



Original Research Article

Antimicrobial Activity of Indian Medicinal Plants: *Moringa oleifera* and *Saraca indica*

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ABSTRACT

Keywords
Antimicrobial activity, *Moringa oleifera*, *Saraca indica*, Leaf extract.

The antibacterial activity of leaf extracts of *Moringa oleifera* and *Saraca indica* was evaluated in vitro, using disc diffusion method. Six different extracts each for both the plants were used which included acqueous, acetone, chloroform, ethanol, methanol and petroleum ether. The study showed that aqueous and ethanol extract of *Moringa oleifera* was inhibitory against *Escherichia coli* (12mm each), *Proteus vulgaris* (10mm each) and *Salmonella typhi* (12mm and 10mm respectively). All the organisms were sensitive to ethanol extract except *Bacillus subtilis* and *Escherichia coli*. Acetone and chloroform extract of *Moringa oleifera* did not show any antibacterial activity. It was observed that methanol extract of *Saraca indica* showed highest antimicrobial activity against *Staphylococcus aureus* (29mm) while petroleum ether extract exhibited greater antimicrobial activity against all the tested bacteria. *Moringa oleifera* leaf extract showed that *Bacillus subtilis* and *Klebsiella pneumoniae* were resistant to all the extracts.

Introduction

Plants contain numerous biologically active compounds, many of which have been shown to have antimicrobial properties (Cowan, 1999). Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years and there is increasing interest in plants as sources of agents to fight microbial diseases (Charlandy et al., 1999). Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents

(Ahmad et al., 1998; Bhavnani and Ballow, 2000). *Moringa oleifera* is medicinal species, belonging to monogeneric family Moringaceae. The "Moringa" tree is considered one of the world's most useful trees, as almost every part of the Moringa tree can be used for food or has some other beneficial properties. In India and other parts of the country the fruit called as *drumstick* (Devendra et al., 2011). The leaves of *Moringa oleifera* are eaten in African countries, such as Ghana, Ethiopia,

Nigeria, East Africa and Malawi. *Moringa* tree is cultivated for foods and medicinal purposes (Olsen, 1987). *Moringa* leaf is a natural antihelmintic, antibiotic, detoxifier, outstanding immune builder used in some countries for the treatment of malnutrition and malaria (Thilza et al., 2010). All parts of the *Moringa* tree are edible and have long been consumed by humans. According to Oluduro (2012), the many uses of *Moringa* include: alley cropping (biomass production), animal forage (leaves and treated seedcake), biogas (leaves), fertilizer (seed-cake), foliar nutrient (juice expressed from leave), green manure (from leaves), gum (from tree trunks), honey and sugar cane juice-clarifier (powdered seed), honey (flower nectar) and medicine (all plant parts).

Moringa leaves are known to have a high content of protein, minerals and vitamin, hence an ideal nutritional supplement (Fletcher, 1998). *M. oleifera* leaves have been used to combat malnutrition, especially among infants and nursing mothers and hasten uterine contraction during child birth in pregnant women. It's antihypertensive, diuretic, antispasmodic, antiulcer, and anticancer and cholesterol lowering activities have been reported (Caceres et al., 1992; Dangi et al., 2002). The leaves are helpful in increasing breast milk in nursing mothers during breastfeeding and leaf decoction has been found useful in the treatment of asthma, back pain and rheumatism. *M. oleifera* tree has in recent times been advocated as an outstanding indigenous source of highly digestible protein, carotenoids and vitamin C suitable for utilization in many of the so called developing regions of the world where under nourishment is a major concern. In some parts of the world for example Senegal and Haiti, health workers have been treating malnutrition in small children, pregnant and nursing women

with *Moringa* leaf powder. The antioxidant activity of various extracts of *M. oleifera* leaf has been reported by several other authors (Bajpai et al., 2005; Singh et al., 2009; Sreelatha and Padma, 2009; Sultana et al., 2009). However, much has not been reported on the antibacterial healing property of *M. oleifera* leaf.

