World Journal of Pharmaceutical ReseaRch

Volume 3, Issue 2, 2772-2788. Research Article ISSN 2277 – 7105

IN VITRO ANTIMICROBIAL POTENTIAL, BIOSAFETY AND BIOACTIVE PHYTOCONSTITUENTS OF *MORINGA OLEIFERA* STEM BARK

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Article Received on
05 January 2014
Revised on 30 January2014,
Accepted on 25 February 2014

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ABSTRACT

M. oleifera is known for its nutritional and numerous medicinal uses that have been appreciated for centuries in many parts of its habitat and introduced ranges. Stem bark of this plant, has been traditionally used for stimulation purposes as well as antimicrobial and other pharmacological disorders. This work evaluated the stem bark of this plant for its antimicrobial potential by determining the minimum inhibitory concentration (MIC), time kill assay and post antibiotic effect (PAE) against some human pathogens of yeast strains, Gram positive and Gram negative bacteria. Its safety was ascertained by Ames test and MTT assay, qualitative and quantitative analysis of phytoconstituents and their antimicrobial activity was established. Multiple means were compared by ANOVA at 5% confidence level. Antimicrobial activity of various solvents extracts indicated acetone to be the best extractant and hence was used to evaluate subsequent *in vitro* studies. The extract exhibited a broad spectrum potential and was neither mutagenic nor toxic. *Klebsiella pneumoniae* 1, *Pseudomonas aeruginosa* and interestingly methicillin resistant *Staphylococcus aureus* (MRSA) were the most sensitive with a minimum inhibitory concentration (MIC) of 0.1mg/ml. The Gram positive bacteria showed susceptibility within a time kill range of 6 h – 10 h as compared to Gram negative bacteria which ranged from 10 h – 24 h. Among the major phytoconstituents established, flavonoids and diterpenoids exhibited broad spectrum properties. These findings indicate that *Moringa oleifera* stem bark, could be a potential source of active principals for development of broad spectrum drugs or drug leads.

Key words: antimicrobial; *Moringa oleifera*; phytoconstituents; pharmacodynamics; broad spectrum; stem bark

1. INTRODUCTION

Plant-based drugs have been used worldwide in traditional medicine system for the treatment of various diseases. It has been estimated that between 60-90% of the population of developing countries use traditional medicines almost exclusively and consider it to be a normal part of primary healthcare.[1] The modern pharmacopoeia contains 25% drugs derived from plants and many others, which are synthetic analogues, built on prototype compounds isolated from plants.[2] The benefits of using plant-based medicines are that, they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.[3]

A vast number of medicinal plants were and are still used for various treatments and *Moringa* is one such genera which belong to the family Moringaceae with around 33 species of which 4 are accepted, 4 are synonym and 25 have not been assessed so far.[4] The traditional knowledge and its use is referenced in more than 80 countries and it's known in over 200 local languages. For instance, the plant has been used by various societies including the Roman, Greek, Egypt, India and many others for thousands of years with writings dating as far back as 150 AD. The history of *Moringa* dates back to 150 B.C. where ancient kings and queens used its leaves and fruit in their diet to maintain mental alertness and healthy skin.[5] Ancient Maurian warriors of India were fed with *Moringa* leaves extract in the warfront. The elixir drink was believed to give them extra energy and relieve them of the stress and pain incurred during war. Amongst the explored *Moringa* species, *M. oleifera* is the most prevalent for its nutritious and numerous medicinal uses that have been appreciated for centuries in many parts of its native and introduced ranges.[6] Amid the different aerial and underground morphological parts of this plant, stem bark has been traditionally used for stimulation purposes as well as antimicrobial.[7] From microbiological perspective, scanty literature is available on antimicrobial potential of this plant part except a few reports at preliminary level.[6, 8, 9] On this basis, the work was designed to evaluated its antimicrobial potential using acetone extract, ascertain its safety and establish the phytoconstituents responsible for the antimicrobial activity which, to the best of our knowledge have not been reported before.

