**Journal of Agri-Food and Applied Sciences**
Available online at jaas.blue-ap.org
©2014 JAAS Journal. Vol. 2(1), pp. 15-21, 31 January, 2014
E-ISSN: 2311-6730

# In vitro Antimicrobial activity of *Rosmarinus officinalis* leave extracts

**Shama IY. Adam[1], Abdullah AY. Ahmed[2], Adam KM. Omer[2], Aldai MA. Bashir[2], Omer AM. Abdel Rahman[2] and Warda S. Abdelgadir[3]***

1- Al- Neelain University, Faculty of Science and Technology, Department of Biochemistry and Molecular Biology
2- Omdurman Islamic University, Faculty of Science and Technology, Department of Botany
3- Food Research Centre, P. O. Box 213, Khartoum North, Sudan

**Corresponding Author:** Warda S. Abdelgadir

*Received: 25 December, 2013* *Accepted: 10 January, 2014* *Published: 31 January, 2014*

**ABSTRACT**

A study was performed in which petroleum ether, methanol and water extracts of *Rosamarinus officinalis* leaves in different concentrations (5, 12.5 and 25%) were evaluated for their possible antimicrobial activity against six standard pathogenic microorganisms, *Staphylococcus aureus* (gram-positive bacteria), *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (gram-negative bacteria) in addition to the fungi *Candida albicans* and *Aspergillus niger*. The aqueous extract in different concentrations exhibited antimicrobial activity against all tested organisms (except *Ps. aeruginosa* at 5%) and that the activity is concentration –dependent. At all concentrations, the petroleum ether extract had no activity against *S. aureus*, *P. aeruginosa* and *C. albicans* but the growth of *E. coli* and *Proteus vulgaris* was not inhibited by 12.5 and 25% concentrations and *A. niger* by 25%. Except for *C. albicans* which was inhibited by all methnol extract concentrations, other organisms grew at 5% concentration and inhibited by 12.5 and 25%. Findings were compared to those produced by Gentamicin and Nystatin (10 µg), reference antibiotic and antifungal respectively..

***Keywords:*** *agar well diffusion method, antimicrobial activity, methanolpetroleum ether, water, Rosemarinus officinalis methanol*.


## INTRODUCTION

Medicinal plants have been used for centuries as traditional health remedies for human diseases because they contain chemical components of therapeutic value (Nostro, *2000*). Their usage is most popular for 80% of world population in Asia, Latin America and Africa and is reported to have minimal side effects (Bibitha, 2002 and Maghrani, 2005).

Natural products are known to play an important role in both drug discovery and chemical biology. In fact, many of the recently available drugs either mimic naturally occurring molecules or have structures that are fully or in part derived from natural motifs (Cheesbrough, 2000). Natural antimicrobials can be derived from different parts of the plant (barks, stems, leaves, flowers and fruits), various animal tissues or from microorganisms (Gordon and David, 2001). Although some therapeutic benefits can be traced to specific plant compounds, herbs contain mainly active constituents that, together, combine to give the plant its therapeutic value. Consequently, it is believed that the whole plant has more effective healing properties than its isolated constituents (Nair and Chanda, 2004). Bioactive compounds such as glycosides, alkaloids and terpenes are examples contained in some plants and could be used as drugs and antimicrobial agents (Edeogal, 2005). Many extracts and

essential oils have been derived from plants and found to have antibacterial, fungicidal and insecticidal properties (Hänsel, 1999).