Saraca indica belongs to the family Caesalpiniaceae is commonly known as Asoka, Sita Asoka and Haempushpam. It is found in India, China, Ceylon and Malaysia. It occurs almost throughout India up-to an altitude of 750 m in the central and in the eastern Himalayas and Khasi, Garo and Lushai hills, wild in Chittagong, Bihar, Orissa, Konkan, Deccan, and Mysore. It has become quite scarce in several localities and is reported to be threatened in North Eastern Region of India. The principal constituents of this tree are a steroid component and a calcium salt. The earliest chronicled mention is in the Ayurvedic treatise, the Charka Samhita (100 A.D.), in which Asoka is recommended in formulations for the management of pain with relation to uterus (Gynecological) as Anodynes. The Bhavprakash Nighantu, commonly known as the Indian Materia Medica (1500 A.D.), cites the plant as a uterine tonic that is effective in regularizing the menstrual disorders (Sainath et al., 2009). Useful parts of the plant are barks, leaves, flowers and seed. The plant is useful in dyspepsia, fever, burning sensation, colic, ulcer, menorrhagia, leucorrhoea, pimples, etc. (Srivastava et al., 1988). Considering both the selected plants as a potential antibacterial agent, the present investigations were undertaken to test the antimicrobial activity of the leaf extract of *Moringa oleifera* and *Saraca indica* against selected bacterial pathogens.

Materials and Methods

Plant Collection

Fresh leaves of *Moringa oleifera* and *Saraca indica* were plucked from the *Moringa* tree and *Asoka* tree respectively growing in Nagpur. The leaves were identified and confirmed by the Botanist at the Botany Department of the present institution. Plant leaves were initially allowed to air-dry in the laboratory for four weeks and then finally ground to a fine powder, using a mortar and pestle, and stored for further use.

Preparation of Extracts

The 25 g of the powdered leaves were separately extracted in 500ml conical flasks with 100 ml of deionised distilled water (aqueous extraction) while 100ml each of acetone, chloroform, ethanol, methanol and petroleum ether (solvent extraction). The conical flasks were plugged with rubber corks, then shaken at 120 rpm for 30 min and allowed to stand at room temperature for 5 days with occasional manual agitation of the flask using a sterile glass rod at every 24 hours. The extracts were separately filtered using Whatman filter paper no. 1. The resulting filtrates were then concentrated in a rotary evaporator and subsequently lyophilized to dryness (Oluduro, 2012).

Test Microorganisms

Pure cultures of microorganisms used for the evaluation of the antimicrobial potential of the leaves extracts were purchased from the National Chemical Laboratory (NCL), Pune. *Staphylococcus aureus* (NCIM 2672), *Bacillus subtilis* (NCIM 2063), *Escherichia coli* (NCIM 5010), *Klebsiella pneumoniae* (NCIM

2707), *Proteus vulgaris* (NCIM 2857), *Salmonella typhi* (NCIM 2501) and *Pseudomonas aeruginosa* (NCIM 5029) where, NCIM= National collection of industrial microorganisms.

Antimicrobial Disc Preparation

Antimicrobial activity of the aqueous and solvent extracts of the leaves was assayed using the paper disc diffusion method (Oluma et al., 2004; Doughari et al., 2007). Discs of about 6mm diameter were made from Whatman filter paper no. 1 using a paper puncher. Batches of 100 discs were transferred into Bijou bottles and sterilized in the oven at 121°C for 15 minutes. The concentrated leaf extracts were redissolved in 5% dimethyl sulfoxide (DMSO) and sterile discs were impregnated with 30µl of 30mg/ml of each extract. The discs were carefully and firmly placed on the Mueller Hinton Agar (MHA) plates lawned previously with the test organism.

Standardization of Inoculum

The inocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4°C and subcultured into nutrient broth using a sterilized wire loop. The density of suspension inoculated onto the media for susceptibility test was determined by comparison with 0.5 McFarland standard of Barium sulphate solution (Cheesbrough, 2002).

Antimicrobial Activity

Disc agar diffusion technique described by Bauer et al (1966) was employed for antibacterial activity. Paper discs were impregnated with 30 µl of a solution of 30 mg/ml and the standard antibiotic gentamicin was used as a control for

comparison. Filter paper discs dipped into sterile distilled water, solvents blank and the standard antibiotic were used as control. The plates were then incubated at 37°C for 24 hours. Antibacterial activity was determined by measurement of zone of inhibition around each paper disc. Inhibition zones were calculated as the difference between the disc diameter (6 mm) and the diameters of zone of inhibition (Sainath et al., 2009).

