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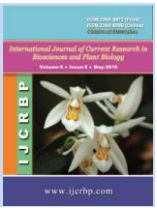

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Isolation and Idendification of Two Triterpenes with Larvicidal Potential in *Launaea taraxacifolia* (Asteracae) on *Anopheles gambiae* by HPLC

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ABSTRACT

This work provides information on the isolation and identification of two bioactive triterpenic compounds in *Launaea taraxacifolia* (Asteracae) and their larvicidal potential. These are oleanolic acid (OA) and lupeol. We prepared a hydro-methanolic extract of *Launaea taraxacifolia* leaves on which we performed a liquid-liquid fractionation with n-hexane. The hexane fraction obtained was subjected to atmospheric pressure chromatography (APC) followed by a series of TLC purifications leading to the isolation of the two triterpenes. Their identification is made by means of a straightforward HPLC-UV method of comparison and superposition with isocratic controls in binary mode. Ursolic, oleanolic and betulinic acids (Sigma) are used as controls for the OA with a mobile phase composed of 20% of H_2O and 80% of a mixture ACN/MeOH (40/35, v/v) and lupeol, α-amyrin, β-amyrin (Sigma) for lupeol with a mobile phase composed of 5% H_2O and 95% ACN. The flow rate was 1000 µL/min with detection at a wavelength of 210 nm. We then carried out susceptibility tests on 3rd instar *Anopheles gambiae* larvae from two genotypes: Kisumu strain of Kenyan origin and the wild strain from Cotonou larval breeding sites. Both triterpenoids were found to be active on both larval origins with lethal concentrations LC_{50} of 38.12 ppm in 24 hours and 2.50 ppm in 48 hours exposure for Kisumu strain; 32.14 ppm and 2.29 ppm respectively in 24 hours and 48 hours for the wild population for the OA. As for lupeol, we obtained LC_{50} of 105.16 ppm and 45.69 ppm respectively in 24 hours and 48 hours on the strain Kisumu; whereas, 103.20 ppm and 32.8 ppm respectively in 24 hours and 48 hours of contact for wild larvae with regard to lupeol.

Introduction

Preventive malaria control is moving increasingly towards mosquito control, of which one of the most prominent methods is larval control. Thus, for integrated and ecologically profitable control, the use of bio-larvicides based on active extracts of plants would contribute to a significant reduction in the population of malaria vectors. The added value of larvicides when integrated with insecticide-treated bednets is that they can induce an additional reduction in infective puncture of up to 73% of the entomological inoculation rate (Fillinger et al., 2009). In addition, the development of pharmacognosy revealed the advantages of several phytochemicals with very rich and varied therapeutic effects. Recent studies conducted in Benin by Ahouansou and colleagues have shown that the hydro-methanolic extract of *Launaea taraxacifolia* (Asteraceae) has an inhibitory effect on the larvae of *Anopheles gambiae* (Ahouansou et al., 2017). *Launaea taraxacifolia* is a leguminous plant, edible and widely used in food in various forms and with many therapeutic effects (Dansi et al., 2008, Koukoui et al., 2015, Owoeye et al., 2015).

This study is undertaken to investigate the phytochemicals responsible for larvicidal activity in *Launaea taraxacifolia*. For this purpose, a liquid-liquid fractionation of the hydro-methanolic extract followed by separation by atmospheric pressure chromatography (APC) was carried out. Chemical analysis by high pressure liquid chromatography (HPLC-UV) after a series of thin-layer chromotography (TLC) purifications allowed us to isolate and identify two triterpenoids that inhibit *Anopheles gambiae* larvae, vectors of malaria: oleanolic acid (OA) and lupeol (Figure 1).

