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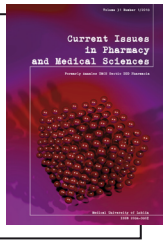

Bioactivity-guided isolation of alkamides from a cytotoxic fraction of the ethyl acetate extract of *Anacyclus pyrethrum* (L.) DC. roots.

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ABSTRACT

Introduction. The alcohol extract of Pellitory (*Anacyclus pyrethrum*) roots has been previously shown to exert anticancer activities on the Human Colorectal Cancer Cell Line (HCT) by targeting apoptosis, metastasis and cell cycle arrest. However, the nature of the cytotoxic molecules associated with this activity remains unexplored.

Aims. This study aims to reinvestigate Pellitory root extract as regard to its cytotoxic activity and to proceed to a bioguided fractionation to explore its active fraction and to give new insight in their phytochemical constituents.

Methods. Powdered roots were subjected to repeated extraction with Petroleum ether (Pe), Chloroform (Ch), Ethyl acetate (Ea) and Methanol (Me). Pellitory extracts were then screened for cytotoxic activity using the Brine Shrimp Lethality (BSL) bioassay.

Results. Ea extract exhibited a marked cytotoxic activity, with LC50 of 249.26 µg/mL in the BSL bioassay. The remaining extracts (Pe,Ch,Me) treated groups exhibited no or low mortality in the range of tested concentrations (1-1000 µg/mL). BSL assay-guided chromatographic fractionation of Ea active Extract revealed a highly cytotoxic fraction (F11) with LC50 of 42.5 µg/mL. Multistep purifications of the active F11 fraction afforded four alkamides, namely N-isobutyldeca-2,4-dienamide or Pellitorine (I), N-propyldodeca--2,8-dienamide (II), N-isobutyltetradeca-2,4-dienamide (III) and N-propylnona-2,5--dienamide (IV).

Conclusions. This study suggests that cytotoxic activity is localized mainly in the ethyl acetate extract (Ea) of pellitory roots. BSL assay fractionation of this active extract leads to the isolation of four alkamides, including pellitorine (I). While this isobutyl alkamide has previously shown strong cytotoxic activities against human cancer cell lines, the other compounds (II to IV) were not previously reported as cytotoxic. Subsequently, the isolated alkamides will be considered in future study as candidates for in depth in-vitro evaluation of their cytotoxicity against cancer and normal cell lines. Finally, through this study, BSL assay demonstrate again its usefulness as bench-top assay in exploring plant extracts for cytotoxic compounds.

Abbreviations

BSL (Brine Shrimp Lethality), Ch (Chloroform), DMSO (Dimethyl sulfoxide), Ea (Ethyl acetate), HCT (Human Colorectal Cancer Cell Line), MCT-7 (Brest cancer strain), Me (Methanol), HL60 (Human promyelocytic leukemia cell line), Pe (Petroleum ether).

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INTRODUCTION

Pellitory (*Anacyclus pyrethrum* (L.) DC., syn.: *Anacyclus officinarum Hayne*), is a perennial procumbent herb which

belongs to the Asteraceae family. The species is widely distributed in North Africa, and elsewhere in the Mediterranean region, in India and in Arabian countries [1]. The root of pellitory is well-known as a medicinal drug. It is used in the ayurvedic and unani systems of medicine for its medicinal properties, such as anti-rheumatic, odontalgia, antibacterial, antiviral, carminative, anti-catarrh, digestive, emmenagogue, febrifuge, vermifuge, sialagogue and anti-cancer [2-8]. In the North Africa region, this herbal drug is known as "*Guentess*", and is indicated among other therapeutic applications, in respiratory infections and in the treatment of hepatic disorders [9-11].

Scientific studies has revealed that extracts from the root exhibit antibacterial, antinociceptive, anti-inflammatory, immunostimulating and antioxidant activities [7,12,13]. Moreover, the phytochemical screening of *Anacylcus pyrethrum* has led to the identification of various secondary metabolites such as alkaloids, tannins, flavonoids, coumarins and lignanes [12]. This species also contains saponins, sesamin, inulin, gum and traces of essential oil [13]. The most important chemical markers present in its root are N-alkamides with pellitorine and polysaccharides [14-16]. Furthermore, LC-MS N-alkamides profiling of an ethanolic *Anacyclus pyrethrum* root extract reported thirteen N-alkamides, including N-isobutyldeca-2,4-dienamide (Pellitorine) and N-isobutyltetradeca-2,4-dienamide [14].

