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Microscopic, phytochemical and antibacterial studies of *Zizyphus oxyphylla* Edgew. leaves

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Abstract: Microscopic, phytochemical and pharmacological profiles are required for correct identification of a plant material to ensure consistent efficacy and safety. But such data are not available for the leaf of an important medicinal plant, *Zizyphus oxyphylla* Edgew. (Family: Rhamnaceae). Therefore, the current study aimed to investigate leaves of the plant for microscopic, phytochemical and antibacterial studies. Powdered material was subjected to microscopy, proximate analyses and estimation of total primary metabolites. Then, different types of extracts prepared using various solvents in order of increasing polarity were screened for antibacterial activity against seven standard strains. The most active extract was hydrolyzed and aglycone enriched fraction was extracted and screened for antibacterial activity. The powder microscopy indicated the presence of vascular bundles filled with cuboidal calcium oxalate crystals, anisocytic stomata and xylary vessels with reticulate and scalariform thickenings. Proximate features and primary metabolites provided characteristic identifying patterns. The most active extract (methanol) upon acidic hydrolysis exhibited higher activity against *B. bronchiseptica* (26.01±0.01 mm), *S. aureus* (26.00±0.00 mm), *P. aeruginosa* (24.03±0.02 mm) and *M. luteus* (24.02± 0.00 mm). The results of the current study provide identifying microscopic and phytochemical profiles that may be useful for correct identification of leaves of the plant. Aglycone enriched extract is having remarkable antibacterial activity hence may be used for activity-guided isolation.

Keywords: *Zizyphus oxyphylla*, antibacterial, acid hydrolysis, hexosides, proximate analyses, phytochemical analysis.

INTRODUCTION

Chemical constituents of plants vary due to genetic makeup, habitat, agro-climatic conditions, harvesting time, postharvest practices and storage conditions. Other factors attributable to these variations include adulteration, misidentification, pesticide residues, microbes and toxic metals (Mukherjee *et al*., 2015). Often, plant materials are adulterated partially or completely with inferior quality ingredients which raises efficacy and safety concerns (Mishra *et al*., 2011; Chikezie and Ojiako, 2015). Adulteration and misidentification also pose legal headaches for herbal and pharmaceutical industries. Hence, these problems need to be addressed following the standard guidelines for the quality control of plant materials (WHO, 1998).

The authenticity of a desired species can be achieved by morphological, microscopic and physicochemical analyses (Joharchi and Amiri, 2012). In practice, crude drugs are most commonly identified by morphological features. However, loss of morphological identity of a plant upon drying and subsequent milling increases the chances of adulteration. Hence, microscopic studies are carried out as a 1st step in the quality control of powdered herbal materials by identifying characteristic cellular and tissue structures (Zhao *et al*., 2006; Zhao *et al*., 2010; Dash *et al*., 2021; Nissar *et al*., 2021). These studies are considered as the simplest and most practical approach mentioned in official compendia for the authentication (Ayurvedic Pharmacopoeia of India, 2001; Xiao, 2002; USP, 2005; Japanese Pharmacopoeia, 2006; European Pharmacopoeia, 2007; British Pharmacopoeia, 2008). Furthermore, proximate analyses provide an insight on the quality of powdered material on the basis of loss on drying, ash contents and total extractives (WHO, 1998). These characters are especially important for identification of a crude drug that has lost the identity during milling and processing (Serrano *et al*., 2010). The phytochemical studies, qualitative and quantitative, indicate chemical nature and amount of primary or secondary metabolites that are directly related to pharmacological activity and safety. Therefore, the present study aimed to find identifying macroscopic, microscopic, physicochemical, phytochemical and antibacterial profiles a medicinally and nutritionally important indigenous plant, *Zizyphus oxyphylla* Edgew. (Rhamnaceae).

In folklore medicine, different parts of the plant are used to treat fever, dysentery, diabetes, jaundice, hepatitis, blood disorders, skin diseases, hypertension, intestinal worms and bleeding gums. Methanolic extracts of stem, root and leaf and their fractions are reported to have activity against some Gram negative and Gram positive bacteria (Nisar *et al*., 2010; Nisar *et al*., 2011; Kaleem *et al*., 2012 a & b; Ahmad *et al*., 2013; Ahmad *et al*., 2016). The activity is reported to be associated with higher

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amounts of polyphenols and cyclopeptide alkaloids. The mass spectra of methanolic extract of aerial parts indicated the presence of aglycones such as coumaric acid, vanillic acid, sinapic acid, ferulic acid and kaemferol in the form of hexosides, but none of the study is reported for antibacterial effect of aglycone enriched extract and glycoside. Such study is needed to select the extract for the isolation of the active compound (s). Traditionally, fresh leaves of the plant are placed in warm water to take bath and particularly to give bath to dead bodies. Leaves are also used for hair washing in rural areas of several Asian countries. This indicates the commercial importance of the plant which may further be enhanced using authenticated material. Hence, the current study was carried out to investigate leaves of *Zizyphus oxyphylla* Edgew for morphological, microscopic, phytochemical and antibacterial studies.

