Original Article

Bulletin of Environment, Pharmacology and Life Sciences

Online ISSN 2277-1808
Bull. Env. Pharmacol. Life Scien. Volume 1 [12] November 2012: 54- 59

Website: www.bepls.com

Two Oils from the Ethyl-acetate Fraction of *Cyathula prostrata* (L.) Blume

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ABSTRACT

*Cyathula prostrata is an herbal recipe used in traditional medicine for the treatment of chest troubles, dysentery, diarrhea, craw-craw, scabies, rheumatism, inflammations and tumours amongst many others. Silica gel column chromatography of the ethyl-acetate fraction yielded two oils whose structures have been established by spectroscopic techniques to be ethyl hexadecanoate **1** (alkyl ester) and 7, 9-Di-tert-butyl-1-oxaspiro n(4, 5) deca-6, 9-diene-2, 8-dione **2** (diketone) respectively. The two compounds demonstrated between marginal and moderate antibacterial and antifungal activities.*
***Keywords**: Cyathula prostrata; chromatography; fraction; ester; diketone.*

INTRODUCTION

Cyathula prostrata (L.) Blume belongs to the family, Amarantheceae [1, 2, 3] and is commonly referred to as pasture weed, pigweed and prickly chaff-flower plant 4. It was originally native to tropical Africa and Asia (Nigeria, Mozambique, Uganda, China, India and Vietnam) but now found growing all over the world including tropical America, Australia and the Pacific Islands [5, 6, 7, 8, 9, 10]. The plant is employed in ethno- medical practice in the treatment of pruritus, dyspepsia, skin ulcers, craw-craw, scabies, diarrhea, dysentery, coughs [11,12, 13, 14, 15, 16], inflammatory conditions such as rheumatism, shingles, wounds and ulcers, sexually transmitted diseases and viral infections [1, 2, 17, 18]. Extracts of the plant are also used as an expectorant, emetic in food poisoning, demulcent and vulnerary. Previously, ecdysterone, a phytoecdysone was isolated from *C. prostrata* [19] while palmitic, stearic, oleic, linoleic and linolenic acids have also been obtained [20]. This present study was undertaken with the aim of isolating other chemical constituents present in the plant using the principles of bio-activity guided column chromatography.

MATERILAS AND METHODS

Plant collection and authentication:

The fresh aerial parts of *C. prostrata* (L.) Blume were collected in the month of August, 2010 on a farmland in Itak Ikot, Ikono Local Government Area, Akwa Ibom State, Nigeria. The plant was identified by Dr. M. Bassey of the Department of Botany and Ecological Studies, University of Uyo, Nigeria. The authentication by comparison was done with herbarium samples of the Forestry Research Institute of Nigeria (FRIN) and the National Institute of Horticulture (NIHORT), both at Ibadan, Oyo State, Nigeria. A voucher specimen of the plant (No H92) was deposited in the herbarium of the Faculty of Pharmacy, University of Uyo, Nigeria. Immediately after collection, the plant was dried in an oven (Gallenkamp, UK) at 40 ^{0}C for 48 h and the resultant dried material powdered on an electric mill (Uniscope, UK).

Bulk extraction and isolation:

The dried powder (1kg) was exhaustively extracted with 50% aqueous EtOH (3 x 5L) at room temperature (27± 2 ^{0}C) for 72h. The resultant crude extract was filtered, concentrated *in vacuo* on a rotary evaporator (Buchi CH-920, Laboratorium Technic, Flawk/SG, Switzerland),

weighed and stored in a desiccator prior to further use. 110g of the extract was partitioned using H_2O:EtOAc (6 x 500 mL) and the ethyl- acetate fraction obtained was evaporated to dryness to give a solid residue from which the two compounds were isolated.

The ethyl acetate fraction (8.5 g) was chromatographed on a silica gel 254 column (Techmel, USA; 10g pre-swollen in 100% toluene; 3g concentration zone + 7g separation zone; 17.5 x 4cm) and eluted with a gradient of 10% $(CH_3)_2CO$: toluene (100mL), 20% $(CH_3)_2CO$: toluene (100mL), 30% $(CH_3)_2CO$: toluene (100mL), 40% $(CH_3)_2CO$: toluene (100mL) and 50% $(CH_3)_2CO$: toluene (100mL). Fractions of 10mL each were collected and monitored on silica plates in $(CH_3)_2CO$: toluene:H_2O (10:20:1) and using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents. Hence, fractions with similar TLC characteristics (R_f, reaction with $FeCl_3$ or vanillin-H_2SO_4) were bulked and five semi-pure residues coded CP-1, CP-2, CP-3, CP-4 and CP-5 were obtained.