Recent studies showed that Anacyclus pyrethrum extract have cytotoxic activity, and can successfully induce apoptosis in human colorectal cancer lines HCT cells [17,18]. Although, these experimental results give relatively some credence to the reported ethnobotanical use of the plant as anticancer [8], the identity of the active compounds associated with this effect is still unknown so far.

On the basis of this information, we decided to investigate Anacyclus pyrethrum roots extracts using brine shrimp lethality (BSL) assay as a predictive test for cytotoxicity [19]. This bioassay will be used to monitor the fractionation steps of the active extract and give new insight in its bioactive fractions and their phytochemical constituents. The isolated compounds can subsequently be tested in future studies using specific antitumor assays of interest.

MATERIALS AND METHODS

Solvents and Reagents

All substances were purchased from Sigma-Aldrich Chemical Co (Strasbourg, France) unless otherwise stated.

Plant material

Anacyclus pyrethrum (L) DC. (Asteraceae) roots were collected nearby Constantine, Algeria. The plant was identified by a Taxonomist (Pr.H. Laouer, Setif University) and voucher specimen (Ref. AN-00301R) was deposited for future reference in the herbarium at the laboratory of botany, University SB Constantine 3, Algeria.

Extraction method

Pellitory extracts were obtained from the air-dried roots (250 g finely ground), by successive maceration (2×1.5 L) with petroleum ether (Pe), chloroform (Ch), ethyl acetate (Ea) and methanol (Me). The extractive solutions were evaporated to dryness under vacuum to obtain 22.8 g (Pe), 18.0 g (Ch), 29.8 g (Ea) and 56.3 g (Me) extracts. Table 1 shows the corresponding yields of the crude extracts, expressed as percentage (w/w) dry powder.

Table 1. Cytotoxicity profile of pellitory extracts

Treatment	Symb	Yield# (%)	Mortality, expressed as percentage Δ							LC 50 (µg/mL)	Toxicity profile ¥
			Concentration (µg/mL)								
			1	10	50	100	250	500	1000		
Petroleum ether	(Pe)	9.12	–	–	–	–	–	10 ±0.5*	20.0 ± 0.5**	2044.65	n/T
Chloroform	(Ch)	7,60	–	–	–	–	–	–	10.0 ±0.0	1391.69	n/T
Ethyl acetate	(Ae)	11.92	–	–		10.0 ±0.0	25.0 ±0.6***	100.0 ±0.0	100.0 ±0.0	249.26	T
Methanol	(Me)	22.52	–	–	–	–	–	–	47,5 ±1.0***	1000.0	n/T

#: yield (Percentage w/w, dry matter)
Δ: Values of mortality after 24h, expressed as mean +/- SD (n=4)
(*p<0.05, **p<0.01, ***p<0.001): value *vs* negative control (1% DMSO)
–: no mortality of nauplii was recorded
¥: LC 50 ≥1000 µg/ml is considered nontoxic (n/T). LC50<1000 µg/ml is toxic (T)

Phytochemical screening

Chemical screening of crude extracts, fractions and subfractions were carried out using well-established staining and precipitation reactions for major groups of bioactive natural products [20].

Thin layer chromatography (TLC) control of fractions and isolated compounds was carried out on precoated silica gel $60F_{254}$ aluminium plates (5×10 mm, ft = 0.25 mm, Merck Germany). Different solvent mixtures were used (S1/ hexane:dichloromethane (9:1), S2/hexane:ethyl acetate (8:2), S3/chloroform:methanol (8:2) and S4/chloroform:methanol (6:4)) and chromatograms were visualized by exposure under UV254/365 light, and by spraying with p-anisaldehyde and vanillin sulfuric reagents followed by heating [20, 21].

Fractionation of Ea extract and isolation of compounds

According to the results of the BSL screening assay (Table 1), ethyl acetate extract (Ea) was selected as the most active (LC = 249.26 µg mL^{-1}) compared to other extracts (Pe, Ch, Me) and, hence, was subjected to chromatographic fractionation. The Ea extract (6 g) was dissolved into small volume (5 ml) of ethyl acetate and the solution was subjected to a LC column (26 mm diameter × 300 mm height) filled with silica gel (type 60Å, 230-400 mesh ASTM, Merck). Elution was carried out with mixtures of hexane and ethyl acetate (90:10®70:30) of increasing polarity as described in Figure 1. Fifteen (50) fractions (100 ml each) were collected.

