

PHYTOCHEMISTRY AND ANTIMICROBIAL ACTIVITY OF EXTRACTS FROM MEDICINAL PLANT *OLEA AFRICANA* AND *OLEA EUROPEA*.

Kemboi Douglas¹

School of Science and Technology, University of Kabianga, Kericho, Kenya.

Kemboidouglas@yahoo.com.

Increase in prevalence of resistant microorganisms especially to synthetic drugs, has necessitated the need to search for new bioactive compounds having natural origin. Thus it was essential to determine the antibacterial activity of hexane, dichloromethane, ethyl acetate and methanol extracts of *Olea africana* and *Olea europea*. Phytochemical investigation of both *Olea africana* and *O. europea* extracts indicated the presence alkaloids, flavones, flavanoids and tannins. *Olea africana* leaves extracts afforded two triterpenoids namely erythrodiol and uvaol which were obtained through repeated column chromatography. The compounds were characterized using Nuclear Magnetic Resonance (NMR) spectroscopy, MS spectroscopy and by comparison with literature values. The isolated triterpenoids exhibited moderate antibacterial activity whereas crude extracts exhibited relatively high antibacterial activity against Gram positive bacterial strains; **methanol extracts inhibited growth and showed 12.4 mm zone** of inhibition against *Staphylococcus aureus*, Erythrodiol exhibited higher antibacterial activity than uvaol against Gram positive bacteria *Staphylococcus aureus* with zones of inhibition of 5.2 mm and 5.0 mm respectively. None of the pure compounds showed significant activity against Gram negative bacteria *Escherichia coli*. The results gives a scientific validity and credence to the ethno-medicinal use of this medicinal plant as a chewing stick.

Key words: *Olea africana*, triterpenoids, antibacterial activity.

INTRODUCTION

Medicinal plants have been identified and used throughout human history because they have the ability to synthesize a wide variety of chemical compounds (Bulunas *et al.*, 2005). These compounds are used to perform important biological functions, and to defend the medicinal plants against attack from predators such as insects, fungus and herbivorous mammals (Fabricant *et al.*, 2001). At least 12,000 such compounds have been isolated so far, a number estimated to be less than 10 % of the total (Tapsell *et al.*, 2006). **According to World Health Organization report**, 80 % of the world's population, mostly from developing countries still rely on traditional medicine for the treatment of common ailments (Shagal *et al.*, 2012).

Oral diseases, like most diseases, are unevenly distributed, with the greatest burden falling on needy and poor populations (Ogunbodede *et al.*, 2015). Thus, lower socioeconomic status populations have higher rates of caries and periodontal diseases than their higher Social Economic Status counterparts (Chidzonga *et al.*, 2015). Elsewhere, there are also disparities in oral health between those from rural and urban areas of Africa (Ogunbodede *et al.*, 2015). According to **WHO** (2008), the main determinants of health in general are the social, economic, and environmental conditions. These factors influences the oral health of individuals and populations. Therefore proper oral hygiene for all implies use of cheaper and readily available alternatives.

Dental caries and periodontal diseases are among the most pursued global oral health problems. They are caused by plaque forming bacteria such as *Myces*, *Actinobacillus*, *Streptococcus* and *Candida* species which reside in the oral cavity (More *et al.*, 2008). These bacteria have been reported to be unsusceptible to antimicrobial agents compared with cultures grown in suspension (Sonkos and Gondson, 2011). It is therefore not surprising that bacteria growing in dental plaque, a natural occurring biofilm show increased resistance to antimicrobial agents. They generate acids that eat away at a tooth or form dental plaque or biofilm. Due to the structure of these biofilms, physical removal is the most effective means of control. Regular and thorough removal of food deposits from teeth also plays a vital role in reducing dental and periodontal diseases especially if accompanied by the use of chewing sticks.

Chemical investigations on chewing sticks and other medicinal plants reported to have medicinal properties has also generated numerous purified compounds which have proven to be indispensable in the practice of modern medicine (Ncube *et al.*, 2012). The use of teeth cleaning agents like tooth brush and tooth pastes or chewing sticks varies from country to country, from urban to rural areas and culture to culture. Its use has been documented in parts of Asia, Africa, the Middle East and South America (Verenne *et al.*, 2006). Chewing stick has remained a common and acceptable teeth cleaning agent in different parts of the world especially in developing countries despite the widespread use of tooth brushes and tooth pastes (Al-Otaibi *et al.*, 2004). Its regular use is based on factors such as availability, cost, therapeutic and social-cultural reasons.

