PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 12 (8): 730-735, 2013
ISSN 1680-5194

Extraction and Isolation Phytochemical and Antimicrobial Activity of Limonoid Compounds from Orange Waste Juice

Ucop Haroen[1], Yetti Marlida[2], Mirzah[2] and Agus Budianyah[1]
[1]Department of Agriculture, Faculty of Animal Husbandry, Jambi University, Jambi 36361, Indonesia
[2]Department of Agriculture, Faculty of Animal Husbandry, Andalas University, Padang 25163, Indonesia

Abstract: Antibacterial activity limonoid compounds of three different solvent extracts (methanol, ethyl acetate and n-heksane) prepared by soxhlet extractor from orange waste juice were screened against two pathogenic bacteria *Escherichia coli* and *Salmonella enteridis*. The highest antibacterial potentiality was exhibited by the limonoid-ethyl acetate followed by the (limonoid-methanol) and (limonoid-n-hexane).The orange waste juice extract can be considered to be as equally potent as the antibiotics. The zone of inhibition, Minimum Inhibitory Concentration (MIC) of the orange waste juice extracts showed that limonoids extracted with ethyl acetate (limonoids-ethyl acetate) resulted in better inhibition of limonoids extracted with (limonoids-methanol) and (limonoids-n-hexane). The concentration 250 ppm of MIC were test showed inhibition of (limonoids-ethyl acetate) higher with a percentage minimum (44.15 for the bacterium *Escherichia coli* and 14.69 for the *salmonella enteridis*) than (limonoids-methanol) (10.67 and 3.84) and (limonoids-n-hexane) (35.58 and 14.10). *In vitro* antibacterial screening of crude (limonoid-ethyl acetate) (limonoid-methanol) and (limonoid-n-hexane) of methanol extract of *orange juice waste* with concentration 250 ppm were test showed the clear zone in millimeters (11.75 for the bacterium *Escherichia coli* and 10.25 for the *salmonella enteridis*) than limonoid-methanol (8.00 and 9.00) and limonoid-n-hexane (8.00 and 9.25). However, when compared to synthetic antibiotics (neomiditril and coleridin), the clear zone of limonoids lower (24%) but still showed his ability as an anti-microbial. (Limonoids-ethyl acetate) dose of 250 ppm showed better activity than doses of (limonoid-methanol) and (limonoid-n-heksana) for the bacterium *Escherichia coli* to *salmonella enteridis* bacterial at 11.918 and 9.158. From these results we can conclude (limonoids-ethyl acetate) extract better than (limonoids-methanol) and (limonoids-n-hexane) and can be used as a substitute for anti biotic synthetic antimicrobial. The phytochemical analysis of orange waste juice extracts showed presence of triter penes, alkaloid, flavonoids, steroid, phenolic, saponin.

Key words: Limonoid compounds, antimicrobial activity, orange juice waste, phytochemical, MIC

INTRODUCTION

Orange is one of the most important commercial fruit crops grown in all continents of the world (FAO, 2004). Orange importance is attributed to its diversified use and growing world demand with about 102.64 million tones total world production and probably stands first largest among the produced fruit.

Orange are mainly used by juice processing industries while the peels are generally wasted. Since the juice yield of citrus is less half of the fruit weight, very large amounts of byproduct wastes, such as peels are formed every year (Manthey and Grohmann, 2001).

Peel waste are highly perishable and seasonal, is a problem to the processing industries and pollution monitoring agencies. There is always an increased attention in bringing useful products from waste materials and orange wastes are no exceptions. Suitable methods have to be adopted to utilize them for the conversion into value-added products Nand (1998). By-product recovery from fruit wastes can improve the overall economics of processing units. Besides this, the problem of environmental pollution also can be reduced considerably. The orange are rich in nutrients and contain many phytochemicals, among others the most important is the limonoid compounds, they can be efficiently used as drugs or as food supplements too. Since there is an increase in the number of antibiotic resistance pathogens, there is always a search of an alternative drug that is regarded as safe. Orange wastes juice if proved to have antibacterial activity, they can be also used in same food industry which generates large peel wastes as a food preservative. Food processors, food safety researchers and regulatory agencies have been increasingly concerned with the growing number of food-borne illness outbreaks caused by some pathogens (Wilson and Droby, 2000; Friedman *et al*., 2002; Sokovic *et al*., 2007). The food industry has tended to reduce the use of chemical preservatives in their products due to increasing pressure of consumers or legal authorities, to either completely remove or to