After TLC control using the different solvent systems S1 to S4 and staining with vanillin sulphuric and p-anisaldehyde reactifs [20], chromatographic fractions with similar TLC patterns were combined to afford sixteen (16) fractions

(F_1-F_{16}), as shown in Figure 1. Subsequent BSL assay screening revealed that the fractions F_4 (56.3 mg), F_8 (37.9 mg), F_9 (259.6 mg), F_{11} (134.9 mg) and F_{16} (862.4 mg) were highly active (LC<100 µg mL^{-1}).

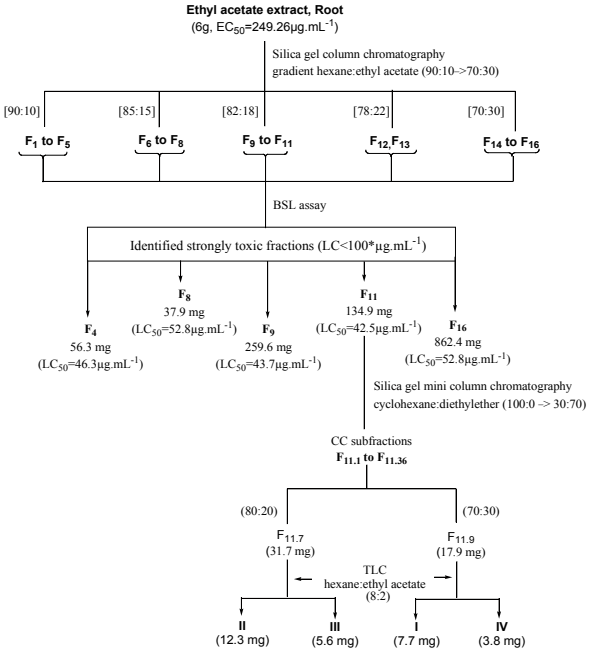


Figure 1. Flow chart of BSL assay-guided fractionation of cytotoxic ethyl acetate extract of *Anacyclus pyrethrum* roots

A phytochemical screening showed a homogeneity in the composition of the fraction F_{11} (LC = 42.5 µg mL^{-1}), with mainly alkamides compounds as revealed upon staining with anisaldehyde reagent [20]. Fraction F_{11} (LC = 42.5 µg mL^{-1}) was subsequently applied to a mini LC column chromatography (15 mm diameter × 200 mm height), filled with silica gel (high purity grade 40, 70-230 mesh ASTM, Merck), and eluted with mixtures of increasing polarity of cyclohexane and diethylether (100:0∃0:100), as described in Figure 1. Thirty six (36) subfractions ($F_{11.1}$ to $F_{11.36}$) were obtained and monitored by TLC. Alkamids were purified by preparative TLC silica gel plates (20×20, Merck) using solvent system S2 from subfraction $F_{11.7}$ (compounds II and III with 12.3 mg and 5.6 mg) and from Subfraction $F_{11.9}$ (compounds I and IV with 7.7 mg and 3.8 mg). Compounds purity was assessed by TLC silica gel using three solvent mixtures of different polarity: hexane:ethyl acetate (2:1), Hexane: chloroform:formic acid (2:6:1) and petroleum ether:ethylacetate:formic acid (7:30:1).

Structural identification

The structures of compounds (I-VI) were characterized by 1H-NMR (BRÜKER Avance 300 MHz, Salerno University, Italy) and comparison with relevant data from bibliography. Samples were dissolved in CDCl3.

Compound (I): White crystals; 1H-NMR δ (CDCl3, 300 MHz) ppm: 0.86 (3H, m, H-10), 0.88 (6H, d, H-3'), 1.32 (6H, m, H-7, H-8 and H-9), 1.88 (1H, m, H-2'), 2.10 (2H, m, H-6), 3.20 (2H ,m, H-1'), 5.00 (1H, br s, N-H), 5.77 (1H, m, H-2), 6.25 (1H, m, H-5); 6.75 (1H, m, H-4); 6.96 (1H, m, H-3).

