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Article

Antibacterial, antibiofilm, hemolytic activity and phytochemical study of various aerial parts of *Moringa oleifera* in Pakistan

Shahnaz Dilawar[1], Afzal Shah[1]*, Hidayat Ullah Khan[1] and Saima imad[2]

[1]Department of Chemistry, University of Science and Technology Bannu-28100, KPK, Pakistan
[2]Chemical Meteorology Division, National Physical Standard laboratory (PCSIR), Plot No. 16, Sector H-9 Islamabad, Pakistan

[*]Corresponding author: Dr Afzal Shah, Department of Chemistry, University of Science and Technology Bannu-28100, KPK, Pakistan. E-mail: afzalnm@yahoo.com

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Abstract: The methanolic extracts of five aerial parts of *Moringa oleifera Lam* were screened for their antibacterial, antibiofilm and hemolytic activity. The phytochemical screening of the methanolic extracts of aerial parts was also evaluated. The antibacterial, antibiofilm activity of the extracts of various aerial parts of *M. oleifera* was evaluated by disc diffusion and micro titer plate assay method against two selected bacterial species. Hemolytic activity of the methanolic extracts was screened against normal human erythrocytes. Comparatively the methanolic extracts of all aerial parts were more effective against Gram positive bacteria *Bacillus subtilis* and Gram negative bacteria *Escherichia coli* (*E. coli*) .The present study suggests that all aerial parts of this plant can be used as an antibacterial, anti-biofilm agent in treating ailments caused by the tested organisms (*Bacillus subtilis* and *Escherichia coli*). Methanolic extracts of aerial parts possessed minimum hemolytic activity. Significant antimicrobial, antibiofilm activity of the extract was found in this study.

Keywords: *Moringa oleifera*; methanol extracts; antibacterial; antibiofilm; hemolytic activity

1. Introduction

The use of natural substances in the treatment of different diseases, including those of infectious disease is currently a challenge in medicine and offered an alternatives, especially in those ailments for which there is no adequate remedy (Domingo and López-Brea, 2003). Medicinal plants are significantly beneficial and economically indispensable. They are rich in a wide variety of secondary metabolites such as tannins, alkaloids flavonoids, vitamins (A, C, E and K), carotenoids, terpenoids, polyphenols, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity (Khan *et al*., 2009). Considerable attention has been paid to bio and eco friendly plants, which can prevent and cure different human diseases (Dubey *et al*., 2004). The antimicrobial potentiality of various medicinal plants is widely studied all over the world (Rojas *et al*., 2006; Arora *et al.*, 2009). *Moringa oleifera Lam* is the most widely cultivated species of monogeneric family, the Moringaceae, as an important medicinal plant, which is native to, Africa, Arabia, South Asia, South America, Himalaya region, India, Pakistan, the Pacific and the Caribbean Islands (Fahey, 2005; Sreelatha *et al*., 2009).

Two species of *Moringa*, i.e., *M. concanensis* and *M. oleifera* are present in Pakistan. *M. oleifera* is cultured extensively all over the moderate areas of the countryside (Qaiser, 1973). In Pakistan, widely cultivated areas of *M. oleifera* are Punjab plains, Sindh, North western Frontier Province and the Baluchistan (Qaiser, 1973). The Moringa plant has been the object of much research due to its multiple uses and well-known bactericidal potential (Gustavo *et al*., 2010). *Moringa oleifera* is known to possess hypoglycemic, hypotensive, anti-microbial, hepatoprotective, immunomodulatory, antioxidant and antitumor activities (Mahajan *et al*., 2010; Sudha *et al*., 2010). It is one of the best known, widely distributed and grown species of a monogeneric family

moringaceae (Anwar *et al*., 2007). The plant is highly regarded and almost every part of the tree is used as food with high nutritional value (Chuang *et al*., 2007). *Moringa oleifera* has been reported to possess antimicrobial properties and this explains the reason for its extensive use in the treatment of human ailments (Lockettet *et al*., 2000). The morphological parts of *Moringa oleifera* against some pathogenic microorganisms have been reported (Doughari, 2007; Nikkon *et al*., 2003; Jamil *et al*., 2007: Kekuda *et al*., 2010). It has been revealed that the antioxidant activity of plants might be due to their phenolic compounds (Cook and Samman, 1996). Flavonoids are a group of polyphenolic compounds associated with the antioxidant activity and anti-inflammatory action (Frankel, 1995). Flavonoids are known as plant pigments for over a century and belong to a vast group of phenolic compounds that are widely distributed in all foods of plant origin. North American diet, flavonoid glycosides are unavoidably consumed daily, with an estimated total consumption of 1 g/d (Formica and Regelson, 1995), which could be much higher if dietary supplements are also consumed. The objective of the current study was, therefore, to determine the antibacterial, antibiofilm hemolytic activities and phytochemical study of aerial parts of *Moringa oleifera.*

