ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 15, Issue 1, 2022

Online - 2455-3891
Print - 0974-2441

Original Article

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY QUANTIFICATION OF OLEANOLIC ACID IN *LAUNAEA TARAXACIFOLIA* AND LARVICIDAL ACTIVITY AGAINST *ANOPHELES GAMBIAE*

AHOUANSOU C AYIDÉ[1,2]*, TOKOUDAGBA JEAN-MARIE D[1], ASSANHOU A GABIN[1], HOUNGUE URSULA[1], HOUNGBEME ALBAN G[2], MEDEGAN FAGLA SÈDAMI R[1], GBAGUIDI A FERNAND[1,2]

[1]Laboratoire de Chimie Organique et Pharmaceutique/Faculté des Sciences de Santé/Université d'Abomey-Calavi, Cotonou, Bénin.
[2]Laboratoire de Pharmacognosie/Centre Béninois de Recherche Scientifique et Technique, Porto-Novo, Benin.
Email: charlahouans@gmail.com

Received: 02 November 2021, Revised and Accepted: 10 December 2021

ABSTRACT

Objective: One of the measures used to prevent malaria is the management of breeding sites. For preventive and ecologically profitable control, the use of bio-larvicides made from active plant extracts would be an asset for the control of malaria vectors, in particular *Anopheles gambiae*. Advances in pharmacognosy have revealed the benefits of several phytochemicals with very rich and varied therapeutic effects. Among the latter, oleanolic acid (OA) is quite remarkable because of its various and multiple properties, much of which is demonstrated with the leaves of *Launaea taraxacifolia*.

Methods: After a liquid-liquid fractionation with different organic solvents of the hydro-methanolic extract of *Launaea taraxacifolia*, we obtained three fractions named Fhex (hexane fraction), FDCM (dichloromethane fraction) and FHM (hydro-methanolic fraction) which were tested on 3rd instar *Anopheles gambiae* larvae.

Results: Fhex proved to be the most active with LC50 of 120.11 ppm and 69.50 ppm respectively in 24 and 48 hours of contact. We then developed a new method of Ultra-Violet High Performance Liquid Chromatography (HPLC / UV) method and determined the quantity of oleanolic acid in the Fhex and FDCM fractions to be respectively 0.46% and 0.23% .

Conclusion: *Launaea taraxacifolia* has a larvicidal potential due to the presence of oleanolic acid whose inhibitory effect against *Anopheles gambiae* larvae.

Keywords: Malaria, *Launaea taraxacifolia*, Oleanolic acid, Larvicidal activity.

 DOI: http://dx.doi.org/10.22159/ajpcr.2022v15i1.43694. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Malaria is a parasitic disease, one of the main vectors of which is *Anopheles gambiae*. Despite the ever-increasing use of insecticide-treated bed nets, the number of malaria cases was estimated at 212 million and 429,000 associated deaths in 2015 [1]. Preventive control measures lean more and more toward mosquito control through larval control. For ecologically profitable control, the use of insecticides of natural origin made from active plant extracts against mosquito larvae emerges a good alternative for the control of malaria vectors. Advanced phytochemical researches have revealed the presence of several bioactive compounds with varied therapeutic effects. Among these, oleanolic acid (OA, Fig. 1) is quite renowned for diverse pharmacological properties.