CP-1 (1.5g, greasy and viscous material) was purified on a much shorter silica gel 254 column (10 x 2cm) successively with 100% toluene (100mL) and 20% $(CH_3)_2CO$: toluene (75mL) resulting in the isolation of ethyl hexadecanoate **1** (upper oil; R_f = 0.72; 270mg). Similarly, CP-3 (1.2g, less viscous substance) was also cleaned on a short silica gel 254 column by isocratic elution using 30% $(CH_3)_2CO$: toluene (180mL) which furnished 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione **2** (lower oil; R_f = 0.63; 310mg). However, attempts made to similarly purify CP-2, CP-4 and CP-5 using both column and preparative thin-layer chromatography (pTLC) were unsuccessful as multi-component profiles were obtained. Subsequently, none of these residues was processed any further.

The optical densities and optical rotation of the isolated compounds were determined on a polarimeter (ADP-220, Bellingham Stanley, UK) while the Refractive indices were obtained using WAY-15 Abbe refractometer (UK).

Antimicrobial tests:

The micro-organisms used in this study, namely; *Bacillus subtilis* (NCTC 8853), *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418) *Pseudomonas aeriginosa* (ATCC 27853), *Shigella dysentriae* (NCTC 8112), *Aspergillus niger* and *Candida albicans* (NCYC 6) were clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fascitis, osteomyelitis, urine, wounds and vaginal swabs obtained from the Medical Laboratory, University of Uyo Health Centre, Uyo. The clinical isolates were collected in sterile bottles, identified and typed by convectional biochemical tests [21, 22] and then refrigerated at 0-5^{0}C at the Microbiology and Parasitology Unit, prior to use. The hole-in-plate agar diffusion method was used

observing standard procedure with Nutrient Agar and Sabouraud Dextrose Agar (Oxoid, UK) for bacteria and fungi respectively. The inoculum of each micro-organism was introduced into each petri-dish (Pyrex, UK). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Pyrex, UK) to produce wells with diameter of approximately 5 millimetres. The wells were equidistant from each other and the edge of the plate [23, 24]. Concentrations of 15mg/mL of crude extract, 5mg/mL of ethyl acetate fraction, 1mg/mL and 5mg/mL of **1** and **2** were introduced into the wells. Also, different concentrations of 10µg/mL of ampiclox (GlaxoWellome, Nigeria), 1mg/mL of nystatin (Rouche, Nigeria) and 100% methanol were introduced into separate wells as positive and negative controls respectively[25-29]. The experiments were carried out in triplicates. The plates were left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37± 2^{o}C for 24 h. Zones of inhibition were measured in millimetres (mm).

RESULTS AND DISCUSSION

Table 1: Optical rotation, optical density and refractive index of compounds 1 and 2

Sample	Optical rotation $[\alpha]^{20}_D$	Optical density $[d]^{20}_D$	Refractive index $[n]^{20}_D$
1	Inactive	0.851	1.4394
2	Inactive	0.564	1.4390

Table 2: Antimicrobial screening of crude extract, ethyl-acetate fraction and compounds 1 and 2 at different concentrations on test microbes in 100% MeOH.

Test microbe	2A 15mg/ml	3C 5mg/ml	1 1mg/ml	1 5mg/ml	2 1mg/ml	2 5mg/ml	Ampiclox 10µg/ml	Nystatin 1mg/ml	100% MeOH
B. subtilis (NCTC 8853)	18.00	11.00	8.00	8.00	7.00	9.00	18.00	5.00	5.00
S. aureus (NCTC 6571)	12.00	14.00	8.00	8.00	7.00	7.00	16.00	5.00	5.00
E. coli (NCTC 10418)	5.00	7.00	9.00	9.00	9.00	9.00	26.00	5.00	5.00
Ps. aeriginosa (ATCC 27853)	5.00	6.00	6.00	6.00	7.00	7.00	14.00	5.00	5.00
S. dysentriae (NCTC 8112)	5.00	8.00	6.00	6.00	7.00	7.00	22.00	5.00	5.00
A. niger	12.00	14.00	6.00	6.00	7.00	7.00	5.00	21.00	5.00
C. albicans (NCYC 6)	5.00	5.00	6.00	6.00	7.00	7.00	5.00	20.00	5.00

Key: The zone diameter recorded is zone of inhibition + size of cup (zone of inhibition +5) mm; 2A = Crude ethanolic extract; 3C = Ethyl-acetate fraction;

1 = Ethyl hexadecanoate;

2 = 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione.