Compound (II): White crystals; 1H-NMR δ (CDCl3, 300 MHz) ppm: 0.92 (6H, t, H-3' and H-12), 1.26 (8H, m, H-2', H-6, H-5 and H-11), 1.54 (2H, dd, H-7), 1.85 (2H, m, H-10), 2.19 (2H ,m, H-4), 3.17 (2H ,d, H-1'), 5.38 (1H, br s, N-H), 5.79 (1H, m, H-2), 6.10 (1H, m, H-9); 6.73 (1H, m, H-8); 6.92 (1H, m, H-3).

Compound (III): White crystals; 1H-NMR δ (CDCl3, 300 MHz) ppm: 0.91 (6H, d, H-3'), 0.94 (3H, m, H-14), 1.28 (14H, m, H-7, H-8, H-9, H-10, H-11, H-12 and H-13), 1.74 (1H, dd, H-2'), 2.36 (2H, t, H-6), 3.20 (2H ,m, H-1'), 5.41 (1H, br s, N-H), 5.78 (1H, m, H-2), 6.18 (1H, m, H-5); 6.83 (1H, m, H-4); 6.98 (1H, m, H-3).

Compound (IV): White crystals; 1H-NMR δ (CDCl3, 300 MHz) ppm: 0.94 (6H, m, H-3' and H-9), 1.30 (4H, d, H-2' and H-8), 1.85 (2H, m, H-7), 2.73 (2H, m, H-4), 3.21 (2H, d, H-1'), 5.02 (1H, br s, N-H), 5.80 (1H, m, H-2), 6.24 (1H, m, H-6); 6.83 (1H, m, H-5); 7.00 (1H, m, H-3).

Brine shrimp lethality (BSL) assay

Eggs of brine shrimp (*Artemia salina*) (Sera®, Bosnia-Herzegovina) were hatched in aqueous brine solution (3.8 g/L at 27-28°C). After 48 hours, the brine shrimp nauplii freed from egg shells were ready for the assay.

The BSL assay was conducted as described earlier with some modifications [22-25]. Briefly, pellitory samples (crude extracts and fractions) were tested at various concentrations (1-1000 µg/mL) in a brine solution containing 1% DMSO (v/v). A suspension solution of 10 nauplii were drawn through a glass capillary and placed in each compartment of a 12-well microplate. Negative control wells contained 1% DMSO in salty water. Pellitory solutions and control were tested in tetraplicate (n=4).

After 24h, the microplates were then examined under light, using a dissection microscope. The number of dead larvae was counted. Nauplii were considered dead if they did not exhibit any internal or external movement during several seconds of observation [26,27].

The percentage of mortality (% M) was calculated by the following formula as previously reported [28]:

$$(\%\ \text{lethality}) = \frac{\text{Total nauplii} - \text{alive nauplii}}{\text{Total nauplii}} \times 100$$

Lethal concentration (LC50) values were determined Finney's Probit regression analysis [29]. The lethality of the extracts on the brine shrimp was classified as previously reported [30,31]: LC50 < 1000 µg/mL was "toxic", LC50 = 500-1000 µg/mL was "weakly toxic", LC50 = 100-500 µg/mL was "moderately toxic", and LC50 < 100 µg/mL was "strongly toxic".

Statistics analysis

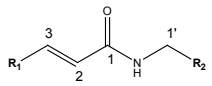
The data were collected from repeated measures (n = 4), and the results were reported as the mean ± standard deviation (SD). Data were analysed using Microsoft Excel 2007 (Redmond, WA, USA) and Microcal 6.0 (Microcal Software®, Inc.) for windows. The level of statistical significance between treated and untreated groups (control) was assessed by Tukey's test. The significance threshold was set at $p < 0.05$.

RESULTS

Pellitory roots extraction with solvents of different polarities yielded extractive materials of different physical consistencies. Both chloroform (Ch) and ethyl acetate (Ea) extracts were of powdery aspect, while those of methanol (Me) and petroleum ether (Pe) were greasy to resinous. Extracts are expressed with by yields, and are reported with their respective cytotoxic activity on the Brine Shrimp Lethality (BSL) assay in Table 1.

The Ae extract of the Pellitory treated group showed 100% mortality at the concentration of 500 µg/mL. The remaining extracts (Pe,Ch,Me) treated groups exhibited no or low mortality in the range of tested concentrations (1-1000 µg/mL). No lethality was found in the negative control (DMSO) group.