MATERIALS AND METHODS

Chemicals, solvents and supplies

Different chemicals and solvents utilized in the present study included copper sulphate, α-naphthol, potassium sodium tartarate, iodine, copper acetate, sodium carbonate, sodium chloride, ferric chloride, barium chloride, sodium hydroxide, Folin-Ciocalteu's reagent, Methanol, ethyl acetate, sulphuric acid, hydrochloric acid, glacial acetic acid, dichloromethane, ethanol, chloroform, petroleum ether, n-hexane, n-butanol, dimethyl sulfoxide and acetone (Merck, Darmstdt, Germany), ninhydrin (BDH, Germany), potassium hydroxide (Omicron, UK), nutrient agar and nutrient broth (Titan Biotech Ltd. India) and Tryptone soya broth (Oxoid Ltd, Hampshire).

The standard bacterial strains including *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 8739), *Bordetella bronchiseptica* (ATCC4617), *Bacillus pumilus* (ATCC 14884), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027) were obtained from Remington Pharmaceuticals (Pvt.) Ltd., Lahore, Pakistan, and the Drug Testing Laboratories, Lahore, Pakistan.

Plant material collection, authentication and processing

The leaves of the plant were collected from Hazara and Swat, Khyber Pakhtoon Khwa, Pakistan, in November, 2017. Prof. Dr. Zaheer-Ud-Din, Department of Botany, Government College University (GCU), Lahore, Pakistan authenticated the plant material. At Herbarium of GCU, Lahore, a voucher specimen number was deposited vide No. bot. 1002. The plant material was dried under shade, pulverized to 180 mesh and stored in polythene bags.

Morphological and microscopic studies

The morphological features of the leaves including color, odor, shape, arrangement, external markings, fracture and internal color were determined by sensory observations. Plant powder (20mg) was put on chloral hydrate drop followed by mixing using fine needle tip. Then, a glass cover slip was placed on the material and the prepared slide was examined under light microscope to locate cellular and tissue structures and document using a digital camera. The images of tissues were identified using the Atalus.

Proximate analyses

Moisture content

The powder (2g) in a tarred china dish was placed in an oven at 105±1°C. After every 30 min, the crucible was placed in a desiccator to cool to room temperature and weighed until two successive weighing were constant (WHO, 1998). The moisture content in the plant powder was determined using following equation:

$$\text{Moisture content (\%)} = \frac{\text{Weight of thermally dried sample}}{\text{Weight of air dried sample}} \times 100$$

Total ash

A dried and clean crucible was weighed. Then, powder (2 g) in a crucible was incinerated in a Muffle furnace at 675±25°C until it became carbon free. The crucible was weighed after cooling at room temperature (WHO, 1998). The total ash content was determined using following equation:

$$\text{Total ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of air dried sample}} \times 100$$

Acid insoluble ash

The total ash obtained in the abovementioned experiment was mixed with dilute hydrochloric acid (25mL), boiled over water bath for 5 min, cooled and filtered. The residue left over filter paper (ashless) was then washed with hot distilled water till filtrate was neutral. The residue along with filter paper was placed in tarred china dish and ignited in the muffle furnace until the material became carbon free. The crucible was weighed after cooling at room temperature (WHO, 1998). The acid insoluble ash was determined using following equation:

$$\text{Acid insoluble ash (\%)} = \frac{\text{Weight of Acid insoluble ash}}{\text{Weight of air dried sample}} \times 100$$

Water soluble ash

The total ash obtained in the abovementioned experiment was dissolved in distilled water (25mL) and then boiled on water bath for 5 min. After filtration of the mixture via ashless filter paper, the residues were washed using hot distilled water. The filter paper containing the residue was placed in a tarred crucible, ignited for 5 min in a furnace at a temperature less than 450°C, cooled in desiccator to room temperature and weighed (WHO, 1998). The water insoluble ash was calculated using the equation mentioned below:

$$\text{Water soluble ash (\%)} = \frac{\text{Weight of total ash} \;\; \text{Weight of water soluble ash}}{\text{Weight of air dried sample}} \times 100$$

Sulphated ash

Two grams powder was moistened with concentrated sulphuric acid in a tarred crucible which was firstly heated on flame until no white fumes were observed and then ignited in a furnace increasing the temperature

gradually to 500-600°C, until material becomes carbon free. The crucible was weighed after cooling it to room temperature in a desiccator. The moistening, heating and igniting process was repeated until two successive weighing were constant (USP, 2005). The sulphated ash was estimated using the following equation:

$$\text{Sulphated ash (\%)} = \frac{\text{Weight of sulphated ash}}{\text{Weight of air dried sample}} \times 100$$

Total extractives

Alcohol soluble extractives: Powder (5g) was extracted with absolute alcohol (100mL) by maceration at room temperature for 24h duration with continuous stirring. After filtration of above mixture, the filtrate (25mL) was transferred to a tarred china dish, evaporated on a water bath and then dried to a constant weight in an oven (105±1°C) (USP, 2005).