Materials and Methods

Plant collection and extract preparation

The leaves of *Launaea taraxacifolia* were harvested in maize fields at Comé, southern Benin, and are identified and certified by the National Herbarium of the University of Abomey-Calavi under number: AA6689/HNB. They are then dried at 16°C in the laboratory for a week before being crushed and reduced to powder. At first, we extracted with 100 g of powder to which we added 500 mL of methanol-water (70:30 v/v) with 0.5% formic acid. This mixture was homogenized and then kept under continuous stirring for 24 hours, filtered and then evaporated to dryness using a rota-vapor (*Heidolph Laborota 4000 efficient*). Then, 2.5 g of the hydro-methanolic extract obtained was dissolved in 150 ml of methanol-water mixture (1:3, v/v). This solution was washed three times with 100 ml of n-hexane by washing with a separating funnel. The hexane fraction (F_{Hex}) obtained after this liquid-liquid fractionation process was reduced to dryness by means of a rota-vapor apparatus.

APC splitting and compound isolation

We poured 100 g of Merck® silica gel (0.063-0.20 mm) into a glass column of 2 cm in diameter and 80 cm in height to which we added 300 ml of methanol, which was left for 24 hours. Conditioning is done with 200 mL of 100% n-hexane cast along the stationary phase and collected in a vial. Then, 300 mg of the F_{Hex} fraction was eluted throughout the stationary phase with increasing solvent polarity gradients of 100 ml of volume each in the following order:

Hexane 100%

Hexane-ethyl acetate-glacial acetic acid (8-1-1, v/v/v)

Hexane-ethyl acetate-glacial acetic acid (6-3-1, v/v/v)

Drain with 100% ethyl acetate

The collection rate is 5 mL / tube at 1 drop / second.

After a regroupment of fractions based on the TLC chromatographic profile of the contents of the tubes numbered T1 to T80, two subfractions named T11-

21 and T31-38, each having two distinct spots were selected on the basis of their physical characteristics.

Then, we did a preparative TLC at the end of which we chose one (1) spot of each subfraction with regard to its sharpness followed by filtration on SPE cartridge. One of the filtrates obtained is concentrated in acetonitrile (ACN) leading to the compound M1 while the other in the n-hexane leading to the compound M2, before being separated by chromatography on Sephadex LH20 gel.

At the end of this series of chromatographic methods, we came up to a white amorphous powder after evaporation to dryness in a test tube of the two compounds M1 and M2, which have the same physical characteristics as TLC parameters oleanolic acid and lupeol (Sigma Aldrich 99%). Spots were evidenced with the well-known sulfuric anisaldehyde reagent which colors the pure compounds M1 and M2 in purplish pink.

HPLC identification of M1 and M2 molecules

The HPLC system used for the identification of M1 and M2 molecules is equipped with a Merck Hitachi pump, an autosampler, a UV detector (LambdaMax, model 481). The column used is Phenomenex Luna C18 250 x 4.6 mm² which contains particles of 5µm in size.

M1 identification

Standards solutions: 500µL of ursolic, oleanolic and betulinic acids (Sigma) at 1mg/mL were spiked with 500µL of M1 solution (1mg/mL).

The identification of M1 was performed using a method recently published to quantitate UA and OA with some modifications (Catteau et al., 2017).

The flow rate was 1000µL/min using an isocratic binary solvent system: solvent A (20%), H_2O pH6 ($CH_3COONH_4$0.02M); solvent B (80%), ACN/MeOH 40:35. Peaks were detected at 210nm.

M2 identification

Standards solutions: 500µL of lupeol, α-amyrin, β-amyrin (Sigma) at 1mg/mL were spiked with 500µL of M2 solution (1mg/mL).

The flow rate was 1000µL/min using an isocratic binary solvent system: solvent A (5%), H_2O pH6 (CH_3COONH_4 0.02M); solvent B (95%), ACN. Peaks were detected at 210nm.