NCTC - National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9, UK.

NCYC- National Collection of Yeast Cultures, UK.

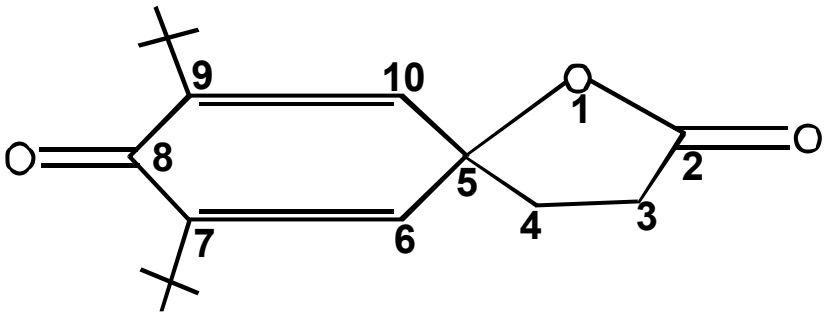
ATCC- American Type Culture Collection**,** Washington, DC.

Spectroscopic data: The data were obtained thus: ES+ - MS on Kratos MS 80, IR on Shimadzu FTIR 8400S, ^{1}H and ^{13}C NMR on Bruker AC 250 operating 300MHz for proton and 75MHz for carbon-13 using $CDCl_3$ as solvent and TMS as internal standard.

Compound 1 : $C_{18}H_{36}O_2$; viscous deep yellow oil; $R_f = 0.72$; $[\alpha]^{20}_D$ (inactive); [d] $^{20}_D$ (0.851); $[n]^{20}_D$ (1.4394); MS [ES+ -MS] m/z (relative intensity): 284 $[M]^+$ (1.0%), 101 $[M\text{-}(CH_2)_2COOCH_5]^+$ (55. 4%), 89 $[M\text{-}(CH_2)_{13}CH_3]^+$ (13.6%), 88 $[M\text{-}(CH_2)_{14}]^+$ (99.9%), 73 $[M\text{-}(CH_2)_{14}CH_3]^+$ (15.6%), 57 $[M\text{-}(CH_2)_{13}COOH]^+$ (21.7%) and 29 $[M\text{-}(CH_2)_{15}COOH]^+$ (22.7%); IR [FTIR] cm^{-1}: 447, 784 and 1732; ^{1}H NMR δ (ppm): 1.44, 1.80, 2.30 and 2.45 ; ^{13}C NMR δ (ppm): 19.76, 22.34, 24.96, 25.84, 26.42, 27.98, 29.18, 30.45, 31.62, 32.22, 34.42, 36.55, 37.20, 47.19, 52.27, 76.60, 77.22 and 168.24 (ester CO).

Compound 2 : $C_{17}H_{24}O_3$; less viscous light yellow oil; $R_f = 0.63$; $[\alpha]^{20}_D$ (inactive); [d] $^{20}_D$ **(**0.564); $[n]^{20}_D$ (1.4390); MS [ES+-MS] m/z (relative intensity): 276 $[M]^+$(1.0%), 220 $[M+ H\text{-}C(CH_3)_3]^+$ (20.2%), 217 $[M\text{-}2H\text{-}C(CH_3)_3]^+$ (25.7%), 205 $[M+H\text{-}CH_3CH_2COOH]^+$ (52.7%), 189 $[M\text{-}C(CH_3)_3\text{-}20]^+$ (24.4%), and 57 $[M\text{-}C_{13}H_{15}O_3]^+$ (99.9%); IR [FTIR] cm^{-1}: 790, 1642 and 1720; ^{1}H NMR δ (ppm): 1.42, 1.72, 2.15, 2.86 and 4.66; ^{13}C NMR δ (ppm): 22.58, 24.50, 26.41, 29.37, 31.61, 34.30, 34.40, 37.44, 39.87, 40.08, 55.01, 61.21, 76.59, 77.44, 124.46 (C=C), 162.12 (CO in conjugation) and 167.67 (lactone or aryl CO).