*Rosmarinus officinalis*, a member of the family *Lamiaceae*, locally known as Rosemary, is a flowering plant that grows in Mediterranean countries, southern Europe and in the littoral region through Minor Asia areas wildly. It is a perennial plant forming a stiff shrub, much branched and densely bushy, with a characteristic aromatic smell. (Khorshid., 2009; Pintore, 2002; Bicchi, 2000) (Fig. $1_a$ Fig $1_b$)

Figure $1_a$. Rosmarinus officinalis L

Figure $1_b$. Rosmarinus officinalis L leaves

A study by Mark, (2003) showed that rosemary produced a significant enhancement of performance for overall quality of memory and secondary memory factors. It was also found to be used as an anti-cramp, diuretic, a tonic for the nerves, digester, anti-rheumatic, for wounds healing and a tonic for the circulation and the heart. It is described in cases of congestion of the liver, inflammation of the gall bladder, gastric lavage, in some cases of jaundice, fatigue, physical and intellectual weakness following the diseases debilitating to the body, migraine, dizziness, palpitations, jittering, strikes, heartburn, carminative and as an anti-antiseptic, useful in the coming flu and asthma, (Antoine, 1998).

The present study on *R. officinals* leaves was planned in order to assess the chemical composition of the leaves and to estimate the possible antimicrobial activities of the leaves petroleum ether, methanol and water extracts at concentrations of 5, 12.5 and 25% against four pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and two fungi ( *Candida albicans*, *Asperagillus nigar*) the antimicrobial activity of plant leaves extracts were compared to reference antibacterial and antifungal drugs.

## MATERIALS AND METHODS

### *Materials*

### *Plant materials*

*Rosemarinus officinalis* leaves were obtained from a local market in Omdurman, Sudan. The plant tissues were cleaned, shade dried and ground using a mechanical grinder.

### *Standard microorganisms*

Four strains of bacteria, yeast and were used as test microorganisms. The bacterial strains included Gram positive Staphylococcus aureus (ATCC/25923), and Gram-negative *Escherichia coli* (ATCC/27853), *Proteus vulgaris* (NCTC/4175), *Pseudomonas aeruginosa* (ATCC/27853), Yeast *Candida albicans* (ATCC/7596) and fungus *Aspergillus niger* (ATCC/9763). All microorganisms were clinical isolates, kindly provided by the scientists at Khartoum National Health Laboratory. All the cultures were maintained and sub cultured on nutrient agar medium.

NCTC: National Collection of Type Culture, Colindale, England.

ATCC: American Type Culture collection, Rockville, Maryland, USA.

Antibiotics: Gentamicin, Roussel Laboratories Ltd, England and Nystatin, Sigma chemical Company, U.S.A.

### *Culture media*

Nutrient broth (Oxoid Ltd, London) form the basis of most media used in microbiological studies and it is prepared according to the manufacturer instructions. Nutrient agar was used to prepare an enriched culture media for bacteria.

For fungi, Sabouraud's agar (Difco, USA) was used as enriched culture media.

***Methods***

***Preparation of plant extracts***

The powdered plant tissues were extracted separately. A sample (50 g plant leaves) was accurately weighed, and extracted with petroleum ether (60-80℃ for 2 hrs) in a soxhlet apparatus. The petroleum extract was evaporated by a Buchi Rota evaporator under reduced pressure. The plant residue was then air- dried, repacked in soxhlet apparatus and extracted with methanol (99.8% for 2 hr). The extract was similarly evaporated exhaustively, air dried and yield was recorded. In a conical flask, the plant residue was further extracted with distilled water over night at room temperature (25-30℃), filtered and freeze dried.

***Preparation of the standard bacterial suspensions***

One ml aliquots of a 24-hours broth culture of the test organisms was aseptically distributed onto nutrient agar slopes and incubated at 37 ºC for 24-hours.The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about $10^8$ -$10^9$ colony forming units per ml. The suspension was stored in the refrigerator at 4ºC till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique (Collee, *et al*. 1996). Serial dilution of the stock suspensions were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micropipette to the surface of dried nutrient agar plates. The plates were allowed to stand for 2-hours at room temperature for the drops to dry, and then incubated at 37ºC for 24 hours .After incubation, the number of developed colonies in each drop was counted. The average number of the colonies per drop (0.02ml) multiplied by 50 and by the dilution factor to give the viable count of stock suspensions, expressed as the number of colony forming units per ml of suspension. Each time a fresh stock suspension was prepared, all the above experimental condition was maintained constant so that suspensions with very close viable count should be obtained.

