. Nutrient availability plays a pivotal role in governing the colonization potential of symbiotic epiphytic microbial communities within the phyllosphere, which, in turn, influences plant fitness and growth. Effective colonization of these symbiotic epiphytic microbial communities is intricately linked to nutrient availability. Epiphytic microorganisms exhibit a remarkable ability to thrive in challenging environmental conditions, characterized by limited nutrients, temperature fluctuations, UV radiation exposure, elevated atmospheric heavy metal concentrations, and exposure to insecticidal agents. Microorganisms engage in passive diffusion mechanisms to leach inorganic ions and organic acids surfaces [1]. Epiphytic bacterial populations significantly contribute to plant by rapid multiplication on plant surfaces, supplying nutrient-rich environment which is rich in carbon and nitrogen sources, and giving protection against pathogenic bacteria and stressful conditions. The presence of these bacteria is primarily determined by nutrient exchanges between host plants and the microbial communities [2]. Epiphytic bacteria provide a range of advantageous contributions to host plants, comprising the augmentation of nutrient accessibility, the synthesis and orchestration of plant growth-regulating hormones, the promotion of heightened plant growth in the presence of stressors, the biosynthesis of antibiotics and lytic enzymes aimed at establishing a nutrient-deprived environment for plant pathogens, and fortification against the incursion of pathogenic organisms [3]. However, the functional, structural, and adaptive aspects of epiphytic microbial communities residing on host plant surfaces remain largely unexplored. The secretion of secondary metabolites represents

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an additional determinant affecting the dispersion of bacteria on plant surfaces, potentially conferring antibacterial characteristics against diverse plant pathogens [4].

The microbiome within the phyllosphere on leaves is directly influenced by the application of antibiotics, as the unintended dissemination and runoff of these substances result in the deposition of antibiotics within the soil. This, in turn, affects the integron-related *IntI1* gene in epiphytic bacteria and the rhizosphere microbiomes [5]. Natural antibiotic resistance genes are anticipated to evolve alongside bacterial antibiotic production functions as a means of self-protection. These mechanisms include hindering antibiotic mobility to target sites, modifying antimicrobial compounds, preventing the active transport of antimicrobial compounds, synthesizing new proteins insensitive to antibiotics, mutating the target site, and protecting the target site [6].

Notably, antibiotic-resistant bacteria are often resistant to heavy metals as well. This dual resistance entails processes, including shifts in membrane permeability, adjustments in the presence of heavy metals and antibiotics, alterations in target sites, and the acquisition of heavy metals and antibiotics [7]. The strong association between antibiotic and heavy metal resistance arises from co-resistance and cross-resistance processes. Recent studies have highlighted the presence of antibiotic resistance not only in bacterial genomes but also in plasmids in heavy metal-contaminated environments [8]. Integrons, featuring integrase (*IntI1*) gene-encoded site-specific recombinases, play a critical role in transferring antibiotic resistance genes in animal and human pathogens and accumulating multiple antibiotic resistance genes into a multi-resistance element [9].

Bacteria represent promising candidates for bioremediation, given their proficiency in detoxifying heavy metals. Microbial remediation, employing microbes for the accumulation and detoxification of heavy metals, stands as a leading method for this purpose [10]. Heavy metal like Chromium (VI) and zinc (II), contribute to elevate reactive oxygen species and oxidative cell impairment during detoxification. Consequently, the combined action of these metals significantly impacts cellular antioxidant properties and the persistence of heavy metal and antibiotic-resistant epiphytic bacteria in host plants [10]. Under such challenging conditions, these bacteria engage in long-term interactions with host plants, promoting plant growth [7]. Recent research has unveiled the potential of plant growth-promoting epiphytic bacteria to enhance the growth and yield of various cereal crops, including wheat [11, 12]. Without the presence of these beneficial epiphytic bacteria, plants would be less competitive against plant pathogens and exhibit reduced tolerance to stressful environments [13].

Recent studies showed that the role of exopolysaccharides (EPS) production as an adaptation mechanism of epiphytic bacteria to safeguard plants against dehydration due to the high-water maintenance capacity of EPS [14]. Additionally, bacterial production of hydrogen cyanide (HCN) provide protection to plants from bacterial pathogens. Epiphytic bacteria also enhancing the host plant growth by solubilization of phosphate, rendering it in a soluble form that is easily taken up by the host plant. Present study is focused on the investigation of the relationship between multiple heavy metals and antibiotic resistance patterns, as indicated by the presence of the integron-related *IntI1* gene, in epiphytic bacterial isolates from selected medicinal plants.

2. MATERIALS AND METHODS

2.1. Collection of Medicinal Plants and Isolation of Epiphytic Bacteria

Medicinal plants (*Withania somnifera, Ficus benghalensis*, *Oleo europeae* and *Aleo vera*) were collected from the Botanical Garden of University of the Punjab, Lahore, Pakistan. Plants were collected with leaves and stems. Sterile polythene bags were used to carry the plant materials to the laboratory and plants were processed within 24 h to avoid the chances of contamination. Epiphytic bacteria were isolated by using stem-leaf-imprinting agar and serial dilution method. Thirty morphologically distinct bacterial colonies were selected from plates of both isolation methods after 48 hours of incubation at 37 °C. Selected isolates were further purified and stored at 4 °C. Selected bacterial isolates were observed for their morphological (Gram staining, colony and cell morphology, motility test) and biochemical

(oxidase, catalase, DNase, starch hydrolysis, TSI, urease, indole, Methyl Red (MR)-Voges Proskauer (VP) and gelatin liquefaction tests) characteristics. Bacterial isolates were identified by comparing 16S rRNA gene sequencing using BLAST against NCBI database. The nucleotide sequences were deposited in the NCBI GenBank to obtain accession numbers. The evolutionary relationships were assessed using MEGA 7 software and neighbor-joining method was used for the construction of phylogenetic tree. Effects of three different pHs (5, 7 and 9), temperatures (28 °C, 37 °C and 46 °C), carbon sources (glucose, fructose, sucrose, and lactose) and nitrogen sources (Peptone, Yeast extract, NH_4Cl, and KNO_3) on growth of bacterial strains were studied for 96 hours [15].

2.2. Heavy Metal and Antibiotic Resistance Profiling of Epiphytic Bacteria

Selected epiphytic bacterial isolates were screened against five heavy metals (K_2CrO_4, $ZnCl_2$, $PbCl_2$, $CuSO_4$ and $NiCl_2$) and two antibiotics of different classes (ampicillin and streptomycin) by agar plate dilution method [7]. Bacterial strains were grown on Luria Broth (LB) agar supplemented with variable concentrations (K_2CrO_4: 100 to 120000 µg/ml, $ZnCl_2$: 100 to 1400 µg/ml, $CuSO_4$ and $NiCl_2$: 100 to 1600 µg/ml, $PbCl_2$: 100 to 5600 µg/ml ampicillin: 100 to 2600 µg/ml, streptomycin: 100 to 1500 µg/ml) of selected metal and antibiotics and incubated at 37 °C for 48 hours. Maximum Tolerable Concentration (MTC) was determined. Six epiphytic bacteria were selected for further study on the basis of their high heavy metal and antibiotic resistance pattern.

2.3. Synergistic Response of Selected Epiphytic Bacteria towards Multiple Metal and Antibiotics Stress

Growth response under single and synergistic conditions for antibiotics (ampicillin and streptomycin) and heavy metals (Cr and Zn) were observed for selected epiphytic bacteria. LB broth was supplemented with 500 µg/ml of antibiotic and heavy metal in single and combination were inoculated with the respective bacteria. Incubations were carried out at 37 °C. Samples were withdrawn under sterile conditions after every 24 h for up to 15 days and optical density (OD) was measured at 600 nm.

2.4. Analysis of Plant Growth Promoting Characters in Epiphytic Bacteria

Solubilization of phosphate salt, HCN and auxin production tests were performed to determine the plant growth promoting potential of bacterial strains. For determination of phosphate solubilization potential of bacteria, Pikovskaya's (PVK) agar medium was used. Lorck method was followed for observing HCN production ability by bacteria and IAA (Indole-3-acetic acid) production by bacterial strains was estimated by using Salkowski's method [16].

2.5. Plant Microbe Interaction (PMI) Studies

In this experiment, healthy cash crop *Triticum aestivum* (FSD-08) was selected due to its economic importance and seeds were bought from Punjab Seed Centre, Lahore, Pakistan. Plate method was used to perform this experiment under controlled conditions of lab by following Mustafa *et al*. [17] with some modification. Different growth parameters of *Triticum aestivum* seedlings were recorded after two weeks of seed germination.

2.6. Exopolysaccharides Production by Selected Epiphytic Bacteria

Selected bacterial strains were analysed for EPS production in the presence of LB media at optimal temperature (37 °C) for one week. Ice cold ethanol precipitation method was used for the extraction of EPS. The total EPS content was calculated by subtracting the dry weight of EPS from its fresh weight [18].

2.7. Preparation of Extracellular and Intracellular Epiphytic Bacterial Extracts for Antibacterial and Antioxidant Profiling

Ethyl acetate was used as a solvent to prepare extracellular and intracellular extracts of epiphytic bacteria. Epiphytic bacteria which were previously selected, were cultured in LB and incubated for 24 hours at 37 °C. For the isolation of extracellular metabolites, cell-free supernatants were combined with ethyl acetate in a 1:1 ratio. The organic layer was evaporated using a rotary evaporator at 37 °C and re-suspended in 20% Dimethyl sulfoxide (DMSO) for future use. In order to extract intracellular bacterial metabolites, ethyl acetate was

also used. The bacterial pellet was sonicated to break the cells open. The resulting lysed cell suspension was centrifuged at 8000 g for 10 minutes, and the supernatant was dried in the rotary evaporator at 37 °C. The dried substance was weighed and re-suspended in 20% DMSO for later use. All extracts of the selected bacteria were tested for antibacterial activity using the agar well diffusion method. *Bacillus* KC881030 and *Pseudomonas* KC881031 test cultures were spread onto Mueller-Hinton (MH) agar plates. Ampicillin at a concentration of 30 µg/ml was the standard, while DMSO served as the control. The plates were then incubated at 37 °C for 24 hours and the diameter of inhibition zones (mm) was measured. The assessment of antioxidant capacity of extracellular and intracellular ethyl acetate extracts of epiphytic bacteria encompassed three techniques: catalase examination, DPPH (2, 2-diphenyl 1-picrylhydrazyl) test, and TPC (total phenolic content) determination. Catalase is an enzyme crucial for combating oxidative stress in bacteria. The catalase activity in specific bacteria was assessed by reacting hydrogen peroxide with a supernatant sample and recording the absorbance at 240 nm for 60 seconds at 15-second intervals. Intra and extracellular extracts of epiphytic bacteria in ethyl acetate solvent were analyzed by the DPPH radical scavenging activity. In presence of stress conditions phenolics compounds are produced by bacteria. The Folin-Ciocalteu method was employed to determine the total phenolic content in extracellular and intracellular extracts of isolated epiphytic bacteria [19].

2.8. Amplification of *IntI1* Gene in Epiphytic Bacteria

Genomic DNA isolation was performed by using Thermo Scientific Gene JET Genomic DNA Purification Kit according to manufacturer instructions. *IntI1* gene amplification was carried out on gradient PCR thermocycler machine amplification using following primers: *IntI1* F (5' CCTCCCGCACGATGATC 3') and *IntI1* R (3' TCCACGCATCGTCAGGC 5'). The program of 30 cycles was run by setting annealing temperature 58 °C. Amplified PCR products were examined by gel electrophoresis. *IntI1* gene was sequenced and different NCBI bioinformatic tools were used for analysis and accession number were obtained by submitting sequences to NCBI GenBank. BioEdit software was used for multiple alignment of gene sequences and phylogenetic tree was constructed by MEGA 7 software using neighbor-joining method [20].

3. RESULTS

3.1. Isolation of Epiphytic Bacteria

Total 30 morphologically different epiphytic bacterial strains were isolated from collected plants by serial dilution method. All selected epiphytic bacteria exhibited highly diverse morphological and biochemical characteristics. All bacterial colonies were round in shape with entire margins except AV2 (irregular) and smooth in texture except AN1 and AV2 (mucoid). Color of bacterial colonies was off-white (AN1, BG4, AV2), white (BG6, OL21), yellow (AN2) with opaque (AN2, BG6, OL21), translucent (BG4, AV2) and transparent (AN1). Most of the strains were gram positive except BG4, AV2 (gram negative); 50% bacteria (AN1, BG4, OL21) were cocci and 50% (AN2, BG4, AV2) were rods. Except AN2 (spore former) and non-motile except AV2 (motile), all bacteria were non-spore formers. Biochemical characterization revealed that 83% bacteria showed positive results for catalase test; 66% positive for starch hydrolysis and VP test; 50% for DNase, methyl red and gelatin liquefaction test; 33% for oxidase and 16% for urease, MR-VP and gelatin liquefaction test. The genetic analysis revealed that the 16S rRNA gene sequences of bacterial strains AN1, AN2, BG4, BG6, OL21, and AV2 exhibited high similarity (ranging from 99 to 100%) with reference strains and accession numbers were obtained (Table 1).

For growth optimization of isolated bacteria, strain AN2 and OL21 preferred to grow at pH 5 whereas, strain BG4 and AV2 grew best at pH 7. Bacterial strain AN1 and BG6 showed best growth at pH 9. Three bacterial strains (AN2, BG6 and OL21) exhibited optimum growth at 46°C while strain AN1 and AV2 preferred 37°C. Only strain BG4 showed good growth at 28°C. Glucose was the best carbon source for the growth of strain BG4 and OL21 but sucrose was best for strain BG6 and AV2. Optimized growth patterns of epiphytic bacteria AN1 and AN2 was observed when media supplemented with lactose and fructose. Peptone was found as best nitrogen source for the growth of all bacterial strains.

Table 1. Bacterial identity along with accession numbers and potential plant growth promoting activities of selected epiphytic bacteria.

Bacterial isolates	Nearest relative	Accession no.	Query cover	Identity	Plant growth promoting activities
AN1	*Staphylococcus pasteuri*	MK875469	98%	99.83%	IAA
AN2	*Microbacterium paraoxydans*	MK875470	99%	99.73%	HCN, IAA
BG4	*Pseudomonas azotoformans*	MK875666	99%	99.80%	HCN, IAA
BG6	*Staphylococcus haemolyticus*	MK874945	96%	100%	PS, HCN, IAA
OL21	*Staphylococcus haemolyticus*	MK875667	99%	99.87%	PS, HCN, IAA
AV2	*Paenobacillus lacti*s	MK874992	99%	99.93%	HCN, IAA

IAA: Indole acetic acid, HCN: Hydrogen cyanide, PS: Phosphate solubilization

3.2. Resistance Profiling of Selected Epiphytic Bacteria

About 20% of bacterial isolates were found to be highly resistant to heavy metals and antibiotics with MTC ranging from 1000-100000 µg/ml for heavy metals and 500-2500 µg/ml for antibiotics. Four bacterial strains (AN1, AN2, BG4 and BG6) exhibited highly diverse resistance pattern to almost all tested heavy metals and antibiotics. Strain AV2 showed resistance to just two heavy metals and two antibiotics tested. Bacterial isolates BG6 and OL21 revealed 80000-100000 µg/ml MTC of chromium whereas AV2 and AN2 were able to tolerate 2500 µg/ml and 1400 µg/ml of ampicillin and streptomycin, respectively (Table 1, supplementary data).

3.3. Synergistic Response of Selected Epiphytic Bacteria towards Multiple Metal and Antibiotics Stress

The two specific heavy metals (Cr and Zn) and antibiotics (ampicillin and streptomycin) were selected due to high resistivity of selected strains. For this, bacterial isolates were exposed to ampicillin, chromium, and zinc, both individually and in combination, at a concentration of 500 µg/ml. The presence of single and combined heavy metal and antibiotic stress distinctly influenced the growth of the selected epiphytic bacterial isolates. The findings featured that these epiphytic bacterial strains possess strong adaptability to both antibiotic and heavy metal stress, whether encountered singly or in combination, illustrating their impressive resistance patterns (Figure S1 supplementary data).

3.4. Analysis of Plant Growth Promoting Characters in Epiphytic Bacteria

In this study, 33% of selected bacterial isolates (BG6 and OL21) had phosphate solubilization ability and 83% showed HCN production except AN1. All bacterial isolates had auxin production ability but highest auxin production was observed by two strains (AN2 and AV2) that was 17 and 28 mg/ml, respectively (Table 1).