Ethyl Hexadecanoate 1



7, 9 - Di-tert-butyl-1-oxaspiro-(4,5)deca-6,9-diene-2,8-dione 2

The rules governing collection of plant parts, extraction and solvent-partitioning were observed thus preventing any changes to the chemical composition of the crude extract or fractions [30, 31]. Two previous studies reported that the crude extract of *C. prostrata* contained saponins, tannins, flavonoids, terpenes and cardiac glycosides while alkaloids, anthraquinones and cyanogenic glycosides were absent [28,29]. Also, further investigations showed that the antibacterial and antifungal activities resided in the ethyl acetate fraction [28,29,32]. In addition, the H_2O/EtOAc partition extracted the largest amount of material, hence the choice of the EtOAc fraction for column chromatography from where compounds **1** and **2** were isolated.

1 and 2 are both oils, hence the optical rotations, optical densities and refractive indices were measured at the λ 589.3nm (Na-D light) and 20 ^{0}C . The results displayed in Table 1 show that both compounds are optically inactive. Consequently, neither would rotate a plane of polarization (either clockwise or anticlockwise) when polarized light passes through either of them. Hence, neither would show dextrorotation (+) nor laevorotation (-)[33,34] . The determined optical densities are 0.851 and 0.564 respectively. Optical density like optical rotation can be used to measure dispersion in a substance. Hence, the results are not surprising because 1 was isolated as a viscous, fixed oil while 2 was obtained as a less viscous, non-fixed oil. Consequently, 1 is expected to be denser than 2 and this fact is corroborated by the determined refractive indices of 1.4394 and 1.4390 respectively. The values, especially those of compound 1 compare favourably with literature values of pure ethyl hexadecanoate at $[d]^{20}_D$ (0.847) and $[n]^{20}_D$ (1.4380) respectively[35-37] . The structures of 1 and 2 were established by a combination of spectroscopic techniques as highlighted above. The obtained MS data were matched with library data of organic compounds [38] , hence, 1 and 2 were identified to be ethyl hexadeacanoate and 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione respectively. The ES+-MS of 1 indicated prominent peaks such as $[M]^+$ at m/z 284 (1.0%) while 101 (55. 4%) and base peak 88 (99.9%) represent the loss of $-(CH_2)_2-$ $COOCH_5$ and $-(CH_2)_{14}$ units respectively from the $[M]^+$. Due to the nature of the matrix, many peaks also appeared in the MS of **2** but those that could readily be identified include: $[M]^+$ at m/z 276 (1.0%) while 220 (20.2%) and 205 (52.7%) indicate the loss of tert-butyl and lactone ring units respectively. The base peak at m/z 57 (99.9%) corresponds to the disintegration of the entire molecular moiety save for one tert-butyl unit. The IR spectra of the two compounds show

diagnostic C=O stretchings at 1732 and 1720 cm^{-1} respectively in addition to the C=C peak at 1640 cm^{-1} noticed especially in spectrum of 2. though, ^{1}H and ^{13}C NMR techniques could not exclusively identify the compounds because 2D-NMR, HECTOR and NOESY experiments were not performed. However, the proton signals at 1-2 ppm indicate methyl proton, while those at 2-3 ppm and 4 ppm respectively account for the methylene and methyne protons in the two compounds. In addition, the diagnostic carbon signals especially of C=C (124.46), esters (168.24) and lactones (167.67) are well accounted for. The results of the antimicrobial tests presented in Table 2 show the two compounds showed moderate antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* while the activity recorded against *Ps. aeriginosa* and *S. dysentriae* was marginal irrespective of concentration of incubation. Also, both demonstrated marginal antifungal activity against *A.niger* and *C. albicans.* These observations are not surprising because gram negative organisms such as *Ps. aeriginosa* usually show a unique resistance to antimicrobial agents. This resistance is believed to be due to the nature of the cell envelope of the organism which unlike gram positive organisms, posses a sophisticated three-layered envelope which does not allow permeation of external agents. Similarly, fungal strains such as *Candida spp*. limit the permeation of substances because of their integral structures which are pleomorphic and facultative in nature hence, resembling those of higher plants [39] .