***Preparation of standard fungal organisms***

The fungal standard cultures from the Medicinal and Aromatic Plant Research Institute were maintained on Peptone water agar, incubated at 25ºC for 4 days. The fungal growth mats were harvested and washed with sterile normal saline and finally suspended in (100 ml) of sterile normal saline and suspension was stored in refrigerator till used.

***Antibacterial Activity***

At the time of testing, the extracts were reconstituted to concentration of 2.5, 5 and 10 per cent in dimethyl sulphoxide (DMSO). Antimicrobial activity was assessed by the agar–well diffusion method (Kinsbury and Wagner, 1990). The inoculums size of each tested bacterium was adjusted to a suspension of $10^6$ cells. The inoculums suspension was spread over a Mueller Hinton agar (MHA) plate, to achieve confluent growth, and allowed to dry. 10 mm- diameter wells were bored in the agar using a sterile cork borer (NO.4) and the agar discs were removed .A 100 µl - aliquot of the reconstituted extract was placed into a well with standard Pasteur pipette and the plate was held for 1 hr at room temperature for diffusion of extract into the agar. Subsequently, the plate was incubated for 18 hr at 37ºC. After incubation, the diameters of the zones of inhibition were measured to the nearest mm. Three replicates were performed and results were recorded.

***Evaluation of antimicrobial activity of Gentamicin and Nystatin***

This was estimated by the agar well diffusion method (Kinsbury and Wagner, 1990). One ml of a 24 hr- incubated broth culture was distributed onto Mueller–Hinton agar. The Gentamicin (Abtech Biologicals Ltd, England) at concentration of 10 µg was used as a reference drug and distributed in the centre of Mueller-Hinton agar and the plate was kept for one hour at room temperature. The plates were then incubated in the upright position at 37°C for 18 hr. After incubation, the diameters of inhibition zones were recorded.

***Statistical analysis***

The experiments were laid out according to randomized block design. Each zone of inhibition experiment usually had three replicates and the mean of three replicates was calculated. The analysis of variance (ANOVA) appropriate for the design was carried out to detect significance of differences among the treatment means.

## RESULTS AND DISCUSSION

***Yield and properties of R. officinalis leaves extracts***

The rosemary (*R. officinals*) leaves water extract had the highest yield (24.3%) which is a white powder. The petroleum ether extract had the lowest yield (8.6%) than methanol and water extracts and it was a yellow sticky paste. A brown sticky

paste extract was obtained when the leaves were extracted with methanol giving a yield of 17.9%. This phenomenon occurred due to the fact that non-polar organic solvent (Petroleum ether) was used to separate lipids before continued with other solvents utilization (Harborne, 1973).

***Evaluation of in vitro antimicrobial activity of R. officinalis leaves extracts***

For screening the extracts of Rosemary for antimicrobial activity, the agar well diffusion method was used. According to Omenka and Osuaba (2000), this method allows better diffusion of the extracts into the medium thus enhancing contact with the organisms. In the paper disc method, the discs may act as barriers between the extract and the organisms thus, preventing total diffusion of active components absorbed by the discs into the medium and may be responsible for the observed differences.

Results of antibacterial activity of different concentrations of Rosemary extracts are shown in Table 1, figures & plates. Results showed that the water was the best solvent for extracting antimicrobial substances from this plant compared to petroleum ether and methanol (organic solvents). The highest activity was demonstrated against *A. niger*, *Candida albicans* and *Proteus vulgaris* at the concentration 25% (zone of inhibition 26, 23 and 21 mm respectively) (Figs. 2&3, plates 1&2) while the lowest activity (zone of inhibition 10 mm) was demonstrated at the concentration 5% against *E. coli.* The concentration of 5% of this extract was not active against *P .aeruginosa.* Antibacterial activity of both aqueous and ethanolic extracts of the 10 plants studied by Nair and Chanda (2007) showed that the aqueous extracts, only that of *Emblica officinalis* Gaertn leaves showed maximum activity against *P. aeruginosa*; none of the others showed any activity.