2. Material and Methods

2.1. Identification and collection of the plant material

Aerial parts of *Moringa oleifera* were collected from different regions of Pakistan and the herbarium specimens were subjected to analysis in Quaid e Azam University, Islamabad Pakistan. Plant samples were identified by (Plant taxonomist) Department of Plant Sciences, Quaid-e-Azam university, Islamabad (Voucher Specimen No. ISL 1367).

2.2. Preparation of plant extracts

The freshly collected different aerial parts of *Moringa olifera* were washed with distilled water and dried at 40 °C. The dried samples were extracted with methanol. The extracts were filtered through Whatman filter paper I and then concentrated on rotary evaporator model (Eyela Tokyo Rikakikakki Co Ltd Japan) at 45 °C. Dried samples were kept at 4°C till used for the assay. The extracts were solubilised and diluted with sterile water to get the desired concentration.

2.3. Bacterial culture and inoculums preparation

The identity and purity of the bacterial strains *Bacillus subtilis* JS-2004 and *Escherichia coli* (*E. coli*) ATCC 25922 used for the test were verified by the institute of Microbiology University of agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37 °C in Nutrient agar (Oxoid, UK).

2.4. Antibacterial activity

The extracts obtained were screened for their antibacterial activity in comparison with standard antibiotic (Ciprofloxacin 30 µg/dish) (Oxoid, UK) *in-vitro* by disc diffusion method using *B. subtilis and E. coli* as a test organisms. The filter discs (6 mm in diameter) were individually impregnated with each extract, placed on the agar plates which had previously been inoculated with the tested microorganisms. The diameter of the growth inhibition zones (zone reader) was measured in millimeters for the organisms and was compared to the control (Bashir *et al*., 2015).

2.5. Anti-biofilm activity of the aerial parts

The effect of the methanolic extracts of the aerial parts of *Moringa oleifera* on biofilm formation was examined following the method of (Yarwood *et al*., 2004) micro titer plate assay with a little modification. The appropriate concentration of plant extracts was added to the wells of 96 microtiter plates having bacterial cultures inoculated in LB (*Luria-Bertani)* broth. Negative and positive controls were also included in the experiment.Plates were incubated for 24 h at 37°C. After incubation the growth medium was discarded, and the wells were washed thrice with sterile physiological saline (0.85% NaCl). The adhered cells were stained with 0.1% crystal violet for 10 min. The excessive stain was removed by washing twice with 0.85% NaCl, while cell-bound dye was eluted with 33% glacial acetic acid and the absorbance of eluted solution was measured at 578 nm using a micro titer plate reader. After incubation, the plant extracts were carefully removed and reduction in biofilm formation was determine by measuring the absorbance of biofilms at 570 nm using a micro titer plate reader.

2.6. Hemolytic Activity

Hemolytic activity of the samples was studied by the method used by (Rubab et al., 2015) Three millimeter human red blood cells were collected from Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. Blood centrifuged for 5 min at 1000 ×g plasma was discarded and cells were washed with thrice with 5 mL of chilled (4^oC) sterile isotonic Phosphate-buffered saline (PBS) pH 7.4. Erythrocytes 10^8 cells per m were preserved for each assay. The concentration of each extract (100µg/mL) was mixed with human (10^8cells/mL) separately. The samples were incubated for 35 min at 37^oC and agitated after 10 min. Immediately after incubation the samples were placed on ice for 5 min then centrifuged for 5 min at 1000x*g*. Supernatant were taken from each tube and diluted 10 time with chilled (4^oC) PBS. Triton X-100 (0.1% v/v) was taken as positive control and phosphate buffer saline (PBS) was taken as negative control and pass through the same process. The absorbance was noted at 576 nm using µQuant (Bioteck, USA). The % RBCs lysis for each sample was calculated as;

Percentage hemolysis = (Absorbance of sample-Absorbance of blank÷ Absorbance of positive control

