

Antimicrobial Potential of *Moringa oleifera* Seed Coat and Its Bioactive Phytoconstituents

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The *in vitro* antimicrobial potential of the unexplored *Moringa oleifera* seed coat (SC) was evaluated against some Gram-positive and Gram-negative bacteria and yeast pathogens. Antimicrobial studies with various solvent extracts indicated ethyl acetate to be the best extractant, which was used for the rest of the antimicrobial studies as it tested neither toxic nor mutagenic. Gram-positive bacteria including a methicillin resistant *Staphylococcus aureus* (MRSA) strain were more susceptible with a minimum inhibitory concentration (MIC) range of 0.03–0.04 mg/ml. The antimicrobial pharmacodynamics of the extract exhibited both concentration-dependent and time-dependent killing. Most of the test organisms exhibited a short post antibiotic effect (PAE) except *Enterococcus faecalis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* 1, which exhibited longer PAEs. Amongst the major phytoconstituents established, flavonoids, diterpenes, triterpenes and cardiac glycosides exhibited inhibitory properties against most of the test organisms. The identified active phytochemicals of the *M. oleifera* seed coat exhibited antimicrobial potential against a wide range of medically important pathogens including the multidrug-resistant bugs. Hence, the *M. oleifera* seed coat, which is usually regarded as an agri-residue, could be a source of potential candidates for the development of drugs or drug leads of broad spectrum that includes multidrug-resistant bugs, which are one of the greatest concerns of the 21st century.

Keywords: Antimicrobial, *Moringa oleifera*, antimicrobial pharmacodynamics, phytoconstituents, MIC, toxicity

Introduction

Plants have been and will remain vital to mankind and animals as well as the environment. They have been found to be the largest biochemical and pharmaceutical stores ever known on earth. These living stores can generate limitless primary and secondary metabolites that encompass a wide array of functions [17], many of which have been subsequently exploited by humans for their beneficial role in a diverse array of applications [9]. Phytomedicine is regaining interest owing to the advantages of using antimicrobial compounds of medicinal plants, which include: fewer side effects, better patient tolerance, lower cost, acceptance due to a long history of use and being renewable in nature [43].

It has been estimated that between 60–90% of the population of developing countries uses traditional medicine almost exclusively and consider it to be a normal part of primary healthcare [45]. Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistant strains [5].

A vast number of medicinal plants have been recognized as valuable sources of natural antimicrobial compounds [28]. *M. oleifera* Lam. is one such plant; it belongs to Morinaceae, a monotypic family of a single genus with around 33 species, of which 4 are accepted, 4 are synonyms and 25 have not been assessed [40]. The plant has been reported to have enormous pharmacological potential as: a cardiac and circulatory stimulant; antitumor; antipyretic; antiepileptic; anti-inflammatory; antiulcer; antispasmodic; diuretic; antihypertensive; cholesterol lowering agent; antioxidant; antidiabetic; hepato-protective and antimicrobial. These properties are also being used for the treatment of

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different ailments in the indigenous system of medicine [14, 2]. The plant's various morphological parts have been implicated to have antimicrobial potential, such as the seed cotyledon, leaves, root bark and stem bark [7]. However, from a microbiological perspective, a lot remains to be explored. The present study thus focuses on the *in vitro* antimicrobial potential of *M. oleifera* seed coat (SC) organic extract, which to the best of our knowledge has not been reported before. It also evaluates the safety and phytoconstituents and establishes the groups responsible for the bioactivity.

Material and Methods

Plant sample, chemicals and microbial cultures

The seeds of *M. oleifera* were procured from the local market, Amritsar, India and verified by the Forest Research Institute, Dehradun, India as *M. oleifera* Lamk. The specimen was deposited in Guru Nanak Dev University, Botanical Herbarium under accession number 6746 HERB. The chemicals and standard antibiotics used in this work were purchased from Sigma-Aldrich Corporation, Bangalore, India and HiMedia Laboratories, Mumbai, India. The reference microbial cultures were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. They were selected to represent a broad spectrum of potential pathogens (Gram positive, Gram negative and yeast) that pose significant threats in the medical field.

Extraction of plant material and antimicrobial activity by agar diffusion assay

To establish the best extractant, different solvents were used for extract preparation. The plant sample was processed according to Arora and Kaur [6] where 5% was mixed with the solvents and extracted for 24 h at 30°C at 80 rpm. Each extracted material was vacuum filtered through Whatman filter paper No. 1. The miscella (filtrate) thus obtained was evaporated in a rotavapour at 45°C and the residue was reconstituted in 6 ml of 30% dimethyl sulphoxide (DMSO). The extracts were used for antimicrobial testing by agar well diffusion assay according to Arora and Kaur [6] with 30% DMSO as a negative control.