2. MATERIALS AND METHODS

2.1 Test sample, chemicals and microbial cultures: *M. oleifera* stem bark was collected from the botanical garden of Guru Nanak Dev University, Amritsar (identified and deposited in Guru Nanak Dev University, Botanical Herbarium under accession number 6746 HERB).[10] The chemicals and standard antibiotics used in this work were purchased from Sigma and Hi-Media, Mumbai, India. The reference microbial cultures used in this study were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh - India. They were selected from Gram positive bacteria, Gram negative bacteria and Yeast strains to represent a broad spectrum of potential pathogens that pose significant threats in the medical field. Gram positive bacteria: *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 740) and *Staphylococcus epidermidis* (MTCC 435); Gram negative bacteria: *Escherichia coli (MTCC* 119), *Klebsiella pneumoniae* 1 (MTCC 109), *K. pneumoniae* 2 (MTCC 530), *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhimurium* 1 (MTCC 98), *S. typhimurium* 2 (MTCC 1251), *Shigella flexneri* (MTCC 1457) and Yeast strains: *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 230).

2.1.1 Inoculum and plant material preparation: Each inoculum was standardized to 0.5 McFarland standard while the stem bark was cut into small pieces, dried in shade and processed according to [11].

2.2 Extraction of plant material and antimicrobial activity by agar diffusion assay: Aqueous and seven organic solvents i.e. hexane, petroleum ether, butanol, chloroform, acetone, ethyl acetate, methanol were used for extract preparation. Five grams of the sample was mixed with the known volume of solvent in the ratio of 1:10(w/v).Extraction was done thrice for 24 h at ambient temperature (30ºC) and 80 rpm. The extracts were combined and filtered through Whatman filter paper No. 1. The miscella (filtrate) obtained was evaporated in a rota vapour at 45ºC and the residue was reconstituted in 6ml of 30% DMSO. Hundred microliter of each extract was tested for antimicrobial activity per test organism by agar well diffusion assay. Acetone extract exhibited maximum antimicrobial activity and was thus selected for subsequent studies.

2.3 Minimum inhibitory concentration: The MIC of the acetone extract was determined by agar dilution method.[11] A stock solution of 12.9mg/ml was prepared and dispensed into suitable agar plates in varying concentrations (0.1 - 3mg/ml). The plates were inoculated with

0.1 ml of a 4h activated test organisms (adjusted to 0.5 McFarland standard) and incubated for 24 hours at 37°C for bacterial strains and 24 – 48 hours at 25°C in case of yeast strains. The lowest concentrations exhibiting complete inhibition of the microbial growth was taken as the MIC. The experiment was performed in duplicate and repeated three times with standard antibiotics (Gentamicin 0.059mg/ml and Amphotericin B 0.750mg/ml) as positive controls.

2.3.1 Total activity potency: Total activity potency was determined according to [12]. Total activity potency is the volume at which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract in mg from 1 g plant material by the MIC in mg/ml of the same extract or compound isolated and is expressed in ml/g. The higher the total activity the higher the potency and it is expressed as; Total activity potency = (Amount extracted from 1g plant material) / (MIC of the extract).

2.4 Time kill assay: The time kill assay for acetone extract (12.9mg/ml) was performed by viable cell count method (VCC) as described earlier [13] with slight modification. The 4h grown inoculum was adjusted to 0.5 McFarland standards and serially diluted to 10^{-3} with suitable double strength broth medium. Each diluted inoculum of 0.1ml was mixed in equal volume of predetermined MIC (Table 1) and incubated at 25^{o}C (yeast) and 37^{o}C (bacteria). Sampling was done at 2 h intervals up to 24 hours by spreading 0.1ml of the mixed suspension on suitable agar plates in duplicate. The time kill assay for standard antibiotics (Gentamicin 0.059mg/ml and Amphotericin B 0.750mg/ml stock solutions) was also determined in the same manner. The mean number of colonies was computed and compared with that of control in which the organic extract/ standard antibiotics were replaced with 30% DMSO.

2.5 Post antibiotic effect: The PAE of acetone extract of stem bark and standard antibiotics was performed according to Alsaba et al. (2011) [14]. The concentrations applied in time kill assay for both acetone extract and standard antibiotics, were mixed with suspension of respective test organism (in equal volume) containing approximately 1x10^{5} CFU/ml. After 2 h exposure, the drug activity was stopped by diluting the mixture to 1:1,000 in a drug free pre-warmed suitable double strength broth. In preliminary experiments, this dilution was seen to be sufficient as the residual drug activity resulted in no significant deviation of the growth curve. CFU sampling was done at an interval of one hour 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.