Water soluble extractives: Plant powder (5g) was macerated with distilled water (100mL) pre-mixed with few drops of chloroform in a conical flask for one day at room temperature with continuous stirring. After filtration of the contents, the filtrate (25mL) was dried at 105°C in an oven. The extract was cooled in a desiccator and weighed. The total extractives (alcohol and water soluble) were determined using the following equation:

$$\text{Total extractives (\%)} = \frac{\text{Weight of extract}}{\text{Weight of air dried sample}} \times 100$$

Estimation of total primary metabolites

Total proteins

The plant material (15 g) was soaked in 100mL distilled water containing Triton-X (5-10 drops) for 10h at room temperature followed by filtration. Supernatant was collected by centrifugation (2700 rpm) of 10mL filtrate for 10 min. One hundred microliters of the supernatant were transferred into a test tube and the volume was made 1mL with distilled water. To this, 3mL of reagents C [50mL of reagent A (2% sodium carbonate in 0.10N sodium hydroxide) and 1ml of reagent B (0.50% copper sulphate in 1% sodium potassium tartrate)] and 0.20mL Folin-Ciocalteu's reagent were added and the mixture was incubated at room temperature for 30 min. Finally, the absorbance of the solution was measured at 600 nm against a blank containing all components except the sample. Bovine serum albumin in the concentration ranges (12.5-100µg/mL) was used as a standard. The experiment was performed in triplicates and total protein contents were estimated from the calibration curve of glucose solution, treated like the sample (Sarkar *et al*., 2020).

Total lipids

The method by AOAC (2007) was used to determine total lipid content. Briefly, 15g material, packed in a thimble, was subjected to continuous extraction at 40-60°C for 24h using 50mL n-hexane. The extract was filtered, poured in a tarred flask, dried in vacuo at 40±1°C and then weighed. The total lipid contents were determined using the following equation:

$$\text{Total lipids (\%)} = \frac{\text{Weight of extract}}{\text{Weight of air dried sample}} \times 100$$

Total carbohydrates

The method of Al-Hooti *et al*. (1997) was used to determine total carbohydrates as:

Total carbohydrates = 100 – (Total moisture + Total ash + Total proteins + Total lipids)

Fluorescence analysis

The powder on treatment with various reagents was observed for florescence behavior in day-light and under long and short UV light.

Sequential extraction

The leaf powder (1 kg) was macerated sequentially using petroleum ether, chloroform and methanol as solvent at room temperature. The powder and solvent were used in a ratio of 2:6, W/V. The maceration was carried out in an air tight flask for two days with occasional shaking followed by filtration. The residue was macerated again and the extraction procedure was repeated thrice. The filtrate was evaporated in vacuo at 40°C and stored in airtight glass bottles.

Fractionation of the methanol extract

Fractionation of the methanol extract was carried out by partitioning using different solvents in the order of increasing polarity such as n-hexane, chloroform, ethyl acetate, n-butanol and water. The first four fractions were dried at 40°C in vacuo while the water fraction was concentrated using rotary evaporator at 70°C and then subjected to freeze-drying.

Phytochemical screening

The methanolic extract stock solution (0.50mg/mL) was subjected to qualitative phytochemical tests to determine nature of the constituting primary and secondary metabolites such as terpenoids, amino acids, unsaturated sterols, protein, alkaloids, coumarins, carbohydrates, phenolics, tannins, saponins, amino acids, anthracene derivatives, cardiac glycosides and flavonoids (Evans, 1996).

Preparation of hydrolyzed extract

The methanolic extract (5 g) on treatment with 5mL of 2 N hydrochloric acid at 100°C for 40 min was extracted by partitioning using ethyl acetate. The fraction was tested for aglycones using paper chromatography. A chromoplate was developed using mobile phase comprising a mixture of chloroform, ethyl acetate and n-hexane (6:3:1, V/V). Chromoplate was dried using warm air and sprayed with reagent A (1g diphenylamine and 1mL aniline in 100mL acetone) and reagent B (10mL of

reagent 1 and 1mL 85% orthophosphoric acid) (Ewais *et al*., 2016).

Antibacterial studies

Preparation of growth media

The growth media were prepared by separately dissolving 15g of TSB and 14g of nutrient agar in 500mL of autoclaved distilled water and gentle heating for homogenous mixing. The media were autoclaved for 15 min at 15 Psi and 121°C.

Sterility test

The test tubes and Petri dishes containing 20mL of the sterilized broth and agar, respectively were incubated at 37±1°C for 24 h. The media were considered sterile if no growth (flocculation, sediments or turbidity) was observed.