Minimum inhibitory concentration

The MIC of *M. oleifera* SC ethyl acetate extract was determined by the agar dilution method [6]. A stock solution

of 4.1 mg/ml was prepared and dispensed into suitable agar plates in varying concentrations (0.03-2 mg/ml). The plates were inoculated with 0.1 ml of 4-h activated test organisms (adjusted to 0.5 McFarland standard) and incubated for 24 h at 37°C for bacterial strains and up to 48 h at 25°C for yeast strains. The lowest concentration exhibiting complete inhibition of microbial growth was taken as the MIC. The experiment was performed in duplicate and repeated three times with standard antibiotics (gentamicin for bacteria and amphotericin B for yeast strains) as positive controls.

Total activity potency

Total activity potency is the volume at which a test extract can be diluted without losing the ability to kill microorganisms. It was determined according to Eloff [22] where the amount of extract in mg from 1 g plant material was divided by the MIC in mg/ml of the same extract and was expressed in ml/g. The higher the total activity, the higher the potency, and in mathematical terms it can be expressed as:

$$\text{Total activity potency} = (\text{Amount extracted from 1 g plant material}) / (\text{MIC of the extract})$$

Time kill assay

The time kill assay for SC ethyl acetate extract (4.1 mg/ml) was performed by the viable cell count method (VCC) as described earlier [21] with slight modification. The 4-h grown inoculum was adjusted to 0.5 McFarland standards and serially diluted to 10^{-3} with suitable double strength broth medium. Each diluted inoculum was mixed with an equal volume of the respective predetermined concentration (Table 1) and incubated at 25°C (yeast) and 37°C (bacteria). Sampling was done at 2-h intervals up to 24 h by spreading 0.1 ml of the mixed suspension on suitable agar plates in duplicate. The time kill assay for standard antibiotics already mentioned in the previous section was also determined in the same manner. The mean number of colonies was determined and compared with that of the control in which the organic extract/standard antibiotics were replaced with 30% DMSO.

Post antibiotic effect

The post antibiotic effect (PAE) of the ethyl acetate extract and the standard antibiotics was determined as

Table 1. MIC and time kill concentrations for SC ethyl acetate extract and standard antibiotics.

Organism	Antibiotic MIC ^b	Extract MIC ^d	Extract TK ^e	TAP ^f
<i>Enterococcus faecalis</i>	0.01	0.036	0.036*	455.5
MRSA ^a	0.00019	0.03	0.06	546.6
<i>Staphylococcus aureus</i>	0.001**	0.03	0.06	546.6
<i>Staphylococcus epidermidis</i>	0.001	0.04	0.04*	410
<i>Escherichia coli</i>	0.001	0.036	0.072	455.5
<i>Klebsiella pneumoniae</i> 1	0.00019	0.04	0.04*	410
<i>Klebsiella pneumoniae</i> 2	0.001	1.0	1.0*	16.4
<i>Pseudomonas aeruginosa</i>	0.01	0.036	0.072	455.5
<i>Salmonella typhimurium</i> 1	0.002	0.048	0.096	341.6
<i>Salmonella typhimurium</i> 2	0.001	1.0	2.0	16.4
<i>Shigella flexneri</i>	0.002	0.048	0.096	341.6
<i>Candida albicans</i>	0.099 ^c	0.3	0.6	54.7
<i>Candida tropicalis</i>	0.0005 ^c	2.0	4.0	8.2

^aMethicillin resistant *Staphylococcus aureus*, ^bstandard antibiotic MIC mg/ml (same concentrations were used in time kill assay except *S. aureus* (**)) which took 2 times MIC value), ^cindicates amphotericin B while the rest of the values in the column are for gentamicin, ^dmg/ml, ^econcentrations used in time kill assay (TK) which represent 2 times MIC except those marked with * which are equivalent to one times MIC values (mg/ml), ^fTAP- total activity potency (ml/g) and was calculated using 16.4 mg (quantity extracted from 1 g of plant powder).

described previously [4] with slight modifications. The various MIC values applied in the time kill assay (Table 1), for both extract and standard antibiotics, were mixed with the respective test organisms (in equal volume) containing approximately 1×10^5 colony forming units (CFU)/ml suspended in suitable broth medium. After 1 h of exposure, the drug activity was stopped by placing a 1 : 1,000 dilution of mixed suspension in a suitable drug-free pre-warmed double-strength broth. The sampling was done at an interval of 1 h until visual cloudiness was noted. The PAE was calculated as follows:

$$PAE = T - C$$

where *T* represents the time required for the count in the test culture to increase $1 \log_{10}$ CFU/ml above the count observed immediately after drug removal and *C* represents the time required for the count of the untreated control tubes to increase by $1 \log_{10}$ CFU/ml.