CONCLUSION

This study reports for the first time the isolation of ethyl hexadeacanoate and 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione from the ethyl-acetate fraction of *C. prostrata.* Also, the observed antimicrobial activities of the compounds have lent credence to the folkloric uses of the plant.

ACKNOWLEDGEMENTS

The kind assistance rendered by Dr. P. Dziemba, Institut Fur Pharm. Chemie, Hittorfstr. 58-62, 48189 Muenster, Germany, Dr. Falodun of the University of Benin, Benin, Edo State, Nigeria and Mr. B. Odjobo of the National Research Institute for Chemical Technology (NARICT) Zaria, Kaduna State, Nigeria in obtaining the MS, ^{1}H , ^{13}C NMR and IR spectra respectively is warmly acknowledged. The contribution of Mr. E. Akpan, Principal Technologist, Pharmaceutical Microbiology Unit, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria is equally appreciated.

REFERENCES

1. Breyer-Brandwikj, MG. (1962). Medicinal and poisonous plants of Southern and Eastern Africa. E and S. Livingstone Limited, UK, 1457.
2. Iwu, MM. Handbook of African medicinal plants. CRS Press, Florida, (1993); 435.
3. Hirt, A and Mpia E. (1995). Natural medicine in the tropics. Action for nature and medicine (Anamed) Germany, 232 .
4. Germplasm Resource Information Network.http://www.ars/grin-cgi-bin/npgs/html/taxon (1999); (2012-10-03).
5. Smith, AC. (1981). Flora of Vitiensis nova: A new Flora of Fiji. Vol. 2, National Tropical Botanical Gardens, Hawaii, 289-290.
6. Townsend, CC. Flora of West Tropical Africa.Vol. 1, Part 1; FZ, Vol.9, Part 1, 1988;28.
7. Steyermark,JA. (1995). Flora of Venezuelan Guayana (F. VeneGuay). http://www.wikipedia.org/wiki.cyathula; http://www.ars-grin/ cgi-bin/npgs/html/taxon,(2010-09-06).
8. Swarbrick, JT. (1997). Weeds of the Pacific Islands. Technical Paper No 209. South Pacific Commission, New Caledonia, 124.
9. McCormack, G. (2005). Cook Islands Biodiversity and Natural Heritage. http://www.ars- grin/cgi-bin/npgs/html/taxon,; (2011-03-06).
10. United States Department of Agriculture., (2007). Agricultural Research Service. National Genetic Resource Program. http://www.ars-grin/cgi-bin/npgs/html/taxon,; (2010- 10-01).
11. Dalziel, JM. (1956).The useful plants of Tropical West Africa. Crown Agents, UK,; 174- 175.
12. Burkill, HM. (1985). Useful plants of West Tropical Africa. Vol.1, Fam.A-D, Royal Botanical Gardens, Kew, 324.
13. Burkill, IM. (1990). The flora of Vavau. One of the Tonga Islands, with a short account of its vegetation by Charles Steele Crosby. Linnaeus Journal of Botany, 35: 20-65.
14. Trease, A and Evans WC. (1996).Pharmacognosy. W.B. Saunders Company, Edition 14, UK, 50, 376 and 422-425.
15. Etukudo, AM. (2000). Forest- Our divine treasure. Dorand Publishers, Uyo, Nigeria,; 164.
16. Etukudo, I. (2003). Ethnobotany. Convectional and traditional uses of plants. The Verdict Press, Uyo, Nigeria,243.