OA is a ubiquitous, pentacyclic, multifunctional triterpenoid (isomer of ursolic acid) widely distributed in several food and medicinal plant species. It possesses antioxidant, anti-inflammatory, spasmolytic, antiallergic, acetylcholinesterase, alpha-glucosidase inhibitor, antimicrobial, antiviral, immunomodulator, cytotoxic, antitumor, antiangiogenic, hepatoprotective, and antiprurigin activities [2]. Other specific studies have shown that OA is active against *Dermatophilus congolensis* (bacteria causing dermatosis) [3] and inhibits the actions of *Mycobacterium tuberculosis* H37Rv [4]. Many of these multiple activities have been demonstrated with extracts of *Launaea taraxacifolia*, the phytochemical screening of which reveals the presence of triterpene compounds [5-7]. In addition, recent studies carried out in Benin by Ahouansou and colleagues showed that the hydro-methanolic extract of this plant inhibits the larval growth of *A. gambiae* [8]. Other studies geared toward the search for biolarvicides for different species of mosquitoes could not pinpoint the main molecule(s) responsible for this activity. This is the case of *Hyptis suaveolens* which inhibits *Culex quinquefasciatus* larvae, vector of lymphatic filariasis [9], *Ficus benghalensis* which is active against the *Aedes aegypti* and *Anopheles stephensi* larvae [10]. These different interests raise up an experimentation with the larvicidal activity of OA on the one hand and the demonstration of its presence in *L. taraxacifolia* on the other hand.

It is with this in mind that this work was undertaken with the objective of evaluating the insecticidal potential of this bioactive molecule against the *A. gambiae* larvae and determining the level of OA in *L. taraxacifolia*. To achieve this, we evaluated the mortality of *A. gambiae* larvae in contact with fractions derived from the active hydro-methanolic extract of *L. taraxacifolia*. We then developed a new ultra-violet high performance liquid chromatography (HPLC/UV) method to assess the amount of OA contained in same.

METHODS

Collection of plants and preparation of extracts

The leaves of *L. taraxacifolia* were harvested in corn fields in Comé in southern Benin and were identified and certified by the National Herbarium of the University of Abomey-Calavi under the number: AA6689/HNB. They are then dried at 16°C in the laboratory for 2 weeks before being crushed and powdered. First, we performed the extraction with 100 g of powder to which we added 500 mL of methanol-water mixture (70:30; v/v) with 0.5% formic acid. This mixture was homogenized and then kept under continuous stirring for 24 h and then filtered and evaporated to dryness using a rotary evaporator (*Heidolph Laborota* 4000 efficient). Then, the obtained hydro-methanolic extract was fractionated with organic solvents following a

polarity gradient. Thus, we dissolved 1 g of this crude extract in 150 mL of methanol-water mixture (1:3; v/v). This solution was washed 3 successively with 100 mL of n-hexane by washing with a separatory funnel. The hexane fraction (Fhex) was recovered after each washing and we then proceeded to thrice successive washes with dichloromethane by following the same process as above. We therefore obtained at the end of this liquid-liquid fractionation process three extract fractions: the Fhex; the dichloromethane fraction (FDCM) and the hydro-methanolic fraction (FHM) which were reduced to dryness using the rota-vapor system. All these operations were repeated 3 times and the dried residues obtained after each operation were weighed to determine the average extraction yield.

Anti-larval tests on *A. gambiae*

The bioassays were carried out on wild larvae collected in larval breeding sites in Cotonou according to the morphological and behavioral criteria of the larvae using taxonomic determination keys [11]. The WHO standard protocol for larval susceptibility testing to insecticides used in the control campaign was used with a slight modification in line with our working conditions [12]. The larvae were treated with solutions of the fractions obtained and a 99% pure OA standard (Sigma Aldrich), at a concentration range of 25–4000 ppm prepared from each extract with 2% Dimethyl sulfoxide (DMSO). The tests were carried out in transparent cups (5 cm in diameter) each containing 100 mL of solution and 20 *A. gambiae* larvae in the 3rd life stage. The same number of larvae were placed in another control beaker containing only 100 mL of 2% DMSO. For each of the concentrations of the extracts, of the standard (OA) and the control, three replicates were carried out. The behavior of the larvae, by counting the number of survivors, was followed for 48 h and the lethal concentrations (LC_{50}) were determined every 24 h. In fact, larvae which remain immobile even in contact with a needle and those which are dying are considered dead.

The data analysis was carried out using the statistical software Statistical Package for the Social Sciences 21.0 in probit model at the risk of 5% ($p<0.05$) in the interest of identifying the average mortality rate of anopheles larvae according to the doses applied and extract the LC_{50}.