2.7. Quantitavive Analysis of phytochemicals

2.7.1. Quantitative determination of flavonoids

Standard solutions of rutin (1000 mg / l) were prepared by diluting 10 mg of rutin in 5 ml methanol to a 10 ml volumetric flask and make up to the mark. Standard solutions of concentration (10, 20, 30, 40 and 50 mg / l) were diluted from above to stock solution and used for routine calibration curve. 0.5 ml of methanol extract solution was mixed with 2.5 ml of methanol, 0.5 ml of 2% aluminum chloride, allowed to stand at room temperature for 60 minutes. The absorbance of the reaction mixture was measured with UV-Visible spectrophotometer at the maximum absorption length 420mn. The result is expressed in mg /g.

2.7.2. Determination of total phenols

This method was established to determine total phenols, known as the colorimetric method, in which the phenolic compounds react with the folin ciocalteu reagent (tungstophosphate and molybdophosphate). At basic pH, resulting in a blue coloration capable of spectrophotometric determination at 700 nm. A 10% solution of gelatin was then used to ensure the sequestration of the tannins, the percentage of tannins whose absorbance was referred to gallic acid and read at 700 nm was obtained from both determination. The standard solution of Gallic acid was prepared by taking 50 mg of gallic acid and dissolved it in 1 liter of water. A series of standard solutions was prepared with concentration of 0.10, 20, 30, 40 and 50 mg / l.

2.7.3. Determination of alkaloids

The method designated by sharma (2008) was followed. 1 gram of sample was extracted with 20 ml ethanol in 20% H2SO4 (1:1) for 5 mins and filtered. Five militers of 60% H2SO4 was added to 1 ml of the filtrate. Five milliliter of 0.5% formaldehyde in 60% H2SO4 was added. After mixing and allowing to stand for 3hrs, the absorbance of the complex in chloroform was measured at 565 nm against target prepared by Spectrophotometer (shimadzu uv mini-1240). Atropine was used as standard.

2.8. Statistical analysis

The results are presented as mean± S.E.M. Data were determined using student's t-test. P values < 0.01 and 0.05 were taken as significant.

3. Results and Discussion

In regards to antibacterial activity the zones of inhibition for different extracts are shown in (Table 1). The result showed that the methanolic extracts of various aerial parts of *M. oleifera* showed variable antibacterial activity against selected bacterial pathogens equivalent to that of standard against the entire tested organism. Methanolic extracts of leaf showed maximum *B. subtilis* (21 mm), *E. coli* (19 mm). In a similar way the results of methanolic bark extract showed maximum *B. subtilis* (19 mm), *E. coli* (18 mm). Flower extract showed maximum *B. subtilis* (15 mm), *E. coli* (14 mm). The Fruit showed maximum *B. subtilis* (14 mm), *E. coli* (12 mm). Stem showed minimum *B. subtilis* (12 mm), *E. coli* (11 mm). Leaf extract of *Moringa oleifera* showed high zone of inhibition against *B. subtilis* (21mm) and (19mm) against *E. coli* equivalent to that of the standard. All the organisms tested were affected by the extract with appropriate zones of inhibition. The diameter of zone of inhibition was greater in case of leaf and bark extract as compared to other extracts of *M. oleifera* as shown in Table 1.

Table 1. Antibacterial activity of different extracts of M.O against *Bacillus subtilis* and *E. coli*.

Sample / Control	Amount µg/disc	Zone of inhibition (mm)	
		B. subtilis	*E. coli*
mLe	100	21±0.10	19±0.05
mBa	100	19±0.11	18±0.09
mFl	100	15±0.12	14±0.04
mFr	100	14±0.15	12±0.06
mSt	100	12±0.19	11±0.09
Ciprofloxacin	30	23±0.02	22±0.01

Values are presented as mean ± S.E. of triplicate experiments.
mLe: methanolic extract of leaf mBa: methanolic extract of Bark; mFl: methanolic extract of Flower; mfr: methanolic extract of fruit; mSt: methanolic extract of Stemciprofloxacin (control)

Table 2. Biofilm inhibition against *Bacillus subtilis.*

Sample / Control	Absorbance (nm)	% Inhibition
mLe	0.376	63.17
mBa	0.428	58.08
mFl	0.110	52.63
mFr	0.727	28.79
mSt	0.753	26.24
Ciprofloxacin	0.195	80.90