3.5. Plant Microbe Interaction (PMI) Studies

Inoculation of four epiphytic bacterial isolates (AN1, AN2, BG4, and AV2) enhanced the percentage seed germination of *Triticum aestivum* up to 100%. Inoculation of these bacterial isolates increased seedling length, shoot length and root length up to 58-70%, 25-37%, 87-125%, respectively; while there was an increase in number of leaves up to 25-50% and roots of *T. aestivum* seedlings as compared to control (Table 2, supplementary data). It was observed from this experiment that epiphytic bacterial isolates had a plant growth promoting potential along with the antibiotic and heavy metal resistance.

3.6. Exopolysaccharides Production by Selected Epiphytic Bacteria

Among six epiphytic bacterial isolates, two strains (BG4 and AV2) produced maximum quantity of EPS ranging from 0.5-0.7 g/L after 7 days of incubation. Texture of EPS of both strains was spongy in wet form and hard in dry form whereas color of EPS was gray and brown, respectively (Figure. 1).

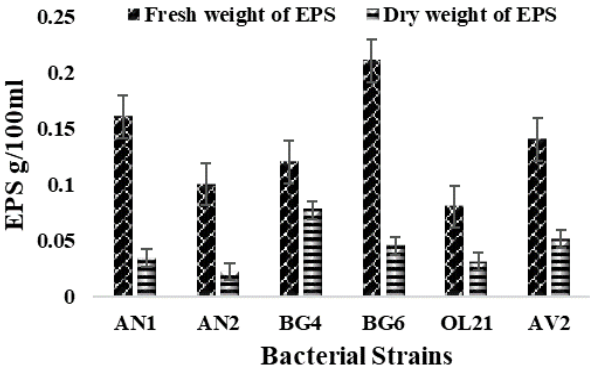

Fig. 1. Quantification of exopolysaccharides (EPS) produced by selected epiphytic bacteria.

3.7. Antibacterial and Antioxidant Activity of Epiphytic Bacteria

All selected bacterial isolates showed antibacterial activity by extracellular extract with 4-6mm inhibition zone against gram positive test bacteria while AN2 strain exhibited 6mm inhibition zone. But, antibacterial activity by intracellular extract was exhibited by only two strains (BG4 and AV2) with 4mm inhibition zone against gram positive test strain. Whereas, none of the selected bacteria showed antibacterial activity against gram negative test strain. Catalase activity profiling, DPPH (2, 2-diphenyl 1-1picrylhydrazyl) and total phenolic content (TPC) were the three methods used for the determination of intracellular and extracellular antioxidative property of epiphytic bacterial isolates. Catalase activity estimation of cell-free bacterial suspension revealed 50% of bacterial isolates showed maximum extracellular catalase activity with OD (0.120 nm) in AN2, (0.106) in OL21 and (0.178) in BG4 (Figure 2(a)). All selected bacterial isolates exhibited significant extracellular DPPH scavenging potential with 33-59% increase in DPPH reduction ability as compared to control. While, only one strain BG6 gave 29% intracellular DPPH scavenging activity (Figure 2(b)). Estimation of phenol concentration revealed that highly significant TPC of 78-173 µg/ml was found in extracellular extract of selected bacteria as compared to control (Figure 2(c)). Hence, this analysis showed that extracellular components of selected bacterial isolates had more antioxidant potential than their intracellular components (Figure 2).

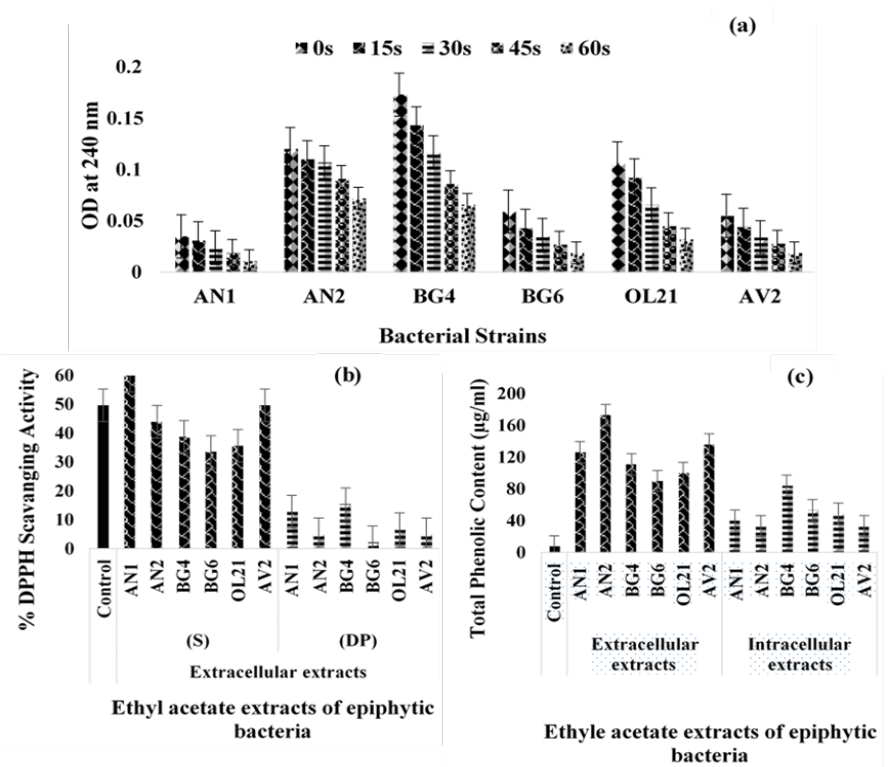

Fig. 2. Antioxidant potential of selected epiphytic bacteria (a) Catalase activity. Strain AN2, BG4 and OL21 had maximum catalase activity. (b) DPPH scavenging capability. Strong extracellular %age of DPPH scavenging capability of all selected epiphytic bacteria. (c) Total phenolic content. Extracellular TPC was significantly higher than intracellular TPC in all selected epiphytic bacteria.

3.8. Amplification of *Int1* Gene in Epiphytic Bacteria

Genomic DNA was isolated from all selected bacterial isolates for the screening of *IntI1* gene. Integrase gene *IntI1* of 280bp size was successfully detected in 66% of selected bacterial isolates (AN1, BG4, BG6 and OL21). The partial sequences of the *IntI1* gene demonstrated complete similarity with bacterial genera known to possess conserved domains of the *XerC* integrase/recombinase superfamily. These domains play roles in replication, recombination, repair, and in elements of the mobilome such as prophages and transposons (Figure 3). The sequences of the *IntI1* gene were submitted to the NCBI GenBank with the accession numbers MK882928 (for BG4) and MK882929 (for OL21).

4. DISCUSSION

Epiphytic microbial populations exhibit diversity in both plant species and the prevailing climate. In the present investigation, about 30 distinct epiphytic bacterial strains from four different medicinal plants. The selected epiphytic bacteria demonstrated resilience across a wide pH range. *Pseudomonas azotoformans* displayed optimal growth at a pH range of 5-8, while pH 7 was observed as conducive for the growth of *Paenobacillus* sp. and *Pseudomonas* sp. [21]. Epiphytic microorganisms are remarkably adaptable to environmental conditions on plant surfaces that undergo temperature fluctuations. Consequently, these bacteria may exhibit varying optimal growth temperatures. Within the scope of this investigation, it was observed that the selected epiphytic bacteria displayed robust growth across a spectrum of temperatures. *Staphylococcus* pasteuri and *Staphylococcus* haemolyticus showcased their ability to grow within broad temperature ranges, spanning from 15-45 °C and 18-46 °C, respectively. *Microbacterium paraoxydans* and *Pseudomonas azotoformans* reported their optimal growth temperatures at 28 °C and 27-31 °C, respectively [22]. Furthermore, *Paenobacillus* lactis displayed vigorous growth within the temperature range of 30-40 °C at pH 7. The selected epiphytic bacteria exhibited optimal growth in the presence of a diverse array of carbon sources, with the capacity to assimilate various carbon substrates from the plant's interior, including glucose, sucrose, and fructose.

The primary objective of this investigation was to probe into the resistance profiles of epiphytic bacteria against various heavy metals and pharmaceutical agents, considering different concentrations and exposures to environmental contaminants in air and water. Our findings unveiled the presence of six epiphytic bacterial strains

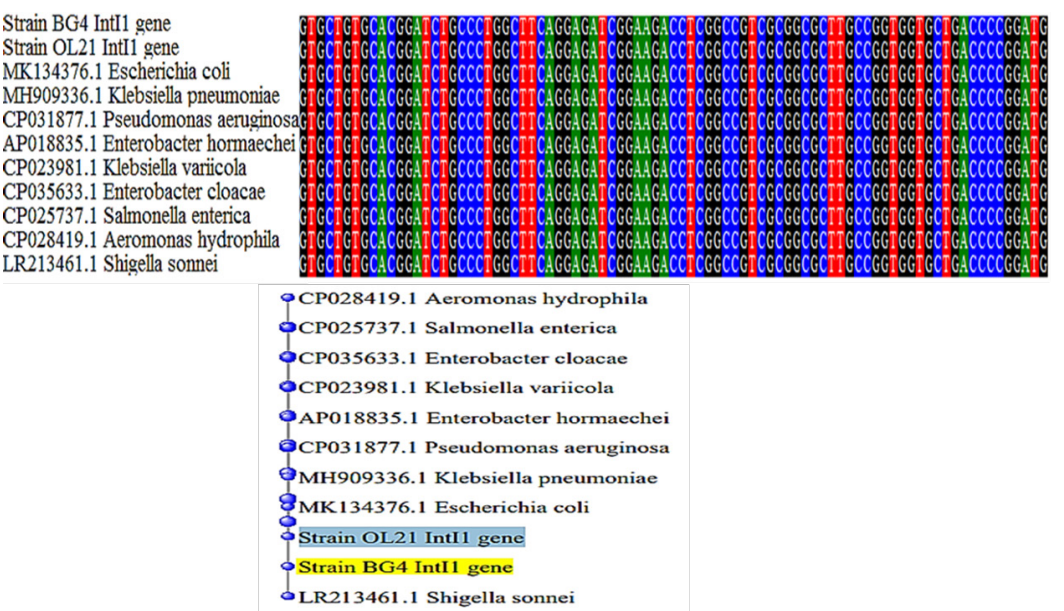

Fig. 3. Multiple alignment and of phylogenetic analysis of nucleotide sequence of *IntI1* gene indicated existence of conserved domain of *XerC* (integrase/recombinases/transposases) superfamily in multiple heavy metal and antibiotic resistant bacteria (BG4 and OL21) with 100% homology.

capable of withstanding multiple heavy metals and antibiotics simultaneously, thereby implying a potential association between resistance to heavy metals and antibiotics. To further elucidate these resistance patterns, a comprehensive analysis of 16S rRNA sequences was undertaken, leading to the identification of six epiphytic strains. These strains exhibited strikingly high similarity percentages of 99-100% with *Staphylococcus pasteuri*, *Microbacterium* paraoxydans, *Pseudomonas azotoformans, Staphylococcus haemolyticus, and Paenobacillus lactis*. Typically, *Microbacterium sp., Pseudomonas sp.,* and *Paenobacillus* sp. showed multiple metal-resistant bacteria [23]. Additionally, *Staphylococcus pasteuri* [24] and *Staphylococcus haemolyticus* [25] are known for their multiple heavy metal and drug resistance. Modification in heavy metal and antibiotic resistance, changes in membrane permeability, increased concentrations, and alterations in target sites may be the principal mechanisms involved in the development of resistance against heavy metals and antibiotics. Enhanced growth and resistance patterns detected when heavy metals like Chromium and Zinc supplemented as stressed elements to epiphytic bacteria. These epiphytic bacteria mitigate toxicity of heavy metals to plant signifying horizontal gene transfer patterns in natural environments which support plant growth under stress conditions.

This resistance pattern in epiphytic bacteria primarily arises from the release of antibiotics and heavy metals into the environment due to their extensive use [7]. Another factor contributing to this resistance pattern is the replacement of the wild-type bacteria population with a larger, resistant bacterial population on the plant surface. Recurrently multiple metal and antibiotic resistance observed in *S. haemolyticus* and *Pseudomonas azotoformans* [26].

Epiphytic bacteria have been shown to be beneficial for enhancing plant growth [27]. Both endophytic and epiphytic bacteria from medicinal plants possess the capability to produce indole-3-acetic acid (IAA), which plays a crucial role in stimulating plant cell development, division, differentiation, and gene regulation [28]. Approximately 33% of selected epiphytic bacteria produce significant amounts of IAA that are beneficial for plant growth. The production of hydrogen cyanide (HCN) is an important factor for good bacteria because it not only supports the growth of plants but also inhibits pathogenic bacteria. This attack protects the host from harmful organisms [7].

Remarkably, 83% of the chosen bacterial strains demonstrated a robust capacity for hydrogen cyanide (HCN) production, underscoring their significance as promoters of plant growth. Bacterial phosphate solubilization showcased their ability to mobilize otherwise insoluble phosphate, fostering improved plant development. Due to their epiphytic origin, only 33% of these bacteria possessed phosphate-solubilizing capabilities. A study has substantiated those endophytic bacteria resistant to heavy metals hold potential for enhancing plant growth due to their plant growth-promoting (PGP) traits [29]. Introducing epiphytic bacteria to plants offers several advantages, including heightened plant growth, disease suppression, and increased crop yields. Epiphytic bacteria also have the capacity to generate exopolysaccharides, associated with various biological functions such as antibacterial, antioxidant, biosurfactant, and immune-modulation. In our study, all selected epiphytic bacteria demonstrated the potential for exopolysaccharide (EPS) production, with a maximum EPS concentration of 77 mg/100 ml recorded. A separate report revealed that endophytic bacteria from *Withania somnifera* produced exopolysaccharides at a concentration of 0.19 mg/ml [30]. Kinetics of exopolysaccharide production is usually synchronized with stressed conditions. In this study epiphytic bacteria show resistance against heavy metals and antibiotics. These bacteria withstand the stressed conditions by producing EPS in its surroundings.

The epiphytic bacteria exhibited more potent extracellular antibacterial properties compared to their intracellular counterparts. Notably, all extracellular extracts from epiphytic bacterial supernatant displayed significant antibacterial activity against gram-positive strains, surpassing their effectiveness against gram-negative strains. This implies that epiphytic bacteria release bioactive compounds extracellularly with the ability to combat harmful pathogens [31]. Epiphytic bacteria play a crucial role in the host plant's antioxidant defense mechanism. In our study, catalase enzyme levels diminished over time in all selected epiphytic bacteria, except for *Microbacterium paraoxydans*,

while *Pseudomonas azotoformans* exhibited high catalase concentration.

In the evaluation of antioxidative activity, all ethyl acetate extracts displayed DPPH scavenging capabilities compared to the control. However, extracellular supernatant extracts exhibited 33-59% greater DPPH scavenging potential than other extracellular and intracellular extracts. The analysis of total phenolic content using the Folin-Ciocalteu assay indicated that extracellular supernatant extracts contained more total phenolic content than their intracellular counterparts. It was demonstrated that the enhanced radical scavenging ability is attributed to the specific action of phenolic compounds, acting as free radical terminators [32]. Molecular screening of resistance to heavy metals and antibiotics in epiphytic bacterial isolates revealed the presence of the Class 1 integrons/ recombinase (*IntI1*) gene. Approximately 66% of bacterial isolates tested positive for the *IntI1* gene, which exhibited 100% similarity to the conserved domains of the *XerC* superfamily (integrase/recombinase) and the alignment shows similarity with ESKAPE pathogens as well [33]. This superfamily is associated with replication, recombination, and repair, as well as the mobilome of prophages and transposons. The presence of multiple heavy metal and drug resistance is strongly correlated with integrons, containing an integrase gene and a cassette integration site where antibiotic resistance genes are integrated [30, 34]. The existence of the *IntI1* gene in the genome of these bacteria aids their survival under conditions of heavy metal exposure and antibiotic stress. In future prospects, these strains could find application in the bioremediation of heavy metals and serve as biofertilizers to promote plant growth under adverse conditions.