Quantitative analysis by HPLC

The HPLC system used was equipped with a HITACHI VWR 5160 HPLC pump; a VWR 5430 HITACHI UV-DAD detector; a 100 µL Rheodyne loop injection valve (USA); a HITACHI VWR 5260 autosampler; a Computer DELL Optiplex 7010 with Microsoft Windows 7 Enterprise, using CHROMASTER SYSTEM MANAGER version 1.1 software for data acquisition and a thermostat for HITACHI VWR 5310 columns where a constant temperature of 25°C prevailed. The method used was inspired by that developed and validated by Gbaguidi and collaborators, with a slight modification [3]. The coefficients of variation intra-day and inter-day analyses and oleanolic acid content of fractions are showed in Tables 3 and 4 respectively.

The stationary phase was a Merck Lichrocart 250–4 mm, Lichrospher 100 RP-18e 5 µm column. The mobile phase (Acetonitrile - milli-Q water, 85:15, v/v) was run at a flow rate of 1 mL/min in isocratic mode, the sample injection of 20 µL and detection wavelength of 215 nm. The analytical time was set at 20 min.

Calibration curve

The calibration curve was established with a series of concentrations of pure OA (Sigma Aldrich): 5, 10, 20 and 25 µg/mL prepared from a stock solution of 1 mg/mL in l HPLC grade acetonitrile. The injection volume was 20 µL, the range of quantities in OA was: 100, 200, 400 and 500 ng. The regression model is a linear function f (x)=ax+b with the amount of OA introduced on the abscissa and the area under the curve of the corresponding peak on the ordinate(Fig. 2).

Preparation of samples to be assayed

We prepared a concentration of 1 mg/mL from each of the extracts obtained by fractionating liquid with acetonitrile. The FHM was not used for the assay due to its non-solubility in acetonitrile. After filtration through a 0.45 µm PTFE membrane, 20 µL of the extracts were injected corresponding amount of 20,000 ng.

Recovery rate

We prepared a mixture of the standard (10 µg/mL) with the Fhex (2 mg/mL) at equal volume to obtain an OA/Fhex solution: 5 µg/ml^{-1} mg/ml. This mixture was carefully filtered and analyzed by HPLC in the same way as the solutions of the standard and the fractions. The recovery rate was evaluated as follows:

$$\text{Recovery rate (\%)} = (A-B)/C \times 100.$$

A is the amount of OA obtained from the Fhex fraction mixture with the addition of the OA. B is the amount of OA in the Fhex fraction without addition and C is the amount of OA added.

RESULTS

Larvicidal activity

The yields obtained from the liquid-liquid extraction by gradient polarity of solvents are given in Table 1.

The hydro-methanolic mixture is the solvent which produced the largest quantity by mass of fraction (81.63%). These results are in accordance with the nature of the *L. taraxacifolia* extract which has been fractionated and which is obtained from the same mixture of solvents but in varying proportions.

The LC_{50} obtained after the biological screening of the fractions and of the standard on the larvae of *A. gambiae* are given in Table 2.

As shown in this table, OA had the lowest LC_{50} (2.29 ppm) after 48 h of exposure. For the fractions tested, Fhex showed the best LC_{50} regardless of the duration of the experiment followed by FDCM. The FHM fraction gave very high LC_{50} values compared to those obtained previously with the hydro-methanolic extract of *L. taraxacifolia* using the same category of larvae: 157.36 and 116.88 ppm in 24 h and 48 h respectively [8].