Values are presented as mean ± S.E. of triplicate experiments.
mLe: methanolic extract of leaf, mBa: methanolic extract of Bark, mFl: methanolic extract of Flower, mfr: methanolic extract of fruit, mSt: methanolic extract of Stem

Table 3. Biofilm inhibition against *E. coli.*

Sample / Control	Absorbance (nm)	% Inhibition
mLe	0.33	66.32
mBa	0.35	64.28
mFl	0.48	51.01
mFr	0.66	32.65
mSt	0.65	27.69
Ciprofloxacin	0.131	86.63

Values are presented as mean ± S.E. of triplicate experiments.
mLe: methanolic extract of leaf, mBa: methanolic extract of Bark, mFl: methanolic extract of Flower, mfr: methanolic extract of fruit, mSt: methanolic extract of Stem

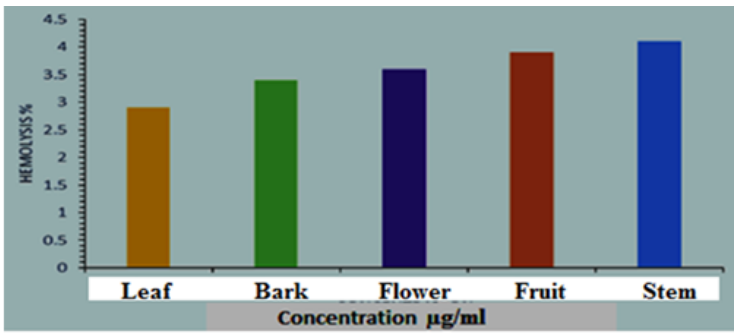

Figure 1. Hemolysis of various aerial parts of *Moringa oleifera.*

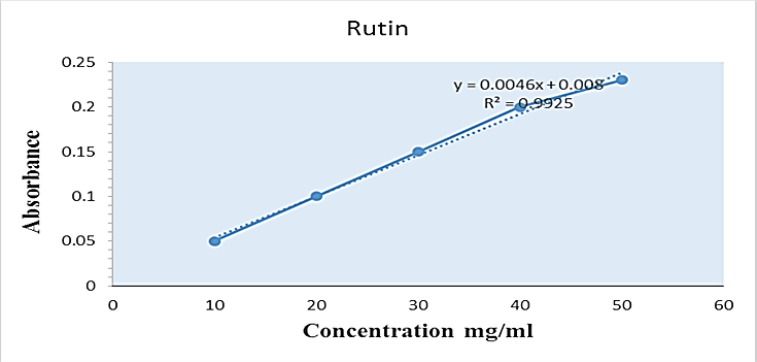

Figure 2. Calibration curve for the quantification of flavonoids.

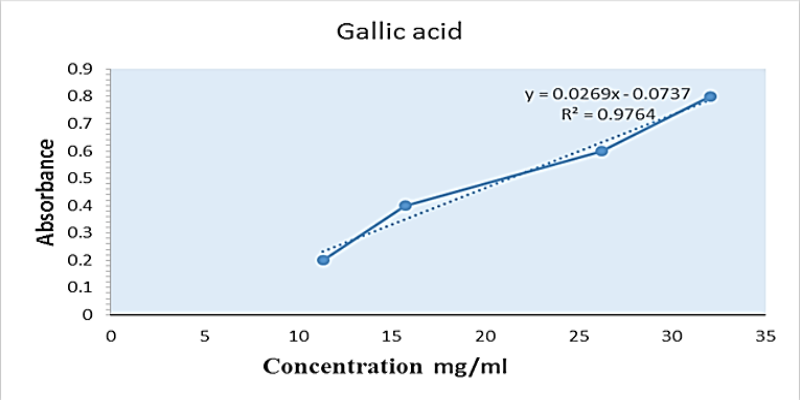

Figure 3. Calibration curve for the quantification of phenols and tannins.

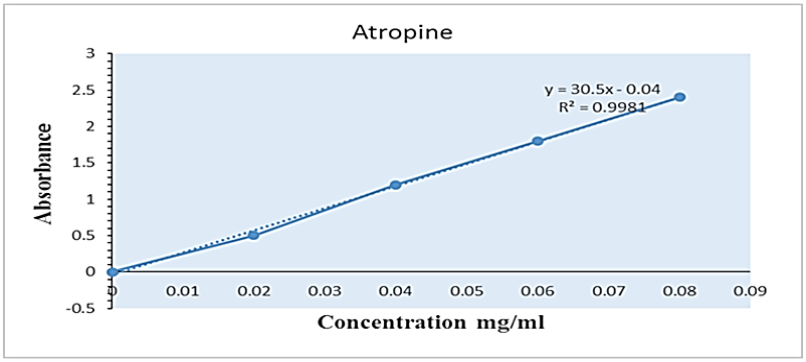

Figure 4. Calibration curve for the quantification of alkaloids.