Biosafety

Ames mutagenicity testing. Ethyl acetate extract was subjected to both spot and plate incorporation methods of the Ames test to evaluate its mutagenicity according to Mortelmans and Zeiger [31] with slight modifications. In the case of the plate method, the inoculum was activated over-

night in nutrient broth at 37°C. Mutagenicity testing was performed by adding 0.1 ml of the activated culture and 0.1 ml of extract equivalent to the MIC (0.048 mg/ml) of *S. typhimurium* (adjusted accordingly) to 5 ml of top agar containing 0.25 ml of 0.5 mM histidine - biotin mixture (1 : 1 ratio) [histidine and biotin were prepared separately according to their respective molecular weights and combined by first dissolving biotin in water at 60°C and then adding histidine]. The contents were mixed and immediately poured onto glucose minimal agar plates. Similarly, the same protocol was followed for the spot method except that the SC extract was impregnated on a disc that was placed in the center of the plate. Sodium azide (5 µl of 17.2 mg/ml) was used as a positive control while 30% DMSO was used as negative control. The plates were prepared in duplicate and incubated at 37°C for 48 h. The number of visible revertant colonies was counted. The mutagenic potential of the extracts was determined on the basis of number of colonies as compared to the positive control.

Cellular toxicity testing using MTT assay. The cellular toxicity of the ethyl acetate extract of *M. oleifera* SC was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay [15] with slight modifications. Ten milliliters of sheep blood was drawn into an injection syringe containing 3 ml Alsever's solution (antico-

agulant) and transferred to sterile centrifuge tubes. The blood was centrifuged at $1600 \times g$ at room temperature ($25 \pm 3^\circ\text{C}$) for 20 min. and the supernatant was discarded. The red blood cells (RBCs) were washed thrice in PBS by centrifugation before re-suspending the final pellet in the same buffer. Various dilutions of these cells were prepared and counted with the help of a hemocytometer under a light microscope so as to obtain cells equivalent to 1×10^5 CFU/ml. The following formula was used to determine the required number of cells: Number of cells/ml = Average number of cells in 5 big squares $\times 25 \times$ dilution factor $\times 10^4 \text{ cm}^3$. The diluted cells were dispensed into 100 μl per well and incubated at 37°C overnight. The supernatant was removed carefully and 200 μl of the highest and lowest MIC ranges of the SC extract were added in separate wells and incubated further for 24 h. The supernatant was discarded again and 20 ml MTT solution (5 mg/ml) was added to each well and incubated further at 37°C for 3.5 h at 60 rpm. After incubation, the supernatant was withdrawn without disturbing the cells and 50 ml DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 595 nm using an automated microplate reader (Biorad 680-XR, Japan). The wells with cells treated with 30% DMSO served as the control.

Phytochemical screening

M. oleifera SC was powdered and subjected to qualitative and quantitative analysis of various secondary metabolites by standard methods as follows:

Alkaloids: Qualitative analysis was performed and the reactions were scored positive on the basis of turbidity or precipitation according to Trease and Evans [42]. Quantitative analysis was performed according to Harborne [25]. The residue obtained is the alkaloid, which was dried to constant weight.

Phenolic compounds and tannins: For phenolic compounds, 1 g of the plant powder was suspended in 30 ml methanol and incubated under shaking conditions at 30°C for 24 h. In the case of tannins, 1 g of plant powder was suspended in 20 ml of 45% ethanol and boiled for 5 min and cooled. The filtrates from both cases were subjected to various qualitative tests according to Tiwari *et al.* [41]. Quantification of flavonoids and tannins was done according to Boham and Kocipai-Abyazan [11] and Vieira *et al.* [44], respectively.

Saponins: The qualitative test was performed according

to Kokate [27] while quantification was done according to Obadoni and Ochuko [32].

Terpenoids: Diterpenes and triterpenes were detected according to Obasi *et al.* [33] and Roopashree *et al.* [39], respectively. Quantification of triterpenes was done according to Bojana *et al.* [12], while diterpenes were quantified according to Mehdi *et al.* [30].

Phytosterols: Qualitative analysis was performed according to Finar [23].