Table 1: Fractions rate

Fractions	Fhex	FDCM	FHM
Yields (%)	4.17±0.29	1.11±0.13	81.63±3.47

Fhex: Hexane fraction, FDCM: Dichloromethane fraction, FHM: Hydro-methanolic fraction

Table 2: LC50 values in ppm of the extracts tested on the larvae

Oleanolic acid		Fhex		FDCM		FHM	
24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
32.14	2.29	120.11	69.50	679.65	172.39	3615.11	1861.22

P<0.05 significant concentrations causing the mortality of half of the larvae, Fhex: Hexane fraction, FDCM: Dichloromethane fraction, FHM: Hydro-methanolic fraction, LC_{50}: Lethal concentrations 50

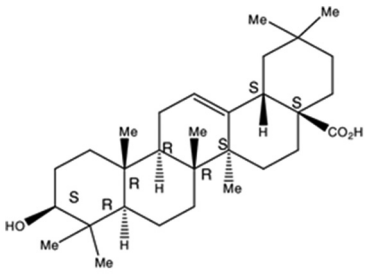

Fig. 1: Chemical structure of oleanolic acid

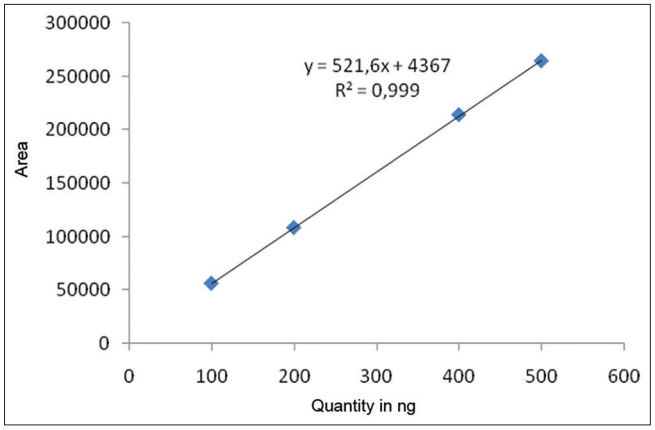

Fig. 2: Calibration curve

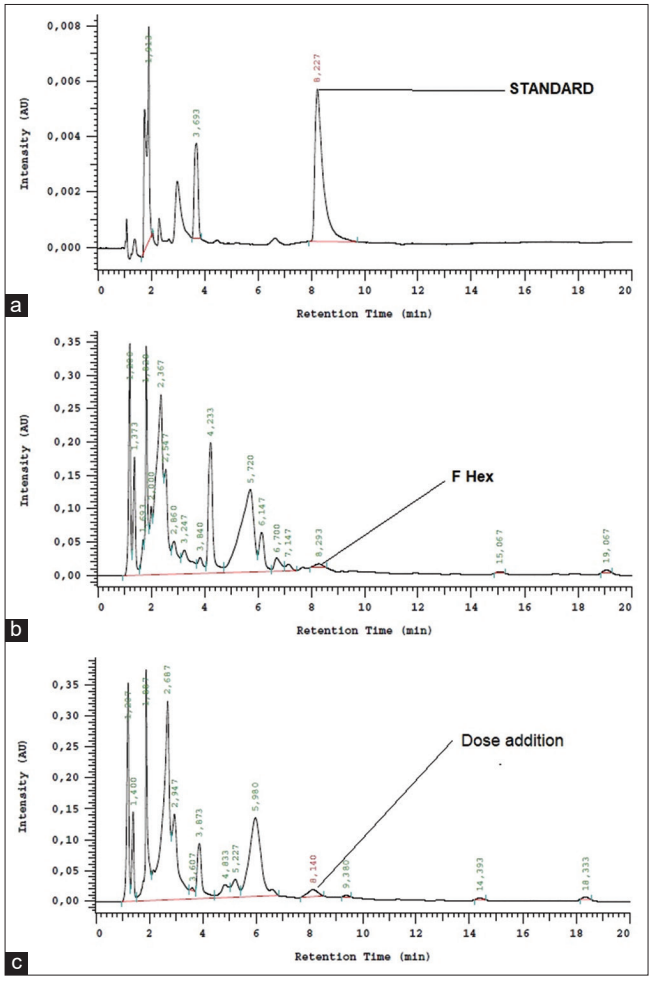

Fig. 3: (a) Standard chromatogram. (b) Chromatogram of the hexane fraction (Fhex) fraction, (c) Chromatogram of the dosed addition (AO/Fhex)

Quantification by HPLC

OA was identified by the HPLC method in the Fhex and FDCM fractions by comparison of their retention times with the standard and found to be 8.22±0.075 min (Fig. 3a-c).