Growth promotion test

The test tubes and Petri dishes containing 20mL of the sterilized broth and agar, respectively were inoculated with bacterial cultures and incubated at 37±1°C for 24h. The media were considered nutritive if growth (flocculation, sediments or turbidity) was observed in the test tubes inoculated with bacteria.

Evaluation of purity and preparation of fresh bacterial cultures

Pre-labeled test tubes containing 10mL of the sterilized broth were inoculated with a wire loop full of the bacterial culture. The test tubes were screw-capped and incubated at 37±1°C for 24h. For confirming the purity of the culture, 10mL of sterilized molten nutrient agar medium was solidified at room temperature in the test tubes inclined to 45°. The slants were inoculated with freshly prepared bacterial cultures and incubated at the abovementioned conditions. The morphology of the colonies ensured the purity of the culture that was further used for preparation of inoculum. The bacterial culture was maintained at 4°C on nutrient agar slants and sub-cultured regularly every 30 days.

Preparation of inoculum

A loop-full of isolated colonies was inoculated into 5mL of TSB and incubated at 37±1°C for 24 h. The suspension was centrifuged at 2700 rpm for 5 min and the cell pellet was reconstituted with sterile normal saline. The turbidity was matched and adjusted equivalent to 0.50 McFarland standard (0.50mL of 1.175% (W/V) barium chloride mixed with 99.5mL of 1% (V/V) sulfuric acid). The final inoculum was equivalent to nearly 1.5×10^8 cfu/mL.

Antibacterial assay (The agar well diffusion method)

The procedure described earlier was used to assess antibacterial activity of extracts (Nair *et al*., 2013; Mummed *et al*., 2018; Prastiyanto *et al*., 2020). Briefly, 20mL of sterile nutrient agar medium was poured into sterile Petri dishes and allowed to solidify for 30 min. The inoculum (100µL) was spread on the solidified agar medium and wells (6 mm in diameter, 2 cm apart) were made using a sterile cork-borer. One hundred microliters of sample were added in assigned wells and the plate was incubated for 24 h at 37±1°C. Cefepime and DMSO were used as standard and solvent controls, respectively. After incubation, diameter of inhibitory zones produced by each sample well was measured, recorded and compared with the standard.

Minimum Inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

For determination of the MIC, two-fold serial dilutions (400, 200, 100, 50, 25, 12.5, 6.25µg/mL) of sample were prepared in tubes containing 1mL of TSB and 30 µL of the bacterial suspension. The test tubes were incubated at 37±1°C for 24 h. The minimum concentration of extract in the broth medium that had inhibited the growth of the test microorganism was taken as MIC (Chandrasekaran and Venkatesalu, 2004; El-Araby *et al*., 2020). Then, a loop full of broth from tubes without visible growth in the MIC assay was cultured on freshly prepared sterile nutrient agar plates which were then incubated at 37±1°C for 24 h. After incubation, the minimum concentration that was able to prevent the colony formation was considered as MBC (Seyydnejad *et al*., 2010).

STATISTICAL ANALYSIS

All experiments were performed in triplicates and the results were expressed as mean ± standard deviation (SD). The experimental data were analyzed using one-way ANOVA followed by Post-hoc Tukey's HSD using SPSS version 22. A p-value below 0.05 was considered as significant difference.

RESULTS

Morphological and microscopic studies

The leaves were green, ovate to lanceolate, non-fleshy, hairy and tough. The fracture was hard and the internal color was light yellowish green. The cellular or tissue components found by light microscopy are shown in fig. 1. The identified characteristic structures were vascular bundles filled with cuboidal calcium oxalate crystals, anisocytic stomata, xylary vessels with sclariform and reticulate thickenings, polygonal parenchyma, and stone cells with pitted lumen.

Proximate analyses

The results of proximate analyses of leaf powder are shown in table 1. The moisture content of the leaf powder was found to be 12.35%. The values of total ash and, acid-insoluble and water-soluble ash were also within the official specification (> 13% and 0.5-5.5%, respectively) (WHO, 1998). The sulphated ash of the leaves was found to be 10.20%. The alcohol soluble extractives were found

to be higher than the water-soluble extractives which indicated that alcohol was more suitable solvent for extraction of the phytoconstituents than water.

Table 1: Proximate analysis of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew

Tests	% content (mean ± SD)
Moisture content	12.35±0.22
Total ash	11.16±0.08
Acid insoluble ash	4.88±0.32
Water insoluble ash	5.02±0.01
Sulphated ash	10.20±0.10
Water soluble extractives	9.08±0.04
Alcohol soluble extractives	20.66±0.36
Total proteins	14.47±0.04
Total lipids	12.25±0.06
Total carbohydrates	73.31±0.00

Data are expressed as mean ± SD (n=3)

Total primary metabolites

The percentage of total protein determined from linear regression equation (fig. 2) of the plot between concentration and absorbance of the solution containing bovine serum albumin was found to be 14.47. Moreover, total lipids and total carbohydrate were 12.25 and 73.31%, respectively. These results indicated that the powder contained more carbohydrates than protein and lipids. It also indicated that the plant material had good nutritive value (Ejelonu *et al*., 2011).

