



Research Paper

Afr. J. Traditional,
Complementary and Alternative
Medicines
www.africanethnomedicines.net

ISSN 0189-6016©2008

ANTIMICROBIAL ACTIVITY OF VARIOUS EXTRACTS OF *OCIMUM BASILICUM* L. AND OBSERVATION OF THE INHIBITION EFFECT ON BACTERIAL CELLS BY USE OF SCANNING ELECTRON MICROSCOPY

Ilhan Kaya¹, Nazife Yiğit², Mehlika Benli^{*3}

¹Yuzuncuyil University, Faculty of Agriculture, Department of Plant Protection, Van, Turkey

²Kirikkale University, Faculty of Science and Arts, of Biology, Kirikkale, Turkey

³Ankara University, Faculty of Science, Department of Biology, Ankara, Turkey

*E- mail: mehlikabenli_76@hotmail.com or mehlikabenli@gmail.com; Phone : +903122126720 / 1505; Fax: +903122232395

Abstract

The antimicrobial activities of chloroform, acetone and two different concentrations of methanol extracts of *Ocimum basilicum* L. were studied. These extracts were tested *in vitro* against 10 bacteria and 4 yeasts strains by the disc diffusion method. The results indicated that the methanol extracts of *O. basilicum* exhibited the antimicrobial activity against tested microorganisms. While the chloroform and acetone extracts had no effect, the methanol extracts showed inhibition zones against strains of *Pseudomonas aeruginosa*, *Shigella sp.*, *Listeria monocytogenes*, *Staphylococcus aureus* and two different strains of *Escherichia coli*. The cells of microorganisms, which were treated and untreated with plant extracts, were observed by using the scanning electron microscope. It was observed that the treated cells were damaged.

Key words: Antimicrobial activity, Disc diffusion method, *Ocimum basilicum*, Plant extract, SEM, Spices.

Introduction

Some medical plants have been used for a wide variety of purposes such as food preservation, pharmaceutical, alternative medicine, and natural therapies for many thousands of years. It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and therefore be more environmentally acceptable. Thus, natural antioxidants, antibacterial, cytotoxic, antiviral, fungicidal agents and nutrients have gained popularity in recent years, and their use and positive image among consumers are spreading. In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have been developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Davis, 1994; Service, 1995). In order to find new therapeutic agents, plants that have antimicrobial activity have attracted attention (Kalembe and Kunika, 2003; Juliani and Simson, 2002; Falerio et al., 2003).

The Labiatae family is one of the most employed medicinal plants as a worldwide source of spices and also as a consolidated source of extracts with strong antibacterial and antioxidant properties. Within this family, the genus *Ocimum* provides various species, and *Ocimum basilicum* is one of the more than 60 *Ocimum* species. These *Ocimum* plants, plant parts, extracts and essential oils are used as spices and flavours for various food products as well as effective drugs for many applications in folk medicine especially in Africa and Asia (Sacchetti et al., 2004; Jirovetz et al., 2003).

Ocimum basilicum, commonly known as basil, is naturally distributed in the East Anatolia region in Turkey. The leaves of these plants are used in traditional cuisine as spices. Basil, which is a popular culinary herb, and its essential oils have been used extensively for many years in flavouring of meats and sausages. Basil oil has also found a wide application in perfumery, as well as in dental and oral products. In addition, because the public nowadays prefers natural food additives, naturally derived antimicrobial agents such as basil, are

becoming more important in antimicrobial packaging as they present a perceived lower risk to consumers (Sappakul et al., 2003).

Leaves and flowering parts of *O. basilicum* are traditionally used as antispasmodic, aromatic, carminative, digestive, galactagogue, stomachic, and tonic agents (Chiej, 1984; Lust, 1983; Duke and Ayensu, 1985). They have also been used as a folk remedy to treat various ailments such as; feverish illnesses, poor digestion, nausea, abdominal cramps, gastro-enteritis, migraine, insomnia, depression, gonorrhoea, dysentery, and chronic diarrhoea exhaustion (Chopra et al., 1986). Externally, they have been applied for the treatment of acne, loss of smell, insect stings, snake bites, and skin infections (Martin and Ernst, 2004).

Medicinal plants have constituted an important source of active biomolecules for centuries. Although medicinal plants in Turkey are quite large in variety, ethnobotanical and pharmaceutical studies on these plants have been made only in recent years (Ates and Erdogru, 2003; Kivcak et al., 2002; Erturk et al., 2003; Ozdemir et al., 2004; Dulger and Gonuz, 2004a; Dulger and Gonuz, 2004b).