Larval susceptibility tests on *Anopheles gambiae*

Bioassays were carried out on two larval strains: wild larvae collected in larval breeding sites in Cotonou according to the morphological and behavioral criteria of the larvae using taxonomic determination keys (Gillies and Coetzee, 1987) and the larvae Kisumu of Kenyan origin obtained at the Entomological Research Center of Cotonou (CREC). Kisumu larvae have been kept in culture at the CREC laboratory for several years and their sensitivity is regularly checked. The WHO standard protocol for larval susceptibility testing against insecticides used in control campaigns has been exploited with a slight modification in line with our working conditions (WHO, 2005). The larvae were treated with solutions of M1 and M2 compounds in a concentration range of 25 to 200 ppm, prepared with 2% DMSO (dimethyl sulfoxide). The tests were carried out in transparent cups 5 cm in diameter, each containing 100 mL of solution and 20 larvae of *Anopheles gambiae* at the 3rd stage of life. The same number of larvae was placed in another control beaker containing only 100 mL of 2% DMSO. For each of the concentrations of M1 and M2 as well as for the control, three replicates were performed. The behavior of the larvae, by counting the number of survivors, was monitored for 48 hours and the lethal concentrations (LC_{50}) were determined every 24 hours. Indeed, are considered as dead, the larvae that remain immobile even in contact with a needle and those also who are moribund.

The analysis of the data is done using SPSS 21.0 statistic software in Probit model at the risk of 5%

(P <0.05) aiming at determining the average mortality rate of anopheles larvae following the doses applied in order to extract the lethal concentrations (LC_{50}).

Results and Discussion

Identification of M1 and M2

Both compounds M1 and M2 were isolated from the hydro-methanolic extract of *Launaea taraxacifolia* by liquid-liquid fractionation followed by APC and identified by HPLC -UV analysis by co-elution with controls (Figure 2 and 3).

Figures 2 and 3 show the chromatographic profile of standards and compounds M1 and M2. The chromatogram obtained with the solution formed by the mixture of the oleanolic acid standard (Sigma) and the compound M1 has a single peak at 38 min which is also present at the level of the other chromatograms at the same retention time with the peak of ursolic, and betulinic acid standards (Figure 2). Compound M1 is therefore oleanolic acid.

The chromatogram obtained with the solution formed by the mixture of the lupeol standard (Sigma) and the compound M2 has a single peak at 64 min which is also present at the level of the other chromatograms at the same retention time with the peak of α-amyrin and β-amyrin standards (Figure 3). Compound M2 is therefore lupeol.

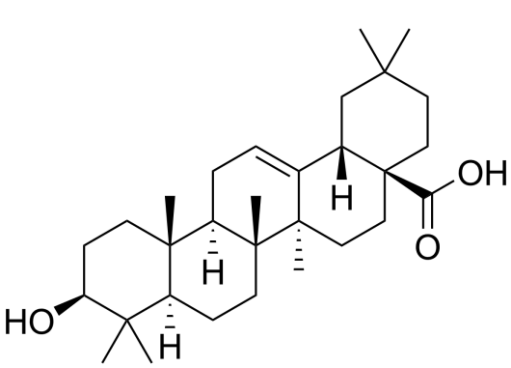
Larvicidal actitvity of M1 and M2

The lethal concentrations 50 (LC_{50}) obtained after the biological screening of the compounds M1 and M2 on the larvae of *Anopheles gambiae* are recorded in Table I. We note from reading this table that the LC_{50} values obtained with the compound M1 is significantly lower than those of the compound M2 regardless of the exposure time and the origin of the larvae considered with the lowest value (2.29 ppm) held after 48 hours of contact.

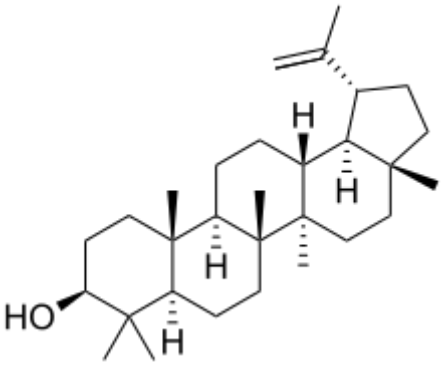
Table 1. LC_{50} values in ppm of the M1 and M2 compounds on the larvae.

	Kisumu				**Wild**			
	M1		**M2**		**M1**		**M2**	
	24h	48h	24h	48h	24h	48h	24h	48h
LC_{50}	38.12	**2.50**	105.16	45.69	32.14	**2.29**	103.20	32.38

p< 0.05 [0.000 – 0.025] significant level Concentrations causing mortality of half of larvae



Oleanolic acid (OA)



Lupeol

Fig. 1: Chemical structure of oleanolic acid and lupeol.

