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Screening of Antimicrobial and Antioxidant Activities of *Moringa oleifera* Lam. Leaf Extracts Against Multidrug Resistant Pathogenic Bacteria

Wesal Waheeb Khouj[1], Munazza Gull[1]*, Sawsan Abdulaziz Rahimuddin[1], Rahma Alshamrani[2], Azhar Abdullah Najjar[2], Ahmed Mahmoud Al-Hejin[2,3] and Galila Faisal Zaher[4]

[1]*Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*
[2]*Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*
[3]*King Fahad Medical Research Center, Microbiology Lab, P.O. Box 80216, King Abdulaziz University, Jeddah, Saudi Arabia,*
[4]*Department of Hematology, King Abdulaziz University, P.O.Box 80215, Jeddah, Saudi Arabia*

ABSTRACT

Antibiotic resistance is a problem that continues to challenge the healthcare sector in a large part of the world. It is very important to control that problem, so the discovery of new active compounds and antibiotic has focused on screening bacteria for new growth inhibitory compounds. This study aimed to investigate the antibacterial, antioxidant potential and phytochemical composition of extracts of *Moringa oleifera* Lam. leaves in methanol, ethyl acetate and hexane against clinically resistant bacterial isolates. A total of 8 bacterial isolates were selected to analyze the antibacterial and antioxidant potential of various *Moringa oleifera* leaf extracts. Antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion technique and MIC and MBC values were also recorded. Antioxidant potential was determined based on the free radical scavenging activity of 2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay. Finally, qualitative and quantitative analyses of phytochemical constituents of *Moringa oleifera* leaf extracts were performed by HPLC. Result showed that ethyl acetate extract demonstrated higher antibacterial activity against *Bacillus subtilus* with zone of inhibition 28 ± 8.2 mm, followed by *Streptococcus viridans* (21.67 ± 5.86 mm). These extracts were not active against *E. coli, Klebsiella pneumonia* and *Salmonella* group B. Hexane extract showed antibacterial activity against all tested bacteria. The extracts showed strong antioxidant activity with 50% efficient concentration (EC50) values of 117.94 and 150.96 µg/ml for the methanol and ethyl acetate extracts respectively. The highest phenolic content was observed in methanolic leaf extract with 140.1 9 ± 0. 0.71 (mg GAE/g) while flavonoid was found 98.67±2.10 (mg QE /g) respectively. In addition, different phenolic and flavonoid compounds were also determined individually. This study concludes that *Moringa oleifera* Lam. leaf extracts have significant antimicrobial and antioxidant properties which authenticate its potential as cure against a wide variety of infectious bacterial diseases.

KEY WORDS: MORINGA LEAVES; PATHOGENIC BACTERIA; ANTIOXIDANTS; PHYTOCHEMICALS; ALKALOIDS; FLAVONOIDS.

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INTRODUCTION

Infectious diseases have appeared as one of the major threats to human health around the world and become the major cause of morbidity and mortality. The research of this line has been fruitful and provided medical science with many of the frontline antibiotics in clinical use (Ilanko, et al., 2019). However, antibiotic resistance becomes a problem that continues to challenge the health care sector in a large part of the world. The rise of untreatable bacterial diseases with more resistance to antibiotics and the cause of increasing evolution of multi-drug resistant (MDR) bacteria that remains a wildly unresolved problem and big challenge to health services (Valle Jr, et al., 2015; Maillard, et al., 2020; Tufa, et al., 2020). The discovery of new active compounds against new targets is very important to control the problem that most pathogenic organisms are becoming resistant to antibiotics. Natural antibacterial and antioxidants compounds produced by plants are becoming a big interest in recent research (Dalukdeniya, et al., 2016). They used as safe therapeutics for a wide range of various diseases in medicinal applications (Busani, et al., 2012; Thirumalai, et al., 2018; Adamczak, et al., 2020).

Moringa oleifera leaves are a well-known source of natural antibacterial and antioxidants. For controlling the pathogenic bacteria, *Moringa oleifera* Lam. has become promising natural antimicrobial agent with potential applications in pharmaceutical industry (Reetu, et al., 2020). The extracts of *Moringa oleifera* Lam. can be used to discover antibacterial agent for developing new pharmaceuticals to control various human pathogenic bacteria responsible for the severe illness. *Moringa oleifera* leaves are providing protection against infections and degenerative diseases by inhibiting and scavenging free radicals (Ashour, et al., 2020).