Fluorescence analysis

The behavior of the powder in UV and visible light with and without treatment with different reagents is given in table 2. These results showed that the powder exhibited various shades of grey, green, yellow and brown in daylight and UV light.

Yield of sequential extracts

The yield of petroleum ether, chloroform and methanol extracts was found to be 0.88, 1.28 and 2.16%, respectively. These results showed that material contained higher contents of phytoconstituents of intermediate polarity.

Table 3: Phytochemical constituents in the ethanolic extract of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew

Phytoconstituents	Phytochemical tests	Observations
Terpenoids	Salkowaski test	+
	Liebermann's test	+
Sterols	Salkowaski test	+
	Liebermann's test	+
	Sulphur test	+
Cardiac Glycosides	Keller Killani test	-
	Bromine water test	-
	Legal's test	-
Flavonoids	Ferric chloride test	+
	Lead acetate test	+
	Alkaline reagent test	+
Alkaloids	Mayer's test	+
	Wagner's test	+
	Hager's test	+
	Dragendorff's test	+
Proteins	Ninhydrin test	+
	Xanthoprotic test	+
	Millon's test	-
	Biuret test	+
Carbohydrates	Molisch's test	+
	Benedict's test	+
	Barfoed's test	+
Saponins	Legal's test	+
	Bromine water test	+
	Foam test	+
Lipids	Soap formation test	+
Tannins	Ferric chloride test	+
Coumarins	Sodium hydroxide test	+

Phytochemical studies

The results of qualitative phytochemical analysis are shown in table 3. The results showed the presence of flavonoids, alkaloids, saponins, tannins, protein, carbohydrates, sterols and terpenoids, coumarins, however, cardiac glycosides were absent. Since, secondary metabolites such as flavonoids, alkaloids, saponins and tannins are considered biologically active especially in wound healing, and as antioxidant, antimalarial, anti-inflammatory and antibacterial, hence,

Table 2: Fluorescent behavior of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew after treatment with different reagents in daylight and UV light

Reagent	Daylight	UV radiation	
		Short wavelength	Long wavelength
70% sulphuric acid	Dark black	Dull green	Dark grey
50% sulphuric acid	Dark grey	Dark green	Light grey
60% nitric acid	Dark brown	Dull green	Dark grey
10% sodium hydroxide	Brown	Light green	Light brown
10% ferric chloride	Yellow	Dull green	Dark brown
Chloroform	Colorless	Light green	Pale yellow
Potassium hydroxide	Light yellow	Dark green	Grey
Aniline	Bright red	Dull green	Dark brown
Water	Greenish yellow	Light green	No color

Table 4: Zone of inhibition (mm) of sequential extracts of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew

Bacterial species	Standard	Petroleum ether	Chloroform	Methanol
B. bronchiseptica	22.93±0.11	8.03±0.02*	16.96±0.06*	22.00±0.00*
P. aeruginosa	24.05±0.04	10.00±0.00*	14.12±0.21*	19.02±0.02*
E. coli	22.03±0.03	7.00±0.00*	11.70±0.20*	18.00±0.00*
B. subtilis	16.00±0.00	9.01±0.01*	15.00±0.00*	16.05±0.05
B. pumilus	14.00±0.00	8.00±0.00*	14.18±0.12*	15.02±0.02*
S. aureus	24.05±0.04	10.12±0.28*	12.04±0.04*	22.10±0.05*
M. luteus	20.06±0.05	-	-	14.04±0.03*

Table 5: Zone of inhibition (mm) of various fractions of methanolic extract of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew

Bacterial species	n-hexane	Chloroform	Ethyl acetate	n-butanol
B. bronchiseptica	15.03±0.03*	17.00±0.00*	23.02±0.02	16.86±0.03*
P. aeruginosa	12.01±0.01*	16.00±0.00*	21.13±0.06*	12.69±0.21*
E. coli	10.02±0.02*	15.01±0.01*	20.02±0.02*	13.01±0.01*
B. subtilis	12.00±0.00*	14.02±0.02*	21.00±0.00*	14.03±0.02*
B. pumilus	-	-	18.05±0.04*	-
S. aureus	14.04±0.04*	16.03±0.03*	22.13±0.06*	15.00±0.00*
M. luteus	-	-	12.04±0.03*	10.00±0.00*

Data are expressed as mean ± SD (n=3), * indicates significant difference in activity compared to standard