Results and Discussion

Fresh leaves of *Moringa oleifera* and *Saraca indica* were collected for the evaluation of their antimicrobial activity against test bacterial pathogens. The antibacterial activity of the aqueous extracts of *Moringa oleifera* was inhibitory against *S. aureus* (10mm), *E. coli* (12mm), *P. vulgaris* (10mm) and *S. typhi* (12mm). All the selected bacterial pathogens were resistant to acetone and chloroform extract of *M. oleifera*. Ethanol extract was effective against *E. coli* (12mm) while both ethanol and methanol extracts were inhibitory against *P. vulgaris* and *S. typhi* (10mm each zone of inhibition respectively) zone of inhibition. Petroleum extract was not effective against all the test pathogens except *Ps. aeruginosa* (12mm) (Table 1) (Figure 1).

Aqueous extract of *Saraca indica* showed inhibitory effect only against *E. coli* (12mm) and *K. pneumoniae* (10mm). Among all the extracts used, Methanol extract showed highest inhibitory effect against *S. aureus* (29mm) followed by Petroleum Ether extract against *E. coli* (26mm), Acetone extract against *S. typhi* (24mm) while chloroform extract against *P. vulgaris* (20mm) and Ethanol extract against *B. subtilis* (18mm) (Table 2) (Figure 2). The susceptibility of the bacterial isolates to the reference antibiotic

is presented in Table 1 and 2. Some of the bacterial pathogens developed resistance to the reference antibiotic. Meanwhile some of them were sensitive to the leaves extracts but resistant to the reference antibiotics.

In this study ethanolic extract of *Moringa oleifera* as well as *Saraca indica* did not exhibit antimicrobial activity on all the tested bacterial pathogens but showed insignificant effect on some of the selected bacterial pathogens at the extract concentration of 30 mg/ml. This might be due to the presence of flavonoids, tannins, glycosides and terpenoids (Vinoth et al., 2012). The findings of this study is however at variance with Doughari et al (2007) who reported in their study on the antibacterial activity from the aqueous, acetone and ethanolic extracts of the leaves of *M. oleifera* that ethanolic extract of the plant demonstrated the highest activity, while the aqueous extract showed the least activity at 100 mg/ml. Bukar et al (2010) reported that *M. oleifera* leaf ethanol extract had the broadest spectrum of activity on the test bacteria.

In the present study no zone of inhibition was observed for chloroform extract, whereas Devendra et al (2011) reported the antibacterial activity of *M. oleifera* leaves chloroform extract. It is clearly noted that aqueous extract of *M. oleifera* leaf possesses significant antimicrobial activity against most of the selected bacterial pathogens, thus signalling its broad spectrum of antibacterial activity, thus rendering water as a good solvent of extraction for the *M. oleifera* leaves, much so, that most people who use this leaf as a traditional mean of treatment of various skin ailments and other diseases make use of water-based extract of the leaf. Another reason for *M. oleifera* aqueous extract might be due to the presence of

flavonoids, saponins, tannins, glycosides and terpenoids (Vinoth et al., 2012). Petroleum ether *M. oleifera* extract did not show any zone of inhibition against all tested bacterial strains except *P. aeruginosa* and the results were strongly correlated with that of Devendra et al (2011). The variations in the findings of this study compared to earlier studies reported may be due to climatic factor as it affects the plants, soil conditions of the geographical locations, solvents used etc. (Oluduro, 2012).

Meanwhile petroleum ether extract of *Saraca indica* showed antimicrobial activity against all the selected bacterial pathogens. The methanolic extract and chloroform extract of *S. indica* showed better and greater inhibitory property on most of the selected bacterial pathogens than aqueous extract, thus making the solvent as the best medium for the extraction of the active components of the *S. indica* leaf. The highest antibacterial activity of all the extracts tested was found against *S. aureus* for methanol extract and it was strongly correlated with the results of Sarojini et al (2011). This may be due to the presence of flavonoids, glycosides, saponins and steroids. Like Pal et al (1985) we also found that the ethanolic extract was active against *B. subtilis*. Moreover, they found that the ethanolic extract was more effective in cases of *S. aureus* and *E. coli*. It has been reported that different solvents have different extraction capabilities (Majorie, 1999; Srinivasan et al., 2001). Effectiveness of *S. indica* ethanol extract was appeared to be due to the involvement of alkaloids, flavonoids, glycosides, saponins and tannins.