Religious reasons have also contributed to the increasing popularity in the use of this form of teeth cleaning. It is often mentioned that the Islamic prophet Mohammed recommended its use as he is quoted in various Hadith advising the use of siwak (*Salvadora persica*) (Almas, 2001; Hyson, 2003). Olive plant (*Olea europea*), Lime tree (*Citrus aurantifolia*), Neem (*Azadirachta indica*) and Orange tree (*Citrus sinensis*) are also used as a chewing stick.

In response to emerging trends of bacterial resistance to antibiotics there is continuous and urgent need to discover new antimicrobial compounds from natural products like chewing sticks with diverse chemical structures and novel mechanisms of the new emerging oral infectious diseases. The current research focused on two medicinal plants *Olea africana* and *Olea europea* which are traditionally used by local inhabitants of Kabianga in Kericho, Kenya as a chewing stick. The aim of the research was to extract, isolate and compare the antibacterial activity of new compounds.

Abbreviations: ATCC, American Type Culture Collection; BCC, Belgian Coordinated Collection Microorganisms; COSY, Correlation Spectroscopy; DCM, Dichloromethane; HMBC, Heteronuclear Multiple-Bond Correlation Spectroscopy; HSQC, Heteronuclear Single Quantum Correlation Spectroscopy; MH, Mueller Hinton; NMR, Nuclear Magnetic Resonance; NOESY, Nuclear Overhauser Effect Spectroscopy; TLC, Thin Layer Chromatograph; WHO, World Health Organization.

MATERIALS AND METHODS.

Plant material

The leaves and twigs of *O. africana* and *O. europea* were collected in January, 2015 from University of Kabianga Botanical garden, Kericho County in Kenya. The plants were identified by a taxonomist, Mr. D. Maritim, University of Kabianga, Kenya and a voucher specimen (kemboi 01) was deposited in the Herbarium, University of Kabianga, Kericho County, Kenya.

Extraction and isolation

The air dried at room temperature and ground plant materials of the plants (1000 g leaves and 1000 g twigs) were sequentially extracted with organic solvents in the order of increasing polarities including; hexane, dichloromethane, ethyl acetate and methanol, using cold extraction for 24 hours in each case. The extracts were then concentrated under a reduced pressure to a minimum volume using a Rotavapor. They were then allowed to evaporate to dryness to a solid mass. The yields obtained were hexane 30.0 g (leaves), 23.0 g (twigs); dichloromethane 24.1 g (leaves), 15.3 g (twigs); ethyl acetate 6.24 g (leaves), 5.22 g (twigs) and methanol 5.35 g (leaves), 4.35 g (twigs) for *Olea africana* and *O. europea*.

Phytochemical analysis

Qualitative tests for terpenoids, tannins, flavanoids, flavones, carotenoids and alkaloids were carried out by standard method as described by Edeoga *et al.* (2005). The tests were based on the visual observations of color change or formation of a precipitate after addition of specific reagent.

Flavones

Flavones were tested by adding 5 mL of ammonium solution to 1 mL of aqueous filtrate of the plant extract followed by addition of 2 mL sulphuric acid. A yellow coloration indicated the presence of flavones.

Alkaloids

Alkaloids were tested for by mixing 5 g of the powder sample extract with 25 mL of 1 % sulphuric acid. It was then allowed to stand and then filtered. About 10 mL of the filtrate was shaken and Meyer's reagent added. Formation of a white precipitate indicated the presence of alkaloids.

Saponins

Tests for saponins was carried out according to the method described by Evans, (1996). About 2 g of the twigs and leaves powder extracts were boiled in 20 mL of distilled water on a water bath and then filtered. About 10 mL of the filtrate was then mixed with 5 mL of distilled water and shaken vigorously to give a stable persistent froth. Three drops of olive oil were then added and the mixture shaken vigorously, then observed for the formation of emulsion.

Flavonoids

The test for flavonoids was carried out according to the method described by Geissman *et al.* (2005), whereby 5 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of the stem and leaves powder extracts. This was followed by addition of concentrated sulphuric acid. Yellow coloration indicated the presence of flavonoids.

Twigs and leaves extracts were subjected to a test for carotenoids whereby 1 g of each extract was boiled in 20 mL water. The filtrate was then treated with concentrated sulphuric acid. Blue color indicated the presence of carotenoids.