Glycosides: Detection was done according to Edeoga *et al.* [20], with further quantification according to Abdel-Azim *et al.* [1]. All quantitatively isolated secondary metabolites were dissolved in predetermined 30% DMSO and 50 μl in each case was subjected to the agar well diffusion assay to assess their antimicrobial potential [6].

Data analysis

Most of the experiments were performed in triplicate and repeated thrice. The data was analyzed by analysis of variance (ANOVA) for comparison of multiple means. The chosen level of significance for the statistical tests was $p < 0.05$. The analyses were carried out using Statgraphics Centurion 16 (Stat Point Technologies, Inc. Warrenton, Virginia, USA) and SigmaStat 3.5 Software[®] (Systat Software Inc., 501 Canal Blvd., Suite E, Point Richmond, CA 94804-2928 USA).

Results

Antimicrobial activity of *M. oleifera* SC organic extracts

The experiment was designed to check the antimicrobial activity of *M. oleifera* SC and determine the best extractant. The antimicrobial potential of *M. oleifera* SC was found to be extractant dependent. One way ANOVA by Fisher's least significant difference (LSD) procedure indicated four homogeneous solvent groups i.e. acetone and aqueous; butanol and methanol; ethyl acetate; chloroform. Though components in each of the first two groups were comparable in activity, a statistically significant difference at 5% confidence level among all the groups was observed (Fig. 1A). Hexane and petroleum ether were less effective as most of the test organisms were resistant to their extracts. Ethyl acetate extract showed a broad spectrum and an outstanding efficacy and thus was selected for subsequent experiments (Fig. 1B). In this case, *Candida albicans* was the most sensitive with an average zone of inhibition of

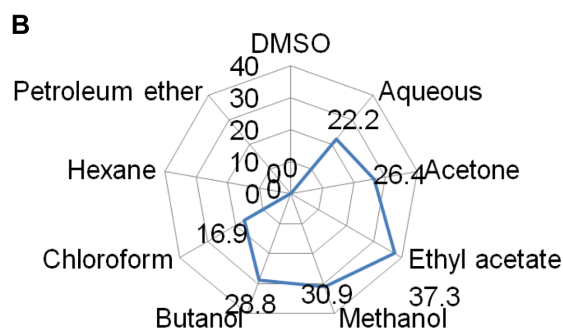
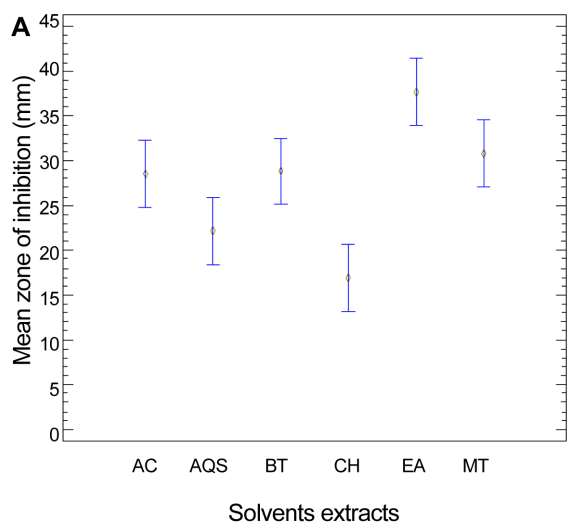


Fig. 1. Antimicrobial activity of *M. oleifera* SC solvent extracts in three independent experiments. (A) One way ANOVA by Fisher's least significant difference (LSD). Columns with similar lower case letters are not statistically different at 5% confidence level. AQ: Aqueous; AC: Acetone; EA: Ethyl acetate; MT: Methanol; BT: Butanol; CH: Chloroform, (B) Grand mean zone of inhibition of the test organisms against various solvent extracts of *Moringa oleifera* SC.

59.67 mm. *Staphylococcus aureus* and *Staphylococcus epidermidis* were the most sensitive Gram-positive bacteria with an average inhibition zone of 45 mm and 47 mm, respectively. On the other hand, *Klebsiella pneumoniae* 1 was the most sensitive among the Gram-negative bacteria with an inhibition zone of 51.33 mm.

Minimum inhibitory concentration

The experiment was designed as described earlier to ascertain the effectiveness of the extract. The results indicated varying MIC values against the test organisms. Gram-positive bacteria exhibited relatively lower MIC concentrations ranging from 0.03 mg-0.04 mg/ml as compared

to those of Gram-negative bacteria, which ranged from 0.036 mg-1.0 mg/ml (Table 1). This result correlates with agar diffusion assay studies of ethyl acetate extract (data not shown). Both reference and methicillin resistant strains of *S. aureus* were the most sensitive with a MIC of 0.03 mg/ml while *K. pneumoniae* 2, *S. typhimurium* 2 and *Candida tropicalis* exhibited higher MIC values ≥ 1 mg/ml (Table 1).