Today, most pathogenic organisms are becoming resistant to antibiotics

(Chandarana et al., 2005). To overcome this alarming problem, the discovery of novel active compounds against new targets is a matter of urgency. Thus, *M. oleifera* and *S. indica* could become promising natural antimicrobial agents with potential applications in pharmaceutical industry for controlling the pathogenic bacteria. Farooq et al (2007) reported that plants occur in varying habitats, a great magnitude of variation in the concentration and composition of phytochemical ingredients in the different parts of such plant is expected. Moreover, Waller and Nowacki (1978) reported that phytochemicals are produced in response to perceived threats by the plants, therefore variation exist in the production of these phytochemicals depending on the type and amount of threat encountered by the plant.

The leaf extracts of *M. oleifera* and *S. indica* showed an appreciable inhibitory activity against the selected bacterial pathogens. *Moringa* leaves have been reported to be a good source of natural antioxidants such as ascorbic acid, phenolics and aryttenoids (Dillard and German, 2000). *Saraca indica* is used in traditional medicine against different ailments like, gynecological disorders, uterine fibroids, burns, diarrhea (Satyavati et al., 1970).

This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases. However, if plant extracts are to be used for medicinal purposes, issues of safety and toxicity will always need to be considered.

Table.1 Antimicrobial activity of *Moringa oleifera* by disc diffusion method

Organisms	Zone of Inhibition (mm)						
	Leaves Extracts (30 mg/ml)						Positive Control Gentamycin (30 mg/ml)
	Aqueous	Acetone	Chloroform	Ethanol	Methanol	Petroleum Ether	
<i>Staphylococcus aureus</i>	10mm	R	R	R	R	R	12mm
<i>Bacillus subtilis</i>	R	R	R	R	R	R	R
<i>Escherichia coli</i>	12mm	R	R	12mm	R	R	15mm
<i>Klebsiella pneumoniae</i>	R	R	R	R	R	R	R
<i>Proteus vulgaris</i>	10mm	R	R	10mm	10mm	R	8mm
<i>Salmonella typhi</i>	12mm	R	R	10mm	10mm	R	10mm
<i>Pseudomonas aeruginosa</i>	R	R	R	R	R	12mm	R

Where, R= Resistant

Table.2 Antimicrobial activity of *Saraca indica* by disc diffusion method

Organisms	Zone of Inhibition (mm)						
	Leaves Extracts (30 mg/ml)						Positive Control Gentamycin (30 mg/ml)
	Aqueous	Acetone	Chloroform	Ethanol	Methanol	Petroleum Ether	
<i>Staphylococcus aureus</i>	R	14mm	16mm	16mm	29mm	18mm	14mm
<i>Bacillus subtilis</i>	R	20mm	18mm	18mm	14mm	16mm	14mm
<i>Escherichia coli</i>	12mm	R	13mm	R	14mm	26mm	12mm
<i>Klebsiella pneumoniae</i>	10mm	12mm	12mm	R	11mm	14mm	11mm
<i>Proteus vulgaris</i>	R	22mm	20mm	14mm	14mm	18mm	12mm
<i>Salmonella typhi</i>	R	24mm	R	R	R	16mm	13mm
<i>Pseudomonas aeruginosa</i>	R	R	14mm	R	R	18mm	13mm

Where, R= Resistant

Figure.1 Antimicrobial activity of *Moringa oleifera* against bacterial pathogens

