



Asian Journal of Pharmacognosy

Research Article

Bioassay guided isolation of anti-inflammatory principles from *Prismatomeris tetrandra* (Roxb.) K. Schum

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Abstract

The objective of this study is to investigate the anti-inflammatory constituents of *Prismatomeris tetrandra* (Roxb.) K. Schum through bioassay-guided fractionation procedures. The methanol extract of the leaves (MLE) displayed marked anti-inflammatory effects on TPA induced mouse ear oedema and hyaluronidase inhibitory assays as compared to the reference compound. The chloroform fraction from MLE (CF) showed the highest potency and was further subjected to bioassay guided fractionation which resulted in ursolic acid (**1**) being the compound responsible for the anti-inflammatory activity, with 103.18 μ M and 0.42 mg/ear IC₅₀ inhibition towards hyaluronidase (at 100 μ g/ml) and TPA-induced inflammation in mice (at 2mg/ear), respectively.

Keywords: *Prismatomeris tetrandra*, hyaluronidase, TPA induced mouse ear oedema, ursolic acid, bioassay guided isolation

Introduction

Prismatomeris (Rubiaceae) is a small genus of shrubs comprising 25 species distributed in tropical areas such as Ceylon, North Eastern India, South Western China and southwards of tropical Australia [Ridley, 1967; Johansson, 1987]. *P. tetrandara* (Roxb.) K. Schum [syn. *P. albidiflora* King and *P. malayana* Ridley] (figure 1) is known to grow from north-eastern India to Sri Lanka, Bangladesh, Burma, southern Laos and China, Vietnam, Thailand, Peninsular Malaysia, Indonesia and Philippines [Lemmens & Bunyapraphatsara, 2003].

Locally, *P. tetrandra* is known as *sepedeh*, *mundess* and *susun kelapa hutan* [Ridley 1967]. This species is also popular as *tongkat haji samat*. The wood is consumed as a tonic and depurative in herbal tea drinks after childbirth. A mixture of the roots or stems with other herbal plants is used in a decoction to treat bloody vomiting [Lemmens & Bunyapraphatsara, 2003]. In Indochina, the decoction has been used in a mixture with coconut and henna to treat bronchitis [Lemmens & Bunyapraphatsara, 2003]. In



addition, the leaves are applied as a poultice to fresh wounds while the roots macerated in water are used to treat snakebites [Lemmens & Bunyaphrathasara, 2003; Burkill, 1966].

Previous phytochemical investigation of *P. tetrandra* have resulted in the isolation of anthraquinones, triterpenes, sterols [Zi-Ming et al., 2005; Pittaya et al., 2008; Dey et al., 2003; Zhang et al., 2010; Tu et al., 1981]. However, only a few biological evaluation studies have been conducted on this species. Dey et al. (2003) reported on the cytotoxicity of the leaf extract of *P. tetrandra* on human tumour cell lines together with the antitumor activity of ursolic acid and prismatomerin [Dey et al., 2003]. Several anthraquinones from this plant were also investigated for their potential as anticancer, antifungal and antimalarial, anti-allergic and antioxidant agents [Pittaya et al., 2008; Krohn et al., 2007; Kwanjai et al., 2005].

To the knowledge of the authors, no biological studies regarding the anti-inflammatory activity of the secondary metabolites isolated from *P. tetrandra* has been reported until present. Thus, in the search for bio-potential activity of this plant, the present study was designed to evaluate the anti-inflammatory effects of the root, leaf and stem extracts via the *in vitro* hyaluronidase inhibitory and *in vivo* TPA induced mouse ear oedema bioassay. Then through bioassay guided fractionation procedures, the active components were identified.

Materials and Methods

Plant material: *P. tetrandra* was collected from the district of Setiu, Terengganu in 2006 and a voucher specimen was deposited in the herbarium of the Forest Research Institute Malaysia (FRIM) (FRI 50080). *P. tetrandra* was identified by a FRIM botanist. The fresh plant materials were separately cut or chopped into smaller pieces and dried in an oven at 40°C.

Solvents and chemicals: Analytical TLC was carried out on Merck 60 F₂₅₄ silica gel aluminium sheets (absorbent thickness: 0.25 mm). Column chromatography was performed using silica gel (Merck 230-400 mesh, ASTM) and Sephadex LH-20 (Sigma-Aldrich). IR spectrum was recorded using a Perkin-Elmer Spectrum 100 Fourier Transform Infrared (FT-IR) spectrometer equipped with a mid-infrared deuterated triglycine sulphate (DTGS) detector. NMR spectra were recorded in C₅D₅N (Merck, Germany) with tetramethylsilane as the internal standard, using a JEOL ECA 400 MHz NMR spectrometer. The mass spectra were recorded on a Thermo Fisher Scientific LTQ Orbitrap mass spectrometer equipped with an electrospray ionization probe. UV spectra were recorded using a Shimadzu 1650 PC UV-Vis Spectrophotometer. A Jasco P-1020 Polarimeter was used to record the optical rotation. All solvents were of analytical grade and were distilled prior to use.