Table 1. MIC and time kill concentrations for stem bark standard antibiotics and organic extract

Organisms	Antibiotics MIC[b]	SB[c] MIC	SBTK[d]	TAP[e]
Enterococcus faecalis	0.01	0.7	1.4(2)	36.9
MRSA [a]	0.00019	0.1	0.3(3)	258
Staphylococcus aureus	0.001*	0.2	0.4(2)	129
Staphylococcus epidermidis	0.001	0.2	0.4(2)	129
Echerichia coli	0.001	0.2	1.2(6)	129
Klebsiella pneumoniae 1	0.00019	0.1	0.2(2)	258
Klebsiella pneumoniae 2	0.001*	1.5	3.0(2)	17.2
Pseudomonas aeruginosa	0.01	0.1	0.3(3)	258
Salmonella typhimurium 1	0.002	0.2	1.2(6)	129
Salmonella typhimurium 2	0.001	1.5	4.5(3)	17.2
Shigella flexneri	0.002	0.2	1.2(6)	129
Candida albicans	0.099**	0.6	1.8(3)	43
Candida tropicalis	0.0005 **	3.0	9.0(3)	8.6

*[a]: Methicillin Resistant Staphylococcus aureus; [b]: 1×MIC was used in time kill assay except * which took 2× MIC in mg/ml, ** represent values for amphotericin B while the rest of the values are for gentamicin ; [c]: SB - Stem bark (mg/ml); [d]:SBTK- Stem bark time kill concentrations in mg/ml (the values in parentheses represent total MIC); [e]:TAP - Total Activity Potency (ml/g) was calculated using 25.8mg/g (quantity extracted from plant powder)*

2.6 Biosafety

2.6.1 Ames mutagenicity testing: Mutagenicity of acetone extract was evaluated by both spot and plate incorporation methods of Ames test according to Mortelmans and Zeiger (2000) [15] with slight modifications. In plate method, equal ratio of 0.1 ml culture (overnight activated at 37˚C) and extract equivalent to 0.2mg/ml MIC of *S. typhimurium* was added to 5

ml of top agar containing 0.25 ml of 0.5 mM histidine-biotin mixture (1:1 ratio). The contents were mixed and poured onto glucose minimal agar plates immediately. Similarly, the same protocol was followed for spot method except that the acetone extract was impregnated on disc which 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 and mutagenic potential of the extracts was determined by comparing with positive control.

2.6.2 Cellular toxicity testing using MTT assay: Cellular toxicity of acetone extract was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay [16] with slight modifications. Ten milliliter sheep blood was taken into injection syringe containing 3 ml Alsever's solution (anticoagulant) and transferred to sterile centrifuge tubes. The blood was centrifuged at 1600 × g at room temperature (25 ± 3°C) for 20 min. to separate the plasma from the cells. The supernatant was removed, 6 ml phosphate buffer saline (PBS) added and centrifuged again. The RBCs were washed thrice with PBS by centrifugation and the pellet was resuspended in 6ml of PBS. Serial dilutions of these cells were prepared using PBS and counted with the help of a hemocytometer under a light microscope so as to obtain cells equivalent to $1x10^5$ CFU/ml. The cells from selected dilution were dispensed into 0.1ml per well and incubated at 37°C for overnight. The supernatant was removed carefully and 200µl of acetone extract adjusted according to highest and lowest MIC range was added and incubated further for 24 hours. The supernatant was removed again and 20 µl MTT solution (5mg/ ml) added to each well and incubated further for 3.5 h at 37°C on orbital shaker at 60 rpm. After incubation, the supernatant was removed without disturbing the cells and 50 µl 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 untreated cells served as control.

2.7 Evaluation of Phytocochemical profile and their antimicrobial activity: *Moringa oleifera* stem bark was powdered and subjected to qualitative and quantitative analysis of secondary metabolites by standard methods as follows; **(i) Alkaloids:** Qualitatively analysis was done according to Trease & Evans (1989)[17] and reactions were scored positive on the basis of turbidity or precipitation. Quantitative analysis was performed according to Harborne (1998) [18]. The alkaloid residue obtained was dried to constant weight. **(ii) Phenolic**