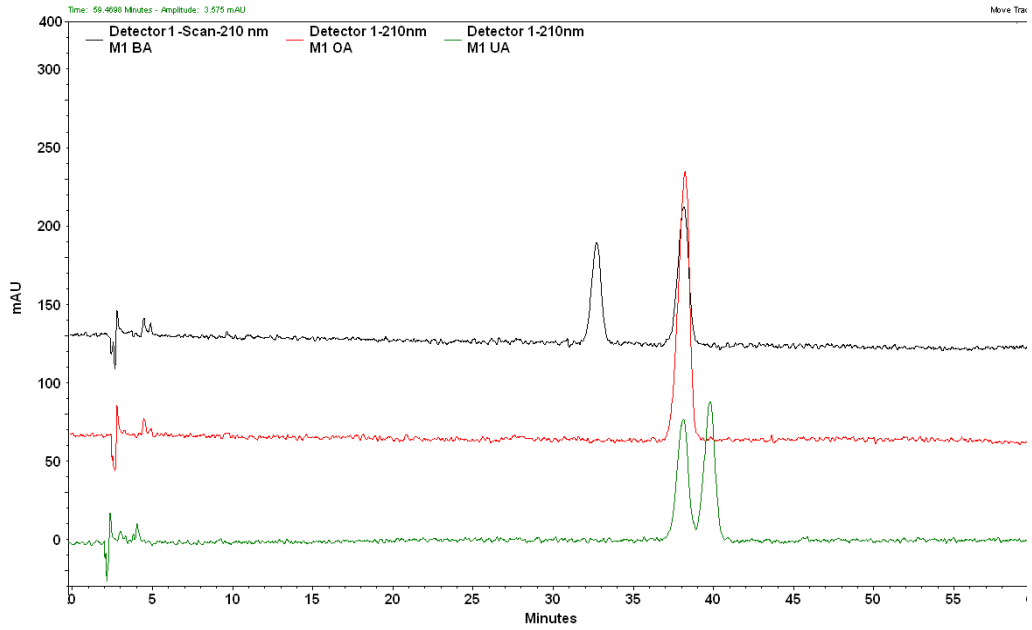


Fig. 2: M1 identification chromatograms.

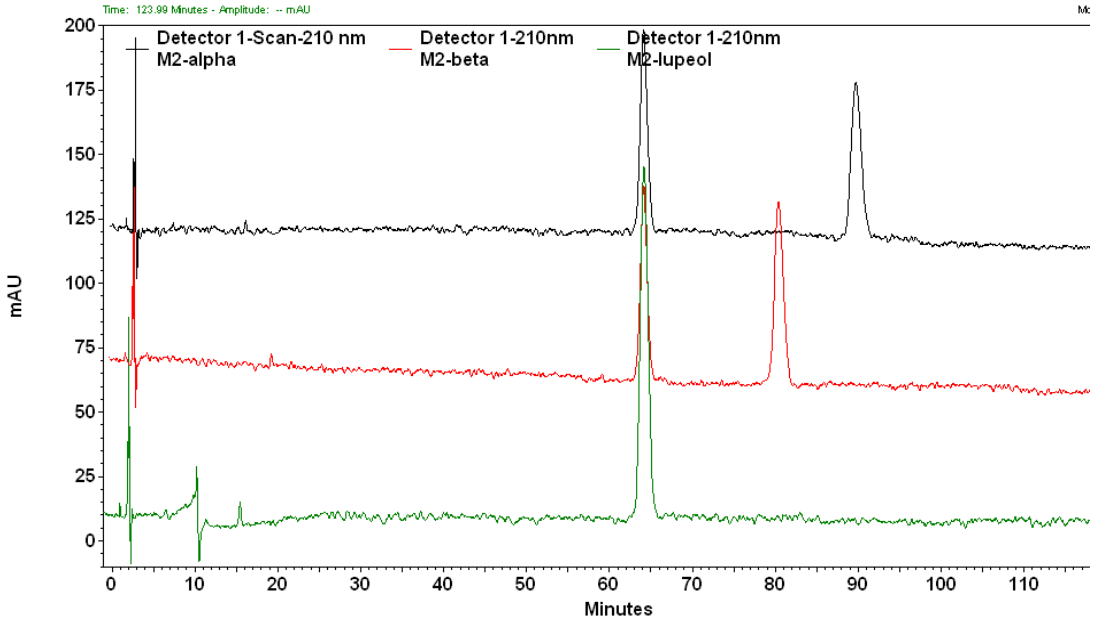


Fig. 3: M2 identification chromatograms.