Phytochemical analyses have shown that its leaves are particularly rich in vitamins especially A, D and C. Also, they are containing essential amino acids, antioxidants, flavonoids, and a lot of minerals that are essential for growth and development (Gopalakrishnan, et al., 2016; Su and Chen, 2020). The extracts from *Moringa oleifera* exhibit multiple nutraceutical or pharmacological functions including anti-inflammatory, antioxidant, anti-cancer, hepatoprotective, neuroprotective, hypoglycemic, and blood lipid-reducing functions (Kou, et al., 2018; Shourbela, et al., 2020). Recent trials revealed that *Moringa oleifera* leaves might contribute to prevent obesity as well as obesity-related complications (Mabrouki, et al., 2020). Considering these facts, the present research work was designed to explore the antimicrobial and antioxidant activities of *Moringa oleifera* Lam. leaf extracts. The present study also evaluates the occurrence of natural antimicrobial and bioactive compounds in *Moringa oleifera* Lam. leaf extracts and characterize it to be used as the alternative therapeutic agent. There are only a few elaborative studies on the bioactive constituents of *Moringa oleifera* leaves and their effect on multidrug resistance bacteria. This study aims to bridge the gap. Moreover, much of the evidence remains anecdotal as there has been diminutive concrete scientific studies done to hold authentic claims about *Moringa oleifera* indicating the need of more exploration of this plant (Fahal, et al., 2018; Suresh, et al., 2020).

MATERIAL AND METHODS

Plant Material Collection: Fresh leaves of *Moringa oleifera* Lam. were collected from Saudi Arabia locally and identified in the laboratory by standard flora identification method and confirmed by plant data base https://www.rbg.vic.gov.au/science/herbarium-and-resources/online-databases. The taxonomic identification of this plant also performed by comparison with existing herbarium in Biology department of King Abdulaziz University, Jeddah Saudi Arabia.

Plant Extracts Preparation: The collected *Moringa oleifera* leaves were directly washed to remove debris and allowed to dry under shade. The dried leaves were grounded by blender to fine powder and 790 g was obtained. For the extract preparation, plant material extracted with hexane, ethyl acetate and methanol (72 h each) using a Soxhlet extractor. Extracts were then filtered with Centrifuge at 4000 G for 5 min to remove any debris and concentrated using a rotary evaporator under vacuum at approximately 40°C. The dried extracts lyophilized in lypholyser and stored in airtight tubes at 4°C for further use.

Pathogenic Bacteria Used for Susceptibility Test: A total of 8 bacterial species were tested including four Gram positive bacteria (*Bacillus subtilus, Staphylococcoccus aureus, Streptococcus viridans* and Methicillin resistance *Staphylococcoccus aureus*) and four Gram negative bacteria (*E. coli, Klebsiella pneumoniae, Salmonella* group B, and *Shigella sonnei*) that were obtained from Microbiology Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. These species were originally isolated from the clinical samples and identified based on standard phenotypic tests according to Bergey's manual of systematic bacteriology.

Determination of Antibacterial Activity: Antibacterial activity of the hexane, ethyl acetate and methanol extracts of the plant was studied by standard paper disc diffusion method. Active cultures of eight bacteria were prepared by transferring a sterile loop swap of culture to 5 ml of nutrient broth and incubated at 37 °C for 24 h. The turbidity was adjusted equivalent to 0.5 McFarland units by spectrophotometry at 600 nm. Final cell concentrations were adjusted to 105 $CFUmL^{-1}$ with reference to the McFarland turbidometry (Burt and Reinders, 2003). The positive control was antibiotic kanamycin (25µg/ml) for antibacterial activity. The susceptibilities of the isolated pathogens were determined by the modified Kirby-Bauer disc diffusion method (Bauer, et al., 1966) with Muller Hinton agar plates (MHA, Merck, Germany). Aliquots of inoculums were spread over the surface of agar plates with a sterile cotton swap.

To test the antimicrobial activity, all extracts were dissolved in DMSO to make a final concentration of 400 µl /ml. Each extract (20 ul) was soaked of each extract soaked separately into sterile discs and dried in open air. Solvents were evaporated and then the discs were placed on bacterial cultures. These discs placed on Muller Hinton agar plates, previously swabbed with the bacterial inoculums. The plates were left at room temperature for 1 hour and then the petri dishes were subsequently incubated for 24 h at 37°C. Each experiment was done in triplicate and mean values were taken. Antimicrobial activity was measured in the diameter (mm) of the clear inhibitory zone formed around the disc.

Determination of Minimum Inhibitory Concentrations (MIC): The minimum inhibitory concentration (MIC) of the extracts was determined for most sensitive bacterial species. A 16-hour culture was diluted with a sterile physiologic saline solution (0.9% (w/v) sodium chloride) with reference to the 0.5 McFarland turbidometry to achieve the inoculum approximately equal to 105 $CFUmL^{-1}$ (Burt and Reinders, 2003). In the tube dilution assay, standard bacterial suspension and different concentration of extracts (5, 10, 20, 40, 80 and 160 mg/mL) were added to tubes containing 1 mL Muller Hinton broth. These tubes were incubated at 37°C for 24 hours. The first tube of the series with no sign of visible growth was considered as the MIC. This process has been done three times (Mahboobi, et al., 2006).