compounds and Tannins: For phenolic compounds, 1g of the plant powder was suspended in 30ml methanol and incubated under shaking conditions at 30°C for 24h. In case of tannins 1g of plant powder was suspended in 20ml 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. 2011) [19].Quantification of flavonoids and tannins was done according to Boham & Kocipai-Abyazan (1974) [20] and Vieira et al. (2011) [21] respectively. **iii) Saponins:** Qualitative test was performed according to Kokate (1999) [22] while quantification according to Obadoni & Ochuko (2001) [23]. **(iv) Terpenoids:** Triterpenes and diterpenes were detected according to Roopashree et al. (2008) [24] and Obasi et al. (2010) [25] respectively. Quantification of triterpenes was done according to Bojana et al. (2000) [26] while diterpenes were quantified according to Mehdi et al. (2011) [27]. **(v) Phytosterols**: Qualitative analysis was performed according to Finar (1986) [28]. **(vi) Cardiac glycosides:** The detection was done according to Edeoga et al. (2005) [29] with further quantification according to Abdel-Azim et al. (1996) [30]. All quantitatively isolated secondary metabolites were dissolved in 30% DMSO and subjected to agar diffusion assay to assess their antimicrobial potential.

2.9 Data analysis

Most of the experiments were performed in duplicate and repeated thrice. The data was analyzed by ANOVA for comparison of multiple means and the chosen level of significance was $P<0.05$. The statistical analyses were done by 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).

3.0 RESULTS AND DISCUSSION

3.1 Antimicrobial activity of M. oleifera stem bark solvents extracts: Different solvents extracts exhibited organism specific antimicrobial activity. Analysis by one way ANOVA revealed acetone solvent to be the most effective extractant against all test organisms. A significant difference was observed at 5% confidence level between the following four homogenous groups; aqueous and chloroform, ethyl acetate, methanol, butanol whereas hexane and petroleum ether were the least effective (Fig. 1). *Candida tropicalis* was resistant to all the extracts except that of acetone. Previous reports of the same plant part using methanol, hexane and aqueous extract exhibited inhibition zone size varying from 8 mm – 15 mm for *Staphylococcus aureus,* and 7 mm – 8 mm for *Escherichia coli* respectively, [9] which are significantly less effective than our present findings. The variations may be attributed to

the difference in mode of extraction. It's remarkable to note that *Klebsiella pneumoniae* 2 and *Salmonella typhimurium* 2 exhibited a good inhibition zone of 26 mm and 27.5 mm, respectively for acetone which otherwise were least sensitive or resistant to other extractants. A reasonably good zone of inhibition (25 mm) shown by MRSA, a medically important strain, could be an indication of potential for other drug resistant bugs. The effectiveness of acetone could be attributed to its better extractive ability for high yield of active constituents. The broad spectrum properties observed could be due to individual or synergistic effect of the active groups which were identified in the later stages of this study.

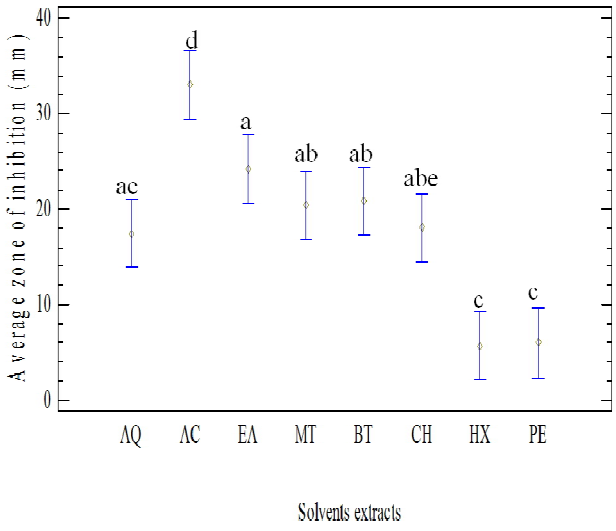

Figure 1 Effect of different solvents on antimicrobial activity of *M. oleifera* stem bark. Values are expressed as grand mean zone of inhibition of three determinations. Different superscript among the columns shows significant difference at 5% level by Fisher's least significant difference (LSD). AQ – Aqueous; AC - Acetone; EA – Ethyl acetate; MT – Methanol; BT – Butanol; CH – Chloroform; HX – Hexane; PE – Petroleum ether