Total activity potency

This experiment was performed to establish the volume at which active constituents extracted from 1 g of dry plant material can be diluted and still be potent enough to kill the pathogens. It was calculated by dividing the extract quantity in mg from 1 g plant material of *M. oleifera* SC by the MIC of various test organisms. The higher the total activity, the higher the potency. Varying potencies were observed in dif-

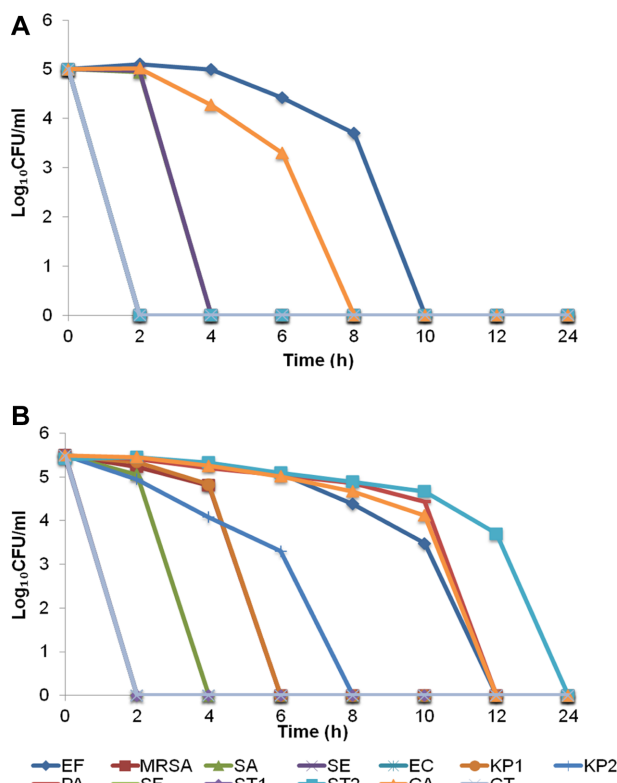


Fig. 2. Time kill assay for different test organisms. EF: *Enterococcus faecalis*, MRSA: Methicillin Resistant *Staphylococcus aureus*, SA: *Staphylococcus aureus*, SE: *Staphylococcus epidermidis*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*, KP: *Klebsiella pneumoniae*, SF: *Shigella flexneri*, ST: *Salmonella typhimurium*, CA: *Candida albicans*, CT: *Candida tropicalis*. (A) *M. oleifera* SC ethyl acetate extract, (B) Standard antibiotics (gentamicin for bacterial pathogens and amphotericin B for yeast strains).

ferent groups of test organisms. Gram-positive bacteria exhibited the highest total activity ranging from 410 ml-546.6 ml/g whereas that of Gram-negative bacteria ranged from 16.4 ml-455.5 ml/g. The potency of the extract against yeast strains was relatively low in comparison to that observed in bacterial pathogens (Table 1).

Time kill assay

The durations and concentrations of *M. oleifera* SC extract that induced total killing of the various test organisms was determined by time kill assay. The concentrations used for this study were predetermined based on MIC values. A log reduction was observed in all test organisms and the concentration range was relatively lower for Gram-positive bacteria as compared to that of Gram-negative and yeast strains (Table 1). However, a total killing of Gram-negative bacteria was observed at 2 h, some of which, such as *E. coli*, *Shigella flexneri* and *S. typhimurium* 1 were comparable to standard antibiotic. *C. albicans* survived up to 8 h while *C. tropicalis* was killed in 2 h (Fig. 2A). The microbicidal nature was confirmed as no regrowth occurred in any of the microorganisms even after 24 h of incubation. Though in crude form, *M. oleifera* SC recorded a maximum kill time of 10 h as compared to 24 h for standard antibiotics such as gentamicin and amphotericin B (Fig. 2B).