Corresponding Author: Ucop Haroen, Department of Agriculture, Faculty of Animal Husbandry, Jambi University, Jambi 36361, Indonesia

adopt more natural alternatives for the maintenance or extension of product shelf life (Nychas and Tassou, 2000). The orange waste juice of orange fruit is a rich source of flavanones, mainly compounds limonoid (Miller *et al*., 2004) and many polymethoxylated flavones, which are very rare in other plants (Ahmad *et al*., 2006). The antimicrobial abilities of essential oils, among which citrus oils, are also shown to be a particularly interesting field for applications within the food and cosmetic industries Caccioni *et al*. (1998). It has also been used as an anti-cancer Guthrie *et al*. (2007), antimicrobial Roy and Shalendra (2006), antifungal, anti-virus, anti bacterial, anti inflamasi Ko *et al*. (2007), hypotensive agent Kumamoto *et al*. (1986), antioxidant Ferguson *et al*. (2002), Paulose *et al*. (2005), Majo *et al*. (2005), carminative, insect repellent, antibacterial, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent Voogd (1981). This study was aimed to focus on waste minimization in fruit juice processing industry. This study investigates the antibacterial activity and the fundamental scientific basis for the use of orange waste juice of orange fruits by determining the chemical constituents as well as quantifying the yield percentage of crude phytochemicals.

MATERIALS AND METHODS

Plant material: The orange waste juice collected in November 2011 from the local fruit juice shops in the city Jambi and rinsed with clean water. After collection, the orange juice waste were shade dried at oven temperature (45-50°C) to constant weight over a period of 5 days and then pulverized using a mortar and pestle. The pulverized orange waste juice were kept separately in an air-tight cellophane bag until used.

Preparation of extracts: Waste mashed with orange juice using a mortal and pestle as much as 2.20 kg. Then at room temperature. Then filtered to obtain the extract, the extract was concentrated using a rotary evaporator at a temperature of 40°C. Exactly 700 g each of the pulverized plant was macerated successively in n-hexane, ethyl acetate and 95% methanol for 36 h each. The mixtures were then filtered under vacuum and the filtrates concentrated using a rotatory evaporator. The methanol concentrate was evaporated to dryness in a water bath. The extracts were stored in an airtight sample bottles and kept in a desiccator until used.

Microorganism used for the activity test: Both gram positive and gram negative bacterial strains were taken for the test. The bacterial strains used for the investigation are listed in Table 1. These organisms were collected from the Microbiology Laboratory of Pharmacy Discipline Andalas University, Padang Indonesia.

Isolation of limonoid compound: Extraction method used was meserasi (Arbain, 1995). Waste mashed with orange juice using a mortal and pestle as much as 2.20 kg. Then at room temperature. Then filtered to obtain the extract, the extract was concentrated using a rotary evaporator at a temperature of 40°C.

Preliminary phytochemical analysis (qualitative test)
Test for alkaloids: Two milliliter filtrate was mixed with 1% HCl and about 6 drops of Mayor's reagents. A Creamish or pale yellow precipitate indicated the presence of respective alkaloids.

Test for flavonoids: Two milliliter filtrate was added to conc. HCl and magnesium ribbon. Pink-tomato red colour indicated the presence of flavonoids.

Test for steroids: Two milliliter of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 mL H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for saponins: Froth test for saponins was used. 1 g of the sample was weighed into a conical flask in which 10 mL of sterile distilled water was added and boiled for 5 min. The mixture was filtered and 2.5 mL of the filtrate was added to 10 mL of sterile distilled water in a test tube. The test tube was stopped and shaken vigorously for about 30 sec. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

Test for terpenoids (Salkowski test): Five milliliter of each extract was mixed in 2 mL of chloroform and concentrated H_2SO_4 (3 mL) was carefully added to form