The objective of the present study was to evaluate the potential antimicrobial activities of *O. basilicum* plant extracted with acetone, chloroform and two different concentrations of methanol.

Materials and Methods

Plant material

Ocimum basilicum L. (voucher No. 1281) was selected as a test plant. Fresh plants of *O. basilicum* were collected from Van (Turkey) during June-September of 2004. These plants were identified and preserved at Yuzuncuyil University, Faculty of Agriculture, Department of Plant Protection, and then all plants were air-dried. The leaves were taken and used in this study to determine its antimicrobial activity against the test microorganisms.

Preparation of plant extracts

To prepare plant extracts three different solvents were used. Dried leaves of plants were mechanically ground, and 2 g of plant was extracted with 20 ml of acetone or chloroform or methanol, then it was gently heated. Upon heating, it was rinsed for 24 h at room temperature. Then the extracts were filtered using Whatman filter paper No. 1 and the filtrates were then evaporated in incubator at 30 °C. The dried extracts of acetone or chloroform were resuspended with 10ml distilled water. The dried extracts of methanol were resuspended with two different volumes (10 ml or 5 ml) of distilled water. The extract of suspension with 10ml distilled water was further diluted than the extract of suspension with 5ml distilled water (Ates and Erdogru, 2003; Kivcak et al., 2002; Ozdemir et al., 2004).

Test microorganisms

In vitro antimicrobial studies were carried out on ten bacteria strains (*Enterococcus gallinarum* CDC-NJ-4, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* RSHI, *Escherichia coli* ATCC 25922, *Shigella sp.* RSHI, *Escherichia coli* RSHI, *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853), and on four yeast strains (*Saccharomyces cerevisiae* Pakmaya, *Candida albicans* ATCC 845981, *Candida crusei* ATCC 6258, *Candida albicans* ATCC 90028). *E. coli*, *B. subtilis* and *Shigella* species were used in this study were pathogens from isolates from Refik Saydam Hifzissihha Institute (RSHI) (Ankara, Turkey). All strains were grown on nutrient broth (Merck).

Determination of antimicrobial activity by disc diffusion method

The disc diffusion method was used to determine the antimicrobial activities by the disc diffusion method. Fresh cultures of microorganisms that were grown for 24 h were used and diluted 10^{-1} with sterile physiological saline solution (0.85% NaCl). 100 μ l of test microorganisms containing 2.0×10^6 colony forming units (cfu/ml) for bacteria were inoculated on the surface of Muller Hinton Agar (Merck) plates. The three sterile discs with a diameter of 6 mm were placed onto each agar plate containing microorganisms. Then 30 μ l of extracts were dropped onto discs under sterile conditions and were incubated at $+37 \pm 0.1$ °C for 24 h. After incubation, the diameters of inhibition zones were measured in millimetres on all plates. All experiments were repeated three times (Phadke and Kulkarni, 1989). Amikacin (30 μ g/ml) (Eczacibasi), vancomycin (30 μ g/ml) (Mayne), penicillin (10 U/ml) (I.E.Ulagay), gentamicin (10 μ g/disc) (I.E.Ulagay), rifamicin (5 μ g/ml) (Aventis), tetracycline (30 μ g/ml) (SIGMA), ampicillin (10 μ g/ml) (SELVA), chloramphenicol (30 μ g/ml) (SIGMA) and erythromycin (15 μ g/ml) (SIGMA) were used as positive controls. Positive control discs were tested on the

same microorganisms under the same conditions. The stock antibiotics mentioned above were prepared in appropriate amounts ($\mu\text{g/ml}$) and 20 μl were dropped on discs (6 mm), and for negative control; acetone, methanol and chloroform were used.

Scanning electron microscopy

The bacteria that were susceptible to the plant extracts were prepared for Scanning Electron Microscope (SEM). Small agar pieces were cut out from the inhibition zone and they were fixed in 3 % (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.2) for an hr at room temperature and then washed four times in sodium phosphate buffer. The pieces were then post-fixed in 1 % (w/v) osmium tetroxide (OsO_4) for an hr and then washed four times in the buffer. They were dehydrated in a graded alcohol series. The last stages of dehydration were performed with propylene oxide ($\text{CH}_3\text{CH}_2\text{CH}_2\text{O}$). The specimens were dried and were mounted onto stubs using double-sided carbon tape, and then were coated with a thin layer of gold by a Polaron SC 502 sputter coater. They were examined in a Jeol JSM 6060 LV Scanning Electron Microscope (Hayat, 1981).