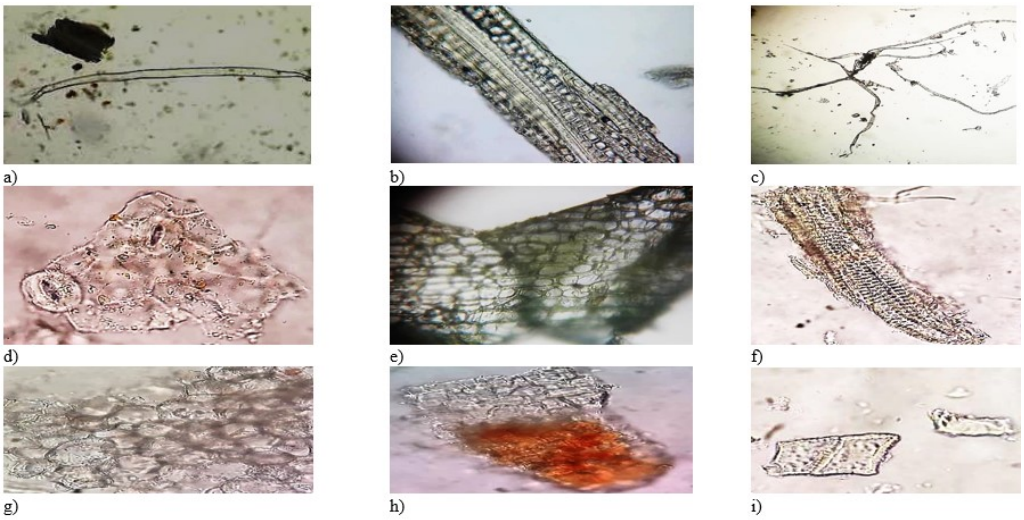

Fig. 1: Microscopic features of leaf powder of *Zizyphus oxyphylla* (ZO), a) bast fiber with tapered ends, b) xylary vessels containing cuboidal calcium oxalate crystals, c) non-pigmented fiber clusters, d) lower epidermal layer showing the presence of anisocytic stomata, e) oblique view of the chlorophyll pigmented epidermal cells, f) xylary vessels with reticulate ans scalariform thickenings, g) thin-walled polygonal parenchyma, h) lateral view of tannin-filled non-isodiamteric epidermal cells, i) stone cells with scalriform thickeneing and large pitted lumen

the plant is expected to show remarkable biological activities (Sadiq *et al*., 2015).

Antibacterial activity

The results of the antibacterial activity of the sequential extracts of leaf are given in table 4. The activity of the methanolic extract was found to be higher as compared to the petroleum ether and chloroform extracts.

Moreover, the order of activity of the methanolic extract for different bacterial strains was found to be 22.10±0.05 mm (*S. aureus*), 22.00±0.00 mm (*B. bronchiseptica*), 19.02±0.02 mm (*P. aeruginosa*) and 18.00±0.00 mm (*E. coli*). The inhibitory effects of the methanolic extract were comparable with the standard drug for *B. bronchiseptica*, *B. subtilis* and *B. pumilus* but lower for *P. aeruginosa*, *E. coli*, *M. luteus and S. aureus*. The chloroform and n-hexane extracts were found to be inactive against *Micrococcus luteus* while methanolic extract showed the inhibitory zone. Due to the remarkable antibacterial effects, methanolic extract was fractioned with a number of solvents in the order of increasing polarity.

The zones of inhibition produced by different fractions against bacterial strains are given in table 5. The results showed that ethyl acetate fraction was most active with a maximum zone of inhibition 23.02±0.02 mm for *B.*

bronchiseptica followed by 22.13±0.06 for *S. aureus* and 21.13±0.06 for *P. aeruginosa*. As shown in fig. 3, the fraction showed inhibitory effects against *B. bronchiseptica* in a concentration-dependent manner. The MIC and MBC of the ethyl acetate fraction against different Gram positive and Gram negative bacterial strains was found to be ranging between 25-200 µg/mL and 50-400 µg/mL, respectively (table 6).

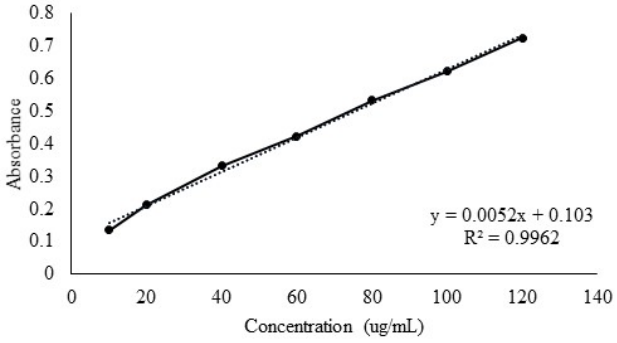

Fig. 2: Standard curve of bovine serum albumin for determination of proteins in leaf powder of *Zizyphus oxyphylla* (ZO)