Post antibiotic effect (PAE)

The experiment was performed to evaluate the ability of

the active components to suppress microbial growth after a brief exposure to the test organisms even in absence of a host defense mechanism. The concentrations used in the PAE study were equivalent to those used in the time kill assay (Table 1). The PAE induced by both plant extract and standard antibiotic varied among the groups of organisms tested. Though the extract was in crude form, its effectiveness against most of the Gram-positive bacteria was obvious as it exhibited longer PAEs as compared to standard antibiotics. *Enterococcus faecalis* showed the longest PAE of about 21 h at a concentration of 0.036 mg/ml as compared to that of gentamicin, which exhibited a shorter PAE of about 4 h at 0.01 mg/ml. *K. pneumoniae* 2 was the only Gram-negative bacterium with a longer PAE of 7 h at 1 mg/ml. In the case of yeast strains, *C. tropicalis* exhibited a longer PAE of about 22 h at a concentration of 4 mg/ml (Table 2).

Biosafety

This experiment was designed to evaluate the safety of the active components by determining their mutagenicity and toxicity by standard methods. The ethyl acetate extract of *M. oleifera* SC exhibited no mutagenicity at both 0.030 mg/ml and 2.0 mg/ml, which represented the established lower and upper range MIC concentrations within various test organisms. This was confirmed by toxicity assay as the level of viability in treated cells was comparable with that of untreated cells (control).

Table 2. PAE of seed coat and standard antibiotics by viable count method after 1 h exposure to antimicrobial agent.

Organism	SC in mg/ml (× MIC)	PAE (h) ^b	A ^c in mg/ml (× MIC)	PAE (h) ^b
<i>E. faecalis</i>	0.036(1)	21.3 ± 1.15	0.01(1)	4.3 ± 0.58
MRSA ^a	0.06(2)	3.67 ± 0.58	0.00019(1)	4.67 ± 0.58
<i>S. aureus</i>	0.06(2)	18.3 ± 1.53	0.002(2)	0.3 ± 0.58
<i>S. epidermidis</i>	0.04(1)	6.0 ± 1.0	0.001(1)	1.3 ± 0.58
<i>E. coli</i>	0.072(2)	2.67 ± 0.58	0.001(1)	20.3 ± 0.58
<i>K. pneumoniae</i> 1	0.04(1)	1.33 ± 0.58	0.00019(1)	4.0 ± 1.0
<i>K. pneumoniae</i> 2	1.0(1)	7.0 ± 1.0	0.001(1)	5.67 ± 0.58
<i>P. aeruginosa</i>	0.072(2)	2.0 ± 0.0	0.01(1)	1.67 ± 0.58
<i>S. flexneri</i>	0.096(2)	1.67 ± 1.15	0.002(1)	2.67 ± 0.58
<i>S. typhimurium</i> 1	0.096(2)	3.67 ± 1.52	0.002(1)	1.0 ± 1.0
<i>S. typhimurium</i> 2	2.0(2)	4.6 ± 0.58	0.001(1)	4.33 ± 0.58
<i>C. albicans</i>	0.600(2)	2.67 ± 0.58	0.099(1)*	5.67 ± 0.58
<i>C. tropicalis</i>	4.0(2)	5.67 ± 0.58	0.0005(1)*	22.3 ± 0.58

^aMethicillin resistant *Staphylococcus aureus*, ^bexpressed as the mean ± S.D. of three determinations, ^cantibiotics (gentamicin and * - amphotericin B). The concentrations used here were based on the final MICs used in the time kill assay (Table 1).

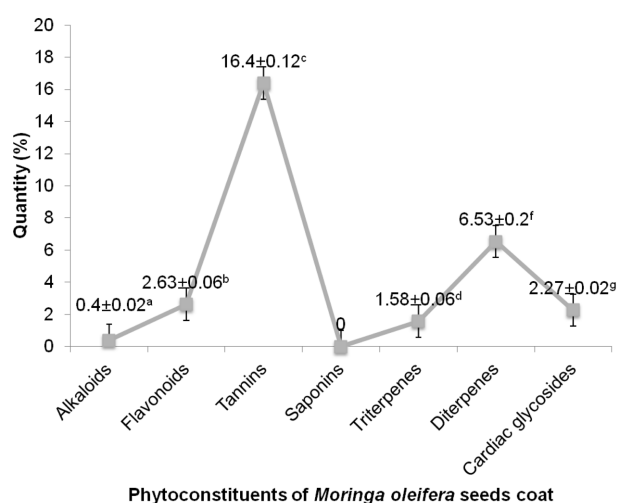


Fig. 3. Percentage quantitative estimates of phytoconstituents of *Moringa oleifera* seed coat. Data points represent mean \pm S.D. of three determinations. Different superscript letters on data points shows statistical difference at 5% confidence level by Holm-Sidak procedure.