3.2 Minimum Inhibitory Concentration (MIC): The extent of inhibitory activity by the best extractant was further evaluated by determining the MIC. The extract exhibited a broad spectrum potential whereby, *Klebsiella pneumoniae* 1, *Pseudomonas aeruginosa* and interestingly MRSA strain were the most sensitive with an MIC value of 0.1mg/ml which correlates their total activity potency values. *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhimurium* 1, *Shigella flexneri* and *Escherichia coli* which belong to the two major groups of bacteria exhibited a similar MIC value of 0.2mg/ml (Table 1). The MIC value reported herein for *Escherichia coli* is much lower than that of methanolic extract 625µg/ml.[8] However, some of the Gram negative bacteria such as *Klebsiella pneumoniae* 2

and *Salmonella typhimurium* 2 exhibited higher concentrations of 1.5mg/ml which may be attributed to the outer-membrane permeability barrier that limit access of the antimicrobial agent to their targets in the bacterial cell.[31] In case of the yeast strains, *Candida albicans* was more sensitive to the extract with an MIC of 0.6mg/ml as compared to *Candida tropicalis* which showed MIC of 3mg/ml. The corresponding MIC values of the standard antibiotics, considering their purity, were comparatively quite low (Table 1). The MIC values reported in this study for most organisms are much lower than those of aqueous extract of the same plant part which ranged between 3 mg/ml – 4.2 mg/ml. [10] Though in crude form, the MIC values obtained were encouraging in most of the organisms tested, hence, further *in vitro* studies such as time kill assay and post antibiotic effect were performed to establish their efficacy.

3.3 Time kill assay*:* The kill kinetics provides more accurate description of antimicrobial agents than does the MIC [32]. [The concentration used for the time kill study was based on predetermined MIC (Table 1). Gram positive bacteria exhibited a minimum time kill of 6 h and a maximum of 10 h at 0.3 mg – 1.4 mg/ml as compared to Gram negative bacteria with time kill range of 4 h – 24 h at concentration of 0.2mg – 3.0mg/ml. In case of yeast strains, *Candida tropicalis* was killed in 6 h at a concentration of 9.0mg/ml while *Candida albicans* survived up to 12 h at 1.8mg/ml (Fig. 2a). Though in crude form, efficacy of acetone extract to some of the test organisms was comparable to that of standard antibiotics (Fig.2b). The performance of acetone extract is quite effective as compared to our previous work with aqueous extract.[10] The microbicidal nature was further confirmed as no re-growth occurred in any of the microorganisms even after 24 h of incubation.

3.4 Post Antibiotic Effect (PAE)*:* 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.[33] PAE is being applied increasingly to allow antimicrobial dosing regimens to be developed on a more scientific basis. Different antimicrobials induce varied duration of PAE against different types of microbes. The concentrations for each test organism used in time kill assay in both acetone extract and standard antibiotics, were applied in PAE studies. Both test and standard antimicrobial agents induced a varied PAE amongst test organism. The extract induced a short or no PAE to the test organisms as compared to standard antibiotics which showed relatively longer PAE. Gram negative bacteria exhibited a comparatively longer PAE than Gram positive bacteria perhaps due to. *Pseudomonas*

aeruginosa belonging to Gram negative group exhibited relatively longer PAE of 3.3h at 0.3mg/ml. On the other hand, Gram positive MRSA which is a medically important resistant strain exhibited a PAE of 2.0h in the same concentration. *Candida tropicalis* showed relatively longer PAE of 2.6h perhaps due to higher concentrations as compared to *Candida albicans* which showed no PAE (Table 2). Usually, more prolonged intermittent dosing regimens apply primarily to antimicrobials that exhibit a prolonged PAE. The present study shows no major difference in PAE as the various groups of test organisms exhibited a shorter or no PAE and could be attributed to cell wall alterations by the active constituents.[34] To the best of our knowledge, this particular work is being reported for the first time.