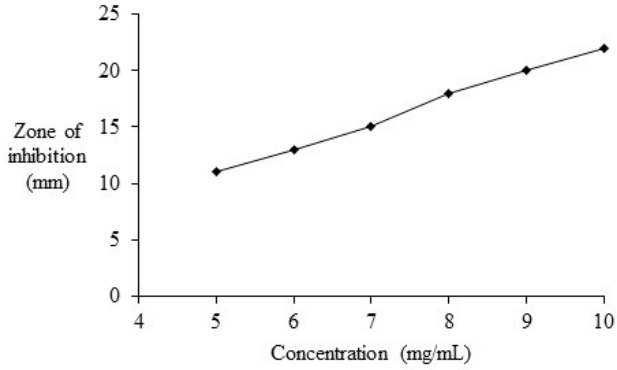

Fig. 3: Concentration-dependent antibacterial activity of ethyl acetate fraction of methanolic extract of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew against *B. bronchiseptica*

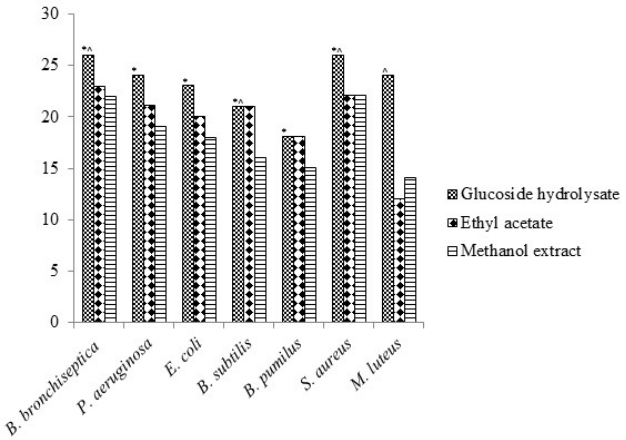

Fig. 4: Comparison of antibacterial activity of methanolic extract and its ethyl acetate fraction and hydrolyzed extract of leaf powder of *Zizyphus oxyphylla* Edgew,* indicates significant difference in activity of glucoside hydrolysate compared to methanol and ^ indicates significant difference in activity of glucoside hydrolysate compared to ethyl acetate.

Table 6: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate fraction of methanolic extract of leaf powder of *Zizyphus oxyphylla* (ZO) Edgew

Bacterial species	MIC (µg/mL)	MBC (µg/mL)
B. bronchiseptica	25	50
P. aeruginosa	100	200
E. coli	100	200
B. subtilis	50	100
B. pumilus	50	50
S. aureus	25	50
M. luteus	200	400

Data are expressed as mean ± SD (n=3)

The methanolic extract was then subjected to acid hydrolysis to separate the glycone and aglycone components and investigate its anti-bacterial activity. The hydrolyzed extract was found to active against different bacterial strains as, *B. bronchiseptica* (26.01±0.01 mm) followed by *S. aureus* (26.00±0.00 mm), *P. aeruginosa* (24.03±0.02 mm) and *M. luteus* (24.02±.0.00 mm). The comparison of the activity of the methanolic extract, ethyl acetate fraction and hydrolyzed extract is shown in fig. 4. The results indicated that aglycone enriched extract had significantly higher antibacterial effects as compared to crude methanolic extract and its ethyl acetate fraction ($p < 0.05$) and must be used for isolation studies.

DISCUSSION

Herbal medicines have been used in medical practice for thousands of years and have made a great contribution to maintain human health. Hence, plant or plant based medicines owing to economy, efficacy and safety are advantageous over synthetic drugs. However, increase in the demand results in less availability of herbs and increases the chances of adulteration. Contrary to the taxonomic method of identification and authentication of a plant based on the morphology of raw/crude part, pharmacognostic study verify and differentiate one species from the other on the basis of characteristics features of dry powder. These features are broadly classified as, microscopic, physicochemical and phytochemical (Chanda, 2014).

The characteristic identifying features of the leaf determined by morphological and microscopic studies may provide useful information for authentication of the plant material in crude form, and after air-drying and milling. The physicochemical studies such as ash

contents, moisture content and total extractives determine the identity, quality as well as purity of a drug. The presence of moisture in crude drugs is believed to provide a medium to accelerate the degradation process by activation of enzymes and microbial growth. Hence, high moisture content can deteriorate the quality and effectiveness of crude drugs during storage. The content of the moisture in the plant material were found to be higher than that was reported in previous study (Mazhar, 2013). The possible reason for this was the collection time of the plant. The harvesting season of plant in Pakistan is May-November (Ahmad *et al*., 2017). However, Mazhar (2013) collected the plant from Kashmir (Pakistan) in March. In contrast to this, we collected the fresh plant in the harvesting season from its natural habitat hence the moisture contents were little higher but within the acceptable range (0-13%) (WHO, 1998).