Determination of Minimum Bactericidal Concentrations (MBC): To determine the MBC, for each set of test tubes in the MIC assay, a loop full of broth was collected from the tubes without any visible growth and cultured at 37°C for 18 - 24 hours. The highest dilution that yields no colony formation on solid medium was considered as MBC (Motamedi, et al., 2009).

Time-Kill Kinetic Study: The time-kill kinetics was studied by culturing one standard loop of the suspension from the tube possessing MBC on MHA from 0 to 36 hours. This was performed at the first hours of intervals for the first 18-hour study, and then at 2-hour intervals for the later 18 hours (Mahboobi, et al., 2006).

Antioxidant Activity: The antioxidant activity of the hexane, ethyl acetate, and methanol leaf extracts of *Moringa oleifera* was determined based on the free radical scavenging activity of 2, 2- diphenyl-1- picrylhydrazyl (DPPH) according to the method described by Brand-Williams, et al., (1995). One hundred and fifty µl of DPPH solution (4.3mg/3.3ml methanol) was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. 50µl of various concentrations (25, 60, 120 and 240 µg/ml) of each extract was taken and the volume was made uniformly to150µl using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each, 150µl DPPH was added (to exclude color factor of sample we performed sample blank to each concentration that contains no DPPH, only methanol added, then we subtract the reacted sample with DPPH from blank sample). Absorbance was taken after 15 min at 517nm using methanol as blank on spectrometer. The % scavenging was calculated as: % scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100.

Phytochemical Screening: Preliminary phytochemical screening was performed by using standard tests. Test for alkaloids was done using Dragendroff's test. To 1ml of extract, 1ml of water and 1ml of Aq. NaOH was added. Yellowish brown precipitate was obtained which confirmed the presence of Glycosides. Presence of flavonoids was confirmed by adding 1ml of 10% lead acetate to 1ml of the extract with the appearance of yellowish green precipitate. A froth was obtained by boiling 1ml of extract with 1ml of distilled water which confirmed the presence of saponins. Few drops of 0.1% $Fecl_3$ was added to 1ml extract. A brownish green precipitate was formed confirming the presence of tannins. A brown precipitate was obtained by adding 1ml of $CHCl_3$, 2ml conc. $H2SO_4$ to 1ml of extract indicating the presence of terpenoids (Evans, 2002). To 1ml of extract was added 1ml of 40% NaOH and 2 drops of 1% $CuSO_4$. A pink color was obtained indicating the presence of proteins. Benedict's test was performed with 1ml extract. A slight red precipitate was obtained showing the presence of carbohydrates.

Estimation of Total Phenolic Contents: The total phenolic content of the extracts was determined using the method described by Kim, et al., (2003) with modification. To start the analysis, 1 ml of the extract (0.1 mg/ml) was mixed with 0.2 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 1 ml of 7.6% Na_2CO_3 solution was added to the mixture followed by the addition of 2 ml of deionised distilled water. The mixture was stirred and allowed to stand for 90 minutes. The mixtures (in triplicate) were incubated at 40°C for 30 min and the absorbance was read at 760 nm. The total phenolic content was determined from extrapolation of calibration curve which was made using gallic acid solution and expressed as milligrams of gallic acid equivalents (GAE) per gram of the dry weight.

High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds: High performance liquid chromatography analysis with UV detection was performed for the estimation of the phenolic compounds in the plant extracts. The shaded dried plant material (200 g) was crushed to make it coarse powder. The coarse powder (20 g) was grinded with 25 ml distilled water of 2 N-HCl. The grinded plant extracts were heated in water bath using air condenser at 100°C for 1 h. The plant extracts were filtered using Whatman filter paper No.1. By using a separating funnel, the filtrate was extracted with diethyl ether. The layer of diethyl ether was washed and separated with distilled water and dried over sodium sulphate (anhydrous). The final evaporated extract was obtained using rotary vacuum evaporator at 25°C. The collected extract re-dissolved in HPLC grade ethanol

(5 ml), prior to the injection into HPLC column. The samples were filtered through 0.22 µm organic filter (Millipore) before use (Joshi, 2011).