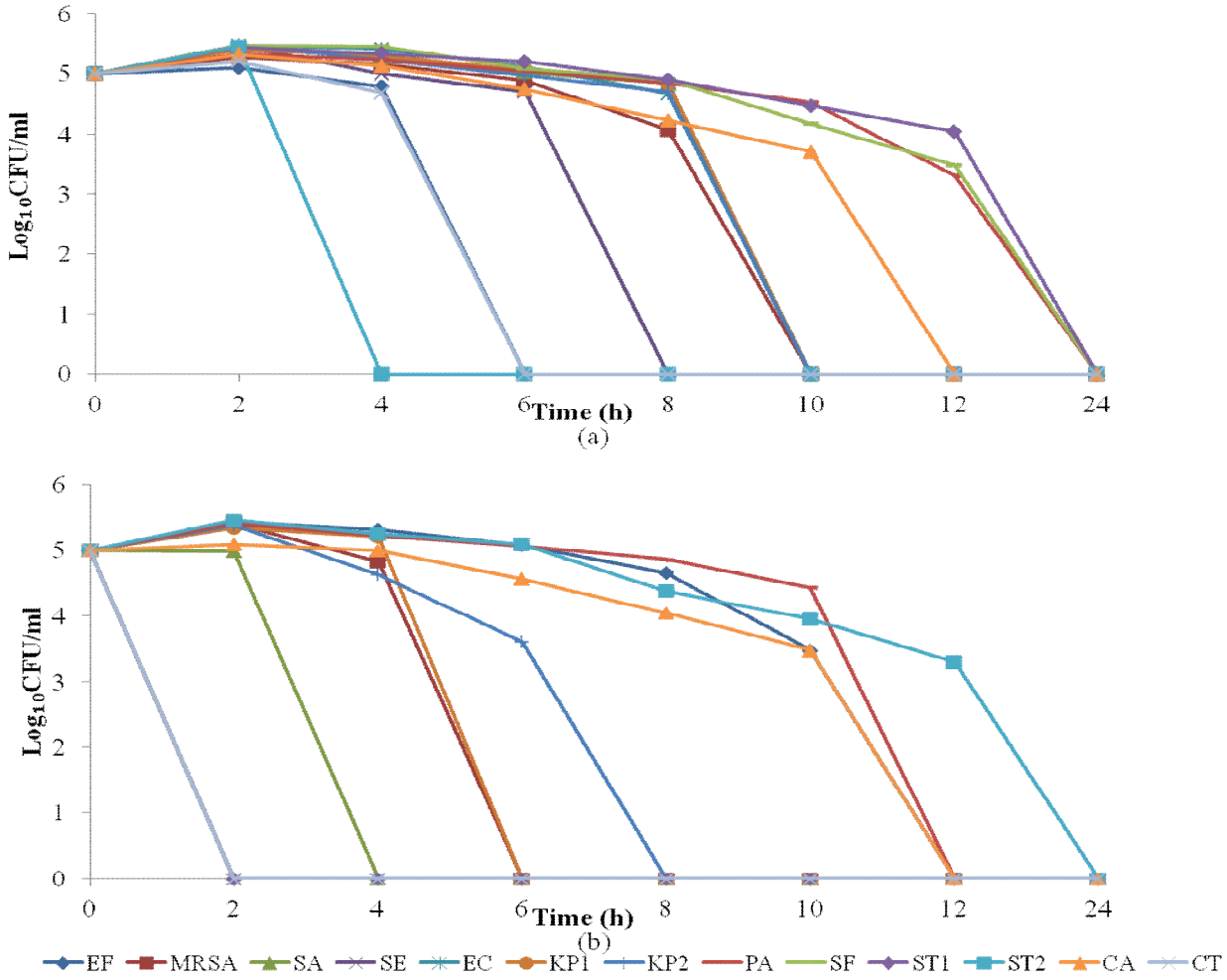

Figure 2. Time kill assay of (a) *M. oleifera* stem bark acetone extract (b) Standard antibiotics against different organisms (Gentamicin for bacterial pathogens and Amphotericin B for yeast strains). EF – *Enterococcus faecalis*; MRSA – methicillin resistant *Staphylococcus aureus*; SA – *Staphylococcus aureus*; SE – *Staphylococcus epidermidis;* EC – *Escherichia coli*; KP – *Klebsiella pneumoniae;* PA – *Pseudomonas aeruginosa*; SF – *Shigella flexneri;* ST – *Salmonella typhimurium;* CA – *Candida albicans*; CT – *Candida tropicalis.*

Table 2 PAE of stem bark and standard antibiotics by viable count after 2 h of exposure to antimicrobial agent

Orgarnisms	Extract (× MIC)	P AE(h)[b]	Antibiotics (× MIC)	PAE(h)[b]
Enterococcus faecalis	1.4(2)	0.0	0.01(1)	4.3±0.58
MRSA[a]	0.3(3)	2.0±0.0	0.00019(1)	4.67±0.58
Staphylococcus aureas	0.4(2)	1.6±0.54	0.001(2)	0.3±0.58
Staphylococcus epidermidis	0.4(2)	0.0	0.001(1)	1.3±0.00
Escherichia coli	1.2(6)	2.3±0.58	0.001(1)	20.33±0.58
Klebsiella pneumoniae 1	0.2(2)	2.3±0.58	0.00019(1)	3.33±0.58
Klebsiella pneumoniae 2	3.0(2)	2.6±0.58	0.001(2)	5.67±0.58
Pseudomonas aeruginosa	0.3(3)	3.3±0.58	0.01(1)	1.67±0.57
Shigella flexneri	1.2(6)	0.0	0.002(1)	2.67±0.58
Salmonella typhimurium 1	1.2(6)	0.6±0.58	0.002(1)	1.0±1.0
Salmonella typhimurium 2	4.5(3)	3.3±0.58	0.001(1)	4.33±0.58
Candida albicans	1.8(3)	0.0	0.099(1)	*5.67±0.58
Candida tropicalis	9.0(3)	2.6±0.58	0.0005(1)*	22.33±0.58