Terpenoids

Test for terpenoids was carried out by adding 1 mL chloroform to the 1 mL of extract and then an equal volume of concentrated sulphuric acid was added. Formation of the bluish red coloration indicated presence of terpenoids.

Tannins

Tannins were tested by mixing 0.5 g of the sample with 20 mL of water in a test a beaker. About 2 mL of 0.1M FeCl₃ was added. Formation of blue black coloration indicated presence of tannins.

Isolation and purification of compounds 1 and 2.

The DCM extracts of the leaves was separated by column chromatography using a step gradient of hexane: dichloromethane: ethyl acetate, starting with 100 % hexane stepped to 10, 20, 30, 50, 80 and 100 % dichloromethane, followed by 20 and 30 % ethyl acetate in dichloromethane. Twenty fractions of 100 mL each were collected in each step. Fractions 1-19 were combined and purified using 80 % dichloromethane to produce erythrodiol (18 mg), fractions 34- 36 were combined and separated with 20 and 30 % ethyl acetate in DCM to afford Uvaol (12 mg).

Erythrodiol (1).

White amorphous solid, The ¹H-NMR spectral data δ_H 5.17 (H-12), δ_H 3.54 (H-3), δ_H 3.20 (H-28), δ_H 1.22, 1.13, 0.96, 0.91, 0.89, 0.85, 0.76 (3H each). The ¹³C-NMR spectral data; δ_C 144.2 (C-13), δ_C 122.4 (C-12), δ_C 79.02 (C-3) and δ_C 69.65 (C-28).

Uvaol (2).

White amorphous solid, The ¹H-NMR spectral data δ_H 5.17 (H-12), δ_H 3.50 (H-3), δ_H 3.14 (H-28), δ_H 1.35, 1.34, 1.34, 1.31, 1.32, 1.07, (3H each). The ¹³C-NMR spectral data; δ_C 138.7 (C-13), δ_C 122.3 (C-12), δ_C 79.0 (C-3) and δ_C 69.7 (C-28).

BIOLOGICAL STUDIES

Antibacterial activity of the extracts were determined by micro-broth dilution assay as described by Buwa and Staden, (2006). The four preserved strains of bacteria used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 87853, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* BCCM 1735. The bacterial strains were cultured for 18 hours at 37°C and were standardized to a cell density of 1.5×10^8 cfu mgL⁻¹ equivalent to 0.5 McFarland Standard. About 10 mL of standardized bacterial strains (*Escherichia coli* ATCC 25922, *Bacillus subtilis* BCCM 1735, *Pseudomonas aeruginosa* ATCC 87853, and *Staphylococcus aureus* ATCC 25923) were seeded in petri-plates containing MH agar by streaking entire MH media surface using sterilized cell spreader under luminous flame. The mixture was then allowed to dry for five minutes. This was followed by the application of plant extract filter paper discs which were placed over the

numbered divided parts on culture media. Two-fold Serial dilutions was used. Concentration was made in range of 1.0 to 0.3 mgL⁻¹. Plates were covered to avoid contamination and were incubated for 24 hours at 37 °C. DMSO impregnated discs were used as the negative control. Zones of inhibitions of growth were measured in millimeters. The minimum inhibitory concentration (MIC) was described as the lowest concentration of the test compounds that completely inhibited the growth of microorganisms.

RESULTS AND DISCUSSION

Phytochemical tests of the extracts revealed the presence of alkaloids, terpenoids, tannins and flavones in *O. europea* and *O. africana* (**Table 1 and 2**). Alkaloids were absent in all the twigs extract while carotenoids were absent in all leaves extracts of *O. africana*, this was the main difference in the phytochemical profile of the twigs and leaves in *O. africana* and *O. europea*. Other researches have shown that quinolone group of alkaloids, cinchonine and cinchonidine were found to be present in the plant ethanol leaves extracts of *Olea europea* (Hansen *et al.*, 2006) while studies on stems showed presence of tannins, alkaloids and flavones (Geissman *et al.*, 2005).