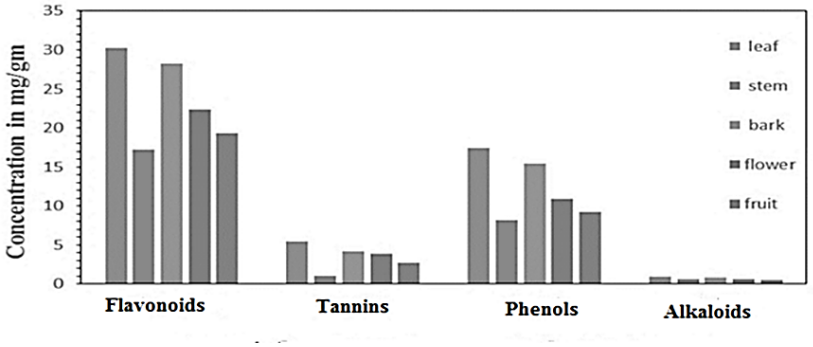

Figure 5. Quantitative analysis of phytochemicals.

Antibiofilm potential of five plant extracts of *Moringa oleifera* was also evaluated in this study. Extracts of leaf, bark and seed exhibited most powerful biofilm inhibition against *E. coli* and *B. subtilis* as compared to other aerial parts of the same plant shown in Table 2 and Table 3. The hemolytic activity of the methanolic extracts of different aerial parts of MO were screened against human blood erythrocytes. Methanolic extracts of leaf, bark, flower, fruit and stem showed low hemolytic activity (at dose 100 µg/ml) toward human erythrocytes as shown in Figure 1. The calibration curve used to quantify the flavonoids present in the moringa is shown in Figure 2. This curve shows a good linearity at concentrations of 10 to 50 mg / l. With this curve we proceeded to quantify the flavonoids present in this plant, the concentration of this metabolite varied significantly in relation to different aerial parts of *Moringa oleifera*. Figure 3 Shows the calibration curve that was made for quantification of phenols and tannins, an excellent linearity is observed at concentrations between 10-50 mg / l of gallic acid. Figure 4 Shows the calibration curve to quantify the alkaloids in *Moringa oleifera*, in which good linearity is observed at concentrations of 0.2 to 1.2 mg / ml Atropine. From this curve we proceeded to quantify the alkaloids in this plant, the development of this metabolite varied little in relation to their different aerial parts. The variability of the concentration of flavonoids, alkaloids, tannins and phenols in different aerial parts of the plant is shown in Figure 5 which is very significant. The total flavonoid contents were calculated from the extracts of leaf, stem, bark, flower and fruit using the standard curve of rutin solution, respectively (Figure 5). The data were interpreted regarding RAE (rutin equivalents) of the extract, respectively. The extract of leaf showed a significantly high flavonoid content (30.26±0.73 mg QAE/g) than the bark whereas the extracts of bark showed flavonoid contents (28.26± 0.73 mg QAE/g) higher than that of flower (22.42±0.98 mg QAE/g), fruit (19.36± 0.18 mg QAE/g) and stem (17.24± 0.08 mg QAE/g). The total flavonoid contents of the different extracts are in the range of leaf extract>bark extract>flower extract>fruit extract >stem extract as shown in (Figure 5). The tannins contents were studied in extracts utilizing the Folin-Ciocalteu's reagent and is expressed in terms of gallic acid equivalent (the standard curve equation: y = 0.0269x – 0.0737, R2 = 0.9764). The values obtained for the concentration of tannin contents are expressed as mg of GA/g of extract. The results of phenolic content of the aerial parts showed a significant difference ($p < 0.05$). The highest concentration of tannins (5.43± 0.10mg of GA/g) was examined in methanolic extract of leaf then the bark whereas the extracts of bark showed tannins contents (4.22± 0.10 mg of GA/g) higher than that of flower (3.85± 0.25 mg of GA/g), fruit (2.70± 0.10mg of GA/g) and stem (0.99±0.01 mg of GA/g). The total phenolic contents (TPC) were calculated from the methanolic extracts of leaf, stem, bark, flower and fruit using the standard curve of Gallic acid solution, respectively (Figure 5). The data were interpreted regarding GAE (gallic acid equivalents). The results of phenolic content of the aerial parts showed a significant difference (p <0.05). The extracts of the leaf showed the highest level of phenolic content of 17.4± 0.20 (mg GAE/g) which was significantly higher than that of the phenolic content of the bark 15.38±0.08 (mg GAE/g) whereas the extracts of bark showed phenolic contents 28.26± 0.73 (mg GAE/g) higher than that of flower 10.88± 0.15 (mg GAE/g), fruit 9.27± 0.0 (mg GAE/g) and the stem 8.22.± 0.11 (mg GAE/g). The total phenolic contents of the different extracts are in the range of leaf extract>bark extract>flower extract>fruit extract >stem extract as shown in (Figure 5).