Table 1. The antimicrobial activity (zone of inhibition) of the tested plant extracts and the tested antibiotic (standard) against pathogenic bacteria by disc diffusion method

Bacteria	Inhibition Zone *mm			
	M	Ea	H	Postive control Kanamycin 25 µg
Bacillus subtilus (c)	9.7±2.52	28±8.2	7±1.73	4.67±0.577
Staphylococcus aureus (C)	0	0	5.67±5.5	6±0
Streptococcus viridans (c)	0	21.67±5.86	8.67±1.53	5.33±0.577
Methicillin-resistant Staphylococcus aureus	4±2	0	8.3±0.58	2.33±0.577
E.coli	0	0	9.3±0.58	5.33±0.577
Klebsiella pneumoniae	0	0	10.67±0.58	5.66±0.577
Salmonella group B	0	0	10.67±0.58	5.66±0.577
Shigella sonnei	0	6±1.73	7±1	5.33±0.577

Values are presented as mean ± SD of triplicate experiments.
Data showed the mean inhibition zone from a triplicate.
c= most sensitive bacteria, C= most resistance bacteria, c= most medium bacteria and 0: No activity.
M: Methanol extract, Ea: Ethyl acetate extract and H: Hexane extract.

Table 1a. Antibacterial activity (MIC and MBC, mg^{-1}) of the plant extracts of *Moringa oleifera* against pathogenic bacteria

Bacteria	Methanol		Hexane		Ethyl acetate	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram Positive						
Bacillus subtilus	10	0	5	40	10	40
Staphylococcus aureus	20	150	0	0	0	0
Streptococcus viridans	15	40	15	40	0	0
MRSA Staphylococcus	30	40	0	0	20	140
Gram Negative						
E. coli	10	20	0	0	0	0
Klebsiella pneumoniae	15	140	0	0	0	0
Salmonella group B	15	20	0	0	0	0
Shigella sonnei	5	150	5	150	0	0

Analysis of Individual Phenolic Acids by HPLC: The reverse phase high performance liquid chromatography (RP-HPLC) analysis was performed for the estimation of phenolic acids compounds like gallic acid, parahydroxy benzoic acid, vanillic acid, syringic acid and ferulic acid. During study, HPLC apparatus was HPLC-Beckman model-322 equipped with 100A model pump, 210 injector, 420 controller, mixer and BD-40 recorder. C18 column (ultrasphere) with specification of 5um (25 cm x 4.6 mm length). Mobile phase was setup strictly with this ratio, methanol: water (1% acetic acid in 20: 80 v/v). Prior to use in HPLC, mobile phase was degased. Flow rate was maintained at 1ml min^{-1} with chart speed 1cm min^{-1}. UV detector was fixed with max 280 nm λ, aufs Attenuation (0.02) and isocratic mode. For individual phenolic compound, the detector response was calibrated and measured with standard phenolic acids strictly as described by Tandon, et al., (2001). All standard phenolic compounds were procured from Sigma-Aldrich chemical company, USA.

Statistical Analysis: The parameters tested in triplicate and the values expressed as the mean ± standard deviation (SD). Statistical analysis was performed with analysis of variance (ANOVA) test and independent sample t-test using the Mega Stat Excel (version 10.3, Butler University) and all columns versus control and the P value < .05 considered as significant.

RESULTS AND DISCUSSION

Moringa oleifera Lam. is reported to have an antimicrobial effect on pathogenic bacteria. The antimicrobial properties of *Moringa oleifera* have been attributed to different parts of the plant, such as the leaves, seeds, pods and stems (Tirado-Torres, et al., 2019) which are known for their antibacterial activity and are counted as rich source of antimicrobial agents (Abadallah and Ali, 2019). The disc diffusion method of antimicrobial susceptibility testing was performed to determine the antibacterial activities of the plant against multidrug resistance pathogenic bacteria through an *in vitro* assessment of finding sensitivity or resistance to an antimicrobial agent (Figure 1). The extracts of *Moringa oleifera* tested showed varying degree of inhibitory activity against the tested pathogens (Table 1).

Comparison of all plant extracts data for their antibacterial potential reveals that ethyl acetate extract showed the highest antibacterial activity with zone size (28 ± 8.2 mm) while the methanol extract exhibited least activity by zone size of (4 ± 2 mm). In the study of Raj, et al., (2011) used different extracts of *Moringa oleifera* Lam. root that tested for antimicrobial activities against some pathogenic bacteria by disc diffusion method and the result showed high antibacterial activity against *Pseudomonas aeruginosa* (18.2 ± 0.2 mm) by ethyl acetate extract.

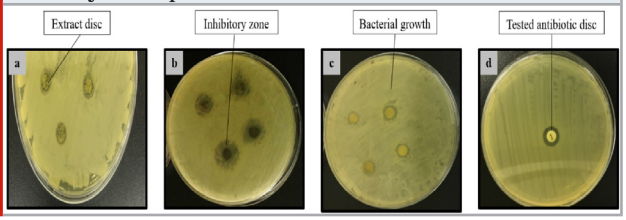
Table 1b. The Time-Kill kinetic of methanolic extract of *Moringa oleifera* at 20 mg/ml concentration against *E. coil*

Hour	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Methanolica extract	+	+	+	+	+	+	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a (+): Growth found
b. (-): Growth inhibition

On the other hand, methanol and ethyl acetate extracts were not active against *E. coli, Klebsiella pneumonia* and *Salmonella* group B. Hexane extract showed antibacterial activity against all tested bacteria. The results were compared with obtained data using standard antibiotics, kanamycin (25 µg/disc that served as reference for inhibition zone diameter. The results of MIC and MBC of hexane, ethyl acetate and methanolic extracts for 8 bacterial species are shown in Table 1a and Time-kill kinetic of methanolic extract of *Moringa oleifera* was 6 hours (Table 1b).