Hyaluronidase (bovine testes, type 1-S), hyaluronic acid (human umbilical cord, sodium salt), bovine, serum albumin, and ammonium acetate were purchased from, Sigma Chemical Co., almond β-glucosidase, boric acid, fisetin, L-lysine, L-leucine, and L-glutamic acid were purchased, from Wako Chemical Co.

Preparation of the methanol, petroleum ether, chloroform, ethyl acetate and water extracts: 200 g of dried leaves, roots and stems, were separately weighed and extracted with methanol at room temperature for three days (1 L, 2X). Following filtration, the combined methanol extract was evaporated to dryness under reduced pressure to yield crude methanol leaf extract (MLE), crude methanol root extract (MRE)



and crude methanol stem extract (MSE). The extracts were subjected to anti-inflammatory activity evaluation.

Evaluation of all the extracts (MLE, MRE and MSE) resulted in MLE being the most potent in inhibition towards the hyaluronidase enzyme and TPA-induced mouse ear oedema activities. Therefore, further investigation towards the above mentioned activities was carried out on the leaves of this plant. The same extraction procedure as mentioned earlier was carried out on 2 kg of the leaves to afford 200 g (10 % w/w of the dried leaves) of MLE. Firstly, the MLE was dissolved in 14 L of MeOH. Next, it was suspended in 1 liter of distilled water and partitioned successively with petroleum ether (5 L), chloroform (5 L) and ethyl acetate (5 L) to yield the respective fractions: petroleum ether (PF; 22.4 g), chloroform (CF; 35.1 g), ethyl acetate (EF; 11.7 g) and water (WF; 49.4 g).

Bioassay-guided fractionation and isolation: The methanol extracts of the roots, stems and leaves were evaluated for their inhibitory activity against hyaluronidase enzyme and TPA mouse ear oedema activities. The leaf extract exhibited the highest anti-inflammatory activity for both assays (Table 1). Therefore the MLE was subjected to bioassay-guided fractionation.

The MLE was re-extracted to give PF, EF, CF and WF. These fractions were also subjected to both bioassays and the CF was found to be the most potent with 72.19 ± 1.25 % and 90% inhibition towards hyaluronidase (100 μ g/ml) and TPA-induced inflammation (2 mg/ear), respectively. The active CF (35 g) was further investigated in order to identify the active components. The CF was dissolved in methanol and subjected to column chromatography using Diaion HP-20 with methanol (2 L) as the eluting solvent. A total of 200 fractions were obtained and their profiles were monitored via TLC using chloroform and methanol as the solvent system. The fractions were pooled into 3 sub-fractions (C1, C2, C3). Sub-fraction C3 (2.79 g) which highly inhibited the activity of hyaluronidase enzyme was further fractionated and purified using silica gel column chromatography (chloroform: methanol; 10:0 \rightarrow 9:1 v/v) to give 8 sub-fractions (C3-1 to C3-8). Sub-fraction 6 (C3-6) exhibited the highest inhibition against the hyaluronidase enzyme and TPA induced mouse ear oedema activity. Column chromatography of this fraction with chloroform : methanol (98:2 v/v) yielded 3 β -hydroxyurs-12-*en*-28-oic acid (ursolic acid) (**1**) (1.5 g, 98:2).

Isolation of compounds from less active fractions: Apart from sub-fraction 6 that yielded ursolic acid (**1**) as the most active compound, the less active fractions 1, 2 and 7 were also investigated for their chemical constituents and were further evaluated for their inhibition property towards hyaluronidase.

Sub-fractions C1 and C2 were purified using silica gel column chromatography eluting with a chloroform : methanol (v/v) system to afford two compounds; 2 (20 mg, eluted with 96:4 v/v), and 3 (9 mg, eluted with 100:0 v/v). Another compound, 4 (4.0 mg, eluted with 98:2 v/v), was isolated from fraction 7 (C3-7)

Animals: The experiments were performed in accordance with the suggested ethical guidelines for the care of laboratory animals. The male strains of albino mice (25-30 g) were purchased from the Animal Centre, Institute for Medical Research, Kuala Lumpur (IMR). The animals were housed in plastic cages at room temperature, 12 h light (08.00 to 20.00 h). Food and water were available *ad libitum*.