In addition to its manifold therapeutic activities (antioxidant, antimicrobial and antiviral properties) widely proven by scientific research (Dansi et al., 2008, Gbadamosi et al., 2012, Arawande et al., 2013, Owoeye et al., 2015, Adimonyemma et al., 2016), *Launaea taraxacifolia* also has an inhibitory effect on *Anopheles gambiae* larvae (Ahouansou et al., 2016, Ahouansou et al., 2017). As part of the development of phyto-larvicides beneficial to the environment and targeting mosquitoes resistant to chemical insecticides, it is necessary to search and identify the main phytochemicals responsible for

larvicidal activity. In this study, we first performed a liquid-liquid fractionation of the hydro-methanolic extract of *Launaea taraxacifolia* with n-hexane. The hexane fraction F_{Hex} obtained was fractionated further by atmospheric pressure chromatography leading to different sub-fractions. The latter have undergone a series of thin layer chromatographies which led to the isolation of two molecules, namely M1 and M2 after purification and revelation with sulfuric anisaldehyde. These isolated molecules of *Launaea taraxacifolia* whose phytochemical screening reveals the presence of triterpenic compounds (Dansi et al., 2012, Koukoui et al., 2015), have the same physical characteristics TLC as the chromatograms of acid oleanolic and lupeol (Sigma Aldrich 99%) used as control molecules. The identification of the molecules M1 and M2 is done by means of a HPLC-UV method in co-elution mode and by comparison and superposition of the chromatograms of each molecule with standards. The conformity of the chromatograms of the isolated compounds and those of the controls confirms the purity of the molecules M1 and M2. Our results showed that the molecule M1 is oleanolic acid and the molecule M2 is lupeol. These two phytochemical molecules of terpenic nature possess important known pharmacological effects. OA is a multifunctional triterpenoid pentacyclic (isomer of ursolic acid) ubiquitous, widespread in several food and medicinal plant species. It is endowed of various properties such as antioxidant, anti-inflammatory, spasmolytic, antiallergic, acetylcholinesterase inhibitor, alpha-glucosidase, antimicrobial, antiviral, immunomodulatory, cytotoxic, antitumor, anti-angiogenic, hepatoprotective, antipruritic (Jesus et al., 2015). Other specific studies have shown that OA is active on *Dermatophilus congolensis* (bacterium causing dermatosis) (Gbaguidi et al., 2005) and inhibits the actions of *Mycobacterium tuberculosis* H37Rv (Jimenez-Arellanes et al., 2013). Lupeol has various medicinal properties, including anti-inflammatory, but also anti-protozoan, antimicrobial, antitumor, and chemotherapic properties (Prasad et al., 2008); it is effective in laboratory models as an inhibitor of skin cancer (Saleem et al., 2004). The identification method used is inspired by that used by Catteau and colleagues for the identification of oleanolic and ursolic acids in *Vitellaria paradoxa* (Catteau et al., 2017). The LC_{50} of the OA and lupeol obtained after the biological screening are significantly lower than those obtained with the hydro-methanolic extract used for the isolation of the compounds: 182.68 ppm and 135.13 ppm respectively in 24 hours and 48 hours on Kisumu larvae against 157.36 ppm and 116.88 ppm on wild larvae respectively in 24 hours and 48 hours of contact (Ahouansou et al., 2017). Our results are similar to those obtained previously with the essential oils of *Cinnamomum osmophloeum* and *Cryptomeria japonica* on the larvae of *Anopheles gambiae* SS with LC_{50} respectively of 58.15 and 63.92 µg/mL at the same exposure times (Mdoe et al., 2014b, Mdoe et al., 2014a). Earlier and similar works were done in Cameroon with the essential oils of a number of plants, the most effective giving an LC_{50} of 18 ppm in 24 hours of activity on 4th instar larvae of *An. gambiae* (Tchoumbougnang et al., 2009). On the other hand, Kisumu and wild larvae practically adopt the same behavior with respect to each compound. OA and lupeol are therefore responsible for larvicidal activity in *Launaea taraxacifolia*. Nevertheless, the LC_{50} values obtained with the compound M1 are significantly lower than those of the compound M2 regardless of the exposure time and the origin of the larvae considered with the lowest value (2.29 ppm) held after 48h of contact. OA is therefore more active on *An. gambiae* larvae. These results are encouraging in view of the resistance observed at the level of *An. gambiae* with respect to certain chemical insecticides of the group of organophosphorus compounds, pyrethroids and carbamates (Djogbenou et al., 2011, Edi et al., 2012, Nwane et al., 2013). Also, we intend to develop a method of direct extraction of these two triterpenoids in order to determine them quantitatively for a better standardization of the use of this plant.