Table 1: Phytochemical analysis of orange juice waste extracts

Secondary metabolites	Reagent	Observation	Result
Alkaloids	Meyer	White mist formed	(+)
Flavonoids	Sianidin test	Orange solution	(+)
Steroids	Liebermann-burchard	Blue solution	(+)
Triterpenoids	Lieberman-burchard	Red-brown solution	(+)
Phenolic	$FeCl_3$	Solution blue/purple	(+)
Saponin	H_2O	Foam	(+)
Coumarin	NaOH/Ethanol/Water	Fluorescence more light on the KLT plate	(+)

Description: (+): contains secondary metabolites (x): does not contain secondary metabolite

a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Test for phenolic compounds: Most of the water-methanol phase were removed with a pipette into a small test tube, then add $FeCl_3$ reagent the formation of blue / purple indicates the content of phenolic compounds.

***In vitro* testing of extracts for antimicrobial activity**

Antimicrobial assay: *Escherichia coli*, *Salmonella enteridis*. The culture medium in this research: GTA+ $CaCO_3$ plate a consisted of 20 g glucose, 2,5 g tripton, 20 g bacto agar (Oxoid) and 15 g $CaCO_3$, MRS broth (Merck), Nutrient Agar (NA) (Merck). A loopful of the test organism was taken from their respective agar slants and sub-cultured into test-tubes containing nutrient broth for bacteria. The test-tubes were incubated for 24 h at 37°C for bacteria. The obtained microorganisms in the broth were standardized using normal saline to obtain a population density of 108 cfu/mL for the bacteria. The spores suspension were standardized to 105 cfu/mL. The medium was prepared according 20 g of Blood Agar were weighed into a conical flask 1000 mL of distilled water was added and capped with a cotton wool. The media were boiled to dissolution and then sterilized at 121°C for 15 min. The media were allowed to cool to 45°C and 20 mL of the sterilized medium was poured into sterile petri-dishes and allowed to cool and solidify. The plates were labeled with the test microorganism (each plate with a test microbe). The microbes were spread evenly over the surface of the medium with the aid of a glass spreader. The plates were dried at 37°C for 30 min and divided into two sets to be used for the well diffusion method and the disc diffusion method, respectively.

Minimum inhibitory concentration-broth dilution method: The MIC was determined using broth dilution method as described in Ibekwe *et al.* (2001). The nutrient broth and sabouraud dextrose liquid were prepared according to the manufacturer's instruction (10 mL of each broth was dispensed into separate test-tube and was sterilized at 121°C for 15 min and then allowed to cool. Two-fold serial dilution of the extract in the broth were made from the stock concentration of the extract to obtain 250 ppm for limonoid-methanol and limonoid-ethyl acetate and the limonoid-hexane extracts. 0.1 mL of the standardized inoculums of the microbes were then inoculated into the different concentrations of the extracts in the broth. The test tubes of the broth were then incubated at 37°C for 48 h and 30°C for 1-7 days for bacteria respectively and observed for turbidity of growth. The lowest concentration which showed no turbidity in the test tube was recorded as the Minimum Inhibition Concentration.

RESULTS AND DISCUSSION

The maceration extract of the orange waste juice using different solvents yielded different results in each of the experiment conducted in the this study. There are existing, a difference in the percentage yield of the extract obtained of the three solvent. Figure 1, shows the comparison of the percentage yield of extracts obtained from different solvent with respect to various sources. For methanol extract percentage yield of 4 times more than the extraction solvent ethyl acetate and n-hexane. This variation in yield of the three ranges of solvents explains that solubility of different plant compound in different solvent. Orange waste juice extracts showed a significant antibacterial activity against all the test organisms. (Limonoid-ethyl acetate) extracts showed a very good antimicrobial activity when compared to (limonoid-methanol) extracts and (limonoid-n-hexane) extracts. (Limonoid-ethyl acetate) extracts showed a maximum zone of inhibition against *E. coli* (11.750 mm) followed by *S. enteridis* (11.500 mm), where as the (limonoid-methanol) extracts showed a maximum zone of inhibition against *E. coli* (8.062 mm) followed by *S. enteridis* (9.125 mm) and (limonoid-n-hexane) extract showed zone inhibition against *E. coli* (8.000 mm) and *S. enteridis* (9.312 mm). This antibacterial activity may be indicative of the presence of metabolic toxins or broad spectrum antibiotic compounds. In case of (limonoid-ethyl acetate) show more or less the same antibacterial activity. (Limonoid-ethyl acetate) proves to be a good solvent for the extraction of antibacterial agents from both the sources as it has shown better yield as well as antibacterial activity relating that higher yield means high concentration of single or variety of phytochemicals and therefore high antibacterial activity. This statement can be validated as (limonoid-ethyl acetate) showed the highest yield as an antibacterial activity in orange juice waste. Ekwenye, Uchechi and Edeha, Oghenerobo (2010) reported the antibacterial activity *E. coli* extract ethanol from citrus sinensis leaf extract water 3 mm and 7 mm. Results microbial test *E. coli* showed that water and ethanol solvents have high levels of antibacterial activity is low when compared with the results of this