Phytochemical investigation of *O. africana* led to the isolation of two triterpenoids 1 and 2 which belongs to classes of oleanane and ursane respectively. Compound 1 (**Figure 1**) was obtained from DCM extracts as a white amorphous solid. The ¹H-NMR spectrum compound showed characteristic peaks of a pentacyclic triterpenoid by correlation. Single olefinic proton signal (a triplet) was observed at δ_H 5.17 and was assigned to H-12, it also revealed two oxygenated protons resonating at δ_H 3.54 and δ_H 3.20. They were assigned to H-3 and H-28 respectively indicating the presence of hydroxyl moiety. Also present were seven methyl peaks at δ_H 1.22, 1.13, 0.96, 0.91, 0.89, 0.85 and 0.76 each 3H. The ¹³C-NMR spectra of compound exhibited the presence of 30 carbon signals (**Table 4**). The DEPT spectrum shows 7 methyl carbons, 11 Methylene, 5 Methine and 7 Quaternary carbon. The Sp^2 signals at δ_C 144.2 and 122.4 were attributed to C-13 and C-12 respectively; characteristic of oleanane triterpenoids. Also present were two oxygenated carbon signals at δ_C 79.02 (C-3) and δ_C 69.65 (C-28) which correlated to the signals at δ_H 3.54 and δ_H 3.20 in the HSQC spectrum. The HMBC spectrum showed correlation of H-23 and H-24 with C-3, H-23 and H-24 with C-5, H-25 with C-9, H-29 with C-19, H-27 with C-8, C-14, H-11 with C-13, H-18 with C-12 and C-15. The NOESY spectrum exhibited correlations of H-3 and H-28, indicating that they are both in the alpha position. Based on these spectral data, the compound was identified as a triterpenoid. The structure was proposed by comparison of data with those found in literature and was identified as the known erythrodiol (Marizeth *et al.*, 2002). Erythrodiol has also been isolated from *Olea europea* (Hansen *et al.*, 2006).

Compound 2 (**Figure 2**) was a white amorphous powder from dichloromethane extracts. The ¹H NMR spectrum showed the presence of a single olefinic proton signal (a triplet) at δ_H 5.11 and was assigned to H-12. This proton correlated to methine carbon signal at δ_C 122.3 in the HSQC spectra. The ¹H NMR also revealed two oxygenated protons resonating at δ_H 3.50 and δ_H 3.14. They were assigned to H-3 and H-28 respectively indicating the presence of 3 β -hydroxyl moiety. Methyl peaks were at δ_H 1.35, 1.34, 1.34, 1.32, 1.31, 1.31 and 1.07 each 3H. The ¹³C-NMR spectrum of compound 2 (**Table 4**) exhibited the presence of 30 carbon signals, the DEPT spectrum showed the presence of 6 methyl carbons, 7 methine, 10 methylene and 7 quaternary carbon. The sp^2 signals at δ_C 138.7 and δ_C 122.3 in the ¹³C NMR spectrum were attributed to C-

13 and C-12 respectively, characteristic of ursane triterpenoids. Also present in the ^{13}C NMR spectrum were two oxygenated carbon signals at δ_{C} 79.0 (C-3) and 69.7 (C-28). The HMBC spectrum showed correlation of H-18 and H-21 with C-29, H-30 and H-11 with C-9, H-9 with C-11. The position of the double bond at C-12, 13 was also evident from the HMBC correlations of olefinic proton with carbons at δ_{C} 138.87 (C-13), δ_{C} 125.30 (C-18). The NOESY spectrum exhibited correlations of H-3 and H-28. HSQC spectrum showed correlations between C-5 and H-5, C-9 and H-9, C-16 and H-16. Based on spectral data and literature values it was identified as the known uvaol (2) (Marizeth *et al.*, 2002).

The MIC values recorded for the crude extracts suggested moderate antibacterial activity (**Table 3**). The most active extracts was methanol with MIC value of 0.3 mg/mL against *S. aureus* and 1.0 mg/mL against *B. subtilis*. The least active extracts were from hexane with MIC values of 1.0 mg/mL against *S. aureus* and *B. subtilis*. This could be because plant-based constituents have also been reported to exhibit different modes of action against bacterial strains which range from interference with the phospholipoidal cell membranes, this has a consequence of increasing the permeability profile and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components and destruction or inactivation of genetic material (Kotzekidou *et al.*, 2008).

Table 1.

Compounds present in stems and leaves of *Olea africana*

	Twigs extract				Leaves extract			
	Hex.	DCM	EtoAc	Meth.	Hex.	DCM	EtoAc	Meth.
Alkaloids	-	-	-	-	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Flavanoids	+	+	+	+	+	+	+	+
Flavones	+	+	+	+	+	+	+	+
Carotenoids	+	+	+	+	-	-	-	-

Key: + denotes present,

Hex: Hexane, **Meth:** Methanol,

- denotes absent.