Results and Discussion

Disc diffusion assay

The antimicrobial activities of *O. basilicum* (acetone, methanol, and chloroform) extracts against the microorganisms were examined in the present study, and their potency, were assessed by the presence or absence of inhibition zones and zone diameter. The results are given in Table 1. Also, the inhibition zones formed by positive control of antibiotic discs are presented in Table 2. As it can clearly be seen in Table 1, the methanol extract has stronger and broader spectrum of antimicrobial activities compared with acetone and chloroform extracts. The results showed that two concentrations of methanol extracts have different antimicrobial effects. The methanol extract suspended with 10 ml deionized water exhibited inhibition effects against 2 bacterial strains; namely *P. aeruginosa* and *Shigella sp.* The methanol extract suspended with 5 ml deionized water show inhibitory effects against 6 bacterial strains; namely *P. aeruginosa*, *Shigella sp.*, *L. monocytogenes*, *S. aureus* and two different strains of *E. coli*. Two different concentrations of methanol extracts were used in our study. It was not surprising that more condensation of methanol extracts resulted in more effective antimicrobial activity. If more concentrations of extracts are used, antimicrobial effect will increase. On the other hand, the chloroform and acetone extracts of basil showed antimicrobial activity against only one bacterial strain, *P. aeruginosa*. Besides, the results showed that three different extracts exhibited inhibitory effects against bacterial strains but had no effect against yeast strains.

Ethanol, methanol, and hexane extracts from *Ocimum basilicum* L. were investigated for their *in vitro* antimicrobial properties by Adiguzel and colleagues (Adiguzel et al., 2005). Their result showed that none of the three extracts tested have antifungal activities, but these extracts have anticandidal and antibacterial effects. Among these three extracts, while hexane was observed to be more effective in a wider spectrum when compared to methanol, no effects were observed in the extracts prepared with ethanol. In our study, methanol extracts have showed the most effective result among the extracts tested. When Adiguzel and his colleagues' findings (2005) are compared to ours, similar results with respect to common bacteria tested were obtained, in our study. While no effects on *Candidas* were observed in our study, anticandidal effect on *Candida albicans* was reported in Adiguzel and his colleagues' study (2005). In the other study, the extract of *O. basilicum* had antibacterial activity against only *P. aeruginosa* (Nascimento et al., 2000). In our study, effective antibacterial activity on the four tested extracts of *O. basilicum* on *P. aeruginosa* was observed. In another study, the extract of *O. basilicum* had antimicrobial properties against *E. coli*, *Salmonella paratyphi* and *Shigella dysenteraea* (Omogbe et al., 1996).

The antibacterial activity of different extracts from the leaves of *O. gratissimum*, another species of *Ocimum*, were tested against pathogenic bacteria that cause diarrhea and effective results on all the four bacteria studied were obtained (Adebolu and Oladimeji, 2005). Even though they are different species of *Ocimum*, it has been observed that these two plants have the same effects on *S. aureus* and *E. coli* strains. Even though the type of plants the antimicrobial effects of which are tested are the same, when the results are compared, there are differences in terms of the bacteria against which they are effective; and in cases where they are effective on the same bacteria, they perform different activities in terms of quality and quantity. It is considered that these differences depend on the individual different chemical contents of the plants and thus, such differences are caused by some factors such as the structure of the soil, daily and seasonal changes that occur during the collection of the plant material, physiological growth period of the plant, part of the plant studied, extraction process, solvent material and the types of bacteria used (Izzo et al., 1995; Martinez et al., 1996; Tunon et al., 1995). When the antimicrobial activity of *O. basilicum* methanol extract is compared with the standard

Table 1: The antimicrobial activity of *O. basilicum* extracts against 14 microorganisms.