5. CONCLUSIONS

This study signifies the integron-mediated multiple heavy metals and antibiotic resistance pattern of epiphytic bacteria isolated from local medicinal plants. Existence of *IntI1* gene in epiphytic bacteria aids in endurance of heavy metal and antibiotic stress. Hence, further investigation at molecular level is needed to identify the further classes of integrons that supports in survival of epiphytic bacterial communities under stress environment. Moreover, these bacteria exhibited beneficial characteristics of plant growth promotion, antioxidant capabilities and EPS production making them suitable candidates to be used as biofertilizers in agriculture sector. These findings have implications for understanding the complex interactions between plants, bacteria, and environmental stressors, and may inform strategies for improving plant growth and stress tolerance in challenging environments.

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Performance of Synthetic Pesticides against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) under Laboratory Conditions

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Abstract: Fall armyworm (FAW), *Spodoptera frugiperda* is a native insect pest of maize crop in South America. It has become an invasive species after its introduction in Sindh, Pakistan. Considering various options for its management, this study was conducted to determine the effectiveness of different pesticides (Proclaim 0.19EC, Coragen 28SC, Match 50EC and Runner 240SC) against 3rd, 4th, and 5th instar larvae of fall armyworm. The pesticides were prepared at half and full recommended doses as per the specifications of the manufacturer. The results regarding mortality percentage were recorded after 24, 48, 72, 96, 120, 144 and 168 hrs. The results indicated that the mortality percentage of FAW in all pesticides at both doses increased with time duration and reached a maximum at 168 hrs. Similarly, all pesticides mostly killed 50 to 100% larval population of FAW after 48 to 168 hrs at half and full doses. The Proclaim shows 100% mortality percentage on half and full dose against 3rd, 4th, and 5th instar larvae, followed by Coragen and Runner. Among pesticides, Match was found least effective. Among doses, maximum mortality of FAW in all pesticides was recorded at full dose as 5th instar larvae were found most susceptible, followed by 4th and 3rd instar. Therefore, it is suggested that a proper application schedule of pesticides, especially Proclaim and Coragen, should be included in the integrated management of FAW in maize to reduce its damage.

Keywords: Maize, Pesticide, *Spodoptera frugiperda,* Synthetic.

1. INTRODUCTION

Maize (*Zea mays* L.) belongs to family Gramineae and native to central America. The crop has always been used by mankind for survival and development, providing medicinal, dietary, herbal, pharmaceutical, economic, industrial, and research benefits [1]. Maize crop is the third cereal crop after wheat and rice in Pakistan and a staple food of many countries [2]. The United States produces 43% of global maize, while China contributes 20%. In comparison, Pakistan produces about 7.5 million metric tonnes annually. Maize is gaining importance in Pakistan due to its use in food, poultry feed, and industry. Unlike the U.S., Pakistan's farming is small-scale and traditional. Therefore, testing pest control products locally is crucial to ensure effectiveness under Pakistani conditions and protect this increasingly valuable crop [3].

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Insect pests are among the most important factors contributing to the low yields facing corn production today. The crop is attacked by 140 different types of insects with varying percentage (%) damage. Out of these insects, 12 species are serious pests of maize, causing damage from sowing to harvest and also under storage conditions [4]. In field condition, maize stem borer is one of the major insect pests causing significant losses but with recent appearance of another new species known as fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) these losses exerted speedily [5, 6].

The application of novel synthetic pesticides is a most effective control method is emergency based that could be a necessary strategy of integrated pest management to bring down invasive *S. frugiperda* in China [7]. For this reason, assessing the efficacy of chemical insecticides in contrast to *S. frugiperda* laboratory populations is a top priority [8]. Agricultural managers and farmers lack of experience with *S. frugiperda*, which is essential for the development of efficient management approaches [9]. As an emergency response to the situation, governments distributed and promoted heavy use of chemical pesticides among smallholder farmers to fight *S. frugiperda* in various countries. The improper application of chemical insecticides by untrained farmers improved concerns for health and the environment. The goal of this study was to evaluate the performance of locally available synthetic pesticides against the 3rd, 4th, and 5th instar larvae of *S. frugiperda*. Thus, the present study was conducted to determine the toxicity of synthetic pesticides against *S. frugiperda* larvae and to determine the most susceptible larval instars against synthetic pesticides.

2. MATERIALS AND METHODS

2.1. Place of Work

The Experiment was conducted at the Stored Grain Research Laboratory, Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University, Tando Jam, Pakistan.

2.2. Insect Collection and Rearing

The larvae were collected from the maize field in the surroundings of Tando Jam and then reared on natural diet (maize) at 27 ± 2 ºC and relative humidity of 60-70%. The different instar larvae were separated in plastic cups to avoid cannibalism. Fresh maize leaves were provided as food to FAW larvae and changed on a daily basis until pupation. The pupae were placed in plastic cages with sand to facilitate adult emergence. After emergence, adults were kept in the cages and given an artificial diet (20% honey and 80% water). The adults were then coupled in insect rearing cages for mating and oviposition, where they were provided with fresh maize leaves for egg laying. As females lay eggs in clusters in leaves, which were separated with the help of scissors and then put into petri dishes for further rearing, which was used for the experiments [10].

2.3. Experimental Set-up and Data Collection and Analysis

The experiment was laid out in a completely randomized design (CRD) with five treatments and each treatment replicated three times. The following pesticides were used in experiment (Table 1). All the pesticides were used at their recommended and half of the recommended dose to determine whether they can even be effective at the reduced dosage to get desired control of FAW larvae with minimum environmental contamination. All the calibrations for the individual insecticides were done accordingly to make 10 ml solutions using the disposable syringes, separate for each dose and insecticide. Afterwards, each dose of the respective insecticides was applied on fresh maize leaves using a disposable micro syringe (50 µL capacity). A volume of 30 µL was uniformly applied on a 4 cm² area of the leaf surface and allowed them to dry completely before the release of FAW larvae.

A control treatment (sprayed with water) on maize leaves was also used. Ten freshly moulted 3rd, 4th, and 5th instar *S. Frugiperda* larvae were transferred in respective treatments separately to avoid cannibalism in plastic cups. Observations on the larval mortality was recorded at 24-hours interval for seven days (168-hours). The collected data on mortality was analysed using Analysis of Variance (ANOVA), whereas the Least Square Difference (LSD) was used for mean comparison. All analyses were done using STATISTIX 8.3 computer software.

Table 1. Details of pesticides used against *Spodoptera frugiperda* larvae during the study.

Treatments	Brand name	Active ingredient	Formulation	Dose (ml/acre)	Distributer	Source of purchase
T1	Coragen 28SC	Chlorantraniliprole 28.8%	Suspension Concentrate (SC)	50	FMC Pakistan Ltd.	Agrochemical Shop, Hyderabad
T2	Proclaim 0.19EC	Emamectin Benzoate 1.9%	Emulsifiable Concentrate (EC)	100	Syngenta Pakistan Ltd.	Agrochemical Shop, Hyderabad
T3	Match 50EC	Lufenuron 5%	Emulsifiable Concentrate (EC)	100	Syngenta Pakistan Ltd.	Agrochemical Shop, Hyderabad
T4	Runner 240SC	Methoxyfenozide 24%	Suspension Concentrate (SC)	100	Bayer Pakistan (Pvt.) Ltd.	Agrochemical Shop, Hyderabad
T5	Control					

3. RESULTS

3.1. Mortality Percentage of 3rd Instar Larvae of *Spodoptera frugiperda*

The effect of different synthetic pesticides at half and full doses against 3rd instar larvae of *S. frugiperda* is shown in (Figure 1). The results showed significant difference ($P < 0.05$) in mortality percentage of *S. frugiperda* at different intervals. In half dose all the pesticides were found effective to cause *S. frugiperda* mortality immediately after their application as maximum mortality after 24 hrs was recorded in Proclaim 90%, followed by, Match 30% and, whereas the lowest mortality was observed in Coragen 10%, followed by Runner 20%. Afterwards, a gradual rise was observed in *S. frugiperda* mortality as 100% mortality was recorded in Proclaim and Coragen after 48-hrs and 120 hrs, respectively. In remaining treatments, the maximum mortality percentage (%) was recorded in Runner 80% and Match 80%, after 120-hrs and 144-hrs, respectively. The all pesticides mostly killed 80% to 100% larval population of FAW at 120, 144 hrs. At full dose, the 100% percentage mortality was recorded in Proclaim within 24 hrs, followed by Coragen with 30% whereas lowest mortality was observed in runner 10% followed by match with 20%. After that, a gradual rise was observed in *S. frugiperda* mortality as 100% mortality was recorded in Proclaim and Coragen after 24 hrs and 96 hrs, respectively. In remaining treatments, the highest mortality percentage of 80% was observed in Runner and Match after 120-hrs and 168-hrs, respectively. The all pesticides mostly killed 80% to 100% larval population of FAW at 120, 144 and 168 hrs.

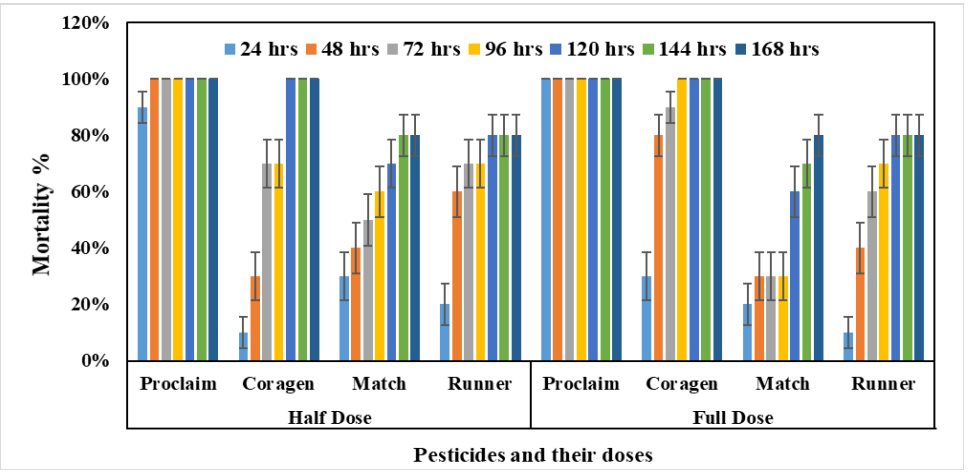

Fig. 1. Mortality of 3rd instar larvae of *Spodoptera frugiperda* on different pesticides at different intervals.

3.2. Mortality Percentage of 4th Instar Larvae of *Spodoptera frugiperda*

The effect of different synthetic pesticides against 4th larval instar of *S. frugiperda* at half and full dose is shown in (Figure 2). The results of mortality percentage were showed significant in *S. frugiperda* difference (P<0.05) at different intervals. In half dose, all the pesticides were found effective to cause *S. frugiperda* mortality immediately after their application as maximum mortality after 24 hrs was recorded in Proclaim 90%, followed by Coragen 50% and the lowest mortality was observed in Runner 10%, followed by Match 20%. Afterwards, a gradual rise was observed in *S. frugiperda* mortality as 90% and 100% mortality were recorded in Proclaim and Coragen after 48 hrs and 96 hrs, respectively. In remaining treatments, the maximum mortality percentage % was recorded in Match 70% and Runner 90%, after 96 hrs and 168 hrs, respectively. The all pesticides mostly killed 70% to 100% larval population of FAW at 72, 96, 120, 144 and 168 hrs. At full dose, the 90% percentage mortality was recorded in Proclaim within 24 hrs, followed by Coragen with 80% whereas lowest mortality was observed in runner 30% followed by match with 40%. After-that, a gradual rise was observed in *S. frugiperda* mortality as 100% mortality was recorded in Proclaim and Coragen after 48 hrs and 72 hrs, respectively. In remaining treatments, the highest mortality percentage was observed in Match 100% and Runner 90%, within 144 hrs. The all pesticides mostly killed 90% to 100% larval population of FAW at 48, 72, 96, 144, and 168 hrs.

3.3. Mortality Percentage of 5th Instar Larvae of *Spodoptera frugiperda*

The effect of different synthetic pesticides against 5th instar larvae of *S. frugiperda* at half and full dose is shown in (Figure 3). The results of mortality percentage were showed significant difference (P<0.05) in *S. frugiperda* at different intervals. In

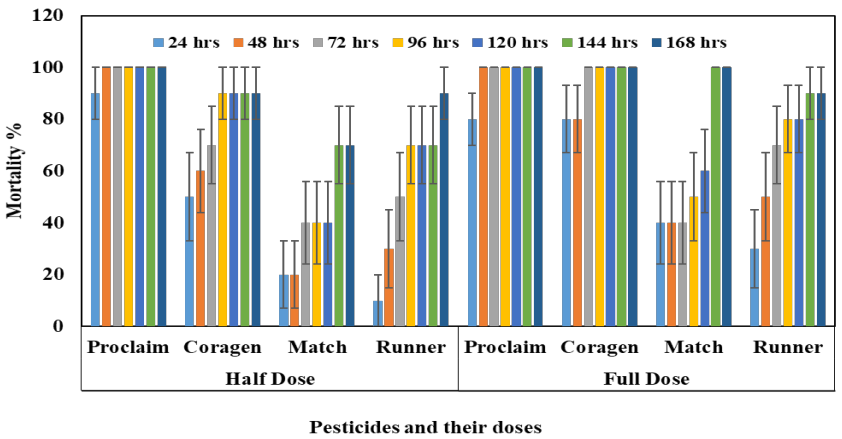

Fig. 2. Mortality of 4th instar larvae of *Spodoptera frugiperda* on different pesticides at different intervals.

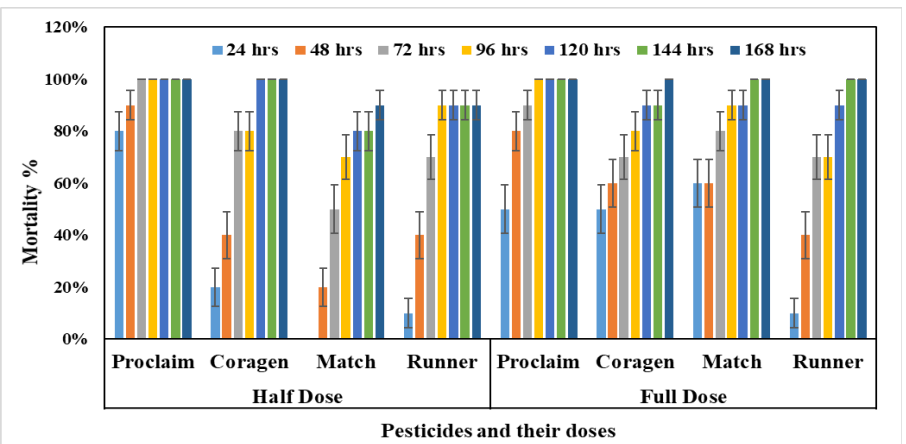

Fig. 3. Mortality of 5th instar larvae of *Spodoptera frugiperda* on different pesticides at different intervals.

half dose all the pesticides were found effective to cause *S. frugiperda* mortality immediately after their application as maximum mortality after 24 hrs was recorded. At half dose, the highest mortality 80, 90 and 100% was observed in Proclaim and the lowest was 0, 20, 50 and 70% mortality in Match at 24, 48, 72 and 96 hrs, followed by Coragen with 20, 40 and 80% and Runner with 10, 40, 70 and 90%. Similarly, after 120, 144, and 168 hrs, the maximum mortality 100% was recorded in Proclaim and Coragen, while the minimum mortality was 80% and 90% in Match and Runner, respectively. At full dose, the highest mortality of 50% was observed in Match at 24 hrs and the lowest was 10% mortality in Runner, followed by Proclaim and Coragen with 50% mortality. Similarly, all pesticides mostly killed the FAW population at 96, 144 and 168 hrs. All the pesticides were found most effective against 5th instar larvae of FAW on half and full doses at all intervals.

4. DISCUSSION

Synthetic insecticides play a vital role in the management of *S. frugiperda*, given confirmed reports of the development of insecticide resistance in FAW populations [11, 12] as well as other adverse effects due to the sole dependence on synthetic insecticides. It is imperative to use an integrated pest management strategy for FAW. The present study conducted to determine the toxicity of synthetic pesticides (Proclaim 0.19EC, Coragen 28SC, Match 50EC, and Runner 240SC) against 3rd, 4th, and 5th instar larvae of *S. frugiperda*. The all pesticides showed that high mortality percentage at half and full dose. Similarly, these results are in accordance with previous findings of Sisay *et al.* [13], as they reported that all of the tested synthetic pesticides were toxic to larvae of *S. frugiperda,* and some pesticides proved high larval mortality in the laboratory.