Phytochemical evaluation

Qualitative and quantitative standard methods were performed to identify and estimate the active phytoconstituents of *M. oleifera* SC. The various biochemical tests indicated the presence of different secondary metabolites. Quantification of the metabolites indicated tannins to be the most

abundant at 16.4% while the quantity of alkaloids was 0.4%, which was very low as compared to the other phytochemicals. The estimated quantity difference of all phytoconstituents was statistically significant at 5% confidence level by the Holm-Sidak procedure (Fig. 3). Saponins, anthranol glycosides and other forms of glycosides tested negative. Flavonoids, diterpenes, triterpenes and cardiac glycosides demonstrated broad-spectrum antimicrobial activity at 1.2 mg, 1.5 mg, 0.058 mg, and 1.6 mg per 50 μ l concentrations, respectively. Flavonoids and diterpenes were found to be more effective and their efficiency in most cases was comparable since they exhibited insignificant difference at the 5% confidence level by the Tukey test (Table 3). On the other hand, cardiac glycosides exhibited antibacterial activity ranging from 15.33-27.67 mm as compared to triterpenes, which ranged from 12-23.33 mm. Most test organisms were sensitive to flavonoids, diterpenes and cardiac glycosides. *C. tropicalis* exhibited resistance to all of the phytoconstituents while *K. pneumoniae* 2 was resistant to cardiac glycosides. Some of the Gram-negative bacteria, such as *S. typhimurium* strains, *K. pneumoniae* 2 and *S. flexneri*, all of which are important pathogens in the food and water industries, were resistant to triterpenes. The most encouraging aspect is that all four phytoconstituents were active against the clinical isolate of MRSA (Table 3).

Table 3. Antimicrobial activity of isolated phytoconstituents of *Moringa oleifera* SC against Gram-positive and Gram-negative bacteria and yeast strains.

Organism	Inhibition zone of phytoconstituents (mm)			
	Flavonoids	Diterpenes	Triterpenes	Cardiac glycosides
<i>Enterococcus faecalis</i>	19.67 ^{b,w} \pm 0.33	27.0 ^{a, st} \pm 0.58	14.0 ^{c, r} \pm 0.00	17.0 ^{d, tu} \pm 0.58
MRSA ^a	27.0 ^{a, tu} \pm 0.58	31.3 ^{b, qr} \pm 0.33	16.67 ^{c, pq} \pm 0.9	27.67 ^{a, p} \pm 1.2
<i>Staphylococcus aureus</i>	33.0 ^{a, s} \pm 0.58	33.67 ^{a, q} \pm 0.33	19.0 ^{b, p} \pm 0.58	20.33 ^{b, rs} \pm 0.33
<i>Staphylococcus epidermidis</i>	38.67 ^{a, p} \pm 0.33	41.67 ^{a, p} \pm 0.33	23.33 ^{c, t} \pm 0.88	18.0 ^{d, st} \pm 0.58
<i>Escherichia coli</i>	26.0 ^{b, tu} \pm 0.58	30.0 ^{a, r} \pm 0.58	12.0 ^{c, r} \pm 0.58	21.0 ^{d, r} \pm 0.58
<i>Klebsiella pneumoniae</i> 1	38.0 ^{a, pq} \pm 0.58	41.0 ^{a, p} \pm 0.58	18.67 ^{c, p} \pm 0.88	5.33 ^{d, pq} \pm 0.88
<i>Klebsiella pneumoniae</i> 2	14.0 ^{a, v} \pm 0.58	14.33 ^{a, v} \pm 0.33	0.0	0.0
<i>Pseudomonas aeruginosa</i>	36.0 ^{b, qr} \pm 0.58	29.0 ^{a, rs} \pm 0.58	14.33 ^{c, qr} \pm 0.33	27.67 ^{b, r} \pm 0.33
<i>Shigella flexneri</i>	27.33 ^{a, t} \pm 0.33	26.0 ^{a, tu} \pm 0.58	0.0	15.33 ^{c, u} \pm 33
<i>Salmonella typhimurium</i> 1	24.33 ^{a, u} \pm 0.33	25.67 ^{a, tu} \pm 0.88	0.0	17.7 ^{c, stu} \pm 0.67
<i>Salmonella typhimurium</i> 2	13.0 ^{a, v} \pm 0.33	13.67 ^{a, v} \pm 0.33	0.0	19.0 ^{c, rst} \pm 0.58
<i>Candida albicans</i>	35.0 ^{b, rs} \pm 0.58	24.0 ^{a, u} \pm 0.58	17.67 ^{c, p} \pm 0.67	24.0 ^{a, q} \pm 0.58

^aMethicillin resistant *Staphylococcus aureus*. Values are expressed as mean \pm SD of three determinations. Different superscript lower case letters before the comma within the row and after the comma within the column show significant difference at 5% confidence level by Holm-Sidak test.