Microorganisms	Diameter of inhibition zone (mm)			
	Chloroform	Acetone	Methanol 10ml	Methanol 5ml
<i>Enterococcus gallinarum</i> CDC-NJ-4	-	-	-	-
<i>Enterococcus faecalis</i> ATCC 29212	-	-	-	-
Bacillus subtilis RSHI	-	-	-	-
<i>Escherichia coli</i> ATCC 25922	-	-	-	13
Shigella RSHI	-	-	14	13
Escherichia coli RSHI	-	-	-	13
<i>Streptococcus pyogenes</i> ATCC 19615	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 29213	-	-	-	15
<i>Listeria monocytogenes</i> ATCC 7644	-	-	-	15
<i>Pseudomonas aeruginosa</i> ATCC 27853	11	12	13	13
<i>Saccharomyces cerevisiae</i> (Pakmaya)	-	-	-	-
<i>Candida albicans</i> 845981	-	-	-	-
<i>Candida crusei</i> ATCC 6258	-	-	-	-
<i>Candida albicans</i> 90028	-	-	-	-

* RSHI: Microbiology Laboratory Culture Collection of Refik Saydam Hifzisiha Institute
 (-): No inhibition zone (resistant)

Table 2. The inhibition zone formed by standard antibiotic disks at same conditions. *RSHI: Microbiology Laboratory Culture Collection of Refik Saydam Hifzisiha Institute (-): No inhibition zone (resistant).

Microorganisms	The diameter of inhibition zone (mm)								
	Amikacin	Vancomycin	Penicillin	Gentamicin	Rifocin	Tetracycline	Ampicilin	Chloramphenicol	Erythromycin
<i>Candida albicans</i> 845981	17	12	-	19	15	-	-	-	12
<i>Candida crusei</i> ATCC 6258	14	11	-	17	17	-	-	-	11
<i>Candida albicans</i> 900628	17	11	-	19	15	-	-	-	11
<i>Enterococcus gallinarum</i> CDC-NJ-4	-	12	-	15	13	-	-	-	11
<i>Enterococcus faecalis</i> ATCC 29212	16	12	-	16	14	-	-	-	11
<i>Bacillus subtilis</i> RSHI	24	19	22	25	23	12	-	13	24
<i>Escherichia coli</i> RSHI	18	-	-	18	-	-	-	-	-
<i>Shigella</i> RSHI	20	-	-	19	-	-	-	-	-
<i>Escherichia coli</i> ATCC 25922	16	-	-	17	-	-	-	-	-
<i>Streptococcus pyogenes</i> ATCC 19615	13	12	-	16	15	-	-	-	12
<i>Staphylococcus aureus</i> ATCC 29213	17	15	19	17	27	12	-	-	18
<i>Listeria monocytogenes</i> ATCC 7644	25	16	-	27	39	-	-	-	19
<i>Pseudomonas aeruginosa</i> ATCC27853	17	-	-	15	-	-	-	-	-
<i>Saccharomyces cerevisiae</i> (Pakmaya)	-	16	-	-	17	10	8	-	11

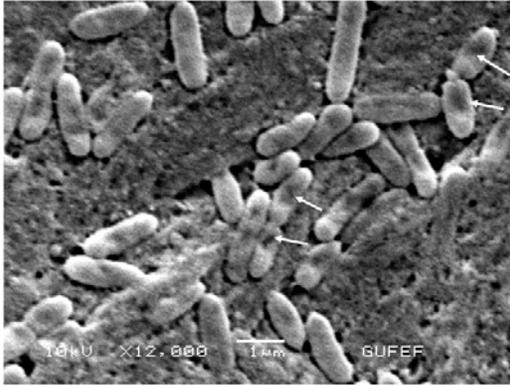


Figure 1. Scanning electron microscope image of damaged *P. aeruginosa* cells (arrows) after treatment with methanol extracts of *O. basilicum*.

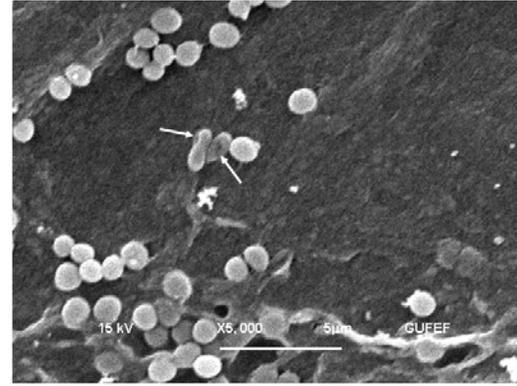


Figure 3. Scanning electron microscope image of damaged *S. aureus* cells (arrows) after treatment with methanol extracts of *O. basilicum*.