Those authors reported in some countries for control with synthetic pesticides, As is common with other insect pest species, synthetic pesticides are important management options for control fall armyworm in the Americas [14]. In Florida, fall armyworm is one of the major insect pests of sweetcorn, and synthetic pesticides are applied in vegetative and reproductive stages of corn to protect by fall armyworm [15] and in southern United States, synthetic pesticides are applied against fall armyworm on sweetcorn, regularly 3 to 4 times weekly. In Mexico, fall armyworm control with chemical method in maize crop is achieved by the application of chlorpyrifos, methyl parathion, phoxim, methamidophos, and along with other synthetic pesticides [16].

Although, our findings indicated that the highest larval mortality % observed in proclaim and Coragen pesticide at half and full doses at all intervals. However, Mallapur *et al.* [17] also observed 96.55% and 94.82% mortality of fall armyworm on Proclaim and Coragen at 72 hours. Similarly, Sisay *et al.* [13] also reported fall armyworm 87.5% mortality after 72 hours in Coragen pesticide. Though, our findings indicated that the maximum mortality of 100% 3rd larval instar of *S. frugiperda* was recorded in Proclaim and Coragen after 72 and 96 hours and minimum 30% was recorded in Match, followed by runner with 60% and 70%. However, the after 144 and 168 hours, the highest mortality 100% of 4th larval instar of *S. frugiperda* was observed in Proclaim and minimum mortality was 70% and 90% in Coragen, runner and Match. At half and full dose, the highest mortality 100% and 90% of 5th instar larvae of FAW was observed in Proclaim and Coragen at 168 hours. All the pesticides found most effective against 3rd, 4th, and 5th instar larvae of FAW on half and full dose at all intervals.

As the pervious findings reported by those authors to effectiveness of various pesticides against fall armyworm, the related of the present study on other lepidopteran insect pests have been studied. Kumar *et al.* [18] who observed mortality 72.82% to 91.88% of *Spodoptera litura* in proclaim. However, the mortality of *S. litura* between various dosages of proclaim ranged from 94.30% to 100% and 88.10% to 100% at 3 and 7 days [19]. Similarly, Karthik *et al.* [20] and Rabari *et al.* [21] also reported 100 and 87.49 mortality percentages of *Helicoverpa armigera* and *S. litura* in proclaim and Spinosad.

5. CONCLUSIONS

It is concluded that all pesticides were effective against 3rd, 4th and 5th instar larvae of *S. frugiperda*. The Proclaim shows that 100% mortality percentage on half and full doses against 3rd, 4th and 5th instar larvae of *S. frugiperda*, followed by Coragen and

Runner. Among pesticides, Match was found least effective. Among doses, maximum mortality of *S. frugiperda* in all pesticides was recorded at full dose as 5th instar larvae were found most susceptible, followed by 4th and 3rd instar larvae. Therefore, it is suggested that a proper application schedule of pesticides, especially Proclaim and Coragen, should be included in the integrated management of *S. frugiperda* in maize to reduce its damage. Further study is much needed to observe more synthetic pesticides against *S. frugiperda* in laboratories as well as fields.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Synthesis, Spectroscopy, Antibacterial and Anti-inflammatory Studies of Homo and Hetero Bimetallic Complexes with Bifunctional (O, S) Ligand

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Abstract: Current studies were performed to synthesize homo- (Sn & Sn) and heteronuclear (Sn & Cd/Zn) complexes (1-**7**) of sarcosine dithiocarbamate and investigate their antibacterial and anti-inflammatory potential. Homobimetallic products, i.e., $Ph_2(Cl)SnSSCLSn(Cl)Me_2$ (1), $Ph_2(Cl)SnSSCLSn(Cl)Me_3$ (2), $Ph_2(Cl)SnSSCLSnBu_3$ (3) were produced by a reaction of sarcosine (HLH), CS_2 and Ph_2SnCl_2 and then with Me_2SnCl_2, Me_3SnCl and Bu_3SnCl, respectively. $Ph_3SnSSCLSn(Cl)Bu_2$ (4) and $Ph_3SnSSCLSnMe_3$ (5) were produced by reacting HLH with KOH, CS_2 and Ph_3SnCl firstly and then with Bu_2SnCl_2 and Me_3SnCl, respectively. The heteronuclear products, i.e., $(Ph_3SnSSCL)_2Cd$ (6) and $(Ph_3SnSSCL)_2Zn$ (7**)** were formed by reaction between HLH, KOH, CS_2 and Ph_3SnCl followed by treatment with $CdCl_2$ or $ZnCl_2$, respectively. Elemental analysis data was agreed well with the molecular composition of the products. Fourier transform infrared (FTIR) spectroscopy have shown the bidentate binding of carboxylate and dithiocarbamate donor sites of ligand. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy of 3 displayed the expected signals of ligand portion and organotin(IV) moieties. TGA data of product 7 verified its heterobimetallic (2Sn, ZnO) composition. All the products except 4 and 6 exhibited significant antimicrobial activities as compared to free ligand. The highest anti-inflammatory potential was displayed by product 3.

Keywords: Homobimetallic (Sn, Sn), Heterobimetallic (Sn, Cd/Zn), Spectroscopy, TGA, Antibacterial, Anti-inflammatory.

1. INTRODUCTION

A significant attention has been focused on the study of homo- [1] and hetero- [2] bimetallic complexes in the last few years. In such products, the cooperative effects of more than one metals can lead to new or increased optic, magnetic, or reactive functions. So, such kinds of products are especially important for designing the catalysts and functional materials [3]. Homobimetallic complexes have been reported for tandem organic transformations [4], as catalysts [5], antibacterial [1], anticancer

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and microbiocidal agents [6]. In heterobimetallic complexes, the bifunctionality of the two different metal atoms may result in important applications of the activating substrates. Such compounds display synergistic reactivity of both metal centers, which is different as compared to that observed for chemical species containing only a single type of metal [7]. The two metal atoms may adopt different roles and may act in a cooperative manner. Such a kind of reactivity is generally called as "cooperative reactivity [7, 8]. The heteronuclear complexes commonly find applications in photochemical and catalytic systems [9, 10]. Mixed metal clusters (in homogeneous or heterogeneous formulations) have been reported to exhibit unusual reactivity due to metal-metal interactions or act as "storehouses for the release of catalytically active fragments [11].

Dithiocarbamates are specifically important among various sulfur ligands due to their applications as high-pressure lubricants, molecular precursors, vulcanization accelerators, in CVD processes, as active ingredients in pesticides, fungicides and pharmaceutical products. Because of close similarities with important biomolecules such as vitamins and amino acids, complexes of metals with sulfur donating ligands (e.g., dithiocarbamates) also continues to increase [12].

Organotin compounds are famous for their numerous structural diversities [13, 14] and coordination with different oxygen [15, 16] and sulfur [17, 18] donor ligands. They have been used as antimicrobial, antioxidant, antileishmanial, anticancer [19], antitumor agents [20], insecticides, acaricides, wood preservatives, ceramic pacifiers, antifouling agents, food additives, textile additives, in coatings of electroconductive materials, in metal finishing operations and paints [21]. Many organotin polymers have been reported as antiviral agents [22]. Organotin(IV) derivatives of carboxylate ligands have been investigated as anticancer agents and find useful applications in the field of cancer chemotherapy [23]. They have been widely investigated for their potential agriculture, materials science, medicinal chemistry and catalysis [24]. Their catalytic activity has been reported in various fields, e.g., formation of polymeric olefins or polyurethanes [25]. Organotin compounds have also been found effective for the stabilization of PVC since they can neutralize the formation of HCl in PVC during high temperature conditions [26]. Some organotin compounds are used to inhibit corrosion in porous materials [27].

A lot of work has been reported on tin(IV) derivatives but heterometallic (Sn, Cd/Zn) derivatives of sarcosine and homometallic products having two different organotin moieties, were rarely reported earlier. In continuation of our previous work on organotin(IV) complexes [28], current studies were conducted to synthesize the homo- (Sn, Sn) and heterobimetallic (Sn, Cd/Zn) complexes. The synthesized products were characterized by elemental analysis (CHNS), FTIR, TGA and ^{1}H NMR studies. They have been tested for their antimicrobial potential by biofilm inhibition method and anti-inflammatory activities.

2. MATERIALS AND METHODS

Dimethyltin(IV) dichloride, dibutyltin(IV) dichloride, diphenyltin(IV) dichloride, trimethyltin(IV) chloride and carbon disulfide were purchased from Sigma-Aldrich-Germany. Sarcosine was procured from Merck, Germany. Remaining chemicals, i.e., potassium hydroxide, zinc chloride and cadmium chloride and solvents of analytical grade were used. All the solvents were used without any further purification.

FTIR spectra were recorded in the range of 4000 to 450 cm^{-1} using KBr discs by Perkin Elmer FTIR spectrometer (L1600301 Spectrum Two FT-IR APV). The ^{1}H NMR spectra were recorded at 300 Hz by a Bruker ARC FT-NMR spectrometer. Thermo-gravimetric analysis was performed by a TGA-7 Perkin-Elmer USA. Antimicrobial activities were performed by biofilm inhibition method using ciprofloxacin as a standard drug [29]. A reported method was used to assess the anti-inflammatory potential of the products while using diclofenac as a positive control (standard drug) and DMSO as a negative control [30].

2.1. Syntheses of Homobimetallic Complexes 1-5

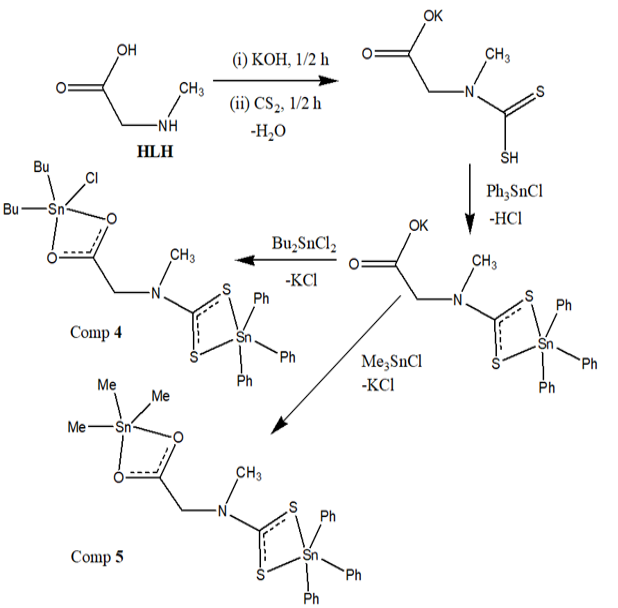
Sarcosine (HLH, 1 mmol, 0.089 g) was stirred with KOH (1 mmol, 0.056 g) for ½ hour in methanol solvent (30 ml) in a 250 ml two necked round bottom flask at room temperature. Then CS_2 (1 mmol, 0.06 ml) was added and the resultant mixture was stirred for half hr. Subsequently, Ph_2SnCl_2 (1 mmol, 0.344 g) was added and stirring was continued for more

1 hour. Finally, Me_2SnCl_2 (1 mmol, 0.220 g) was added and the solution was stirred for four hours. The precipitated KCl was removed after filtration. The filtrate was rotary evaporated to leave behind the solid product 1 which was recrystallized from methanol after addition of few drops of petroleum ether. Use of Me_3SnCl (1 mmol, 0.199 g) or *n*-Bu_3SnCl (1 mmol, 0.325 g) in place of Me_2SnCl_2 in the above reaction has resulted in the formation of products 2 or 3, respectively. The whole reaction route has been displayed in Scheme 1.

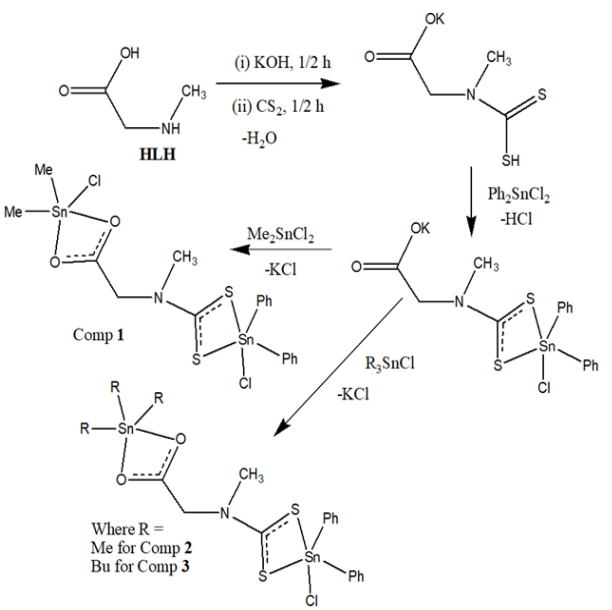
For the synthesis of products 4 and 5, sarcosine (HLH, 1 mmol, 0.089 g) was first stirred with KOH (1 mmol, 0.056 g) for ½ hour in methanol (30 ml) and then with CS_2 (1 mmol, 0.06 ml) for ½ hour followed by stirring with Ph_3SnCl (1 mmol, 0385 g) for 1 hour at room temperature. Finally, the reaction mixture was stirred for four hours with Bu_2SnCl_2 (1 mmol, 0.304 g) or Me_3SnCl (1 mmol, 0.199 g), filtered and the filtrate was rotary evaporated to leave behind the solid product 4 or 5, respectively which was recrystallized from methanol after addition of few drops of petroleum ether. The whole reaction route has been displayed in Scheme 2.

2.2. Syntheses of Heterobimetallic Complexes 6-7

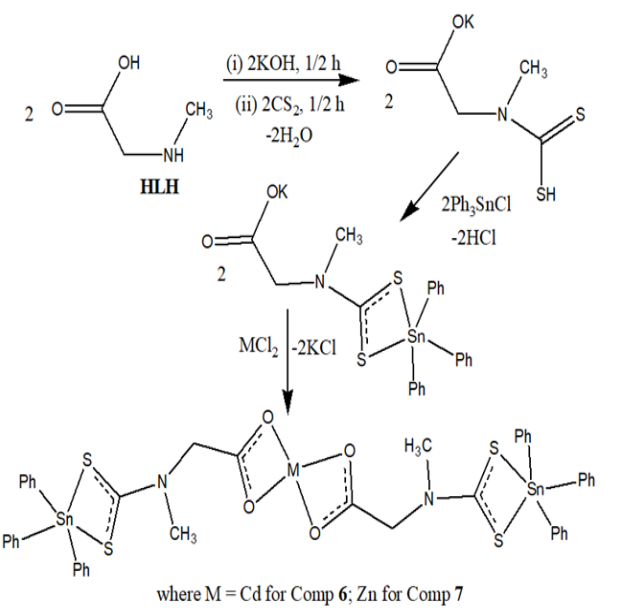
A mixture of sarcosine (2 mmol, 0.178 g) and KOH (2 mmol, 0.112 g) was stirred in methanol (20 ml) for ½ hour in a round bottom flask (250 ml) at room temperature; it was followed by addition of CS_2 (2 mmol, 0.12 ml) and subsequent stirring for more ½ hour. Then 2 mmol of Ph_3SnCl (0.770 g) was added and stirring was continued for 3 hours. Finally, there was addition of aqueous solution of a either $CdCl_2$ (1 mmol, 0.183 g) or $ZnCl_2$ (1 mmol, 0.136 g) with stirring for ½ hr for the formation of $(Ph_3SnSSCL)_2Cd$ (6) or $(Ph_3SnSSCL)_2Zn$ (7), respectively. The whole reaction path has been summarized in Scheme 3.



Scheme 2. Reaction scheme for **the** formation of complexes 4 and 5.



Scheme 1. Reaction scheme for the formation of complexes 1-3.



Scheme 3. Reaction scheme for the formation of complexes 6 and 7.

3. RESULTS AND DISCUSSION

Sarcosine (HLH) was firstly treated with KOH, CS_2 and Ph_2SnCl_2 in equimolar ratio in methanol and then with dimethyltin dichloride, trimethyltin chloride or tri-*n*-butyltin chloride to produce the complexes 1, 2 or 3, respectively. The use of HLH, KOH, CS_2 and Ph_3SnCl in first step and further reaction with either Bu_2SnCl_2 or Me_3SnCl in second step most probably produces the product 4 or 5, respectively. If the reactants (Ph_2SnCl_2 or Ph_3SnCl) in the second step are replaced by $CdCl_2$ and $ZnCl_2$, then the result is the formation of $(Ph_3SnSSCL)_2Cd$ (6) and $(Ph_3SnSSCL)_2Zn$ (7), respectively. The synthesized compounds 1-7 have shown stability in air and have sharp melting points. They are in whitish crystalline solids and have shown good solubility in some organic solvents. The results of elemental analysis were agreed well with the molecular composition of the compounds. Their physical data have been given in Table 1.