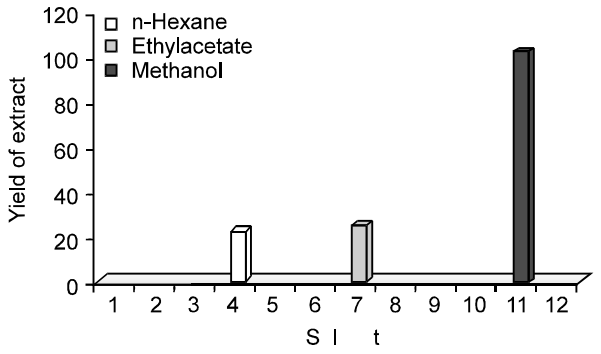

Fig. 1: Percentage yield of orange juice waste extracts

Table 2: *In vitro* antibacterial screening of crude (limonoid-ethyl acetate) (limonoid-methanol) and (limonoid- n-hexane) of methanol extract of Orange juice waste

	Diameter of zone of inhibition in mm				
Bacterial strain	Neomiditril 250 ppm/disc	Coleridin 250 ppm/disc	Limonoid-methanol 250 ppm/disc	Limonoid-ethyl acetate 250 ppm/disc	Limonoid n-hexane 250 ppm/disc
Escherichia coli	18.25	24.50	8.00	11.75	8.00
Salmonella enterisdi	20.00	23.75	9.00	10.25	9.25

Table 3: Minimum Inhibition Concentration of Orange juice waste

	Percentage of minimum inhibition concentration		
Bacterial strain	Limonoid-methanol 250 ppm/MIC	Limonoid-ethyl acetate 250 ppm/MIC	Limonoid n-hexane 250 ppm/MIC
Escherichia coli	10.67	44.15	35.58
Salmonella enterisditis	3.84	14.69	14.10

study microbial test *E. coli* to extract (limonoid-ethyl acetate), (limonoid-methanol) and (limonoid-n-hexane) from waste orange juice waste namely: 11,500 mm, 9,125 mm and 9.135 mm. MIC and Disc of different solvent extracts of orange waste juice shown in Table 1 and Table 2, respectively. The extracts showed significant activity. Results microbial test *E. coli* that (limonoid-ethyl acetate), (limonoid-methanol) and (limonoid-n-heksane) extract showed the highest antibacterial activity when compared with result of research Ekwenye, Uchechi and Edeha, Oghenerobo (2010). This difference is due to differences in the phytochemical composition of various crops or perhaps also due to the extraction method used or the environmental factors and genetic factors orange used. Orange waste juice extract in this study showed that the antibacterial activity is lower than that of synthetic antibiotic use. Inhibition zone for the (limonoid-ethyl acetate) extract of orange waste juice to *E. coli* and *salmonella*, respectively 11,750 mm and 11,500 mm. Ashok *et al.* (2011) reported the inhibition zone for citrus sinensis peel extract against *E. coli* 13 mm. Furthermore Gülay Kirbaslar *et al.*, (2009) reported the inhibition zone for citrus sinensis peel extract and lemon citrus against *E. coli*, respectively 13 and 14 mm. While research Singh Amandeep *et al.* (2009) reported that the inhibition zone citrus sinensis peel extract 13 and 17 mm against *E. coli and salmonella enteridis* respectively. The difference in the antibacterial activity with the same source when extracted with different solvent has proven that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent. Hence solvents of different polarity should be employed as discussed in this study (polar: water, acetone, ethanol; non-polar: ethyl acetate, petroleum ether). Sequential or successive solvent extraction is as good option for better solubility of many of the phytochemical but it is always necessary to know the phytochemicals extracted by each individual solvent so as to avoid the inclusion of unnecessary solvents for extraction process as well as to understand the role of each solvent in the extraction of an individual or class of phytochemicals. With no antibacterial activity, extracts may be active against other bacterial species which were not tested Shale *et al.* (1999). The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body Akinmoladun *et al.* (2007). The preliminary phytochemical investigation revealed the presence of various constituents of orange waste juice. The results are shown in the Table 3. Different solvent showed different class of phytochemicals they showed the presence of flavonoids, saponins etc. anthraquinones were completely absent in both the orange waste juice. These constituents could account for the antibacterial activity but it is difficult to correlate their action to a specific phytochemical. The presence of phenol further indicated that orange waste juice could act as anti-inflammatory, anti clotting, antioxidant, immune enhancers and hormone modulators. Orange waste juice have high quantity of saponin which has hemolytic activity and cholesterol binding properties. Therefore, in addition to their use as drugs, citrus peels can be used as a food preservative or even as food supplement as many literature says that they are highly nutritive.