The BSL screening of Pellitory extracts showed that the most nauplii mortality was localized in the ethyl acetate extract (Ea), which displayed an LC50 = 249.3 µg/mL. To further investigate the active Ea extract, BSL assay-guided chromatographic fractionation (Figure 1) led to sixteen fractions, among which fraction F11 was revealed to be highly cytotoxic, with LC50 = 42.5 µg/mL. Multistep purifications of the active F11 afforded four compounds (I-IV) (Figure 2).



	Compound names	R_1	R_2
(I)	N-isobutyldeca-2,4-dienamide (Pellitorine)	$CH_3(CH_2)_4$-CH=CH-	$-CH(CH_3)_2$
(II)	N-propyldodeca-2,8-dienamide	$CH_3(CH_2)_2$-CH=CH-$(CH_2)_4$-	$-CH_2CH_3$
(III)	N-isobutyltetradeca-2,4-dienamide	$CH_3(CH_2)_8$-CH=CH-	$-CH(CH_3)_2$
(IV)	N-propylnona-2,5-dienamide	$CH_3(CH_2)_2$-CH=CH-CH_2-	$-CH_2CH_3$

Figure 2. Alkamides and their chemical structures

Compound I was isolated as white crystals. The 1-H NMR spectrum exhibited characteristic signals for a N-isobutylamide group at δ 3.20 ppm (2H,m, H-1'), δ 1.88 ppm (1H,m, H-2') and δ 0.88 ppm (6H,d, H-3'), together with amide proton signal at δ 5.00 ppm (1H, br s) as previously reported for isobutylamide groupe [32]. In the low field region four olefinic methines signals (1H, m) are observed at δ 5.77 (H-2), 6.25(H-5), 6.75(H-4) and 6.96 (H-3) ppm. Multiplet signals at δ 1.32 (6H) and 2.10 (2H) are compatible with the germinal protons (H-7,H-8 and H-9) and H6, respectively. High field signals at δ 0.86 (3H, t) is compatible with one methyl protons (H-10). The structure of compound I is assigned to the well-known pellitorine or N-isobutydeca-2,4-dienamide, on the basis of H-NMR data which were in full agreement with literature values [33-38].

Compound II was isolated as white crystals. The 1-H NMR spectrum exhibited signals for a N-propylamide group: δ 3.17 ppm (2H, H-1'), δ 1.26 ppm (2H, H-2') and δ 0.92 ppm (3H, H-3'), together with that at δ 5.38 ppm (1H, br s) assigned to the amide proton. Four olefinic methines signals were observed at δ 5.79, 6.10, 6.73 and 6.92 ppm (1H each) as previously reported for isolated double bonds in alkamides [39]. On the basis of this reference, we deduced the presence of two double bonds in position 2 and 8. By comparing the 1H NMR data with previously published data, compound II was identified as: N-propyldodeca-2,8-dienamide.

Compound III was isolated as white crystals. The 1-H NMR spectrum exhibited characteristic signals for a N-isobutylamide group at δ 3.20 ppm (2H), δ 1.74 ppm (1H) and δ 0.91 ppm (6H), together with amide proton signal at δ 5.41 ppm (1H, br s) as previously reported [40]. Four olefinic methine signals are observed at δ 5.78, 6.18, 6.83 and 6.98 ppm (1H each), which are compatible with the presence of two double bonds conjugated with the amide carbonyl as previously recorded for alkamides [32,40]. On the basis of previously 1-H NMR published data, compound III is identified as: N-isobutyltetradeca-2,4-dienamide.

Compound IV was isolated as white crystals. The 1-H NMR spectrum was some points similar to that of compound II. It shows signals for a N-propylamide group: δ 3.21 ppm (2H), δ 1.30 ppm (2H) and δ 0.94 ppm (3H), together with that at δ 5.41 ppm (1H, br s) assigned to the amide proton as previously reported [40]. The four olefinic methine signals were observed at δ 5.80, 6.24, 6.83 and 7.00 ppm (1H), as recorded for alkamides with double conjugations in position 2 and 5 [40]. By comparing the 1-H NMR data with previously published data [32,40], compound 3 was identified as: N-propylnona-2,5-dienamide.