3.1. FTIR Spectroscopy

FTIR spectroscopy is an important technique which is used to find the functional groups present in the compounds. The newly synthesized samples were characterized via FTIR spectroscopy. The spectra were recorded in the region of 4000-400 cm^{-1} by a FTIR spectrophotometer. The obtained data are summarized in Table 2. The spectra are shown in Figures S1-S7 (Supplementary Information). For the free ligand (HLH), COO asymmetric (υ_{asym}) and symmetric vibrations (υ_{sym}) are reported earlier in

Table 1. Physical data of complexes 1-7.

Comp. no.	Molecular formula	Melting point (°C)	Mol. wt (g/mol)	% Yield	Color	Elemental analysis %age (Calculated/Found)				Solubility
						C	H	N	S	
1	$C_{18}H_{21}NO_2S_2Sn_2Cl_2$	251	655.82	87	White crystalline	32.97/ 32.46	3.23/ 3.14	2.14/ 1.99	9.78/ 9.83	DMSO, Methanol, Ethanol
2	$C_{19}H_{24}NO_2S_2Sn_2Cl$	207	635.40	78	White crystalline	35.91/ 35.78	3.81/ 3.94	2.20/ 2.08	10.09/ 9.98	DMSO, Chloroform
3	$C_{28}H_{42}NO_2S_2Sn_2Cl$	210	761.64	74	White	44.15/ 44.23	5.56/ 5.43	1.84/ 1.73	8.42/ 8.35	DMSO
4	$C_{30}H_{38}NO_2S_2Sn_2Cl$	235	781.63	82	White crystalline	46.10/ 45.95	4.90/ 4.83	1.79/ 1.84	8.20/ 8.09	DMSO, Chloroform
5	$C_{25}H_{29}NO_2S_2Sn_2$	200	677.05	89	White	44.35/ 44.16	4.32/ 4.19	2.07/ 1.94	9.47/ 9.35	DMSO, Methanol, Ethanol
6	$C_{44}H_{40}N_2O_4S_4Sn_2Cd$	275	1138.89	73	White	46.40/ 46.23	3.54/ 3.47	2.46/ 2.59	11.26/ 10.96	Chloroform, Ethanol
7	$C_{44}H_{40}N_2O_4S_4Sn_2Zn$	266	1091.87	71	White	48.40/ 48.11	3.69/ 3.74	2.57/ 2.68	11.75/ 11.43	Methanol, Ethanol

Table 2. FTIR data (cm^{-1}) of complexes 1-7.

Comp. no.	COO			υC-N	υC-S	υSn-C	υM-O	υSn-S
	υ_{asym}	υ_{sym}	Δυ					
HLH	1621	1407	214	-	-	-	-	-
1	1590s	1430w	160	1504m	971s	730s	693m	517w
2	1591s	1479w	112	1508m	997s	729s	659m	519w
3	1610s	1459w	151	1479m	997s	692s	565m	454w
4	1624s	1429w	195	1462m	996s	694s	580m	526w
5	1621s	1463w	158	1479m	997s	642s	563m	453w
6	1655s	1430w	225	1509m	962s	726s	691m	551w
7	1653s	1480w	173	1509m	997s	728s	692m	549w

literature to be 1621 and 1407 cm^{-1}, respectively [31]. In the coordinated products 1-7, the value of υ_{asym} and υ_{sym} was appeared in the ranges of 1590-1655 and 1429-1480 cm^{-1}, respectively. Thus, there was a significant rise of υ_{sym} value to 1429-1480 cm^{-} in complexes [1] as compared to that (1407 cm^{-1}) of free ligand precursor (HLH), this shift in υ_{sym} evidently verifies the ligand metal coordination. The value of $\Delta\upsilon$ (υ_{asym}- υ_{sym}) depends upon the mono-/bidentate coordinating nature of carboxylate ligand. $\Delta\upsilon$ was appeared in the range of 112-195 cm^{-1} indicating bidentate coordination mode in the complexes 1-5 and 7 [32]. However, $\Delta\upsilon$ value in product 6 was appeared at 225 cm^{-1} which indicates monodentate coordination of the carboxylate moiety for its binding with a metal [33]. FTIR data thus supports a penta-coordinated configuration of tin at -COO donor site in the solid state of all products except **7**. The υ(C–N) vibrations were observed at 1462-1509 cm^{-1} which were lied between the ranges of C=N double bonds (1640-1690 cm cm^{-1}) and C–N single bonds (1250–1360 cm^{-1}) indicating a bidentate coordination fashion of the dithiocarboxylate group [34]. Bidentate coordination mode of the dithiocarboxylate group was further verified by the appearance of a solitary υCS band at 962-997 cm^{-1} [35]. The obtained FTIR results thus demonstrate a penta- and tetra coordinated geometry of tin and Cd/Zn, respectively with the dithiocarbamate donor site of products 1-7 in the solid state. Metal ligand linkage was further verified due to the appearance of Sn-C, Sn-O and Sn-S vibrations in the ranges of 642-730, 563-693 and 453-526 cm^{-1}, respectively.

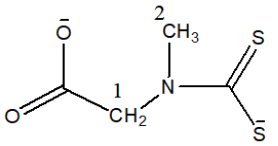
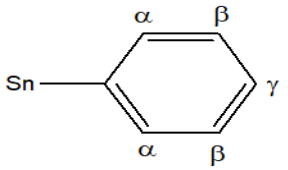
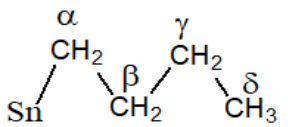
3.2. ^{1}H NMR Spectroscopy

Compound 3 was subjected to proton NMR spectroscopy in DMSO solvent. The chemical shifts are summarized in Table 3 while the spectrum has been displayed in Figure 1. The numbers of protons calculated by incremental method are in good agreement with the experimentally observed data. The product has shown the ^{1}H NMR chemical shifts for ligand portion as well as for both the organotin (phenyltin and butyltin) moieties, thus verifying homobimettalic (Sn, Sn) complexation in product 3.

3.3. Thermogravimetric Analysis (TGA)

Compound 7 was subjected to thermogravimetric analysis (TGA) up to a temperature of 800 °C. The obtained TGA curve is shown in Figure 2.

Table 3. H^{1} NMR data of complex 3.

Portion of complex	**Chemical shift** (ppm) **and protons**
	2.085-2.093t and 1.587-1.618t for protons 1 and 2, respectively.
	8.29d, 7.27-7.77m and 7.03-7.05m for α, β and γ-protons, respectively.
	1.096-1.127t for α protons; 1.29-1.33m for β and γ protons; 0.86-0.89m for δ-protons.

[#]t = triplet, d = doublet, m = multiplet

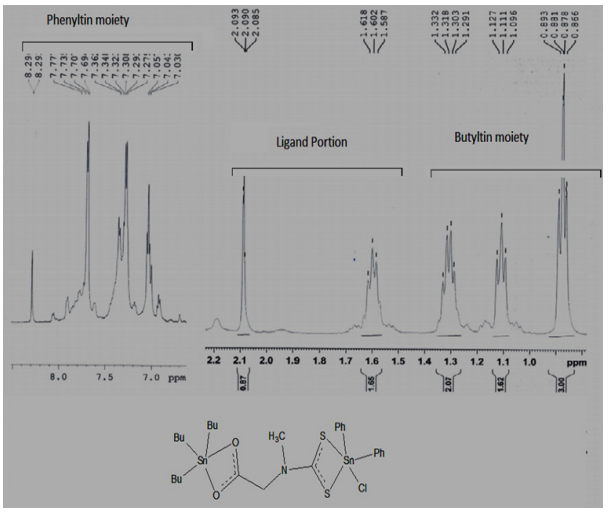


Fig. 1. ^{1}H NMR spectrum of compound 3.

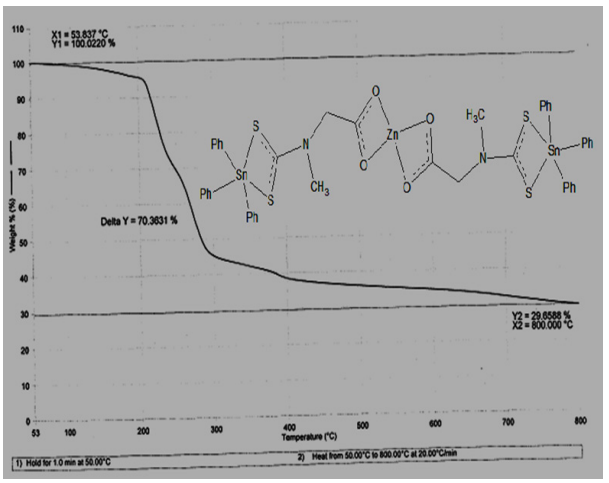


Fig. 2. Thermogram of Compound 7.

The product **7** has shown thermal stability up to a temperature of about 200 °C. However, when it was heated up to 800 °C, it lost 70.35% mass as organic components leaving behind 29.65% residue (Figure 2) in the form of two tin atoms and a zinc oxide molecule. The obtained TGA data thus strongly supports the heterobimetallic composition of the product 7.

3.4. Antibacterial Activities

The free ligand (HLH) and the coordinated products 1-7 were tested for their antimicrobial potential by biofilm inhibition method. Ciprofloxacin was used as a standard drug. The recommended concentration of 1mg/1ml in DMSO was used for each test. The obtained data are displayed in Table 4.

The free ligand has shown no inhibition against both the bacterial strains (*E. coli* and *Staphylococcus*). However, all the complexes except 4 & 6 displayed significant inhibitions of *E. coli* and *Staphylococcu*s biofilms. It is reported in literature that partial sharing of positive charge of a metal atom with the ligand, results in the lowering of its polarity which thus facilitates the permeation of the resultant metal complex through the lipid layer of a membrane [36, 37]. The coordination of the ligand with a metal increases the lipophilic character of consequent complexes, which is responsible for destruction of cell membranes of bacteria and their ultimate death and thus lipophilic character defines the extent of antibacterial activity [38]. The antibacterial potential of our investigated complexes was found to depend upon the nature of target bacterial strain, the substitution pattern at tin and homo- (Sn & Sn) or heteronuclear (Sn & Cd/Zn) nature of a complex.

Table 4. Bacterial biofilm inhibitions (%) by HL and complexes 1-7.

Comp. No	*E. Coli*	*S. Aureus*
HL	-	-
1	30.36	30.61
2	59.51	61.79
3	52.63	62.08
4	-	-
5	66.8	61.79
6	-	-
7	46.15	61.47
Ciprofloxacin	67	69

The highest activity (66.8% inhibition) against *E. coli* was exhibited by compound 5, which may be owed to its homobimetallic (Sn & Sn) nature as well as coordinated triorganotin (trimethyltin and triphenyltin) moieties since triorganotin(IV) complexes in general have been reported to be more active as compared to diorganotin(IV) complexes [39]. By changing the bacterial strain to *S. aureus*, the highest activity (62.08% inhibition) was shown by coordinated product 3 which is also homobiemtallic (Sn & Sn) and is comprised of tributyltin(IV) and chlorodiphenyltin(IV) moieties. The compound 1 containing chlorodimethyltin and chlorodiphenyltin moieties has shown moderate activity with 30.36 and 30.61 % inhibitions against *E. coli* and *S. aureus*, respectively. The coordination products 4 and 6 were totally inactive against each bacterial strain, i.e., *E. coli* and *S. aureus*. Among these products, the homobimetallic complex 4 contains chlorodibutyltin(IV) and triphenyltin(IV) moieties whereas heterobimetallic product 6 consists of two triphenyltin(IV) moieties and one cadmium metal. In going from a heterobimetallic product 6 to 7, there was replacement of Cd metal with zinc which resulted in significant antibacterial potential of 7 against both the tested bacterial strains.

3.5. Anti-inflammatory Activities

Inflammation is the body's defensive reaction to injury, marked by visible signs, e.g., pain, heat, redness and disruptions in normal functions of the body. It is triggered by numerous factors such as microbial invasion, physical trauma and chemical exposure. Its main goals are to facilitate tissue repair, eliminate irritant and neutralize invading pathogens. However, chronic inflammation is associated with numerous health diseases such as cancer, neurodegenerative conditions, autoimmune issues and cardiovascular disorders and is needed to be address. Organotin(IV) complexes have recently attracted the attention of the researchers to address such issues [40]. Rahim et al., 2024 reported the potent anti-inflammatory effects (comparable to indomethacin as a standard) of $\{(n\text{-}C_4H_9)_3SnL$ and $(CH_3)_3SnL$ complexes derived from 4-bromophenoxyacetic acid (HL) [19].

The free ligand (HLH) and our investigated coordinated products 1-7 were tested for their anti-inflammatory activities. Diclofenac was used as

a standard drug (positive control) while DMSO was used as a negative control. The recommended concentration of 1mg/1ml in DMSO was used for each test. The obtained data are summarized in Table 5.

The free ligand (HLH) has shown no anti-inflammatory. However, coordination of ligand with the metal atoms significantly induced the anti-inflammatory potential in all the complexes except 5. The anti-inflammatory potential of the investigated products 1-7 was found to depend upon the substitution pattern at tin and homo- (Sn & Sn) or heteronuclear (Sn & Cd/Zn) nature of a complex. The homobimetallic products 1 to 4 displayed 70.32-89.67% anti-inflammatory potential whereas heterobimetallic product 7 has shown 71.61% anti-inflammatory activity. The highest anti-inflammatory activity (89.67%) was displayed by the coordinated product 3 which is homobiemtallic (Sn & Sn) and contains tributyltin(IV) and chlorodiphenyltin(IV) moieties. The homobimetallic product 5 containing trimethyltin(IV) and triphenyltin(IV) was totally inactive whereas lowest anti-inflammatory activity was displayed by heterobimetallic (Sn & Cd) product 6.

Table 5. Anti-inflammatory activity data of ligand and products 1-7.

Comp. no.	Negative control (DMSO)	Positive control (Diclofenic)	Anti-inflammatory (%)
HLH	0.155	0.042	-
1	0.155	0.042	70.32
2	0.155	0.042	86.45
3	0.155	0.042	89.67
4	0.155	0.042	89.03
5	0.155	0.042	-
6	0.155	0.042	10.96
7	0.155	0.042	71.61

4. CONCLUSIONS

Bimetallic complexes were produced at room temperature by treating sarcosine, KOH and CS_2 in 1:1:1 molar ratio under stirring conditions and subsequent reaction with a di- or tri-phenyltin chloride and then with Me_2SnCl_2/Me_3SnCl/Bu_3SnCl/Bu_2SnCl_2/$CdCl_2$/$ZnCl_2$. The synthesized products were analyzed by elemental analysis, IR, ^{1}H NMR and thermo gravimetric analysis. Elemental analysis data was agreed well with the molecular composition of the products. It was concluded that carboxylate and di thiocarbamate donor sites of the ligand act in a bidentate fashion. The central tin atom exhibits trigonal bipyramidal geometry in solid state with both the oxygen and sulfur donor sites whereas a square planar geometry was assigned around cadmium and zinc ions. ^{1}H NMR data verified the the incorporation of two organotin (phenyltin and butyltin) moieties, thus verifying homobimettalic (Sn, Sn) complexation. Thermo gravimetric analysis verifies the hetero bimetallic nature of complex 7. The investigated complexes exhibit significant antimicrobial activities as compared to free ligand. Anti-inflammatory results show the compound 5 is inactive while the coordinated product 3 had displayed the highest anti-inflammatory activity.

5. SUPPLEMENTARY INFORMATION

FTIR spectra of compounds 1-7 are given in Figures S1-S7, respectively.

6. CONFLICT OF INTEREST

It is hereby declared that there is no conflict of interest among the authors.