Figure 1: Inhibition zones shown by *Moringa oleifera* extracts (a) Methanol extract against *Bacillus subtilus*, (b) Ethyl acetate extract against *Streptococcus viridans*, (c) Hexane extract against *E. coli*, (d) Tested antibiotic Kanamycin as positive control

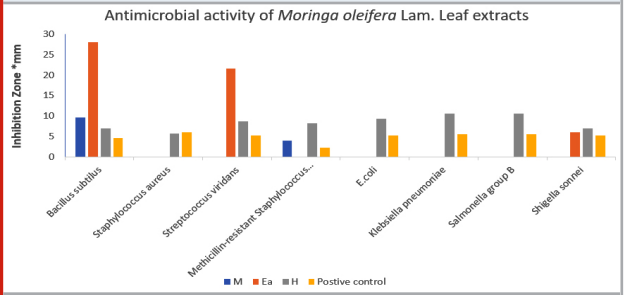


Like this study, Ojiako (2014) showed that ethyl acetate possesses the highest zone of inhibition 10 mm in *Staphylococcus aureus* and *Salmonella typhi* followed by *E. coli* 8 mm, *Candida albican* 4 mm, and *Mucor* 2 mm. Among all tested bacteria *Bacillus subtilus* was more sensitive and showed higher inhibition zone (28 ± 8.2 mm) with ethyl acetate extract, while *Staphylococcoccus aureus* was most resistance bacteria, showed no activity with methanol and ethyl acetate extracts and less zone of inhibition (5.67±5.5 mm) with hexane extract.

Hexane extract showed antibacterial activity against all tested bacteria. The higher zone of inhibition (10.67 ± 0.58) was shown against both *Klebsiella pneumoniae* and *Salmonella* group B and lowest zone of inhibition (5.67 ± 5.5 mm) against *Staphylococcoccus aureus* (Table 1). *Streptococcus viridans*, Methicillin resistance *Staphylococcoccus aureus* and *Shigella sonnei* showed antibacterial activity with two different extracts. Comparing among these bacteria, *Streptococcus viridans* and *Shigella sonnei* showed inhibition zone (21.67±5.86 mm and 6±1 mm) respectively with ethyl acetate extract, while Methicillin resistance *staphylococcoccus aureus* showed less inhibition zone (4±2 mm) with methanol extract. So, *Streptococcus viridans* was the medium sensitive bacteria.

Against *Bacillus subtilus*, ethyl acetate extract showed highest antibacterial activity (28 ± 8.2 mm), while the least activity was (9.7 ± 2.52 mm and 7 ± 1.73 mm) showed by methanol and hexane extracts, respectively. Hexane extract was less sensitive against *Staphylococcoccus aureus* (5.67 ± 5.5 mm) and no activity showed with methanol and ethyl acetate extracts. Also, the ethyl acetate showed antibacterial activity against *Streptococcus viridans* (21.67 ± 5.86 mm) and less activity with hexane extract (8.67 ± 1.53 mm) but no activity with methanol extract. Methanol extract was most resistance against Methicillin resistance *Staphylococcoccus aureus* (4 ± 2 mm) and no activity with ethyl acetate extract, while the hexane extract showed antibacterial activity with zone size 8.3 ± 0.58 mm. Methanol and ethyl extracts showed no activity against *E. coli*, but the hexane extract showed antibacterial potential against *E. coli* (9.3 ± 0.58 mm). *Klebsiella pneumoniae* and *Salmonella* group B showed no activity with methanol and ethyl acetate extracts but high activity in hexane extract with same zone size 10.67 ± 0.58 mm. Ethyl aetate and hexane extract showed activity against *Shigella sonnei* (6 ± 1.73 mm and 7 ± 1 mm) respectively but showed no activity with methanol extract. Similar results regarding antimicrobial activity of Moringa leaves were also reported by (Singh, et al., 2013).