The methanolic extract had no antibacterial activity at the concentration of 5% but only inhibited *Candida albicans* with an inhibition zone of 9 mm. The concentrations of 12.5 and 25% were found to have antibacterial (Plate 2) as well as antifungal activity, except for *Pseudo. aeruginosa* which was inhibited only by the highest concentration (25%) with an inhibition zone of 10 mm. In a study by Panthy and Chaudhary (2006) on the methanol extract of *Justicia adhatoda* L leaves of Nepal, it was found that the extract was active against *Staph. aureus* and *Pseudo. aeruginosa* but not against *E. coli.*.

A narrow range of antimicrobial activity was exhibited by the petroleum ether extract. At all concentrations, it was not active against *staph. aureus*, *Pseudo.aeruginosa and C. albicans*. The highest activity was shown against *E. coli* at 25% with an inhibition zone of 15 mm.

In classifying the antibacterial activity as gram-positive or gram-negative, it would generally be expected that a much greater number would be active against gram-positive than gram-negative bacteria (McCutcheon *et al.* 1992). Even though, in the present study both Gram- positive and Gram–negative bacteria were inhibited by the tested extracts indicating that the antimicrobial activity is dependent on diversity of active ingredients in the plant extract. However, the numbers of bacteria used in screening have been restricted to four: three gram-negative *(Pseudo. aeruginosa, E. coli* and *Proteus vulgaris*) and one gram-positive *(S. aureus)* due to limitation of resources. In previous studies it was shown that Gram positive bacteria had high resistance to the activity of the extracts used (Suresh Kumar *et al.,* 2010). The authors attributed this phenomenon to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergents and hydrophilic dyes as indicate by Nikaido and Vaara, (1985).

Table 1. Antimicrobial activity of *Rosmarinus officinalis* extracts

| Microorganism | Extract concentration % | (Inhibition zone mm) | | |
|---|---|---|---|---|
| | | Petroleum ether extract | Methanolic extract | Aqueous extract |
| *Staph. aureus* | 5 | (-) | (-) | 11±0.14 |
| | 12.5 | (-) | 13±0.26 | 16±0.12 |
| | 25 | (-) | 16±0.17 | 20±0.12 |
| *E. coli* | 5 | (-) | (-) | 10±0.20 |
| | 12.5 | 12±0.14 | 10±0.11 | 15±0.16 |
| | 25 | 15±0.12 | 18±0.22 | 20±0.24 |
| *Proteus vulgaris* | 5 | (-) | (-) | 11±0.10 |
| | 12.5 | 10±0.24 | 9±0.18 | 16±0.14 |
| | 25 | 12±0.11 | 12±0.20 | 21±0.18 |
| *Pseud. aeruginosa* | 5 | (-) | (-) | (-) |
| | 12.5 | (-) | (-) | 12±0.22 |
| | 25 | (-) | 10±0.20 | 17±0.20 |

(-) = No inhibition zone observed.