DISCUSSION

Pellitory (*Anacyclus pyrethrum*) roots has been known since ancient times and has been and is used as medicinal plant applied – among other ailments – against cancer [8]. Of special pharmacological interest, pellitory produces several N-alkamides [14], which are secondary metabolites in plants. Because of their wide structural diversity, these compounds have attracted several research groups to study their pharmacological behaviours [41]. Numerous reports have dealt with the anesthetic, analgesic and anti-inflammatory, but also with the anticholinesterase, antidiabetic, antiparasitic, anticancer, molluscicidal, antiprotozoal and insecticidal activities of N-alkamides [36,41,42].

Among the multiple pharmacological effects of alkamides, its anticancer property is the most promising. Thus, many alkamides isolated from the Asteraceae and other botanical families were shown to exhibit *in vitro* cytotoxic activity against cancer cell lines [7,41,42].

Worth noting is the pellitorine (occurring in pellitory roots) has been shown to have strong cytotoxic activities against HL60 (Human promyelocytic leukemia strain) and MCT-7 (Brest cancer strain) cell lines, with IC50 values of 13.0 µg/mL and 1.8 µg/mL, respectively [43]. Piplartine and analogues have exhibited potent effects in human breast carcinoma MCF-7 cells, whilst being relatively non-toxic to non-tumorigenic MCF-10a cells [44,45]. Other alkamides, as capsaicin was reported to induce the apoptosis of prostate cancer cell lines [46], while pharnilatin did so for those of skin melanoma, as well as lung, ovary and colon [47]. These reports and many involving others alkamides, make these

type of compounds a relatively new and promising group of natural products as source of new anticancer agents [41].

BSL assay is recognized as a general test for bioactive compounds screening [19,48-53]. The technique is described as easily mastered, costs little, and utilizes small amount of test material [50]. Since its introduction in 1982, by Meyer and al. [30], it appears that BSL test is predictive of cytotoxicity and has been successively employed for bioassay-guided fractionation of active cytotoxic and antitumor agents as reported by several authors [48,52-56]. A significant correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines has been demonstrated by the National Cancer Institute (NCI, USA) [51].

In our toxicity evaluation of pellitory root's extracts (Pe, Ch, Ea, Me), the BSL test showed that the most of the nauplii mortality was localized in the ethyl acetate extract (Ea), which displayed an LC50 = 249.3 µg/mL. This value is considered as "toxic" [30,31], and hence bioguided fractionation of Ea active extract of *Anacyclus pyrethrum* root was undertaken and revealed a highly cytotoxic fraction (F_{11}) with LC50 of 42.5 µg/mL. Multistep purifications of the active (F_{11}) fraction have afforded four alkamides (Figure 2): namely N-isobutyldeca-2,4-dienamide or pellitorine (I), N-propyldodeca-2,8-dienamide (II), N-isobutyltetradeca-2,4-dienamide (III) and N-propylnona-2,5-dienamide (IV). The compounds (I) and (III) were previously reported from *A. pyrethrum* roots and other species [14]. The two remaining alkamides (II) and (IV) are isolated for the first time from pellitory, but were cited previously in the natural alkamides database [41].

In our study, the BSL test appears to be effective in the detection of potentially toxic fractions containing N-alkamides, such as pellitorine a well known cytotoxic agent [43]. Hence, our approach of using the *Artemia salina* mortality test to guide the fractionation of the pellitory root Ea extract was productive, and thus supports its appropriateness as a pre-screening tool for cytotoxic compounds.

CONCLUSION

Pellitory roots have been cited as a cancer remedy in folk medicine. Recent study have shown that pellitory alcoholic extract have cytotoxic activity against human colorectal cancer cell lines. Our study suggests that cytotoxic activity is localized in the ethyl acetate extract (Ea) of pellitory root, and this effect might be associated with the presence of alkamides, pellitorine and its analogues (II-IV). Although pellitorine (I) has previously shown strong cytotoxic activities against human cancer cell lines, no studies have been reported on cytotoxic activities of other isolated compounds (II to IV). Subsequently, these alkamides will be considered in future study as candidates for in depth in vitro evaluation of their cytotoxicity against cancer and normal cell lines. Finally, through this study, BSL assay demonstrate again its usefulness as bench-top assay in exploring plant extracts for cytotoxic compounds.

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