EtoAc: Ethyl acetate, **DCM;** Dichloromethane

Table 2

Compounds present in stems and leaves of *Olea europea*

	Twigs extract				Leaves extract			
	Hex.	DCM	EtoAc	Meth.	Hex.	DCM	EtoAc	Meth.
Alkaloids	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Flavanoids	+	+	+	+	+	+	+	+
Flavones	+	+	+	+	+	+	+	+
Carotenoids	+	+	+	+	+	+	+	+

Key: + denotes present,

Hex: Hexane, **Meth:** Methanol,

- denotes absent.

EtoAc: Ethyl acetate, **DCM;** Dichloromethane

Table 3.

The Minimum Inhibition Concentration of Methanol, Hexane, DCM and ethyl acetate against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Extract	Minimum Inhibition Concentration (mgmL ⁻¹)					
	<i>E.-coli</i>		<i>S. aureus</i>		<i>B. subtilis</i>	
	<i>O. africana</i>	<i>O. europea</i>	<i>O. africana</i>	<i>O. europea</i>	<i>O. africana</i>	<i>O. europea</i> <i>a</i>
Methanol	1.50	1.30	0.30	0.31	1.00	1.20
Hexane	2.30	2.20	1.00	0.41	1.00	1.00
DCM	2.30	2.30	0.50	0.54	0.30	0.30
Ethyl acetate	2.30	2.10	1.00	1.00	-	-

Table 4.**¹³C data of compounds 1 and 2.**

Carbon position	Compound 1 (ppm)	Compound 2 (ppm)
1	38.8	39.3
2	27.2	27.2
3	79.0	79.0
4	38.8	38.5
5	55.2	54.0
6	18.3	17.3
7	32.8	32.5
8	39.8	40.0
9	47.6	47.5
10	36.9	36.9
11	23.6	23.4
12	122.3	125.0
13	144.2	138.7
14	41.7	42.0
15	25.9	25.5
16	22.0	23.3
17	36.9	35.2
18	42.3	54.0
19	46.5	39.7
20	31.0	39.4
21	34.1	29.7
22	30.9	35.2
23	28.1	28.1
24	16.7	16.7
25	16.7	16.7
26	16.8	16.8
27	25.9	23.3
28	69.9	69.7
29	33.2	17.3
30	23.6	21.3

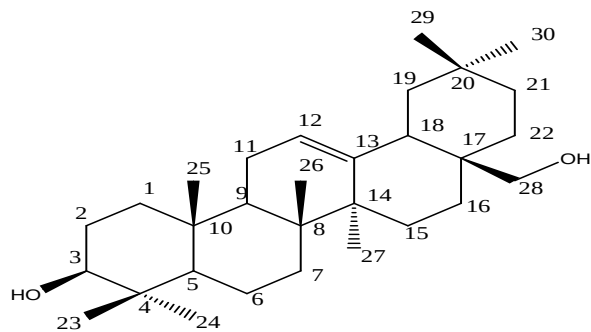


Figure 1

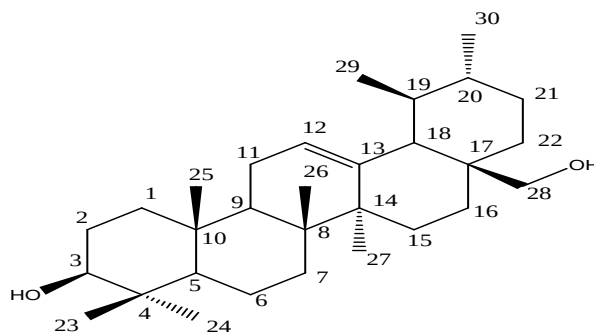


Figure 2

CONCLUSION

Olea africana and *O. europea* are used in Kenyan traditional medicine as a chewing sticks and for treatment of other periodontal diseases. Previous phytochemical studies have found presence of alkaloids, flavanoids, carotenoids and terpenoids among others in *O. europea*. Our study has revealed that the leaves crude extracts contains terpenoids, tannins, flavones and flavanoids while carotenoids were absent in *O. africana* but were present in *O. europea*. Stem extracts tested positive for terpenoids, tannins, flavones and flavanoids while alkaloids tested negative. Purification of the *O. africana* leaves extracts afforded two triterpenoids, identified as erythrodiol and uvaol which belongs to oleanane and ursane classes respectively. They exhibited relatively good antibacterial activity against the bacterial strains used in this study. The triterpenoids have been reported to be used as antibacterial, antifungal, anti-inflammatory, anti-leishmanial and antimalarial. The plant could therefore be used in traditional medicine to treat the symptoms of inflammation and infections by bacteria and as a chewing stick upon further investigation.