The alkaloid contents were examined in leaf, stem, bark, flower and fruit extracts and expressed in terms of atropine equivalent as mg of AE/g of extract (the standard curve equation: y = 30.5x – 0.04, R2 = 0.9981). The highest concentration of alkaloid (0.88± 0.02mg of AE/g) was found in leaf extract follow by the bark (0.80± 0.73mg of AE/g), flower (0.60± 0.0 mg of AE/g) and fruit (0.56.± 0.01 mg of AE/g) while least concentration of alkaloids (0.54.± 0.0 mg of AE/g) was found in the stem extract respectively.

Bacteriological studies in this present work reveal that the antimicrobial activity of the aerial parts of *M. oleifera* affected predominantly bacterial species. The antimicrobial activity of extract might be due to the presence of lipophilic compounds that might bind inner to the cytoplasmic membrane (Jabeen *et al*., 2008). Several authors have reported antimicrobial activities of plant extracts on food borne pathogens (Moreira *et al*., 2005; Kotzekidou *et al*., 2007; Afolabi, 2007; Atiqur Rahman and Sun, 2009). Owing to the significant use of antibiotics, a variety of micro-organism strains with multi-drug resistance have developed (Khan *et al*., 2009). Millions of people die every year due to infectious diseases (Dubey *et al*., 2012). Medicinal plants are precious natural reservoir as they are assumed to have supernatural effects on the mankind and being used by almost all nations of the world. Most of the people in developing an developed countries use traditional medicines originated from medicinal plant (Igbinosa et al., 2009). Various parts of Moringa oleiferaahave been recognized as being good sources of phenolic acids glucosinolates and flavonoids (Amaglo *et al*., 2010; Coppin *et al*., 2013), carotenoids tocopherols (Saini *et al*., 2014).

In the present investigation, the antimicrobial and antibiofilm activity of the extracts of aerial parts of *Moringa oleifera* was assayed against two pathogenic microorganisms *Bacillus subtilis* and *E. coli* at

same concentrations the extracts of *Moringa oleifera* showed a broad-spectrum antibacterial and antibiofilm activity.

The findings of the present study showed potential antibacterial and antibiofilm activity against *Bacillus subtilis* and *Escherichia coli*. The antimicrobial and antibiofilm activity of various aerial parts of *Moringa oleifera* was in order: mLe > mBa >mFl>mFr>mSt and these activities were due to the presence of phytochemical compounds like saponins, terpenoids, alkaloids, carbohydrates, fats, tannins, flavonoids and sterols. The various phytochemicals and secondary metabolites have been reported to exhibited antimicrobial activities (Trentin *et al*., 2013; Manner *et al* 2013). The leaf and bark showed highest zone of inhibition at the concentration of 100 µg/disc and as an antibiofilm against bacterial pathogens while at same concentration of 100 µg/dics the other extracts showed least antibacterial and antibiofilm activity against the studied bacteria.

4. Conclusions

All aerial parts of *Moringa oleifera* showed varying antibacterial, antibiofilm activities. The result also revealed that the methanolic extracts of aerial parts of *M. oleifera* possess very low hemolytic activity and these results may contribute to the growing information about different aerial parts of *M. oleifera* which can be used to discover an antibacterial agent for developing new pharmaceuticals. Their intake could be useful in the prevention of diseases. The extracts of different aerial parts of *M. oleifera* can provide a low-cost and maintainable method toward disease reduction and can ultimately restore the quality of life of the rural and rurban people in developing countries like Pakistan.

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Conflict of interest

None to declare.

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