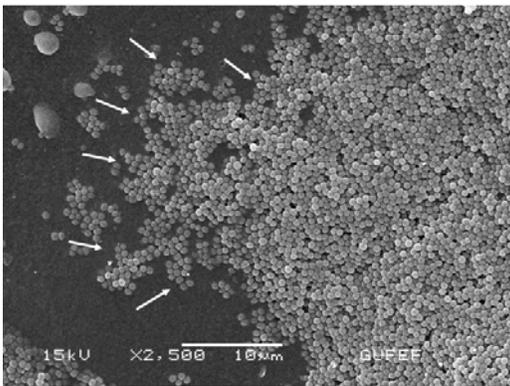


Figure 2. The region of inhibition zone (arrows) of *S. aureus* after treatment with methanol extracts of *O. basilicum*.

antibiotics that were tested, as it can be seen in Table 2, *Shigella sp.*, *P. aeruginosa*, *Escherichia coli* ATCC 25922, *Escherichia coli* were resistant to a variety of standard antibiotics tested but methanol extract of *Ocimum* had antimicrobial activities on these microorganisms. On the other hand, *L. monocytogenes* and *S. aureus* were sensitive to a variety of antibiotics as well as *O. basilicum* methanol extract. Consequently, *O. basilicum* has an inhibitory activity on these bacteria that is as effective as antibiotics. It would be possible to produce new effective antibiotics using the effective material to be refined from this plant.

Scanning electron microscope observations

To observe morphological alteration after the cell was treated with plant extracts we used scanning electron microscope. When the bacterial cells treated with plant extracts were compared with untreated cells, the treated cells appeared to be shrinking and there was a degradation of the cell walls (Figures 1, 2, 3). As the case is in our study, Burt and Reinders (2003) found that oregano and thyme essential oil exhibit strong antimicrobial properties against *E. coli* O157:H7 and observed cells were damaged when treated with essential oil using SEM and the damage in the cells was similar. Burt (2004) tried to explain the mechanism of action for essential oil components in bacterial cells. The mechanism of action was thought to be the degradation of the cell wall,

damage to cytoplasmic membrane proteins, the binding of proteins, leakage of cell contents, and coagulation of cytoplasm and depletion of the proton motive force.

All these findings indicate that *O.basilicum* extracts possess antibacterial activity and they cause lysis and eradicate bacteria by degrading bacterial cell walls. By the findings and purification of the active agent that is present in the extract of *O.basilicum*, it will be possible to discover new natural drugs serving as chemotherapeutic agents for treatment with nosocomial pathogens and could control antibiotic-resistant bacteria. This study will be a base to our further investigations on advanced purification and effect mechanism of its active compounds.

Conclusions

The methanol (suspended with 5 ml deionized water) extracts of *O.basilicum* showed antimicrobial activity against the test microorganisms and caused degradation of the cell walls of sensitive bacteria.