17. Duke, JA. Borderline Herbs. C.R.C. Press, Florida, (1984); 21-24.
18. Kayode, J and Kayode GM. (2008). Ethnomedicinal survey of botanicals used in treating sexually transmitted disease in Ekiti State, Nigeria. Ethnobotanical Leaflets, ; 12: 44-45.
19. Shah, CV and De Souza JN. (1971).Ecdysterone from *Cyathula prostrata*. Phytochemistry, 10: 1398-1399.
20. Unni, BG, Borah A, Warm SB, Singh HR, Devi B and Bhattacharjee M. (2009).Phytochemical and antibacterial study of traditional medicinal plants of North East India on *Escherichia coli*. Asian Journal of Experimental Science, 23(1): 108.
21. Gibson, L and Khoury J. (1986).Storage and survival of bacteria by ultra-freeze. Letters of Applied Microbiology, 3: 127-129.
22. Murray, P, Baron E, Pfaller M, Tenover F and Yolken R. In: Manual of clinical microbiology. American Society of Microbiology Press, Washington, D.C., (1995); 343-345.
23. Washington , J. (1995). The agar diffusion method . In: Manual of clinical microbiology. Edition 4. (Eds). E. Lannelte, A. Balows, W. J. Haasler and H. J. Shadowy. American Society of Microbiology Press, Washington, D.C., 967-970.
24. National Committee for Clinical Laboratory Standards.(2003). Performance standard for antimicrobial susceptibility test. Edition 8, Approved standard. Villanova (PA): The Committee, 130.
25. Oladimeji, HO. (1997). Bioactivity-guided fractionation of *Acalypha wilkesiana* . Unpublished M.Sc. Thesis, Obafemi Awolowo University, Ile-Ife, Nigeria,; 154p.
26. Nia, R, Oladimeji HO, Ubulom PME and Ekpo BAJ. (2006). Larvaecidal and antimicrobial potential of *Anthocleista djalonensis*. L. (Gentinaceae). African Journal of Pharmaceutical Research and Development,; 2(1): 97-101.
27. Adesina, SKO, Idowu O, Ogundaini OA, Oladimeji H, Olugbade TA, Onawunmi GO and Pais M. (2000). Antimicrobial constituents of the leaves of *Acalypha wilkesiana* and *Acalypha hispida.* Phytotherapy Research, 14: 371-374.
28. Oladimeji, HO, Attih, EE and Usifoh, CO. (2012a). Preliminary *in-vitro* investigations into the larvicidal and antimicrobial potentials of *Cyathula prostrata* (Linn.) Blume. Journal of Pharmacognosy and Phytotherapy, 4: 12-15.
29. Oladimeji HO. (2012b). Chemical and biological studies on *Cyathula prostrata* (L.)Blume. Unpublished. Ph.D. Thesis, University of Uyo, Uyo, Nigeria,; 189p.
30. Odebiyi, OO and Sofowora A. (1978). Phytochemical screening of Nigerian medicinal plants- Part I. Lloydia, 1978; 41: 234.
31. Odebiyi, OO and Sofowora, A. (1979).Phytochemical Screening of Nigerian medicinal plants- Part II. 2nd OAU/STRC Inter-African symposium on traditional pharmacopoeia and African medicinal plants. OAU/STRC Publishers, Lagos, Nigeria, 115: 216.
32. Oladimeji, OH, Nia, R, Ajebesin, K, Igboasoiyi, AC and Dalandi, B. (2005). Phytochemical and antibacterial studies on the aqueous and non-aqueous extracts of *Cyathula prostrata* (L.) Blume. Global Journal of Applied and Medical Sciences,; 4(1& 2): 9-12.
33. Olaniyi, A and Ogungbamila, FO. (1991). Experimental pharmaceutical chemistry. Shaneson C.I. Limited, Ibadan, Nigeria, 49-52.
34. Olaniyi, AA. (2000).Principles of quality assurance and pharmaceutical analysis. Mosuro Publishers, Ibadan, Nigeria, 2000; 151-158, 216-217, 264-269 and 443-457
35. Environment Canada. Domestic substances list categorization. Canadian Environmental Protection Act (CEPA). Environmental Registry, http://www.ewg.org/ skindeep/site/about.php; http://www.chemindustry. com/index/ html 2008; (2011-04-08).
36. Food and Drug Administration. (2008). EAFUS [Everything Added to Food]: A food additives database. FDA Office of Food Safety and Applied Nutrition. http://www.ewg.org/skindeep/site/about.php; http://www. chemindustry.com/index/html ; (2011-11-07).
37. International Fragrance Association. IFRA fragrance ingredient list based on 2008 use survey. http://www. ewg. org/skindeep /site/about. php; http://www.chemindustry.com/index/html, 2010; (2011-10-10).
38. Lopez- Avila, V. Organic Mass Spectrum, (1987); 22: 557.
39. Brown, MR. (1975).A question of resistance. The Pharmaceutical Journal, 215: 239-242.