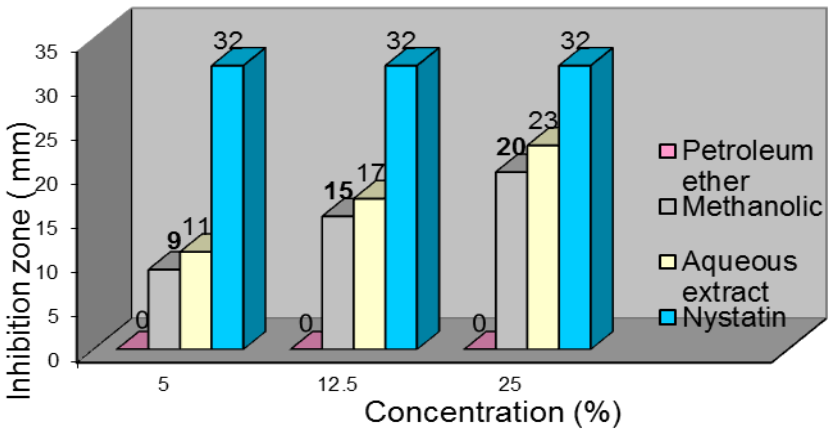


Figure 1. Antifungal activity of R.officinalis extracts against candida albicans

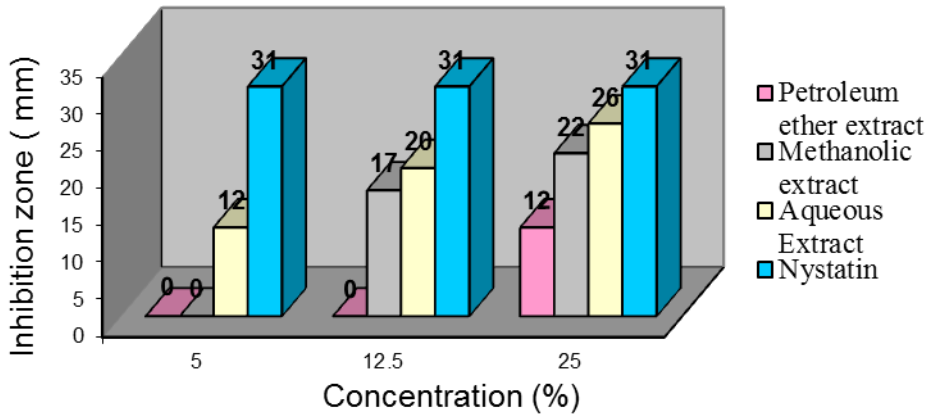


Figure 2. Antifungal activity of R. officinalis extracts against aspergillus niger

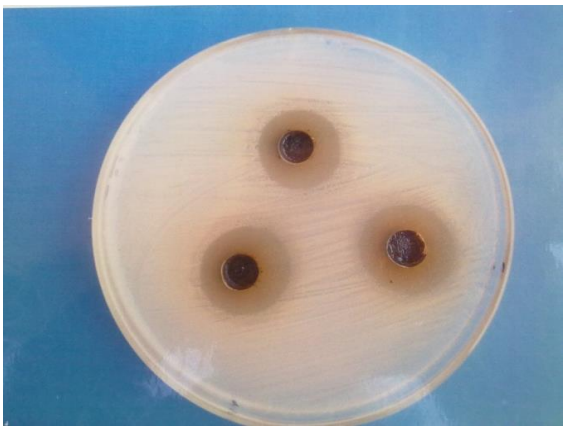

Figure 3. Inhibition zone (20 mm) produced by aqueous extract (25%) of *R. officinalis* against *Candida Albicans*

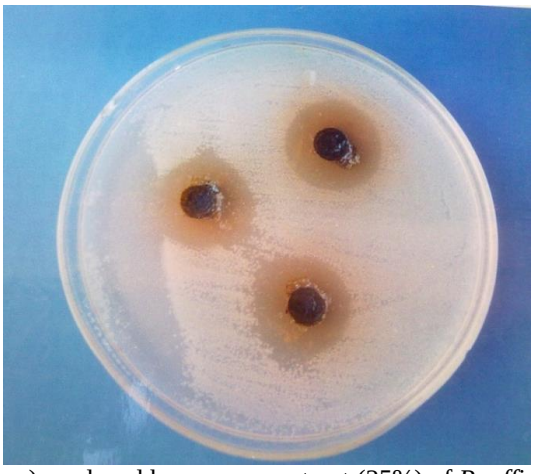

Figure 4. Inhibition zone (21 mm) produced by aqueous extract (25%) of *R. officinalis* against *Proteus vulgaris*