Discussion

To the best of our knowledge, this is the first study to report on the antimicrobial activity and establish the active secondary metabolites of *M. oleifera* SC. The antimicrobial activity of various organic solvent extracts revealed ethyl acetate to be the most effective extractant, perhaps due to a high yield of active constituents. The broad spectrum properties observed could be attributed to individual or synergistic effects of the active groups, which were identified in the later stages of this study. Although no work has been reported on the SC, previous reports on other morphological parts of this plant including seeds, leaves, root bark and stem bark prepared in different organic solvents have demonstrated significantly less activity [2, 7, 18, 37] as compared to the current findings, with the likelihood that most of the effective constituents are accumulated in the SC, which plays a protective role for the seed cotyledon.

The MIC values recorded in this study are being reported for the first time and moreover, the values are relatively lower than the MICs of other morphological parts of this plant including leaves, seeds, and stem bark [7, 13, 38]. Comparatively, the ethyl acetate extract MIC values observed in this study are far lower than the values of the aqueous extract in our previous study [36] and the only reported study of the SC of *Detarium microcarpum* [19]. The seed coat has been known to play a protective role against invading phytopathogens. The susceptibility of various test groups to SC extract indicated Gram-positive bacteria to be the most sensitive, which was confirmed in the total activity potency study. Since the MIC values observed were encouraging in most of the organisms tested, other *in vitro* studies including time kill assay and post antibiotic effect were performed to establish their efficacy and the mode of action. In the time kill assay, the Gram-negative bacteria were inhibited by 2 h while Gram-positive bacteria took more than 2 h except MRSA. This result could be an indication that the active components have concentration-dependent as well as time-dependent killing properties [35]. PAE is persistent suppression of bacterial growth after their brief exposure (1 or 2 h) to an antimicrobial agent even in the absence of host defense mechanisms [16]. The longer PAE observed in Gram-positive bacteria could be attributed to DNA alteration by the active principles as suggested in previous studies that showed most nucleic acid and protein synthesis inhibitors induce long-term PAE against suscepti-

ble bacteria. It could also be an indication of the permeability potential of the active components through the cell membrane. All Gram-negative bacteria exhibited shorter PAE, conceivably an indication of cell wall alterations by the active constituents [34].

The biosafety of this plant extract was determined by Ames test and MTT assay. The Ames test is a reverse mutation test that is based on histidine dependence and mutations in *S. typhimurium* (TA 98/ MTCC 1251, IMTECH, Chandigarh) while the MTT assay is a colorimetric assay based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate MTT into an insoluble, purple-colored formazan product that is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is measured by the viability of the cells. The non-mutagenicity and non-toxicity of ethyl acetate extracts of *M. oleifera* SC are being reported for the first time and this correlates with similar studies on other morphological parts of the same plant. For instance, Grabow *et al.* [24] in their evaluation of the toxicity and mutagenicity of water coagulated with 200 mg/l of *M. oleifera* seed preparations using fish, protozoan, bacterial, coliphage, enzyme and Ames test assays displayed no mutagenic effect. In addition to this observation, different extracts of other morphological parts of this plant including leaves, stem bark and roots have been reported to be safe [3, 8, 26, 29].

The composition of phytoconstituents of *M. oleifera* SC is being reported for the first time. However, stem bark, roots, seeds and leaves of the same plant extracted in different solvents have been reported where the constituents were solvent as well as plant part specific [26, 29]. In this study, we tested the antimicrobial activity of phytoconstituents by agar well diffusion assay where diterpenes exhibited an average zone of inhibition ranging from 13-41.67 mm while flavonoids ranged from 13-38.67 mm and their effectiveness was organism specific. The antimicrobial properties observed here concur with the general overview in previous studies associating such phytochemicals with the defense system of the plant [10].

In conclusion, since ethyl acetate was the best extractant, the active constituents could be polar in nature. The longer PAE observed in Gram-positive bacteria as compared to Gram-negative bacteria could be an indication that the resulting product (s) could have a long dosing regimen

for the former group of organisms. On the other hand, although one multidrug resistant organism was tested, its sensitivity to SC active constituents could be indicative of the potential against such bugs. Interestingly, *M. oleifera* SC are usually regarded as agri-residues. However, this study has revealed them to be a source of potential candidates that could be applicable in the development of drugs or drug leads of broad antimicrobial spectrum as well as a promising remedy for the emerging antimicrobial resistance that is one of the great concerns of the 21st century.

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