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Research Article

Comparative Analysis of Selenium and Quercetin Nanoparticles for their Antioxidant and Hepatoprotective Effects Against Acrylamide-Induced Liver Toxicity in Male Albino Wistar Rats

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Abstract: Acrylamide, a potential occupational carcinogen, is a natural by-product formed during the thermal processing of starchy foods and roasted coffee beans. Recent studies have also reported high levels of acrylamide in various thermally treated fast foods in Saudi Arabia. This study aims to meet the critical need for effective antioxidant therapies to mitigate acrylamide-induced liver damage. By comparing the protective effects of selenium and quercetin nanoparticles, it seeks to identify the more potent nano-antioxidant, thereby contributing to the advancement of safer and more efficient strategies for preventing chemically induced hepatotoxicity. Twenty adult male Albino Wistar rats were randomly divided into four groups: control, acrylamide-treated, acrylamide + SeNP, and acrylamide + QNP. Acrylamide exposure (Acrylamide exposure (50 mg/kg/day, orally for 21 days)) significantly elevated serum levels of cholesterol (CHO), triglycerides (TG), low-density lipoprotein (LDL), alanine transaminase (ALT), aspartate transaminase (AST), creatinine, and urea, cholesterol (233.33 ± 7.50 mg/dL), (238.33 ± 4.93 mg/dL), (67.33 ± 2.51 mg/dL), (80.33 ± 3.51 U/L), and AST (80.00 ± 3.00 U/L) respectively, compared to the control group (CHO: 155.33 ± 8.02, TG: 150.00 ± 7.93, LDL: 39.33 ± 4.16, ALT: 16.66 ± 3.78, AST: 22.66 ± 2.08). while significantly reducing glutathione (GSH) and superoxide dismutase (SOD) levels in liver tissues compared to the control group. Treatment with SeNPs and QNPs led to a marked reduction in these altered biochemical parameters and improved liver histopathology. In conclusion, selenium and quercetin nanoparticles exhibited a protective effect against acrylamide-induced hepatotoxicity in male Albino Wistar rats, suggesting their potential use in mitigating liver damage caused by environmental toxins.

Keywords: Acrylamide, Selenium, Quercetin, Hepatotoxicity, Nanomedicine, Oxidative Stress.

1. INTRODUCTION

Acrylamide is a substance that can be found in both the environment and various industrial processes, and it is considered a potential occupational carcinogen [1]. It naturally forms when starchy foods, like potatoes and grains, or roasted coffee beans are heated, especially during cooking methods like frying, baking, or roasting [2]. In addition to its occurrence in food, acrylamide is also

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used in several industrial applications, such as the production of plastics, paper, and even in sewage treatment and cigarette smoke [3]. Some consumer products, including food packaging and adhesives, may also contain acrylamide [4]. Although it is likely that acrylamide has been present for as long as humans have cooked starchy foods, it wasn't until April 2002 that the Swedish National Food Administration (SNFA) made the public aware that certain food preparation methods could lead to the formation of acrylamide [5]. This discovery highlighted how prolonged heat treatments of certain foods at temperatures of 120 °C or higher could result in significant amounts of acrylamide [6]. Though no official maximum concentration has been set for acrylamide in food, the World Health Organization (WHO) suggests that the acceptable limit for acrylamide in drinking water is 0.5 mg/L [7]. Studies conducted by SNFA and Stockholm University found varying levels of acrylamide in different foods, with protein-rich foods containing moderate amounts (5-50 mg/kg) and carbohydrate-rich foods containing much higher levels (150-4,000 mg/kg) [8]. Foods that are typically free from acrylamide include those that are boiled or prepared without heat [9]. It is now well-established that there is a link between dietary habits and the development of various diseases, including cancer. The consumption of processed foods has been shown to expose individuals to a variety of harmful substances, such a s heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, acrylamide, and nitrosamines. These compounds are known for their mutagenic and carcinogenic properties, which can have detrimental effects on health. As a result, ensuring the production of safe and healthy food has become a critical focus within the food industry [10].

Acrylamide forms in starchy foods when heated above 120 ºC, such as fried potatoes, bread, cookies, and coffee. This compound is primarily produced through a reaction between the amino acid asparagine and a carbonyl-containing substance during high-temperature cooking [11]. A recent study assessing acrylamide levels in thermally-treated foods from Saudi Arabia found that chips contained acrylamide levels ranging from 28 to 954 µg/kg, while labneh and mint had lower levels (28 µg/kg). Acrylamide concentrations in nuts and dried fruits ranged from 2 to 93 µg/kg, and in products like cookies, pastries, cocoa, chocolate, olives, cheese, and grains, levels varied from 26 to 234 µg/kg [12]. The variation in acrylamide levels can be attributed to factors such as food type, cooking methods, and temperature and duration of heating [13]. Another study highlighted a higher risk of acrylamide exposure through cafeteria foods in Jeddah schools, which poses a significant health concern.

In Denmark, the average daily dietary intake of acrylamide is estimated to be 0.27 mg/kg body weight for females and 0.36 mg/kg body weight for males [14]. Similarly in the United States, the estimated average acrylamide exposure is around 0.44 mg/kg body weight per day, which is comparable to levels in the Netherlands [15]. Laboratory research has shown that acrylamide exposure can cause neurological and reproductive toxicity, and it is associated with an increased risk of cancer. Recognized as a neurotoxin over 60 years ago [16], acrylamide exposure in both humans and animals has been linked to symptoms such as ataxia, muscle weakness, weight loss, peripheral edema, and degeneration of axons in both the peripheral and central nervous systems [17]. In pregnant animals, exposure to acrylamide resulted in significant retinal abnormalities in offspring, including ganglion cell degeneration at early stages of development [18]. Additionally, acrylamide has been shown to be toxic to human retinal pigment epithelium cells [19]. Acrylamide is also considered a potential carcinogen, largely due to its genotoxic effects [20]. Researchers at the Fred Hutchinson Center for Cancer Research showed that eating French fries, fried chicken and donuts at least once a week is associated with an increase in the risk of prostate cancer in men. Oral Acrylamide exposure in Albino Wistar Rats resulted in tumors in multiple organs, whereas Acrylamide exposure in humans increases the risk of acquiring cancer [21]. The nanomedicine concept has emerged as a new rising star in the therapeutics field due to its numerous distinct advantages. Nanomedicine is based on a variety of techniques and is related with many types of medications. The increased safety of nanomedicine is a well acknowledged benefit [22]. Nanoparticles are highly distributed solid supramolecular structures composed of organic or inorganic components that are preferably less than 500 nm. The small surface area of nanoparticles provides more accessibility for improved surface functionality within a given volume [23]. These structures have the potential to significantly improve the pharmacokinetics and therapeutic

indices of a wide range of medications, including small compounds, genes, peptide- and protein-based therapies, and diagnostic agents [24-26].

Nanotechnology is an interdisciplinary field that bridges science and medicine, with diverse applications in molecular imaging, diagnostic methods, and precision therapy [27]. Nanoparticles within the mesoscopic size range of 5–100 nm possess vast surface areas and functional groups, enabling their conjugation with therapeutic agents. As a result, they function as adaptable delivery vehicles capable of transporting huge amounts of pharmaceuticals and natural ingredients with better efficacy [28]. Nanotechnology refers to the interactions between cellular and molecular components, artificial materials, and clusters of atoms or molecular fragments, which are engineered into extremely small particles ranging from 1 to 100 nm. Nanometer-sized particles exhibit unique optical, electrical, and structural properties that are not present in individual molecules or bulk materials. This concept of nanoscale devices has paved the way for the development of biodegradable, self-assembled nanoparticles, which are being designed for the targeted delivery of medicinal agents [29]. Since prehistoric times, humans have employed natural goods (natural active substances) to treat a variety of ailments. Natural items have also been employed in the prevention and treatment of different ailments in recent times, which remains an elusive objective in medicine. Nanotechnology can be exploited to increase the systemic delivery and bioavailability of any natural product. Nanoparticle-mediated delivery can also be employed for sustaining the release of natural products, prolonging their action and reducing their frequency of administration [30, 31]. Herein, we assessed the utilization of nanoparticles for the delivery of natural products to target sites to protect and prevent the deleterious health effects of Acrylamide on different body organs and their functions. Quercetin nanoparticles (QNP) are well known anti- inflammatory, antioxidative and anti-allergy agents, which prevent the body from releasing histamine and other inflammatory factors [32]. Selenium nanoparticles (SeNP) have gained much interest compared to other selenium-containing compounds due to their low toxicity and selectivity on cancer cells and minimum or no effect on normal cells. Selenium nanoparticles are studied and used for their potential in recent decades. They play several important roles in treatment of many diseases including immune diseases, diabetes mellitus and, various neurological diseases Furthermore, the polyvalent surface of selenium nanoparticles enables them to engage through covalent and non-covalent bonds with a wide range of chemical substances. Any charges on their surface can be attached to different positive and negatively charged groups, indicating their high adsorption capacity [33]. The aim of our study is to reduce the side-effect related with the Acrylamide consumption using different nanoparticle coated natural molecules (Quercetin and selenium nanoparticles). This study highlights the significant hepatotoxic effects of acrylamide and demonstrates the protective role of selenium and quercetin nanoparticles in mitigating oxidative stress, liver damage, and lipid peroxidation. By using nanoparticle formulations, the study enhances the therapeutic potential of these natural antioxidants through improved bioavailability. These findings provide a strong preclinical basis for developing nanotechnology-based interventions to counteract chemical-induced liver toxicity, with promising implications for preventive healthcare and future clinical applications.

2. MATERIALS AND METHODS

2.1. Chemicals

Acrylamide dry crystals (A8887-100G) were obtained from sigma chemicals Co, USA. Quercetin and selenium nanoparticles (7782-49-2) (40 nm) were obtained from Sigma-Aldrich, USA. All chemicals were of commercially grade and kept at 4 ºC.

2.2. Experimental Animals

Twenty adult male Albino Wistar Rats (160-220 g) were maintained on a standard pellet diet and housed in appropriate cages in controlled environmental condition with free access to water a*d libitum*.

2.3. Experimental Design

Animals were divided into four groups (5 animals each):
Group 1 (control): Animals were administered with 1 ml of physiological saline orally by gavage.
Group 2 (Acrylamide): Animals were administered with Acrylamide (3 mg/kg per day) orally by

gavage for 14 days.
Group 3 (Acrylamide+ Q): Animals were administered with Acrylamide plus QNP (Quercetin nanoparticles) (3 mg/kg per day) orally by gavage for 14 days.
Group 4 (Acrylamide+ S): Animals were administered with Acrylamide plus SeNP (Selenium nanoparticles) (3 mg/kg per day) orally by gavage for 14 days.

2.4. Liver Sample

Ether was used to anesthetize before surgical dissection and examination of the liver. Light microscope was employed to study the histopathological changes; the tissues were fixed in 10% formalin for future use [34].

2.5. Biochemical Analysis

Lipid profile was done by using colorimetric kits supplied by Bio-diagnostic, Egypt [35, 36]. The total protein was determined by Biuret method [37]. The estimation of aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) was carried out according to the method originally developed by Reitman and Frankel [38].

2.6. Determination of Liver Antioxidant Enzymatic Biomarkers

MDA (Malondialdehyde) (nmoL/g tissue) was determined in liver homogenate by a colorimetric assay according to the method established by Satoh [39]. Hepatic GSH (mg/g tissue) was assayed in liver homogenate according to the method developed by Moron *et al*. [40]. Superoxide dismutase (SOD) was evaluated according to the method described by Marklund and Marklund [41].

2.7. Histopathological Investigations

Hepatic tissue slices embedded in paraffin (5 µm) were cut using a sliding microtome (Leica RM2135 Rotary Microtome, Wichita, KS, USA) and stained with hematoxylin and eosin (H&E) stain for a later light microscope histological analysis using the light microscope 46 [42].

2.8. Statistical Analysis

Values are presented as Mean ± SD. Statistical analysis was performed using one-way ANOVA, with a significance level set at $P < 0.05$.

3. RESULTS

3.1. Biochemical Analysis

3.1.1. Levels of lipid profile

Acrylamide treatment in male Albino Wistar rats resulted in a significant elevation in serum cholesterol (CHO), triglycerides (TG), and low-density lipoprotein (LDL) levels, with mean ± SE values of 233.33 ± 7.50, 238.33 ± 4.93, and 67.33 ± 2.51 mg/dL, respectively, compared to the control group (155.33 ± 8.02, 150.00 ± 7.93, and 39.33 ± 4.16 mg/dL, respectively). However, co-administration of selenium led to a notable reduction in these lipid parameters (CHO: 219.66 ± 11.67, TG: 218.33 ± 4.50, LDL: 50.33 ± 17.50 mg/dL), while quercetin treatment also demonstrated a similar lipid-lowering effect (CHO: 219.66 ± 11.71, TG: 213.00 ± 8.15, LDL: 55.66 ± 10.69 mg/dL), when compared to the acrylamide-only treated group. These findings suggest a potential protective role of selenium and quercetin against acrylamide-induced dyslipidemia (Figure 1).

3.1.2. Liver function markers

Administration of acrylamide to male Albino Wistar rats resulted in a marked and significant elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, with values of 80.33 ± 3.51 and 80.00 ± 3.00 U/L, respectively, compared to the control group (16.66 ± 3.78 and 22.66 ± 2.08 U/L, respectively). However, treatment with selenium and quercetin nanoparticles effectively attenuated this increase. Selenium nanoparticles reduced ALT and AST levels to 57.66 ± 8.02 and 68.00 ± 4.35 U/L, respectively, while quercetin nanoparticles brought them down to 59.00 ± 4.58 and 65.00 ± 5.56 U/L, respectively (Figure 2). These results suggest a protective effect of selenium and quercetin nanoparticles against acrylamide-induced hepatic injury.

3.2. Oxidative Stress Biomarkers

Acrylamide administration significantly reduced the levels of key antioxidant enzymes glutathione (GSH) and superoxide dismutase (SOD), with

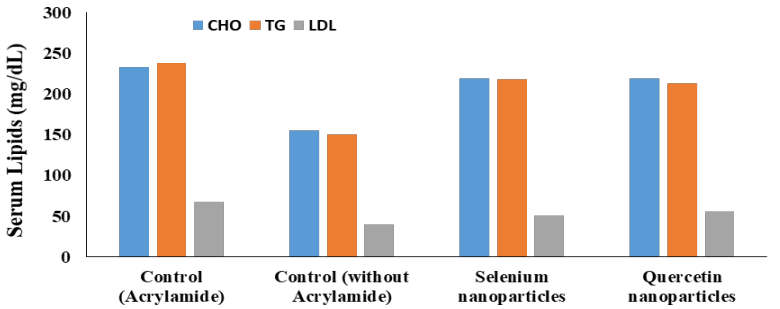

Fig. 1. Lipid profile of male Wistar rats treated with acrylamide, selenium and quercetin nanoparticles.

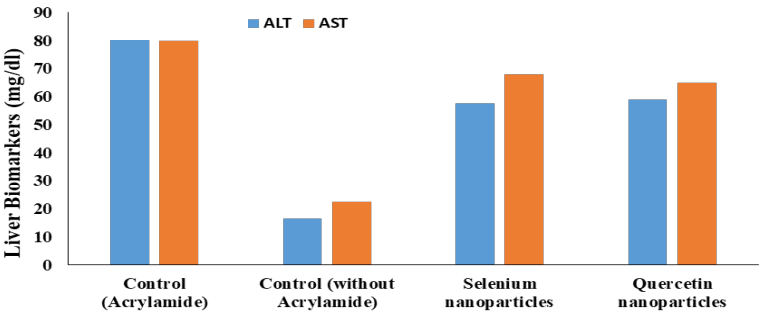

Fig. 2. Liver enzyme biomarkers of male Wistar rats treated with acrylamide, selenium and quercetin nanoparticles.

mean ± SE values of 306.33 ± 5.03 and 568.66 ± 8.50 U/mg protein, respectively, compared to the normal control group (365.00 ± 12.76 and 652.33 ± 14.04 U/mg protein, respectively). Co-treatment with selenium and/or quercetin nanoparticles markedly attenuated oxidative stress, as evidenced by improved levels of these antioxidants compared to the acrylamide-only group. Furthermore, malondialdehyde (MDA), a key indicator of lipid peroxidation, was significantly elevated in the acrylamide-treated group (2.06 ± 0.37 nmol/mg protein) relative to the control group (0.66 ± 0.04 nmol/mg protein. Treatment with selenium and quercetin nanoparticles resulted in a noticeable reduction in MDA levels to 1.05 ± 0.16 and 1.47 ± 0.14 nmol/mg protein, respectively. These findings highlight the considerable protective effect of selenium and quercetin nanoparticles in mitigating acrylamide-induced oxidative damage (Figure 3).