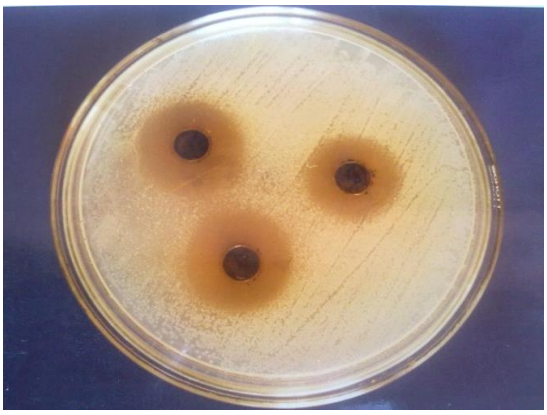

Figure 5. Methanol extract of *R. officinalis* (25%) against *S. aureus* inhibition zone (21 mm)

Table 3. Evaluation of antimicrobial activity of Gentamicin and Nystatin

| Microorganism | Inhibition zone (mm) |
|---|---|
| Gentamicin (10 µg) | |
| Staph. aureus | 22±0.16 |
| E.coli | 22±0.20 |
| Proteus .vulgaris | 19±0.20 |
| Pseudo..aeruginosa | 26±0.12 |

Phytochemical screening of the plants Family Laminaceae revealed the presence of alkaloids, saponins, tannins, anthraquinones, cardiac glycosides, flavonoids and phlobatannins (Sofowora, 1993). The presence of more bioactive phytochemical compounds may increase the antimicrobial activity of the plant, since it is believed that the active compounds produced by the plant inhibit the life processes of microbs especially the disease causing ones (Luc and Amold, 2005)

Antimicrobial activity may involve complex mechanisms, like the inhibition of cell membranes, nucleic acids and proteins synthesis as well as inhibition of nucleic acid metabolism (Oyaizu *et al.,* 2003). It seems likely that the active substances in the extract act separately or in concert to exert these effects. Taking into consideration the properties of organic solvent used for the extraction, the extracts seem to contain diverse substances, ranging from polar to non-polar compounds Moniharapon and Hashinaga 2004.

In this study the antibacterial activity of *R. officinalis* leaves petroleum ether, methanolic and water extracts has been compared with that of gentamycin, a well known aminoglycoside antibiotic and Nystatin the antifungal drug (Table 3). The antibacterial activity of *R. officinalis* leave aqueous extract at 25% concentration against *staph. aureus*, *E. coli and Proteus vulgaris* was slightly lower than that of Gentamycin. The antifungal inhibitory activity of the same extract at the same concentration was stronger when compared to its antibacterial one.

## CONCLUSION

Since this preliminary study of petroleum ether, methanolic and aqueous extracts of *R. officinalis* detected their antimicrobial activity against the pathogens *Staph. aureus*, *E. coli*, *Proteus vulgaris*, *pseudo. Aeruginosa*, *C. albicans* and *A. niger*, they can be recommended for use in folkloric medicine However the specific antimicrobial principles inherent to the plants as well as the mode or mechanism of action of these active compounds need to be fully investigated.

## ACKNOWLEDGEMENT

Thanks are due to the head, Department of Biochemistry and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, for facilities and sincere advice.

## REFERENCES

Antoine BK 1998. Hungarian Musuotai of medicinal plants, Part I: Plants: Nature drug store, the 1st edition p: 63-67.

AOAC 1990. Assoiciation of Official Analystical Chemists, Official Methods of Analysis 15th ed. Assoc., of Analytical Chemists, Washington, DC, USA.

Bibitha B, JishaVK, Salitha CV, Mohan S and Valsa AK. 2002. Antibacterial activity of different plant extracts. Short Communiaction. Indian J Microbiol. 42: 361-363.

Bicchi C, Binello A and Rubliolo P. 2000. Determination of phenolic diterpene antioxidants in Rosemary (*Rosmarinus officinalis L*) with different methods of extraction and analysis, phytochemical analysis: Phytochemical Analysis; 11:236-242.