This work revealed the presence of two triterpene compounds known for their pharmacological activities in *Launaea taraxacifolia*. The discovery of their larviciding potential strengthens their

therapeutic potential in the integrated fight against malaria. *Launaea taraxacifolia* is therefore a good candidate in the formulation of a bio-larvicide. This method of control will be able to solve the question of resistance of malaria vectors on the one hand and the protection of the environment on the other hand.

Conflict of interest statement

The authors have no conflict of interest.

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References

Adimonyemma, N. R., Chukwuma, O. M., Akachukwu, E. E., Iroka, F. C., 2016. Phytochemical analysis and antibacterial activity of *Launaea taraxacifolia* ethanolic leaves extract. Scholars Acad. J. Biosci. 4(3A), 193-196.

Ahouansou, C. A., Fagbohoun, L., Mèdégan Fagla, S., Houngbèmè, G. A., Kotchoni, S., Gbaguidi, A. F., 2017. Inhibition effects of *Elaeis oleifera* (Arecaceae) and *Launaea taraxacifolia* (Asteraceae) on two genotypes of *Anopheles gambiae* larvae. Int. J. Curr. Res. Biosci. Plant Biol. 4, 39-46.

Ahouansou, C. A., Fagbohoun, L., Mèdégan Fagla, S. R., Tchètchè, J., Kotchoni, S., Gbaguidi, A. F., 2016. Phytochemical analysis, toxicity and larvicidal activity of extracts from *Launaea taraxacifolia* (Asteraceae) on *Anopheles gambiae*, a malaria vector. Int. J. Vector Borne Dis. 107, 130-138.

Arawande, J. O., Amoo, I. A., Lajide, L., 2013. Chemical and phytochemical composition of wild lettuce *Launaea taraxacifolia*. J. Appl. Phytotechnol. Environ. Sanit. 2, 25-30.

Catteau, L., Reichmann, N. T., Olson, J., Pinho, M. G., Nizet, V., Van Bambeke, F., Quetin-Leclercq, J., 2017. Synergy between ursolic and oleanolic acids from *Vitellaria paradoxa* leaf extract and beta-lactams against methicillin-resistant *Staphylococcus aureus*: *In vitro* and *in vivo* activity and underlying mechanisms. Molecules. 22(12), pii: E2245.

Dansi, A., Adjatin, A., Adoukonou-Sagbadja, H., Faladé, V., Yedomonhan, H., Odou, D., Dossou, B., 2008. Traditional leafy vegetables and their use in the Benin Republic. Genet. Resour. Crop Evol. 55, 1239-1256.

Dansi, A., Vodouhe, R., Azokpota, P., Yedomonhan, H., Assogba, P., Adjatin, A., Loko, Y. L., Dossou-Aminon, I., Akpagana, K., 2012. Diversity of the neglected and underutilized crop species of importance in Benin. Scient. World J. 2012, 932947.

Djogbenou, L., Pasteur, N., Akogbeto, M., Weill, M., Chandre, F., 2011. Insecticide resistance in the *Anopheles gambiae* complex in Benin: a nationwide survey. Med. Vet. Entomol. 25, 256-267.