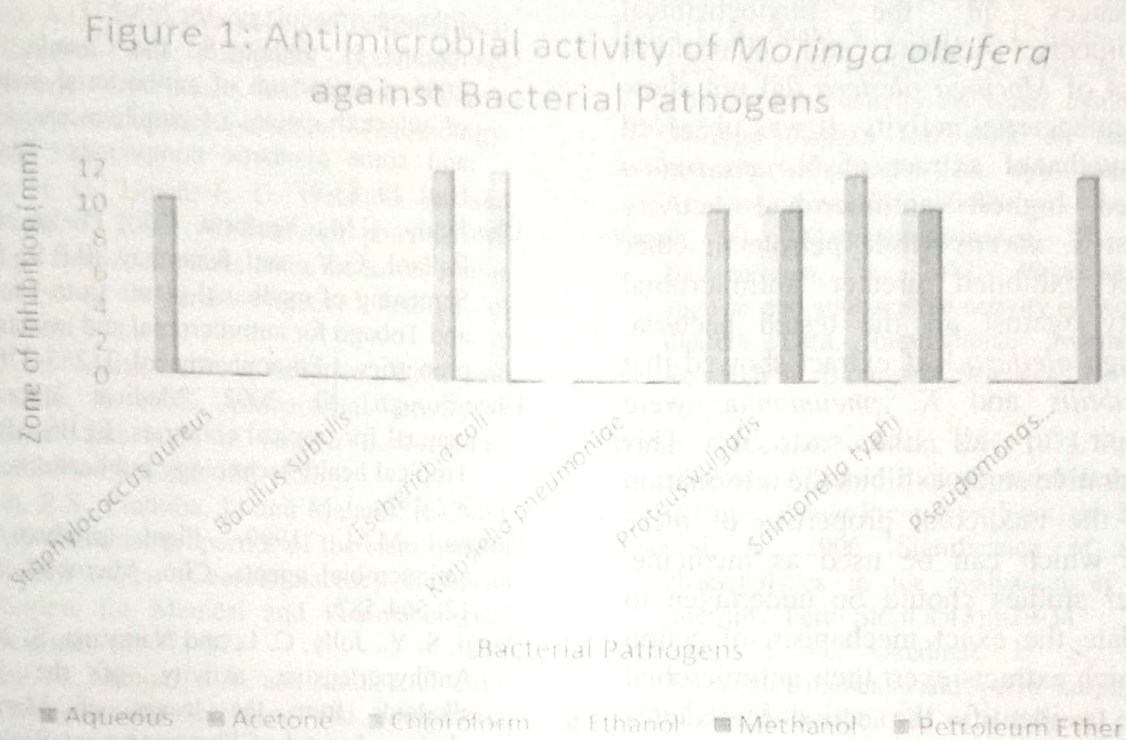
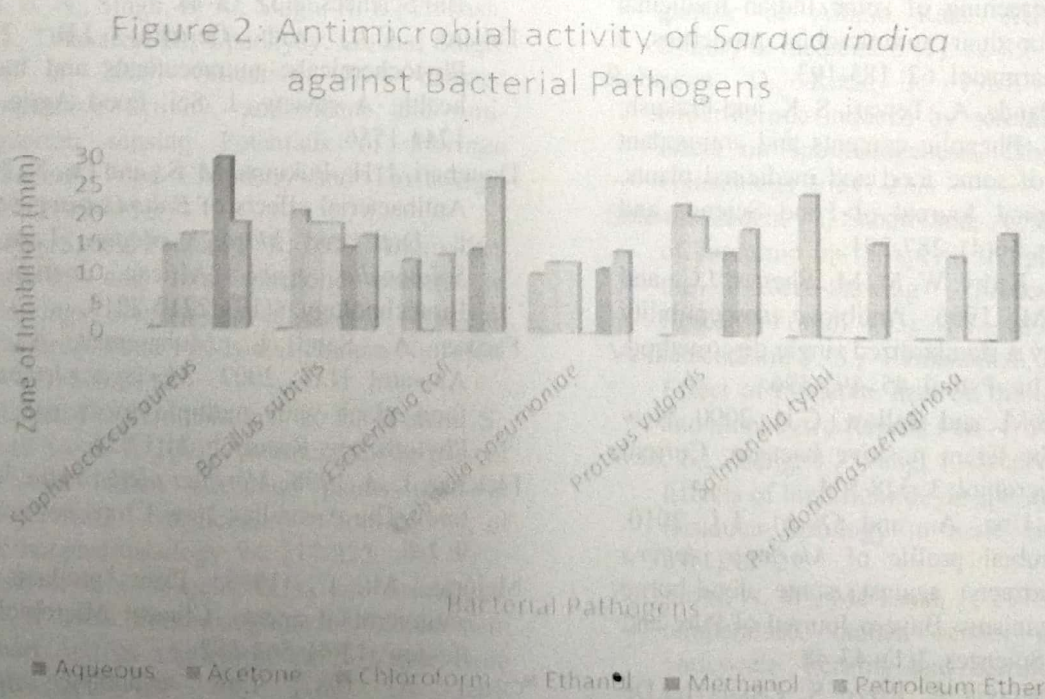


Figure.2 Antimicrobial activity of *Saraca indica* against bacterial pathogens



In conclusion, the extracts studied here have displayed variable antibacterial activities most probably due to the differences in the phytochemical constituents. Acetone and chloroform extract of *Moringa oleifera* did not show any antibacterial activity. It was observed that methanol extract of *Saraca indica* showed highest antimicrobial activity against *S. aureus* while petroleum ether extract exhibited greater antimicrobial activity against all the tested bacteria. *Moringa oleifera* leaf extract showed that *B. subtilis* and *K. pneumoniae* were resistant to all the extracts. This comparative study exhibits the information about the medicinal properties of these plants which can be used as medicine. Further studies should be undertaken to elucidate the exact mechanism of action by which extracts exert their antimicrobial effect to identify the active ingredients which can be used in drug development program for safe health care services.