Figure 2: Comparison of antimicrobial potential of the *Moringa* extracts tested by inhibition zone (mm) using Disc Diffusion method. M: Methanol extract, Ea: Ethyl acetate extract, H: Hexane extract and Kanamycin as positive control



The antimicrobial potential of the experimental plant extracts was evaluated by their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with activity of standard, Kanamycin (25 µg). The *Moringa oleifera* leaf ethyl acetate extract was found most effective as compared to the standard Kanamycin 25 µg against *Bacillus subtilus* (28 ± 8.2 mm), *Streptococcus viridans* (21.67±5.86 mm) and *Shigella sonnei* (6±1.73mm). The inhibition zones produced by hexane extracts were more effective than standard against all tested bacteria except for *Staphylococcoccus aureus* (5.67±5.5 mm) which was most resistance bacteria. Methanol extract was most effective as compared to standard against *Bacillus subtilus* and Methicillin resistance *Staphylococcoccus aureus* (28 ± 8.2 mm and 4 ± 2 mm) respectively (Figure 2).

In the study of Yee (2019), different parts of *Moringa oleifera* were tested for the antibacterial activity of five extracts (PE, EtOAc, MeOH, 95% EtOH and H_2O) and were investigated on 5 strains of bacteria which include *Bacillus Subtilis*, *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumalis* and *Escherichia coli* by agar disc diffusion method. Among these five crude extracts, EtOAc (ethyl acetate) extract showed the inhibition zone diameters in the range of 35-45mm has highest antimicrobial activity than the other extracts.

These results support the data of present investigation. Further the resistance and susceptibility testing of our study was quite similar with the results of Abadallah and Ali (2019) showed that ethanol extracts of *Moringa oleifera* leaf demonstrated higher antibacterial activity with average zone of inhibition of 12.49 mm than aqueous extracts (8.00 mm). Based on the susceptibility of the microorganisms to the extracts, *Shigella spp* was found to be the highest susceptible microorganism with average zone of inhibition of 12.46 mm, followed by *Staphylococcus aureus* (11.47 mm), *Salmonella typhi* (10.81 mm), *E. coli* (10.81 mm) while low average zone of inhibition is shown by *Enterococcus faecalis* (9.76 mm) in their study.

Table 2. Absorbance values of *Moringa oleifera* extracts with different conecentration

Methanol zero blank 0.0	DPPH control absorbance at 517nm (0.414)	*DPPH Conc. 1.3mg/ml Methanol*		
Sample concentration	**1µl (25µg/ml)**	**2.5µl (60µg/ml)**	**5µl (120µg/ml)**	**10µl (240µg/ml)**
Mean methanol Abs.	0.377	0.272	0.181	0.038
Mean ethyl acetate Abs.	0.454	0.318	0.271	0.068
Mean hexane Abs.	0.667	0.375	0.345	0.135

Table 3. Antioxidant activity of *Moringa oleifera* extracts

Sample concentration	Methanol fraction	Ethyl acetate fraction	Hexane fraction
1µl (25µg/ml)	8.9%	No inhibition	No inhibition
2.5µl (60µg/ml)	34.3%	23.2%	9.4%
5µl (120µg/ml)	56.3%	34.5%	16.7%
10µl (240µg/ml)	90.8%	83.6%	67.4%

Many other researchers highlighted the positive and effective antibacterial activity of aqueous extracts, chloroform extracts and methanol extracts obtained from leaves, bark and roots of *Moringa oleifera* (Lam.) against four food borne microbial pathogens, *Salmonella enteritica, Vibrio parahaemolyticus, Escherichia coli* and *Listeria monocytogenes*. The main finding of these studies was obtained that all extraction methods showed antimicrobial activity against all tested microorganisms. Lowest and highest antibacterial activity was shown by aqueous extraction and chloroform extraction of residue obtained after aqueous extraction. Highest antibacterial activity was shown by chloroform extraction of residue obtained after aqueous extraction against *Salmonella enteritica*. *Listeria monocytogenes* was found to be the most resistant microorganism to all types of extracts (Dalukdeniya, et al., 2016; Chakraborty, et al., 2019).

The Moringa plant is mainly ascribed to the presence of antioxidant constituents such as phenolic acids and flavonoids. Due to the high concentrations of antioxidants present in *Moringa oleifera* leaves, they can be used in patients with inflammatory conditions, including cancer, hypertension, and cardiovascular diseases. The antioxidants have the maximum effect on the damage caused by free radicals only when they are ingested in combination. A combination of antioxidants found in *Moringa oleifera* leaves was proven to be more effective than a single antioxidant, possibly due to synergistic mechanisms and increased antioxidant cascade mechanisms (Vergara-Jimenez, et al., 2017; Yakoub, et al., 2018). Phytochemical and antimicrobial analysis of *Moringa oleifera* leaf was also screening by Oladeji, et al., (2020).