[a]: Methicillin Resistant Staphylococcus aureus; [b]:Expressed as the mean ± S.D. of three determinations; represent amphotericin B while the rest of the values in the column represent gentamicin.*

3.6 Biosafety*:* Ames test is a reverse mutation test which is based on histidine dependence and mutations in *S. typhimurium* (TA 98/ MTCC 1251, IMTECH, Chandigarh) while MTT assay is a colorimetric assay which is 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 which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Acetone extract of *M. oleifera* stem bark exhibited neither mutagenicity nor toxicity in both concentrations i.e 0.1mg/ml and 3.0mg/ml representing the lower and upper range MIC as pre-established. The observations are in consonance with the previous study where ethanolic and aqueous extracts of the same plant part did not show any adverse effect on growth related and biochemical parameters in rats. [35] Similarly, extracts of other morphological parts of this plant such as leaves, roots and seeds have also been reported to be safe.[6]

3.7 Phytochemical evaluation*:* The various biochemical tests on *Moringa oleifera* stem bark indicated the presence of alkaloids, flavonoids, tannins, saponins, diterpenoids, tritepenoids, and cardiac glycosides (Fig. 3) which correlates to the previous work on ethanolic and

aqueous extracts of the same plant part.[36] In another study, Sholapur and Patil (2013) [37] reported the same phytoconstituents with ethanolic extract while in case of petroleum ether only steroids were detected. This is an indication that no single solvent can be reliable for extraction of all phytochemicals in a given plant sample thus proper selection of solvent and extraction procedure is of immense importance in phytoconstituents profiling.

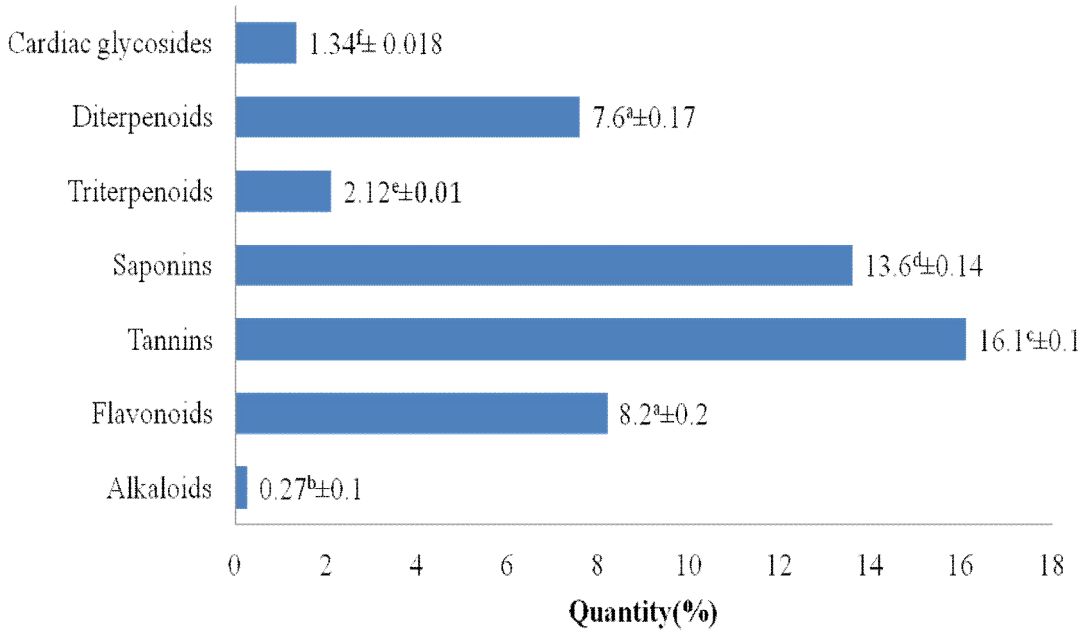

Figure 3 Quantitative analyses of Phytoconstituents of Moringa oleifera stem bark. Values are expressed as mean ± S.D. of three determinations. Different superscript among the columns shows significant difference at 5% confidence level by Holm- Sidak test.