Conclusions: Recycling of fruit waste is one of the most important means of utilizing it in a number of innovative ways yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry. This work has identified the antibacterial activity against the test organisms and phytochemical constituents in Orange juice waste extracts obtained from different solvents. However, further evaluation performed with the pure compounds is required for the definite conclusion of the bioactive compounds contributing to the antimicrobial activity, although the nature and number of active components involved in each extract are not clear, however they are promising. This finding can form the basis for further studies to prepare an optimize preparation of the herbal extract.

ACKNOWLEDGEMENT

The author are very grateful to Directorate general of Higher Education that was funded this experiment by Hibah Doktor Project 2013 Directorate General of Higher Education, Department of National Education Republic of Indonesia.

REFERENCES

Ahmad, M.M., Salim-ur-Rehman, Z. Iqbal, F.M. Anjum and J.I. Sultan, 2006. Genetic variability to essential oil composition in four citrus fruit species. Pak. J. Bot., 38: 319-324.

Amandeep Singh, Ahmed R. Bilal and Anil Devgun, 2009. *In vitro* antibiotic activity of isolated volatile oil of Citrus Sinensis, 7: 1-4.

Ashok, K. Kumar, M. Narayani and M. Subanthini and Jayakumar, 2011. Antimicrobial Activity and Phytochemical Analysis of Citrus Fruit Peels-Utilization of Fruit Waste. Int. J. Engineering and Tech., 6: 5414-5421.

Akinmoladun, AC., E.O. Ibukun, E. Afor, E.M. Obuotor and E.O. Farombi, 2007. Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum*. Sci. Res. Essay., 2: 163-166.

Arbain. D., 1995, "Phytochemical Survey One Way Approach", Heds-Usaid project Unand.

Caccioni, D.R., M. Guizzardi, D.M. Biondi, A. Renda and G. Ruberto, 1998. Relationship between volatile components of citrus fruit essential oils and antimicrobial action on Penicillium digitatum and Penicillium italicum, Int. J. Food Microbiol., 43: 73-79.

Ekwenye Uchechi, N. and V. Edeha Oghenerobo, 2010. The antibacterial activity of crude leaf extract of Citrus sinensis (sweet orange), Int. J. Pharma and Bio. Sci., 1: 742-750.

FAO, 2004. FAOSTAT data 2005. Fooa and Agricultural Organisation of the United Nations, 00111, Rome, Italy. February, 2005.

Ferguson, J.J. 2002. Medicinal Use of Citrus (File medicinal sitrus).

Friedman, M., R.P. Henika, E.R. Mandrell, 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against campylobacter jejuni, *Escherichia coli*, Listeria monocytogenes and *Salmonella enterica*. J. Food Protection. 65: 1545-1560.

Gülay Kirbaslar, F., Aydin Tavman, Basaran Dülger and Gülen Türker, 2009. Antimicrobial activity of turkish citrus peel oils. Pak. J. Bot., 41: 3207-3212.