3.3. Liver Histopathology

Histological evaluation of liver sections from the control group revealed normal hepatic architecture, characterized by well-organized hepatocytes (H), centrally located central veins (CV), and regularly spaced, open sinusoids (S), reflecting normal hepatic function [34]. Conversely, the liver tissues from the acrylamide-treated group demonstrated substantial pathological alterations. These included congested blood vessels (red arrows), swollen hepatocytes (H), focal necrotic areas (black arrows), and signs of focal perivascular fibroplasia with mild leukocytic cell infiltration (arrowhead). Additionally, sinusoids appeared narrowed or completely occluded (S). These changes are indicative of hepatocellular degeneration, inflammation, and

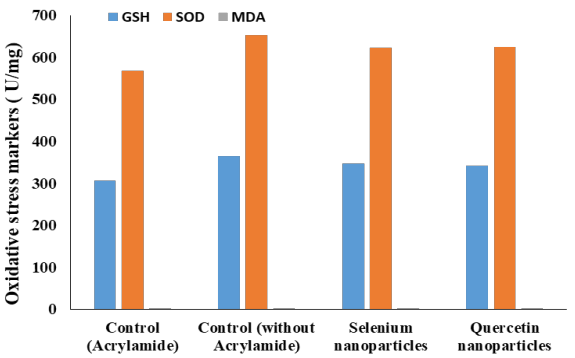

Fig. 3. Oxidative Stress biomarkers of male Wistar rats treated with Acrylamide, selenium and quercetin nanoparticles.

oxidative stress-induced damage, which are well-documented consequences of acrylamide toxicity. In the group treated with selenium nanoparticles, partial histological improvement was observed. Although congested central veins (CV), swollen hepatocytes (H), and occluded sinusoids (S) were still present (red arrows), the degree of tissue damage was noticeably less severe than in the acrylamide-only group. Selenium's antioxidant and anti-inflammatory properties likely contributed to this protective effect. The quercetin nanoparticle-treated group exhibited near-normal liver histology. Hepatocytes (H), central veins (CV), and sinusoids (S) appeared mostly intact, with only mild residual lesions. This suggests that quercetin nanoparticles conferred a more pronounced hepatoprotective effect, possibly due to their potent free radical scavenging and membrane-stabilizing. These histological findings corroborate the biochemical results and underscore the potential of selenium and quercetin nanoparticles in mitigating acrylamide-induced hepatotoxicity (Figure 4). The negative control group represents the normal hepatocytes (H), central veins (CV) and opened sinusoids (S). The Acrylamide treated group showed congested blood vessels (red arrows), swollen hepatocytes (H), focal necrotic area (black arrow), and focal perivascular fibroplasia with few leukocytic cells infiltration (arrowhead), narrowed or occluded sinusoids (S). Selenium treated group showed congested central veins (CV) (red arrows), swollen hepatocytes (H), and occluded sinusoids (S). Quercetin treated group has showing normal hepatocytes (H), central veins and opened sinusoids (S) with partially relieved lesions (Figure 4).

4. DISCUSSION

Acrylamide poses significant risks to human health due to its toxic properties. Acrylamide causes the generation of free radicals through upsetting the balance between oxidative stress and antioxidants in the cells [42]. Acrylamide is a well-known agent which causes the disturbance in blood and cellular lipid ratio [43], which are considered to be risk factors for cardiovascular diseases [44]. The present study stated that, the exposure of male Albino Wistar Rats with Acrylamide induced a significant rise in lipid profile (TCH, TG, and LDL) compared to control group. Acrylamide causes the enhanced production of free radicals resulting in the accumulation of cholesterol due to biosynthesis and decreased cholesteryl ester hydrolysis [45]. In addition, the selenium and/or quercetin nanoparticles treatment

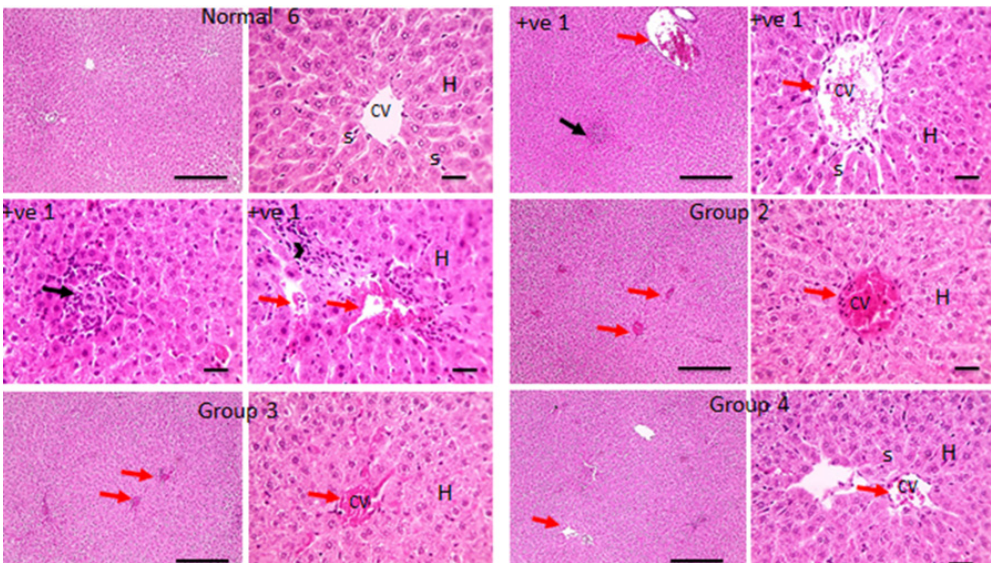

Fig. 4. In the negative control group (Without any treatment) (Group 6), liver sections displayed normal architecture, with healthy hepatocytes (H), clear central veins (CV), and open sinusoids (S). In the positive-control (acrylamide treated) (Group 1), however, there was evidence of vascular congestion (red arrows), swollen hepatocytes, focal necrosis (black arrow), perivascular fibroplasia with sparse leukocyte infiltration (arrowhead), and narrowed or occluded sinusoids. Groups 2 and 3 (treated with selenium and quercetin respectively) still showed congested central veins, swollen hepatocytes, and sinusoidal blockage, although to a slightly lesser degree. In Group 4, these lesions were partially alleviated, with reduced congestion and cellular swelling. Finally, Group 5's liver sections closely resembled normal tissue, exhibiting healthy hepatocytes, unobstructed central veins, and fully open sinusoids.

induced reduction the lipid profile when compared to Acrylamide alone. Our finding was in consistence with the result of similar studies [46, 47]. Urea and creatinine are nitrogenous by-product of body metabolism. In present study, Acrylamide treatment to male Albino Wistar Rats significantly increases the serum urea and creatinine levels in comparison to control group while the selenium and quercetin nanoparticles administration mitigated these effects. The results come to an agreement with results of Uthra *et al.* [44] who stated that the levels of urea and creatinine were disrupted by Acrylamide and the treatment with quercetin restored theses indices towards normal levels. Our findings are consistent with the study by Sengul *et al.* [48], which reported a significant decrease in urea and creatinine levels due to acrylamide intoxication. These acrylamide-induced changes were effectively mitigated by selenium treatment. Similarly, the results of this study revealed a notable increase in liver enzymes following acrylamide exposure compared to the control group, likely attributable to oxidative stress and hepatic inflammation caused by acrylamide [49]. However, treatment with nanoparticles resulted in a significant reduction in these levels, highlighting the liver-protective effects of both quercetin and selenium. When compared with controls, the acrylamide-treated rats showed a pronounced rise in ALT and AST activities, reflecting liver injury induced by acrylamide exposure. These results are coincided with the finding of Hamdy *et al.* [50] and Rivadeneyra-Domínguez *et al.* [51]. Since the human body's primary organs for detoxification are the liver, the degenerative alterations were seen in this study point to a variety of Acrylamide effects [52]. Our study showed that MDA in the hepatocytes was increased significantly, due to Acrylamide exposure and increase was inhibited upon treatment with selenium and quercetin nanoparticles. The present study results are consistent with the studies carried out by various researchers such as Liu *et al.* [53], Sengul *et al.* [48] and Karimi *et al.* [54]. It was observed that acrylamide exposure led to oxidative stress, which was effectively mitigated by the use of antioxidants. In the group exposed to acrylamide, a notable reduction in SOD and GSH levels was detected. However, treatment with nanoparticles restored these levels when compared to the group that received acrylamide alone. These finding were in agreement with other scientists as well [55], who reported that both selenium and vitamin C prevent the damage in the liver and boosted up the redox state in male mice. In addition, these results parallel to those verified by Sengul *et al.* [46] and Uthra *et al.* [43]. Mahdavinia *et al.* [56] reported that quercetin exhibits a protective role against bisphenol-A-induced mitochondrial damage in the liver of Albino Wistar rats. Furthermore, a recent study demonstrated that quercetin, whether administered alone or in combination, effectively corrected the altered parameters associated with the toxicity of copper oxide nanoparticles in rats [57]. In the present study, histopathological analysis corroborated the biochemical findings of acrylamide-induced toxicity in male Albino Wistar rats, aligning with the observations reported by Uthra *et al.* [43]. In the same way, other reports supported our finding and revealed substantial changes in mice and Albino Wistar Rats, and these changes were mitigated using quercetin and selenium nanoparticles respectively [55, 57, 58]. Overall, this study confirms the significant health risks associated with acrylamide exposure, particularly its detrimental effects on lipid metabolism and liver health. Acrylamide-induced oxidative stress leads to elevated lipid profiles, including total cholesterol, triglycerides, and LDL, as well as increased urea and creatinine levels, signaling hepatic and renal impairment. These outcomes support earlier findings on acrylamide's capacity to disrupt metabolic balance and induce oxidative damage. Importantly, the study demonstrates that selenium and quercetin nanoparticles exhibit marked hepato- and nephro-protective effects. Through their potent antioxidant properties, both nanoparticles effectively reduced malondialdehyde (MDA) levels and normalized key antioxidant enzymes, thereby mitigating acrylamide-induced oxidative damage. Biochemical and histopathological analyses confirmed that nanoparticle treatment significantly restored liver and kidney function, reduced lipid abnormalities, and corrected enzyme imbalances. From a practical standpoint, these findings underscore the value of nanoparticle-based interventions in waste recycling, where agricultural or industrial sources of selenium or quercetin could be converted into effective nanotherapeutics. This not only provides economic benefits by lowering material costs and enhancing product value, but also supports sustainable biomedical innovation. However, the potential toxicity of nanoparticles to biological systems necessitates thorough safety evaluations and dose standardization before clinical or commercial use. In comparing

the two nanoparticles, selenium nanoparticles demonstrated slightly superior efficacy in restoring biochemical parameters, likely due to their role in the glutathione peroxidase system, while quercetin nanoparticles offered broader antioxidant effects and better cellular tolerance. This suggests that while both are effective, their mechanisms and safety profiles may be suited to different clinical contexts. Overall, the results advocate for further exploration into nanoparticle-based antioxidants as a cost-effective, scalable, and clinically relevant approach to counteracting environmental toxin-induced damage.

5. CONCLUSIONS

In conclusion, this study confirms the significant health risks associated with acrylamide exposure, particularly its detrimental effects on lipid metabolism, and liver health. Acrylamide-induced oxidative stress leads to a rise in lipid profiles, including total cholesterol, triglycerides, and LDL, as well as increased levels of urea and creatinine, which are indicative of impairment. These findings align with previous research indicating that acrylamide disrupts normal metabolic processes and promotes oxidative damage. It was apparent from our results that selenium and quercetin nanoparticles have hepato-protective and nephro-protective effects through its antioxidant and ameliorative effect against Acrylamide induced toxicity by decreasing the MDA level and normalization of other antioxidative enzymes. The administration of selenium and quercetin nanoparticles effectively reduced the negative effects of acrylamide. Both nanoparticles significantly reduced elevated lipid profiles, normalized urea and creatinine levels, and alleviated liver enzyme abnormalities. The protective effects observed with selenium and quercetin nanoparticles are consistent with their known antioxidant properties, which counteract acrylamide-induced oxidative stress and support cellular health. Histopathological analysis further confirmed the biochemical improvements brought about by these treatments, reinforcing their potential as therapeutic agents against acrylamide toxicity. Overall, this study highlights the protective efficacy of selenium and quercetin nanoparticles in mitigating acrylamide-induced damage and suggests their potential for therapeutic use in combating oxidative stress-related disorders. The present study holds promising translational potential, particularly in advancing therapeutic strategies for specific disease or condition, e.g., neurodegenerative disorders, cancer, metabolic syndromes. By bridging the gap between preclinical research and clinical application, this work lays the foundation for future interventions that can be tailored for human use. The nanoparticle-based delivery of quercetin and selenium in this study not only enhanced their bioefficacy but also validated their protective roles against acrylamide-induced oxidative and histological damage. By leveraging the unique properties of nanotechnology; such as improved solubility, targeted delivery, and enhanced cellular activity; this study provides compelling evidence for the future use of QNPs and SeNPs as viable therapeutic interventions against toxin-induced metabolic and organ-specific pathologies.

6. ETHICAL STATEMENT

The study was conducted in accordance with ethical guidelines and principles for the care and use of laboratory animals. All experimental procedures involving the use of male Albino Wistar rats were approved by Egyptian Network of Research Ethics Committees. The study adhered to the guidelines set by ENREC. Efforts were made to minimize animal suffering and ensure humane handling throughout the course of the experiment.

7. ACKNOWLEDGMENTS

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8. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary Data

Integron Mediated Multiple Heavy Metal and Antibiotic Resistance in Plant Growth Promoting Epiphytic Bacteria

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Supplementary Table 1. Heavy metals and antibiotic resistance profiling of selected epiphytic bacteria illustrating MTC of selected heavy metals (1000-150000 µg/ml) and antibiotics (500-2500 µg /ml).

Heavy metal / antibiotic used (µg/ml)	**Bacterial strain**					
	AN1	**AN2**	**BG4**	**BG6**	**OL21**	**AV2**
K_2CrO_7	3400	3400	2900	80000	100000	1100
	3500	3500	3000	100000	120000	1200
$ZnCl_2$	1100	1000	1000	1000	1000	1000
	1200	1100	1100	1100	1200	1100
$CuSO_4$	1500	700	1200	1000	600	600
	1600	800	1300	1100	700	700
$NiCl_2$	1500	300	1500	600	400	600
	1600	400	1600	700	500	700
$PbCl_2$	4200	4400	2900	3600	3600	2900
	4300	4500	3000	3700	3700	3000
Ampicillin	900	90	900	2300	90	2500
	1000	100	1000	2400	100	2600
Streptomycin	90	1400	400	90	400	600
	100	1500	500	100	500	700

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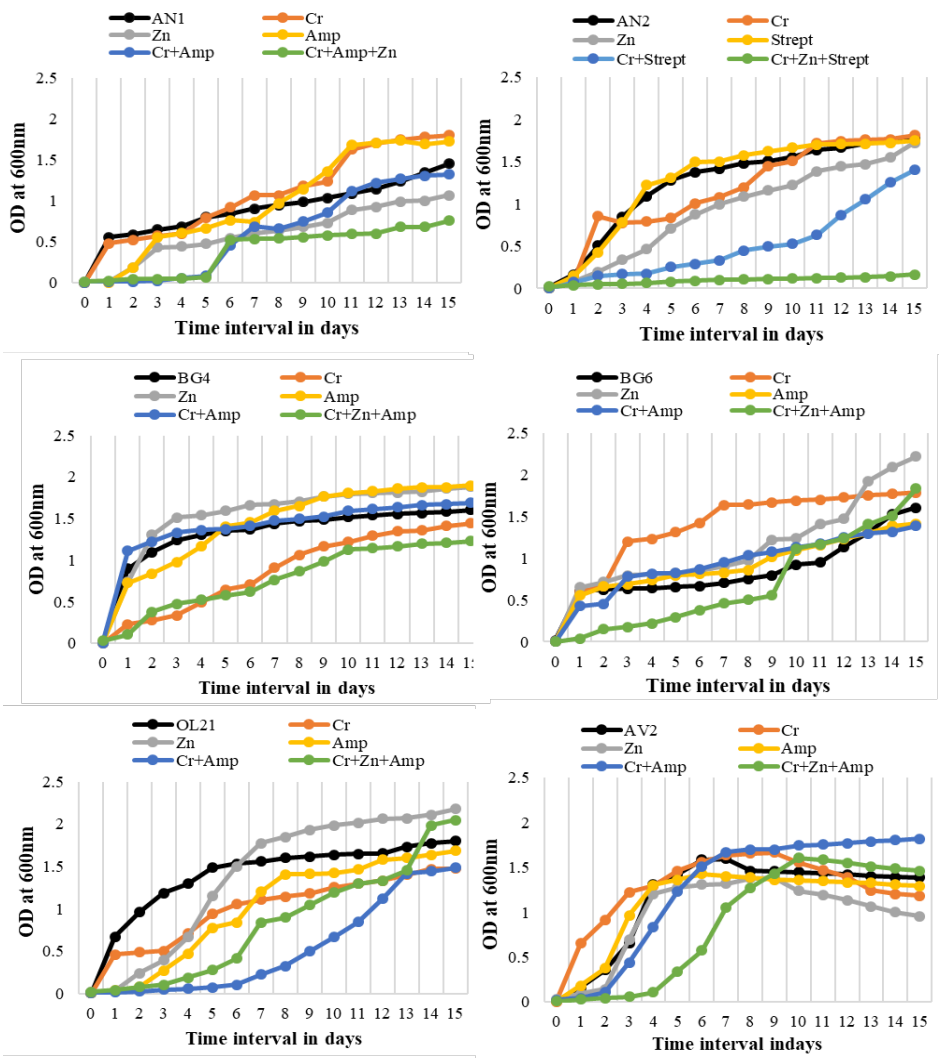
Supplementary Table 2. Effect of selected epiphytic bacterial isolates on the growth promotion of *Triticum aestivum*.