The calibration curve was established for a series of quantities of 100–500 ng for the molecule. The method showed a good linear relation between the areas under curves of the peaks and the series of quantities of OA. The equation for the 3-day run calibration curve was written: y=521.6×+4367 with R^2=0.999. This testifies to a good linearity of the analytical procedure used.

The recovery rate of oleanolic acid by the dosed addition method was 123.2%.

Table 3: CV for intra-day and inter-day analyses

Standard quantity (ng)	CV (%)	
	Intra - day (*n*=3)	Inter - day (*n*=3)
100	3.18	1.65
200	0.18	3.11
400	0.50	1.11
500	0.14	1.40

CV: Coefficients of variation

Table 4: Oleanolic acid content of fractions

Assayed fractions	Oleanolic acid content	
	Quantity in ng	Rate in%
Fhex	92.57±4.14	0.46
FDCM	45.17±0.75	0.23
FHM	ND	ND

ND: Not determined, Fhex: Hexane fraction, FDCM: Dichloromethane fraction, FHM: Hydro-methanolic fraction

DISCUSSION

Synthetic chemical insecticides such as organophosphates, pyrethroids, and carbamates are commonly used for vector control against malaria. Their misuse is increasingly causing the emergence of resistance in target vectors including *A. gambiae* [13,14]. In addition, these synthetic insecticides are not very selective and cause environmental pollution. As part of the search for bio-insecticides, we tested the larvicidal activity of OA through fractions of the active extract of *L. taraxacifolia*. The lowest LC_{50} obtained after 2 days of contact with *A. gambiae* larvae was from pure OA (Sigma Aldrich) at 32.14 and 2.29 ppm, respectively, after 24 h and 48 h of exposure. These values are significantly lower than those obtained with the fractions from the hydro-methanolic extract of *L. taraxacifolia*. Nevertheless, the Fhex fraction driven by n-hexane gives the best larvicidal activity with LC_{50} (120.11 and 69.50 ppm) clearly lower than that of the crude extract, followed by the FDCM. These results are close to those obtained previously with the essential oils of *Cinnamomum osmophloeum* and *Cryptomeria japonica* on *A. gambiae* S.S. larvae with LC_{50} respectively of 58.15 and 63.92 µg/ml at the same exposure times [15,16]. The fairly high LC_{50} of the hydro-methane fraction are justified by the nonpolar nature of the active ingredients which had already been eluted initially with n-hexane then the remainder with dichloromethane which are moreover the solvents used to extract the OA in a vegetable powder [3].

OA quantification performed by HPLC enabled us to confirm its presence in *L. taraxacifolia*. The analytical method of Gbaguidi and collaborators for the determination of OA and ursolic acid in *Mitracarpus scaber* resulted in the homogeneity of the chromatograms of the standard and the sample at the same retention time and at the same wavelength [3]. In addition, the chromatogram resulting from the dosed addition confirms this homogeneity with a recovery rate of 123.2%. This method exhibits good repeatability and precision for the range of quantities of OA analyzed, as evidenced by the coefficients of variations obtained. The limits of detection and quantification were not determined since this analytical method has previously been validated. To provide further confirmation of the presence of OA in *L. taraxacifolia*, we have in prospect to isolate this molecule by chemical methods of purification and isolation and to resort to nuclear magnetic resonance coupled with mass spectrophotometry to determine its chemical structure.

CONCLUSION

Aside from its already established nutritional, metabolic and antimicrobial properties, *L. taraxacifolia* also has a larvicidal potential due to the presence of OA whose inhibitory effect against *A. gambiae* larvae has just been demonstrated in this study.