References

1. Adebolu, T.T. and Oladimeji, S.A. (2005). Antimicrobial activity of leaf extracts of *Ocimum gratissimum* on selected diarrhoea causing bacteria in southwestern Nigeria. Afr. J. Biotechnol., 4: 682 - 684.
2. Adiguzel, A., Gulluce, M., Sengul, M., Ogutcu, H., Sahin, F. and Karaman, I. (2005). Antimicrobial effects of *Ocimum basilicum* (Labiatae) extract. Turk J. Biol., 29: 155 - 160.
3. Ates, D.A. and Erdogrul, O.T. (2003). Antimicrobial activity of various medicinal and commercial plants extracts. Turk J. Biol., 27: 157 - 162.
4. Burt, S.A. (2004). Essential oils: their antibacterial properties and potential applications in foods- a review. Int. J. Food Microbiol., 94: 223 - 253.
5. Burt, S.A. and Reinders, R.D. (2003). Antimicrobial activity of selected plant essential oils against *Escherichia coli* O157:H7. Lett. Appl. Microbiol., 36: 162 - 167.
6. Chiej, R. (1984). Encyclopaedia of Medicinal Plants. MacDonald. ISBN 0-356-10541-5, Little, Brown.
7. Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1986). Glossary of Indian Medicinal Plants (Including the Supplement). Council of Scientific and Industrial Research. New Delhi.
8. Davis, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. Science, 264: 375 - 382.
9. Duke, J.A. and Ayensu, E.S. (1985). Medicinal Plants of China. Reference Publications. Inc. : ISBN 0-917256-20-4. Reference Publications, Algonac, MI (USA).
10. Dulger, B. and Gonuz, A. (2004a). Antimicrobial activity of some endemic *Verbascum*, *Salvia*, and *Stachy* species. Pharm. Biol., 42: 301 - 304.
11. Dulger, B. and Gonuz, A. (2004b). Antimicrobial activity of certain plants used in Turkish traditional medicine. Asian J. Plant Sci., 3: 104 - 107.
12. Erturk, O., Kati, H., Yayli, N. and Demirbag, Z. (2003). Antimicrobial activity of *Viscum album* L. subsp. *abietis* (Wiesb). Turk J. Biol., 27: 255 - 258.
13. Falerio, M.L., Miguel, M.G., Laderio, F., Venâncio, F., Tavares, R., Brito, J.C., Figueiredo, A.C., Barroso, J.G. and Pedro, L.G. (2003). Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. Lett. Appl. Microbiol., 36: 35 - 40.
14. Hayat, M.A. (1981). Principles and techniques of electron microscopy, Vol. 1. Edward Arlond Lt., London, 522 p.
15. Izzo, A.A., Di Carlo, G., Bicardi, D., De Fusco, R., Mascolo, N., Borrelli, F. and Capasso, F. (1995). Biological Screening of Italian Medicinal Plants for Antibacterial Activity. Phytotherapy Res. 9: 281 - 286.
16. Jirovetz, L., Buchbauer, G., Shafi, M.P. and Kaniampady, M.M. (2003). Chemotaxonomical analysis of the essential oil aroma compounds of four different *Ocimum* species from southern India. Eur. Food Res. Technol., 217: 120 - 124
17. Juliani, H.R. and Simon, J.E. (2002). Antioxidant activity of basil. In: Trends in new crops and new uses. (Janick J and Whipkey A eds.), ASHS Press, Alexandria, V A, pp. 575 - 579.
18. Kalembe, D. and Kunicka, A. (2003). Antimicrobial and antifungal properties of essential oils. Curr. Med. Chem., 10: 813 - 829.
19. Kivcak, B., Mert, T. and Ozturk, H.T. (2002). Antimicrobial and cytotoxic activity of *Ceratonia siliqua* L. extracts. Turk J. Biol., 26: 197 - 200.
20. Lust, J. (1983). The Herb Book. Bantam books: ISBN 0-553-23827-2, New York.
21. Martin, K.W. and Ernst, E. (2004). Herbal medicines for treatment of fungal infections: a systematic review of controlled clinical trials. Mycoses., 47: 87 - 92

22. Martinez, M.J., Betancourt, J., Alonso-Gonzales, N. and Jauregui, A. (1996). Screening of some Cuban medicinal plants for antimicrobial activity. *J. Ethnopharmacol.*, 52: 171 - 174.
23. Nascimento, G.G.F., Locatelli, J., Freatas, P.C. and Silva, G.L. (2000). Antibacterial Activity of Plant Extracts Phytochemicalson Antibiotic Resistant Bacteria. *Braz. J. Microbiol.*, 31: 247 - 256.
24. Omoregbe, R.E., Ikube, O.M. and Ihimire, I.G. (1996). Antimicrobial activity of some medical plants extract on *Escherichia coli*, *Salmonella paratyphi* and *Shigella dysenteriae*. *Afr. J. Med. Med. Sci.*, 25: 373 - 375.
25. Ozdemir, G., Karabay, N.U., Dalay, M.C. and Pazarbasi, B. (2004). Antimicrobial activity of volatile component and various extracts of *Spirulina plantensis*. *Phytother. Res.*, 18: 754 - 757.
26. Phadke, S.A. and Kulkarni, S.D. (1989). Screening of in vitro antibacterial activity of *Terminalia chebula*, *Eclapta alba* and *Ocimum sanctum*. *Indian J. Med. Sci.*, 43: 113 - 117.
27. Sacchetti, G., Medici, A., Maietti, S., Radice, M., Muzzoli, M., Manfredini, S., Braccioli, E. and Bruni, R. (2004). Composition and functional properties of the essential oil of Amazonian Basil, *Ocimum micranthum* Willd., Labiatae in comparison with commercial essential oils. *J. Agric. Food Chem.*, 52: 3486 - 3491.
28. Sappakul, P., Miltz, J., Sonneveld, K. and Bigger, S.W. (2003). Antimicrobial properties of basil and its possible application in food packaging. *J. Agric. Food Chem.*, 51: 3197 - 3207.
29. Service, R.F. (1995). Antibiotics that resist resistance. *Science*, 270: 724 - 727.
30. Tunon, H., Olavsdotter, C. and Bohlin, L. (1995). Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF - induced exocytosis. *J. Ethnopharmacol.*, 48: 61 - 76.