Bacterial strain	Germination (%)	Seedling length (cm)	Shoot length (cm)	Root length (cm)	Number of leaves	Number of roots
Control	70±0.81	17.65±4.04	8.8±2.01	8.76±2.09	2±0.12	4±0.57
AN1	80±0	20.66±1.56	11.1±0.16	9.56±1.39	3±0.10	4±0.20
AN2	100±0	27.39±1.31	10.86±0.65	16.53±0.66	2±0.08	6±0.08
BG4	80±0	25.84±1.96	10.43±1.39	15.4±0.57	2±0.10	5±0.10
BG6	80±0	19.45±1.53	9.25±1.40	10.2±0.12	2±0.10	4±0.40
OL21	80±0	16.69±0.62	10.41±0.29	6.27±0.32	2±0.10	5±0.61
AV2	70±0.81	29.60±4.83	10.92±1.85	18.67±2.98	2±0.10	5±0.24

Mean of triplicates with standard errors.



Supplementary Fig. S1. Single and synergistic effect of antibiotic and heavy metal stress on growth kinetics of selected epiphytic bacteria (AN1, AN2, BG4, BG6, OL21 and AV2).

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Supplementary Data

Synthesis, Spectroscopy, Antibacterial and Anti-inflammatory Studies of Homo and Hetero Bimetallic Complexes with Bifunctional (O, S) Ligand

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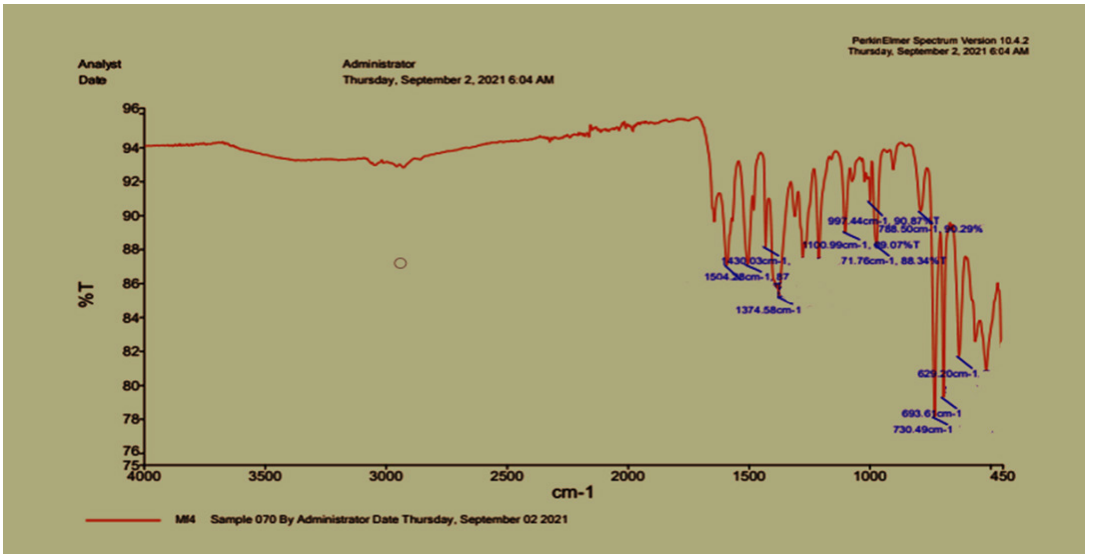

Fig. S1. FTIR spectrum of compound 1.

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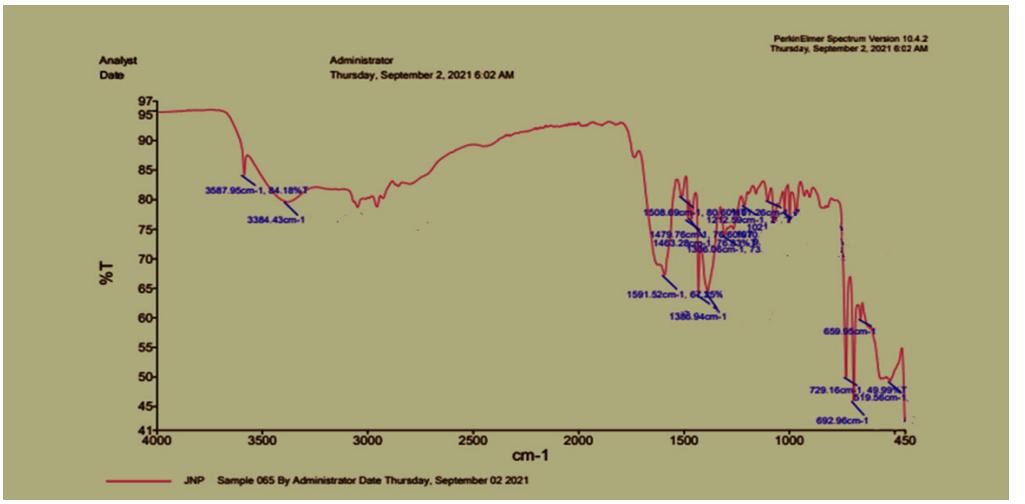

Fig. S2. FTIR spectrum of compound 2.

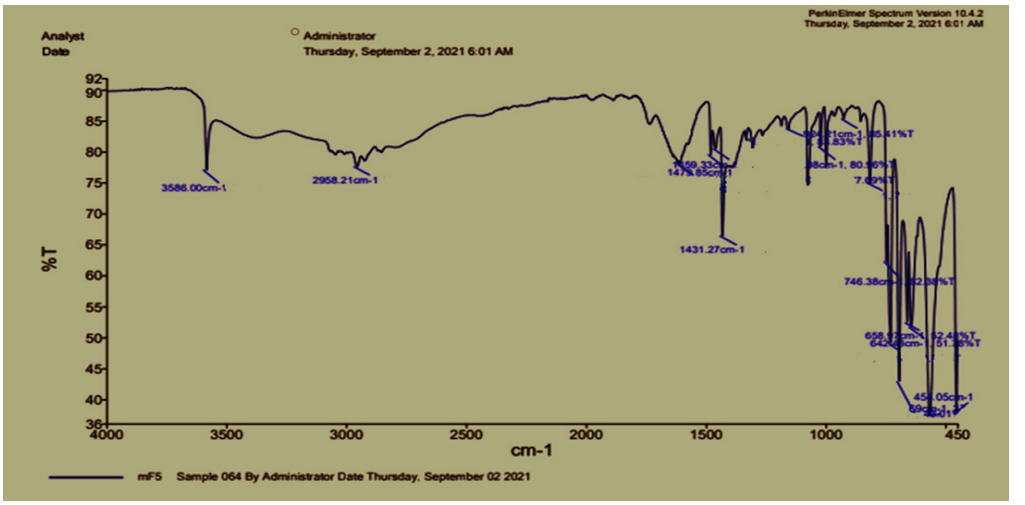

Fig. S3. FTIR spectrum of compound 3.

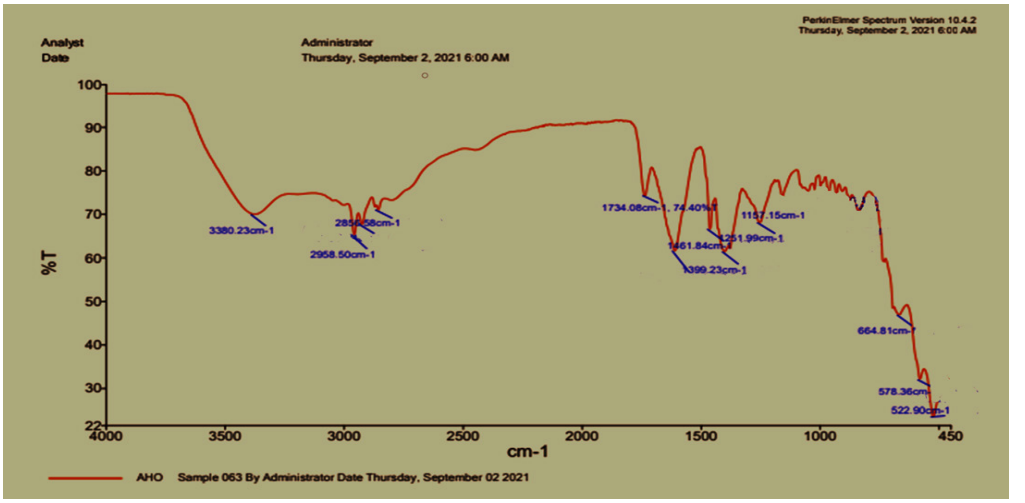

Fig. S4. FTIR spectrum of compound 4.

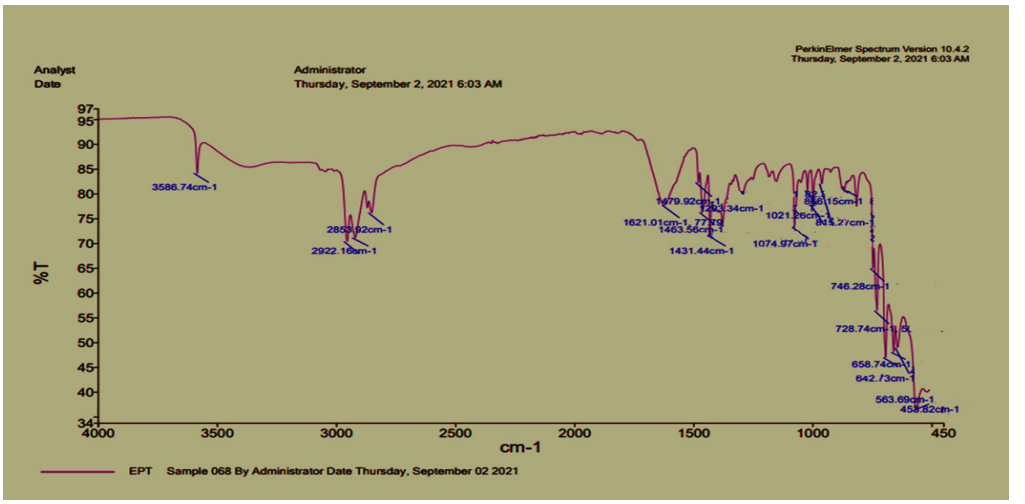

Fig. S5. FTIR spectrum of compound 5.

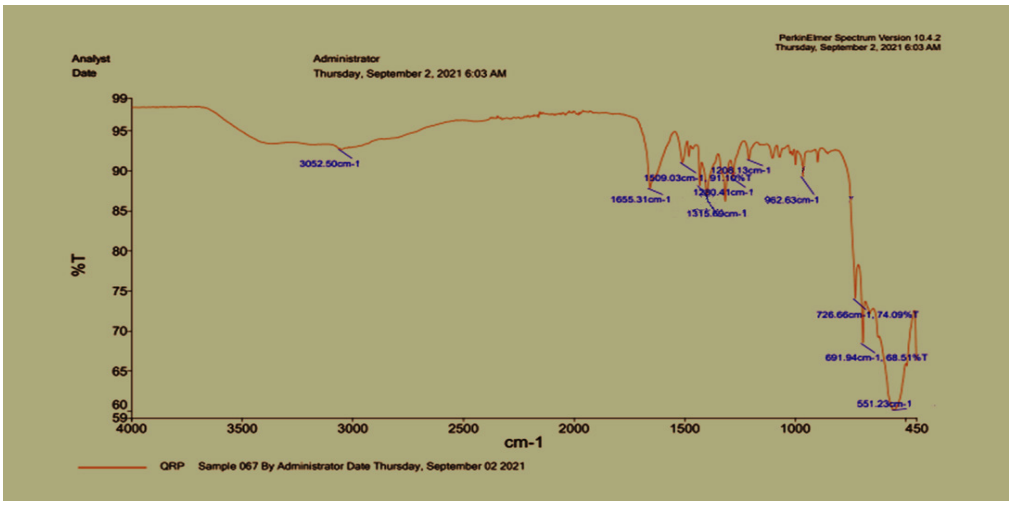

Fig. S6. FTIR spectrum of compound 6.

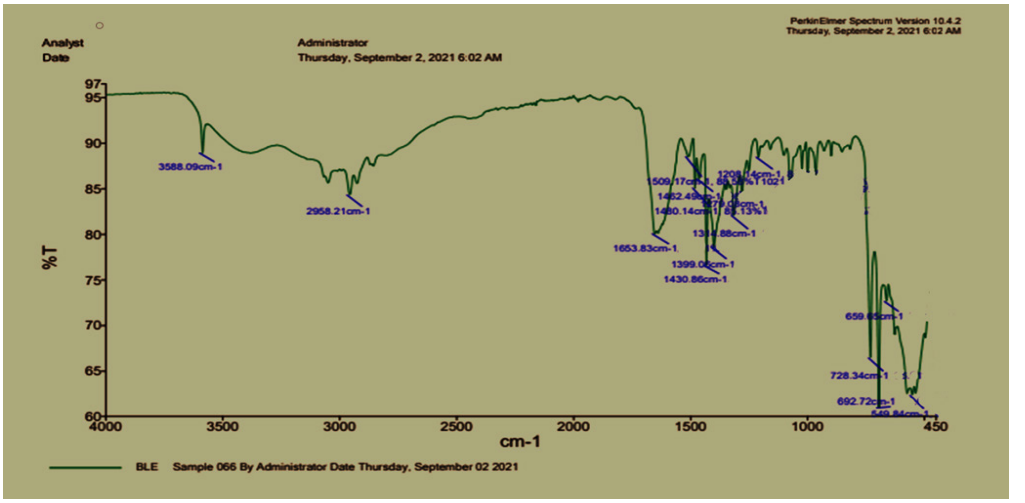

Fig. S7. FTIR spectrum of compound 7.

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b. Books

4. W.R. Luellen (Ed.). Fine-Tuning Your Writing. *Wise Owl Publishing Company, Madison, WI, USA* (2001).

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c. Book Chapters

6. M.S. Sarnthein, J.E. Smolen, and J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: The Northern North Atlantic: A Changing Environment. P.R. Schafer and W. Schluter (Eds.). *Springer, Berlin, Germany* pp. 365–410 (2000).

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d. Reports

8. M.D. Sobsey and F.K. Pfaender. Evaluation of the H_2S method for Detection of Fecal Contamination of Drinking Water. Report No.-WHO/SDE/WSH/02.08. *Water Sanitation and Health Programme, WHO, Geneva, Switzerland* (2002).

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9. UNESCO. Global Education Monitoring Report 2024/5: Leadership in education–Lead for learning. *United Nations Educational, Scientific and Cultural Organization, Paris, France* (2024). https://digitallibrary.un.org/record/4066661?ln=en&v=pdf

10. L.M. Highland and P. Bobrowsky. The landslide handbook–A guide to understanding landslides. Circular 1325. *US Geological Survey, Reston, Virginia* (2008).

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11. M. Khalid, A.B. Majid, F. Mansour, and C.R. Smith. Word Representations with Recursive Neural Networks for Morphology. *27th European Conference on Signal Processing, (2nd - 6th September 2021), Madrid, Spain* (2021).

g. A Degree Thesis

12. M. Afzal. Investigation of structural and magnetic properties of nanometallic Fe-Mn Alloys. Ph.D. Thesis. *Quaid-i-Azam University, Islamabad, Pakistan* (2023).

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