Edi, C. V., Koudou, B. G., Jones, C. M., Weetman, D., Ranson, H., 2012. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, Southern Cote d'Ivoire. Emerg. Infect. Dis. 18, 1508-1511.

Fillinger, U., Ndenga, B., Githeko, A., Lindsay, S. W., 2009. Integrated malaria vector control with microbial larvicides and insecticide-treated nets in western Kenya: A controlled trial. Bull. World Health Organ. 87, 655-665.

Gbadamosi, I. T., Alia, A. E. & Okolosi, O. 2012. In vitro antimicrobial activities and nutritional assessment of roots of ten Nigerian vegetables. New York Sci. J. 5(12), 234-240.

Gbaguidi, F., Accrombessi, G., Moudachirou, M., Quetin-Leclercq, J., 2005. HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. J. Pharm. Biomed. Anal. 39, 990-995.

Gillies, M. T., Coetzee, M., 1987. A supplement to the Anophelinae of Africa South of the Sahara. Publ. of the South African Institute for Medical Research. pp.55-143.

Jesus, J. A., Lago, J. H., Laurenti, M. D., Yamamoto, E. S., Passero, L. F., 2015. Antimicrobial activity of oleanolic and ursolic acids: an update. Evid. Based Compl. Altern. Med. 2015, 620472.

Jimenez-Arellanes, A., Luna-Herrera, J., Cornejo-Garrido, J., Lopez-Garcia, S., Castro-Mussot, M. E., Meckes-Fischer, M., Mata-Espinosa, D., Marquina, B., Torres, J., Hernandez-Pando, R., 2013. Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment. BMC Compl. Altern. Med. 13, 258.

Koukoui, O., Agbangnan, P., Boucherie, S., Yovo, M., Nusse, O., Combettes, L., Sohounhloué, D., 2015. Phytochemical study and evaluation of cytotoxicity, antioxidant and hypolipidemic properties of *Launaea taraxacifolia* leaves extracts on cell lines HepG2 and PLB985. Am. J. Plant Sci. 6, 1768-1779.

Mdoe, F. P., Cheng, S. S., Lyaruu, L., Nkwengulila, G., Chang, S. T., Kweka, E. J., 2014a. Larvicidal efficacy of *Cryptomeria japonica* leaf essential oils against *Anopheles gambiae*. Parasit. Vectors. 7, 426.

Mdoe, F. P., Cheng, S. S., Msangi, S., Nkwengulila, G., Chang, S. T., Kweka, E. J., 2014b. Activity of *Cinnamomum osmophloeum* leaf essential oil against *Anopheles gambiae* s.s. Parasit. Vectors. 7, 209.

Nwane, P., Etang, J., Chouasmall Yi, U. M., Toto, J. C., Koffi, A., Mimpfoundi, R., Simard, F., 2013. Multiple insecticide resistance mechanisms in *Anopheles gambiae* s.l. populations from Cameroon, Central Africa. Parasit. Vectors. 6, 41.

Owoeye, O., Femi-Akinlosotu, O. M., Adejuwon, S. A., 2015. *Launaea taraxacifolia* aqueous extract attenuates cisplatin-induced neurotoxicity by decreasing oxidative stress and neuronal cell death in rats. Arch. Basic Appl. Med. 3, 71-78.

Prasad, S., Kalra, N., Singh, M., Shukla, Y., 2008. Protective effects of lupeol and mango extract against androgen induced oxidative stress in Swiss albino mice. Asian J. Androl. 10, 313-318.

Saleem, M., Afaq, F., Adhami, V. M., Mukhtar, H., 2004. Lupeol modulates NF-kappa B and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. Oncogene. 23, 5203-5214.

Tchoumbougnang, F., Dongmo, P. M. J., Sameza, M. L., Mbanjo, E. G. N., Fotso, G. B. T., Zollo, P. H. A., Menut, C., 2009. Activité larvicide sur *Anopheles gambiae* Giles et composition chimique des huiles essentielles extraites de quatre plantes cultivées au Cameroun. Biotechnol. Agron. Soc. Environ. 13, 77- 84.

WHO 2005. Guidelines for Laboratory and Field Testing of Mosquito Larvicides, Geneva.

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