Figure 3: Antioxidant activity of *Moringa oleifera* extracts M: Methanol extract, Ea: Ethyl acetate extract and H: Hexane extract

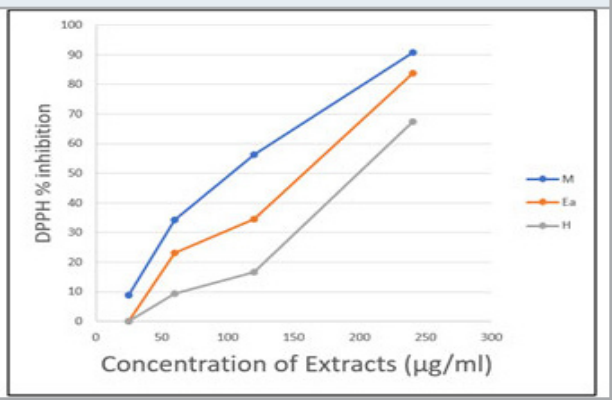


Table 4. Qualitative phytochemical analysis of *Moringa oleifera* leaf extracts

Phytochemical constituents	Extracts Methanol	Ethyl acetate	Hexane
Alkaloids	+	+	+
Glycosides	+	-	+
Flavanoids	+	+	+
Saponins	+	+	-
Tannins	+	+	+
Terpenoids	+	-	+
Proteins	+	+	+
Carbohydrates	+	-	+
Phlobatannins	-	-	+

- indicate absence, + indicates presence

The secondary metabolites in *Moringa oleifera* leaf were extracted by maceration using chloroform, ethyl acetate and ethanol. Some important bioactive metabolites in the leaf extracts, such as steroids, saponins, tannins, flavonoids, terpernoids and phlobatannins were analyzed. The ethanolic leaf extract was observed to show the highest antimicrobial activity when compared to chloroform and ethyl acetate extracts. It also compared favorably to nystatin, streptomycin and gentamicin (standard antibiotics). The study affirmed the therapeutic potency of the plant, indicated by its high antimicrobial effects on some pathogens like *Klebsiella sp, P. aeruginosa, Trichoderma sp, Aspergillus flavus,*

Bacillus cereus, S. pneumoniae, Candida. sp, and *E. coli.*

Quantification of antioxidant activity using DPPH free radical scavenging showed a dose-dependent antioxidant activity for the methanol, ethyl acetate and hexane extracts (Figure 3). From the results obtained, the highest antioxidant activity of 56.3% was exhibited by the methanol extract after 15 min incubation at a concentration of 120 µg/ml. Also, ethyl acetate extract showed good scavenging activity. The extracts showed strong antioxidant activities with EC50 values of 117.94 and 150.96 µg/ml for methanol and ethyl acetate extracts respectively (Table 2). Similar study was reported by Igbo, et al., (2015) using DPPH free radical scavenging for antioxidant activity. From the results obtained, the highest antioxidant activity of 89.1% was exhibited by the methanol extract at a concentration of 125 µg /ml. The extracts showed strong antioxidant activity with 50% efficient concentration (EC50) values of 24 and 44 µg/ml for the ethyl acetate and methanol extracts respectively (Table 3). The study of Fitriana, et al., (2016) provided that *Moringa oleifera* leaves possess antioxidants.

Table 5. Total phenolic (TP) and flavonoids (TF) contents of the *Moringa oleifera* extracts

Extract Samples	Mean Gallic acid Equivalent (mg/g in GAE) Mean	TPC Quercetin Equivalent (mg/g in QE) TFC
Methanol	140.1 9 ± 0. 0.7	98.67±2.10
Ethyl acetate	130.9 ± 0.9	65.77 ± 1.01
n-Hexane	119.4 + 0.5	32.98±2.12

Values are the means ± SD of triplicate

The extracts have been evaluated for its antioxidant activity by 1,1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay and an improved 2,2'-azino-bis- [3-ethylbenzothiazoline sulphonate] (ABTS) radical cation decolorization assay *in vitro*. The methanol extract showed the highest free radical scavenging activity with IC50 value of 49.30 µg/mL in DPPH assay and 11.73 µg/mL in ABTS assay supporting the data of present investigation (Table 2 and 3). Also, the experiments of Vyas, et al., (2020) was clearly indicated that *Moringa oleifera* leaves showed effective free radical scavenging activity which can be attributed to the presence of flavonoids and phenolics along with other compounds.

Preliminary phytochemical investigation showed the presence of diverse phytochemicals which are the bioactive components that can be of use. The result of phytochemicals in the present investigation showed that the plant contains components such as alkaloids, flavonoids, glycosides, tannins, saponins, terpenoids, proteins, carbohydrates (Rodríguez-Pérez, et al., 2015; Udofia, et al., 2020). The phenolic compounds, particularly flavonoids, and terpenoids were abundant in these extracts (Table 4). The total phenolic and flavonoid contents of methanolic extract revealed higher values than ethyl acetate and hexane extracts. The highest phenolic content was observed in methanolic leaf with 140.1 9 ± 0. 0.71 (mg GAE/g) while flavonoid leaf extract was found 98.67±2.10 (mg QE /g) respectively. The result revealed that phenolic content of methanolic extract is higher than that of ethyl acetate and hexane extracts (Table 5). This may be due to different polarity of the solvents used, and phenolics are mainly extracted in higher quantity especially in more polar solvents.