References

- Ahmad, I., Mehmood, Z. and Mohammad, F. 1998. Screening of some Indian medicinal plants for their antimicrobial properties. *J Ethnopharmacol.* 62: 183-193.
- Bajpai, M., Pande, A., Tewari, S. K. and Prakash, D. 2005. Phenolic contents and antioxidant activity of some food and medicinal plants. *International Journal of Food Science and Nutrition.* 56(4): 287-291.
- Bauer, A. W., Kirby, W. M. M., Sherris, J.C., and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* 45: 493-496.
- Bhavnani, S.M. and Ballow, C.H. 2000. New agents for Gram positive bacteria. *Current Opin. Microbiol.* 3: 528-534.
- Bukar, A., Uba, A., and Oyeyi, T.I. 2010. Antimicrobial profile of *Moringa oleifera* Lam. extracts against some food-borne microorganisms. *Bayero Journal of Pure and Applied Sciences.* 3(1): 43-48.
- Caceres, A., Saravia, A., Rizzo, S., Zabala, L., De Leon, E., and Nave, F. 1992. Pharmacologic properties of *Moringa oleifera*. 2: Screening for antispasmodic, anti inflammatory and diuretic activity. *Journal of Ethnopharmacology.* 36: 233-237.
- Chandarana, H., Baluja, S., and Chanda, S. V. 2005. Comparison of antibacterial activities of selected species of zingiberaceae family and some synthetic compounds. *Turk. J. Biol.* 29: 83-97.
- Charlandy, C.M., Seaforth, C.E., Phelps, R.H., Pollard, G.V., and Khambay, B.P.S. 1999. Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. *J Ethnopharmacol.* 3: 265-270.
- Cheesbrough, M. 2002. Medical laboratory manual for tropical countries. ELBS edition. Tropical health technology publications, UK. 2:2-392.
- Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12: 564-582.
- Dangi, S. Y., Jolly, C. I., and Narayana, S. 2002. Antihypertensive activity of the total alkaloids from the leaves of *Moringa oleifera*. *Journal of Pharmaceutical Biology.* 2 (40): 144-148.
- Devendra, B.N., Srinivas, N., Talluri, V.S.S.L. Prasad, and Latha, P.S. 2011. Antimicrobial activity of *Moringa oleifera* Lam., leaf extract, against selected bacterial and fungal strains. *International Journal of Pharma and Bio Sciences.* 2(3): 13-18.
- Dillard, C.J., and German, J.B. 2000. Phytochemicals: nutraceuticals and human health: A review. *J. Sci. Food Agric.* 80: 1744-1756.
- Doughari, J. H., Pukuma, M. S., and De, N. 2007. Antibacterial effects of *Balanites aegyptiaca* L. Drel. and *Moringa oleifera* Lam. on *Salmonella typhi*. *African Journal of Biotechnology.* 6(19): 2212-2215.
- Farooq, A., Sajid, L., Muhammad, A., and Anwarul H.G. 2007. *Moringa oleifera*: a food plant with multiple medicinal uses. *Phytotherapy Research.* 21:17-25.
- Fletcher, L. J. 1998. *Moringa oleifera* (the kelor tree). *The Australian New Crops newsletter.* 9: 1-6.
- Majorie, M. C. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Review.* 12(4): 564-582.
- Olsen, A. 1987. Low technology water