Quantification of established stem bark metabolites indicated tannins to be the most abundant with 16.1 percent of dry weight while alkaloids represented the least concentration (0.27%). The quantity of flavonoids and diterpenes was statistically insignificant at 5% confidence level (Fig. 3). The antimicrobial studies, revealed flavonoids and diterpenes to be the most effective against Gram negative, Gram positive and yeast strains except *Klebsiella pneumoniae* 2 and *Candida tropicalis* which were resistant at a concentration of 1.67mg and 1.58mg per 0.05ml, respectively. Though diterpene concentration was lower than that of flavonoids, it showed higher antimicrobial activity in most of the test organisms with an average zone of inhibition ranging from 17.5 mm – 40mm whereas that of flavonoids ranged from 14mm – 29mm. The difference in activity of the two phytoconstituents against *Salmonella typhimurium* 2 was statistically insignificant at 5% confidence level (Table 3).

Table 3 Antimicrobial activity of isolated phytoconstituents of *Moringa oleifera* stem bark against Gram positive, Gram negative bacteria and yeast strains

Test organisms	Zone of inhibition of Phytoconstituents (mm)	
	Diterpenoids	**Flavonoids**
Enterococcus faecalis	$23.0^{d, st}$±1.4	$14.0^{h, s}$±0.0
MRSA*	$27.5^{c, q}$±0.7	$22.5^{i, q}$±0.7
Staphylococcus aureus	$28.0^{k, q}$±1.4	$18.0^{l, r}$±1.4
Staphylococcus epidermidis	$37.5^{j, p}$±0.7	$26.0^{b, p}$±1.4
Escherichia coli	$26.5^{g, qr}$±0.7	$21.5^{e, q}$±0.7
Klebsiella pneumoniae 1	$40.0^{f, p}$±0.0	$28.0^{f, p}$±1.4
Klebsiella pneumoniae 2	0.00	0.00
Pseudomonas aeruginosa	$26.5^{e, qr}$±0.7	$20.5^{g, qr}$±0.7
Shigella flexneri	$23.5^{i, rs}$±0.7	$20.5^{c, qr}$±0.7
Salmonella typhimurium 1	$20.5^{h, stu}$± 0.7	$17.5^{d, r}$±0.7
Salmonella typhimurium 2	$17.5^{a, u}$±0.7	$17.5^{a, r}$±0.7
Candida albicans	$20.0^{b, tu}$±0.0	$29.0^{j, p}$±1.4
Candida tropicalis	0.00	0.00

**: Methicillin Resistant Staphylococcus aureus; Values are expressed as Mean ± S.D of three determinations. Different superscripts before the comma within the row and after the comma within the column show significant difference at 5% confidence level.*

Interestingly, a clinical isolate of MRSA was sensitive than most of the test organisms for both diterpenes and flavonoids with an inhibition zone of 27.5 mm and 22.5 mm, respectively. This could be an indication that the active constituents have potential against similar drug resistant bugs. To the best of our knowledge, no similar work has been reported. However, other studies though of different plant species, have implicated similar groups of phytoconstituents to have broad spectrum activity which correlates to our findings.[38]

CONCLUSION

Though *Moringa oleifera* stem bark has a vast array of phytochemicals, only diterpenoids and flavonoids have shown broad spectrum antimicrobial potential at individual level. By the

fact that MRSA, a multi drug resistant organism was remarkably susceptible to the active constituents, could be an indicative of their potential against such bugs. Therefore, the active constituents of *Moringa oleifera* stem bark could be considered potential candidates for development of drugs or drug leads of broad antimicrobial spectrum including drug resistance microbes which are currently of great concern. Elucidation of active compounds and subjecting them to *in vivo* studies to ascertain their mode of action is underway.

ACKNOWLEDGEMENT

The scholarship offered to Jemimah Gesare Onsare by the Government of India through Indian Council of Cultural Relations and support by the Government of Kenya, to pursue her Ph. D studies is duly appreciated. The SAP and UPE grants by University Grants Commission, India are also acknowledged.

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