Crude drugs incineration removes the organic matter leaving behind the inorganic compounds like chlorides, phosphates, carbonates, alkali metals, silica and silicates. The water soluble ash removes alkali chlorides and leaves behind the water insoluble ash. Treatment with dilute sulphuric acid produces sulphated ash which converts metal oxides and carbonates into sulphates, hence ignition at high temperature produces more consistent form of ash. The ash values of the leaf powder indicated that the inorganic impurities in the sample were higher but within prescribed limits. The high percentage of ash contents in the sample has been supported by a study according to which the plant contained inorganic metals such as Na, K, Fe, Zn and Mg in abundance (Ahmad *et al*., 2017).

The extractive values provide an insight into solubility profile of most of the constituents of crude drugs which helps in making choice of a solvent to obtain maximum yield and, evaluation of phytochemistry and biological activity. Water is the most commonly used solvent for extraction of polar phytoconstituents, however, we have observed lower water soluble than the alcohol soluble extractives. Hence, ethanol is the solvent of choice for obtaining extracts in higher yield.

Fluorescence analysis gives an idea of purity of powders thus can be used as diagnostic tool for testing the adulteration. The characteristic fluorescence behavior of the powder may serve the purpose of preliminary quality control of the material using the non-instrumental technique.

The phytochemical studies, qualitative and quantitative, indicate chemical nature and amount of primary or secondary metabolites. Knowledge on the phytochemistry is desirable not only for the discovery of therapeutic agents but may also be important in disclosing new sources of metabolites as flavonoids, saponins, alkaloids, tannins and glycosides. For the synthesis of complex chemical substances, these metabolites serve as precursors (Akrout *et al*., 2010). The results of the present study indicated the presence of phenolics, alkaloids, terpenoids, saponins, tannins and coumarins in the methanolic extract. Regarding the presence of coumarins in the plant material, our results were quite contradictory to the previous study (Ali *et al*., 2015). However, a study has indicated that genus Zizyphus contained alkaloids, terpenoids, saponins, tannins, flavonoids and coumarins (Singh *et al*., 2014).

The genus Zizyphus (Rhamnaceae) is comprised of 100 species distributed worldwide and approximately six species are indigenous to Pakistan (Qayum *et al*., 2012). In folklore medicine, different species of Zizyphus are used for skin and urinary tract infections, hepatic and gastrointestinal tract disorders, weakness, insomnia, and overweight (Kritikar 1984, (Alsayari and Wahab, 2021). Scientifically, these species have been proven anti-pyretic, anti-nociceptive, antioxidant, antidiabetic, anti-listerial, antispasmodic, antimicrobial and larvicidal (Adzu *et al*., 2001; Nazif, 2002; Abdel-Zaher *et al*., 2005; Omena *et al*., 2007; De Nisar *et al*., 2007; Wahida *et al*., 2007; Al-Reza *et al*., 2009; Borgi and Chouchane, 2009). In Asian countries, fresh leaves of the plant are added in warm water to bathe dead bodies and, routine hair and body cleansing. These practices indicate the antimicrobial potential of the leaves. Hence, the present study was planned to determine and compare the dose dependent anti-bacterial effects of the sequential extracts, fractions and hydrolyzed extract of the most active (methanolic) extract against a number of Gram negative and Gram positive bacterial strains.

Amongst sequential extracts, methanolic extract was found to be the most active in inhibiting the growth of bacteria. Moreover, antibacterial activity effects of the fractions of the methanolic extract against seven Gram positive and Gram negative were comparable to the standard drug. These results indicated that our findings were contradictory to the previous studies which reported a moderate antibacterial activity of various parts of the plant against *S. aureus* and *B. subtilis.*

A number of studies report that the antibacterial effects of methanolic extract of the plant and its fractions against *B. bronchiseptica*, *E. coli*, *P. aeruginosa*, *S. aureus, B. pumilus, B. subtilis* and *M. luteus* are associated with higher amounts of polyphenols and cyclopeptide alkaloids (oxyphylline-B, oxyphylline-D, oxyphylline-C, nummularin-R and nummularin-C) (Nisar *et al*., 2010; Nisar *et al*., 2011; Kaleem *et al*., 2012 a & b; Ahmad *et al*., 2013; Ahmad *et al*., 2016). Moreover, mass spectral characteristics of the aerial parts of *Zizyphus oxyphylla* methanolic extract have indicated that other than caffeic acid, majority of the polyphenolic compounds such as coumaric acid, vanillic acid, sinapic acid, ferulic acid and

kaemferol exist in the form of hexosides. Hence, we evaluated the contribution of the glycone component in the antibacterial activity of the methanolic extract in the present study. Our findings have confirmed that aglycone – upon hydrolysis of hexosides – has significantly higher inhibition effects as compared to the crude extract, hence, the latter must be used for activity-guided isolation of the active aglycone components.

CONCLUSION

The broad spectrum antimicrobial potential of this plant supports the utilization of this plant in the treatment of infectious diseases and a useful alternative of currently available antimicrobial drugs.

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