Guthrie, N., S. Hasegawa, G. Manners and T. Vandenberg, 2000. Inhibition of human breast cancer cells by citrus limonoids. In: Citrus Limonoids: Functional Chemicals in Agriculture and Foods, Ed. Berhow, M., Hasegawa, S. and Manners, G. ACS Symposium Series. 758: 164-174.

Ibekwe, V.I., N.F. Nnanyere and C.O. Akujobi, 2001. Studies on Antibacterial Activity and Phytochemical qualities of Extracts of Orange peels. Int. J. Environ. Health and Hum. Dev., 2: 41-46.

Kumamoto, H., Y. Matsubara, Y. Iizuka, K. Okamoto and K. Yokoi, 1986. Structure and Hypotensive Effect of Flavonoid Glycosides in Orange (*Citrus sinensis* OSBECK) Peelings. Agri. and Biologi. Chem., 50: 781-783.

Ko HC., Y.H. Wang, K.T. Liou, C.M. Chen, C.H. Chen, W.Y. Wang, S. Chang, Y.C. Hou, K.T. Chen, C.F. Chen and Y.C. Shen, 2007. Anti-inflammatory effects and mechanism of the ethanol extract of *Evodia rutae carpa* and its bioactive components on neutrophils and microglial cells Eur. J. Pharmacol. 555: 211-7.

Manthey, A. and K. Grohmann, 2001. Phenols in citrus peel byproducts: concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. J. Agric. Food Chem., 49: 3268.

Majo, D.D., M. Giammano, M.L. Guardia, E. Tripoli, S. Giammanco and E. Finotti, 2005. Flavanones in citrus Fruit: antioxidant activity relationships. Food Res. Inter: 38: 1161-1166.

Marina Sokoviæ, Petar D. Marin, Dejan Brkiæ, Leo J.L.D. van Griensven, 2007. Chemical Composition and Antibacterial Activity of Essential Oils of Ten Aromatic Plants against Human Pathogenic Bacteria Global Science Books Food, 1.

Miller, E.G., J.L. Porter, W.H. Binnie, I.Y. Guo and S. Hasegawa, 2004. Further Studies on the anticancer activity of citrus limonoids. J. Agric. Food Chem., 52: 4908-4912.

Nand, K. 1998. Recent advances in the treatment of liquid and solid wastes of food processing industries for biogas production and pollution abatenront. Proc. 4th International. Food Convention, Mysore: 35.

Nychas, G.J.E. and C.C. Tassou, 2000. Traditional preservative - oils and spices. In : Robinson RK, Batt CA, Patel PD (Ed). Encyclopedia of Food Microbiology Volume.2. Academy Press. London.

Poulose, S.M., E.D. Harris and B.S. Patil, 2005. Citrus Limonoids ynduce apoptosis in human neuroblastoma cells and have radical scavenging activity. J. Nutr., 135: 870-877.

Roy. A. and Saraf, Shailendra, 2006. Limonoids Overview Of Significant Bioactive Triter penes Distributed in Plants Kingdom. Biol. Pharm. Bull., 29: 191-201.

Shale, T.L., W.A. Strik, and J. van Staden, 1999. Screening of plants used by southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity. J. Ethnopharmacol, 64: 9-14.

Singh Amandeep, Ahmed R. Bilal and Anil Devgun, 2009. *In vitro* antibiotic activity of isolated volatile oil of Citrus Sinensis, 7: 1-4.

Sokovic, M., Petar D. Marin, Dejan Brkic, Leo J.L.D. van Griensven, 2007. Chemical Composition and Antibacterial Activity of Essential Oils of Ten Aromatic Plants against Human Pathogenic Bacteria Global Science Books Food., 1.

Ekwenye, Uchechi N.N and Edeha, Oghenerobo V., 2010. The antibacterial activity of crude leaf extract of *Citrus* sinensis (sweet orange), Int. J. Pharm. Bio. Sci., 1: 742-750.

Voogd, C.E. 1981. On the mutagenicity of nitroimidazoles. Mutat. Res., 86: 243-277.

Wilson C.L. and G.G. Droby, 2000. Microbial Food Contamination, CRC Press, Boca Raton, FL, 1-304 149-171.