- purification by bentonite clay and *M. oleifera* seed flocculation as performed in Sudanese villages: effects on *Schistosoma mansoni* cercariae. *Water Res.* 21: 517-522.
- Oluduro, A.O. 2012. Evaluation of Antimicrobial properties and nutritional potentials of *Moringa oleifera* Lam. leaf in South-Western Nigeria. *Malaysian Journal of Microbiology*, 8(2): 59-67.
- Oluma, H. O., Umeh, E. U., Onekutu, A., and Okolo, J. 2004. Antibacterial potentials of eight medicinal plants from the lower Benue valley of Nigeria. *Nigerian Journal of Botany*. 17: 1-11.
- Pal, S.C., Maiti, A.P., Chatterjee, B.P., and Nandy, A. 1985. Antibacterial activity of flower and buds of *Saraca indica* Linn. *Ind.J.Med.Res.* 82: 188-189.
- Sainath, R.S., Prathiba, J., and Malathi, R. 2009. Antimicrobial properties of the stem bark of *Saraca indica* (Caesalpinaceae). *European Review for Medical and Pharmacological Sciences*. 13: 371-374.
- Sarojini, N., Manjari, S.A., and Kanti, C.C. 2011. Phytochemical screening and antibacterial activity study of *Saraca indica* leaves extract. *International Research Journal of Pharmacy*. 2(7): 176-179.
- Satyavati, G.V., Prasad, D.N., Sen, S.P., and Das, P.K. 1970. Further studies on the uterine activity of *Saraca indica* Linn. *Indian J. Med. Res.* 58: 947-960.
- Singh, B. N., Singh, B. R., Singh, R. L., Prakash, D., Dhakarey, R., Upadhyay, G., and Singh, H. B. 2009. Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing Potentials of *Moringa oleifera*. *Food Chemistry and Toxicology*. 47: 1109-1116.
- Sreelatha, S., and Padma, P. R. 2009. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. *Plant Foods and Human Nutrition*. 64(4): 303-311.
- Srinivasan, D., Perumalsamy, L. P., Nathan, S., and Sures, T. 2001. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *Journal of Ethnopharmacology*. 94: 217-222.
- Srivastava, G.N., Bagchi, G.D., and Srivastava, A.K. 1988. Pharmacognosy of Ashoka stem bark and its adulterans. *Int. J. Crude Drug Res.*, 26(2): 65.
- Sultana, B., Anwar, F., and Ashraf, M. 2009. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves. *Molecules*. 14(6): 2167-2180.
- Thilza, L. B., Sanni, S., Zakari, A. I., Sanni, F. S., Talle, M., and Joseph, B. M. 2010. *In vitro* Antimicrobial activity of water extract of *Moringa oleifera* leaf stalk on bacteria normally implicated in eye diseases. *Academia Arena*. 2(6): 80-82.
- Vinoth, B., Manivasagaperumal, R., and Balamurugan, S. 2012. Phytochemical analysis and antibacterial activity of *Moringa oleifera* Lam. *International Journal of Research in Biological Sciences*. 2(3): 98-102.
- Waller, G.R., and Nowacki, E.K. 1978. Role of alkaloids. *Alkaloid Biology and Metabolism in Plants*. Plenus Press, New York, pp. 249.
- Agarwal, A., 2006. Significance of sperm characteristics in the evaluation of male infertility. *Fertil Steril* 85(3):629-34
- Oyeyemi, M. O., Okediran, B. S., 2007. Testicular parameters and sperm morphology of chinchilla rabbit fed with different planes of Soymeal- *Int. J.Morphol*, 25 (1): 139-144.
- Parhizkar, S., Yusoff, M. J., Dollah, M. A., 2013. Effect of *Phaleria macrocarpa* on Sperm Characteristics in Adult Rats. *Advan Pharmace Bullet* 3(2): 345-352.
- Purushottam Pramanik, Snenangshu Biswas, 2012. Traffic noise: A silent killer of male gamete of Albino Rats. *AUS National Library of medicine* 5 (1): 82-89.
- Robinson, D., Rock, J., 1967. Intrascrotal hyperthermia induced by scrotal insulation: effect on spermatogenesis. *Obstetrics and Gynecology* 2: 217-233.
- Soleimanzadeh, A., Saberivand, A., 2013. Effect of curcumin on rat sperm morphology after the freeze-thawing process *Veterinary Research Forum*. 4 (3): 185 – 189.
- Venkatachalam, P. S., Ramanathan, K. S., 1962. Effect of moderate heat on the testes of rats and monkeys. *J Reprod Fert* 4: 51.
- Wan, S., Zhang, J., Wang, J., Shanxi, B., 2006. Effects of high fluoride on sperm quality and testicular histology in male rats. *Fluoride* 39(1):17-21.
- Zorgniotti, A. W., McLeod, J., 1973. Studies in temperature, human semen quality and varicocele. *Fertility and Sterility* 24: 854-863.