Cheesbrough M. 2000. Medical Laboratory Manual for Tropical Countries, Butterworth, Oxford, pp. 260, 2000.

Collee GJ, Fraser GA, Marmion PB and Simmon A. 1996. Practical MediclMicrobiology. 14th Edn., Churcill Livinstone, Edinburg, pp: 163-174.

Edeogal H, Okwu D and Mbaebie B. 2005. Photochemical constituents of some Nigerian medicinal plants. Afr. J. Biotech., 4: 685-688.

Gordon MC and David JN. 2001. Natural product drug discovery in the next millennium. Pharm Biol 39: 8-17.

Hänsel R, Sticher O and Steinegger E. 1999. Pharmakognoisie-Phytopharmzie, 6th ed., Springer-Verlag, Berlin, pp.692-695.

Harborne JB. 1973. Phytochemical methods. A Guide to Modern Techniques of Plant Analysis. Chapman and Hall, London.

Khorshidi J, Mohammadi R, Fakhr MT and Nourbakhsh H. 2009. Influence of Drying Methods, Extraction Time, and Organ Type on Essential Oil Content of Rosemary (*Rosmarinus officinalis L*.). Nature and Science. 7(11): 42-44.

Kingsbury DT and Wagner GE. 1990. Microbiology. Williams and Wilkins. Maryland, p. 36.

Luc P and Amold JV. 2005. Bioguided isolation of pharmacologically active compounds still a valuable strategy for the finding of new lead compounds. J. Ethnopharmacol. 100:57-60.

Maghrani M, Zeggwah N, Michel J and Eddouks M. 2005. Antihypertensive effect of *Lepidium sativum* in spontaeneously hypertensive rats. J Ethnopharm, 102 (1-2):193-197.

Mark M, Jenny C, Keith W and Paul D. 2003. Aromas of rosemary and lavender essential oils differentially affect cognition and mood in healthy adults. The International Journal of Neuroscience, 113 (1): 15–38.

McCutcheon AR, Ellis SM, Hancock REW and Towers GHN. 1992. Antibiotic screening of medicinal plants of the British columbian native peoples. Journal of Ethnopharmacol. 37:213-223.

Moniharapon E and Hashinaga F. 2004. Antimicrobial activity of Atung (*Parinarium glaberrimum* Hassk) Fruit Extract. Pakistan J. of Biological Sciences, 7(6): 1057-1061.

Nair R and Chanda S. 2004. Antibacterial activity of some medicinal plants of Saurashtra region. J. Tissue Res. 4: 117-120.

Nikaido H and Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49: 1-32.

Nostro N, Germano M, Ngelo VD and Cannatelli M. 2000. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett. Appl. Microbiol. 30: 379-384.

Omenka CA and Osuoha JO. 2000. Antimicrobial potency of Grapefruit seed extract on five selected pathogens. Nigerian Journal of Microbiology. 14 (2): 39-42.

Oyaizu M, Fujimoto Y, Ogihara H, Sekimoto K, Naruse A and Naruse U. 2004. Antioxidative and antimicrobial activities of extracts from several utility plants. Food Preserv. Sci. 29:33-38.

Panthi MP and Chaudhary RP. 2006. Anibacterial activity of some selected folklore medicinal plants from West Nepal. Scientific World. 4(4):16-21.

Pintore G, Usai M, Bradesi P, Juliano C, Boatto G, Tomi F, Chessa M, Cerri R and Casanova J. 2002. Chemical composition and antimicrobial activity *of Rosmarinus officinalis L*. oil from Sardinia and Corsica. Flav. Fragr. J. 17: 15-19.

Sofowora A. 1993. Phytochemical screening of medicinal plants and traditional medicine in Africa, 2nd Edition. Spectrum Books Ltd Nigeria, pp. 150 – 156.

Suresh K, TV, Negi PS and Udaya SK. 2010. Antibacterial Activity of *Nigella sativa* L. Seed Extracts. British J. of pharmacol. Toxicol. 1(2): 96-100.