AUTHORS' CONTRIBUTIONS

Ahouansou CA: Principal investigator, manuscript preparation and analysis supervision. Tokoudagba JM: Elaboration of technical methods. Assanhou AG: Contribution to the writing of the manuscript. Houngue U: Assisted in the developpement of the technical protocols. Houngbeme AG: Elaboration of technical methods. Medegan Fagla SR: Elaboration of technical methods. Gbaguidi AF: General supervisor.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. OMS. Rapport Sur Le Paludisme Dans Le Monde 2016: Résumé. Genève: WHO/HTM/GMP; 2017. p. 4.
2. Jesus JA, Lago JH, Laurenti MD, Yamamoto ES, Passero LF. Antimicrobial activity of oleanolic and ursolic acids: An update. Evid Based Complement Alternat Med 2015;2015:620472.
3. Gbaguidi F, Accrombessi G, Moudachirou M, Quetin-Leclercq J. HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*. J Pharm Biomed Anal 2005;39:990-5.
4. Jiménez-Arellanes A, Luna-Herrera J, Cornejo-Garrido J, López-García S, Castro-Mussot ME, Meckes-Fischer M, *et al*. Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment. BMC Complement Altern Med 2013;13:258.
5. Dansi A, Vodouhè R, Azokpota P, Yedomonhan H, Assogba P, Adjatin A, *et al*. Diversity of the neglected and underutilized crop species of importance in Benin. ScientificWorldJournal 2012;2012:932947.
6. Koukoui O, Agbangnan P, Boucherie S, Yovo M, Nusse O, Combettes L, *et al*. 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 2015;6:1768-79.
7. Owoeye O, Femi-Akinlosotu OM, Adejuwon SA. *Launaea taraxacifolia* aqueous extract attenuates cisplatin-induced neurotoxicity by decreasing oxidative stress and neuronal cell death in rats. Arch Basic Appl Med 2015;3:71-8.
8. Ahouansou CA, Fagbohoun L, Fagla SM, Houngbèmè GA, Kotchoni S, Gbaguidi AF. Inhibition effects of *Elaeis oleifera* (*Arecaceae*) and *Launaea taraxacifolia* (*Asteraceae*) on two genotypes of *Anopheles gambiae* larvae. Int J Curr Res Biosci Plant Biol 2017;4:39-46.
9. Murugesan S, Palani G, Murugesan S, Arivoli S, Raveen R, Tennyson S. Mosquito larvicidal activity of *Hyptis suaveolens* (L.) Poit (*Lamiaceae*) aerial extracts against the filarial vector *Culex quinquefasciatus* Say (*Diptera*: Culicidae). J Med Plants Stud 2015;3:1-5.
10. Govindarajan M. Larvicidal efficacy of *Ficus benghalensis* L. plant leaf extracts against *Culex quinquefasciatus* say, *Aedes aegypti* L. and *Anopheles stephensi* L. (*Diptera*: Culicidae). Eur Rev Med Pharmacol Sci 2020;14:107-11.
11. Gillies MT, Coetzee M. A supplement to the Anophelinae of Africa South of the Sahara. Publ South Afr Inst Med Res 1987;55:1-143.
12. WHO. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. Geneva: WHO; 2005.
13. Djogbenou L, Pasteur N, Akogbeto M, Weill M, Chandre F. Insecticide resistance in the *Anopheles gambiae* complex in Benin: A nationwide survey. Med Vet Entomol 2011;25:256-67.
14. Nwane P, Etang J, Chouasmall UM, Toto JC, Koffi A, Mimpfoundi R, *et al*. Multiple insecticide resistance mechanisms in *Anopheles gambiae* S.L. populations from Cameroon, Central Africa. Parasit Vectors 2013;6:41.
15. Mdoe FP, Cheng SS, Msangi S, Nkwengulila G, Chang ST, Kweka EJ. Activity of *Cinnamomum osmophloeum* leaf essential oil against *Anopheles gambiae* s.s. Parasit Vectors 2014;7:209.
16. Mdoe FP, Cheng SS, Lyaruu L, Nkwengulila G, Chang ST, Kweka EJ. Larvicidal efficacy of *Cryptomeria japonica* leaf essential oils against *Anopheles gambiae*. Parasit Vectors 2014;7:426.