ACKNOWLEDGEMENT

The author is thankful to the division of research, University of Kabianga for funding the research and Prof. Matasyo of Egerton University for allowing bioassay tests to be conducted in his research laboratory.

REFERENCES

- Almas, K. (2001). The antimicrobial effects of seven different types of Asian chewing sticks. *Odontostomatol Tropical*. 24; 17 – 20.
- Al-Otaibi, M., Al-Harthy, M., Gustafsson, A., Johansson, A., Claesson, R. and Angmar, B. (2004). Sub-gingival plaque microbiota in Saudi Arabians after use of miswak chewing stick and toothbrush. *Journal of Clinical Periodontal*. 31; 1048 – 1053.
- Bulunas, J., Kerry, N. and Kinghorn, D. (2005). Drug discovery from medicinal plants. *Journal of Life Science*. 78; 431 - 444.
- Buwa, V. and Staden, V. (2006). Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa. *Journal of Ethnopharmacol*. 130; 139 - 143.
- Chidzonga, M., Carneiro, C., Kalyanyama, M., Kwamin, F. and Oginni, O. (2015). Determinants of oral diseases in the African and Middle East region. *Advanced Dental Resources*. 27; 26 – 31.

- Edeoga, O., Okwu, E. and Mbabie, O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African journal of biotechnology*. 4; 685 - 688.
- Geissmann, A., Dauter, D. and Victor, S. (2005). Anthocyanins, Flavones and related water soluble plants pigments. 'Modern Methods of Plants analyses'. Vol III, Paech K and Tracey MV. (Editors). Springer Verlag Gottingen, Pp.405 - 495.
- Hansen, K., Kompany, R. and Kamazawa, P. (2006). Isolation of an Angiotensin Converting Enzyme (ACE) inhibitor from *Olea europaea* and *Olea lancea*. *Phytomedicine*. 2; 319 - 325.
- Hyson, M. (2003). History of the toothbrush. *Journal of Dental Health*. 51; 73 – 80.
- Kotzekidou, P., Giannakidis, P. and Boulamatsis, A. (2008). Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens *in vitro* and on the fate of inoculated pathogens in chocolate. *Food Science and Technology*. 41; 119 - 127.
- Marizeth, L., Barreiros, J., David, M., Pedro, A., Pereira, P., Maria, H., Guedes, S. and Jucen. D. (2002). Fatty acids Esters of Triterpenes from *Erythroxylum passernum*. *Journal of Brazilian Chemical Society*. 13; 5669 - 5673.
- More, G., Tshikalangea, E., Lall, N., Botha, F. and Meyer, M. (2008). Antimicrobial activity of medicinal plants against oral microorganisms. *Journal of Ethnopharmacology*, 119; 473 - 477.
- Ncube, B., Finnie, F. and Van Staden, J. (2012). *In vitro* antimicrobial synergisms within plants extracts combinations from three South African medicinal bulbs. *Journal of Ethnopharmacology*. 139; 87- 89.
- Ogunbodede, O., Kida, A., Madjapa, S., Amedari, M., Ehizele, A., Mutave, R., Sodipo, B., Temilola, S. and Okoye, L. (2015). Oral health inequalities between rural and urban populations of the African and Middle East region. *Advanced Dental Resources*. 27; 18 – 25.
- Shagal, H., Kubmarawa, D. and Alim, H. (2012). Preliminary phytochemical investigation and antimicrobial evaluation of roots, stem-bark and leaves extracts of *Diospyros mespiliformis*. *International Research Journal of Biochemistry and Bioinformatics*, 2; 11 - 15.
- Sonkos, N. and Gondson, M. (2011). Photodynamic therapy in control of biofilms. *Periodontology*. 55; 143 - 166.
- Tapsell, C., Hemphil, I. and Cobiac L. (2006). Health benefits of herbs and spices; the past, present and future. *Medical Journal*. 185; 54 - 64.
- Verenne, B., Petersen, E and Quattaras. S. (2006). Oral health behavior of children and adults in urban and rural areas of Bukina Faso. *International Journal of Dental Health*. 56; 61 - 70.
- World Health Organization (WHO). (2008). Commission on Social Determinants of Health-final report. Closing the gap in a generation: health equity through action on the social determinants of health. Geneva (Switzerland): WHO.