Ethyl acetate extract also showed the good content of phenolic compounds with total phenolic content of 130.9 ± 0.9 mg GAE/g compared to the extract n-hexane that had 119.4 + 0.5 mg GAE/g, respectively (Table 5). The results are in agreement with the findings of Vongsak ,et al., (2013) determined the quantitative analysis of active compounds was accomplished through high-performance liquid chromatography (HPLC). The extract promoted with maximum amounts of total phenolics (13.23 g chlorogenic acid equivalents/100 g extract) and total flavonoids (6.20 g isoquercetin equivalents/100 g extract) also, exhibited high DPPH-scavenging activity (EC50 62.94 µg/ml).Present result indicates satisfactory phenolic contents. Hence they correspond to the results obtained by (Shih, et al., 2011) and (Srivastava, et al., 2020) where the highest total phenolic content was found in the leaves extract of *Moringa oleifera*.

Table 6. HPLC analyses of bioactive compounds of *Moringa oleifera* plant leaf extract

Plant crude extract sample	Phenolics								Flavonoids				
	Gallic acid	Catechin	Epicatechin	Chlorogenic acid	Ellagic acid	Syringic acid	Gentistic acid	Synapic acid	Quercitrin	Isoquercitrin	Quercetin	Kaempferol	Rutin
	105.67 ± 0.01	20.19 ± 0.03	29.73 ± 0.01	79.31 ± 0.02	52.95 ± 0.02	ND	ND	ND	74.90 ± 0.01	75.65 ± 0.02	137.81 ± 0.01	106.75 ± 0.03	60.38 ± 0.02

ND=not detected; concentration of samples is in mg/g
Values represent means ± standard deviation of triplicate readings.

Phenolic compounds have been reported to be an important class of secondary metabolites, found in medicinal plants and used tremendously as a source of anti-infection agent. Nevertheless, they help to reduce the risk of many diseases owing to their antioxidant power (Abdulkadir, et al., 2015; Zhu, et al., 2020). Analyses of individual phenolic and flavonoid compounds by HPLC showed the presence of diverse biochemical constitution. These compounds were identified by the comparison of their retention times and UV spectra to those of authentic standards analyzed under identical conditions. Qualitative and quantitative analyses of *Moringa oleifera* leaf extract depicted that gallic acid, catechin, epicatechin, chlorogenic acid, ellagic acid, quercitrin, quercetin, kaempferol and rutin were detected with different concentrations (Table 6) indicating its medicinal prospective.

The medicinal uses of *Moringa oleifera* may be due to the antibacterial and antioxidant activities of its bioactive phytochemicals particularly phenolic compounds that showed the significance relevance of *Moringa oleifera* in prevention of different diseases by reducing and /or preventing free radicals. This potential may translate into prevention of chronic diseases associated with antibacterial and oxidative stress for humans who consume various parts of *Moringa oleifera* plant (Chhikara, et al., 2020), Also, the work of Rocchetti, et al., (2020) has revealed great abundance of flavonoids and phenolics acids by using different *Moringa oleifera* leaf extracts.

The global emergence of multidrug resistant bacterial strains is increasing, limiting the effectiveness of current drugs and treatment failure of infections. A novel approach to the prevention of antibiotic resistance of pathogenic species is the use of new compounds that are not based on existing synthetic antimicrobial agents. Based on the findings of this study it could be recommended that the extracts of this plant should be further analyzed to isolate the specific antibacterial compounds and defense mechanisms working in it. Speedy clinical trials should be carried out to explore the pharmaceutical potential of the medicinal plants in the treatment of bacterial and fungal infectious diseases.

CONCLUSION

This study concluded that *Moringa oleifera* leaf extracts have antibacterial activity against both Gram positive and negative bacteria, also revealed high potential free radical scavenging activity. The antibacterial activity showed that *Staphylococcus aureus* was more resistant bacteria while, *Bacillus subtilus* and *Streptococcus viridans* were found among more sensitive against all extracts. The highest antioxidant activity of 56.3% was exhibited by Moringa leaves in the methanol extract. This revealed that the leaves contain considerable concentration of antioxidants with good free radical scavenging activity. This result also indicates satisfactory phenolic and flavonoid contents in leaf extracts. It is interesting to conduct more research in depth on Moringa leaves in order that consumers benefit from them as food additive or nutraceutical and biopharmaceutical industries. This study also provides useful information about possibility of discovering new compounds with more effectiveness against multidrug resistance pathogenic bacteria.

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