Hyaluronidase inhibitory assay: The assay was carried out according to the Sigma protocol [Ling et al., 2003]. The medium of the assay consists of 1.00-1.67 U hyaluronidase in 100 μ l of 20 mM sodium phosphate buffer at pH 7.0 with 77 mM sodium chloride and 0.01% Bovine Serum Albumin (BSA). The medium was pre incubated with 25 μ l of each test compound in DMSO (dimethyl sulphoxide) for



10 min at 37°C. Then, the assay was started by adding 100 µl of hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) to the incubated medium and incubated for another 45 min at 37 °C. The undigested hyaluronic acid was precipitated with 1 ml of acid albumin solution which is made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, at pH 3.75. After standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm. The absorbance in the absence of the enzyme was used as the reference value for maximum inhibition. The inhibitory activities of the test compounds were calculated as the percentage ratio of the absorbance in the presence of the test compounds vs. absorbance in the absence of enzyme. The enzyme activity was checked by a control experiment which was carried out simultaneously, in which the enzyme was pre incubated with 25 µl of DMSO, followed by the assay procedures described earlier. In this case, the percentage ratio of the absorbance in the presence of enzyme vs. that in the absence of enzyme was in the range of 15-20%. The performance of the assay was verified using apigenin as a reference under identical experimental conditions. The extracts, fractions and sub-fractions were tested at a concentration of 100µg/ml each in the final reaction mixture. The results were expressed as the mean of the inhibitory values ± S.D. (standard deviation) of three separate experiments measured in triplicates.

TPA induced mouse ear oedema assay: 12-*O*-tetradecanoylphorbol-13-acetate, TPA (1 µg) dissolved in acetone (20 µl) was applied to the ear of the male strain mice (25-30 g) by means of a micropipette. TPA stock solution of 1 mg/ml was prepared from 1 mg of 12-*O*-Tetradecanoylphorbol 13-acetate (Sigma-Aldrich: P1585-1MG) dissolved in 1 ml of ethanol. 50 µl of the stock solution was aliquot into each amber vial and kept at -20 degree C before being used. Each vial with 50 µg of TPA will be top up to 1 ml volume, with acetone to get working solution of 50 µg/ml. The volume of 20 µl of the 50 µg/ml working solution was applied into the ear of the mice. Therefore each mice ear will be treated with 1 µg of TPA. The plant extracts, fractions and sub-fractions were applied topically to the inner surface of the right ear at concentrations of 2 mg/ear or 0.5 mg/ear of the compound(s), about 30 min before each TPA treatment. The other ear which acted as a control was applied with sample vehicle. The resulting oedema was measured eight hours after TPA treatment. The results were expressed as percentage inhibition (IE%), taken to mean the complete suppression of erythema in the test animals. Each value was the mean of individual determinations from seven mice. Indomethacin, a known inflammatory inhibitor, was used as the positive control for this study. The inhibitory effects (IE%) of each extract was calculated as the ratio of the increase in the weight of the ear sections, according to the following formula:

$$\text{Inhibitory effect (IE\%)} = \frac{[(L-R) / (L-C^*)] \times 100}{= [(L-R)/(L-(L/2.41))] \times 100}$$

where L = weight of the left ear which was treated with TPA only

R = weight of the right ear which was treated with TPA plus extracts

*C = weight of the normal ear (untreated ear)

*C is the calculated weight. It has been found that treating a normal ear with 1 µg of TPA resulted in a 2.41 times increase in the weight of the ear [Carlson et al., 1985].

Statistical analysis: Data obtained were expressed as the mean standard error. The data were analyzed for statistical significance using one way analysis of variance (ANOVA) (for hyaluronidase) and Duncan's multiple range tests for TPA induced mouse ear oedema inhibitory activity. *P*-values less than 0.05 were considered to be significant [**p*<0.05; ***p*<0.01; ****p*<0.001].



Structural identification of isolated compounds: Identification of the isolated compounds (Figure 1) was performed by comparison of their spectroscopic data with those reported in the literature [Seebacher et al., 2003; Jie Ping et al., 2006; Dong Hyun et al., 2005; Chen et al., 2007; Kazuhiko et al., 1997].

Results

In this study, an investigation on the *in vivo* and *in vitro* anti-inflammatory activities of the Malaysian medicinal plant, *P. tetrandra*, was carried out. The leaves, roots and stems were extracted with methanol at room temperature to give methanol leaf extract (MLE), methanol root extract (MRE) and methanol stem extract (MSE). The MLE, MRE and MSE were subjected to the *in vitro* hyaluronidase inhibitory bioassay and topical anti-inflammatory bioassay in the TPA-induced ear oedema model.

The effect of *P. tetrandra* extracts in both experiments is shown in Table 1. The extracts, fractions and compound were prepared at 100 µg/ml and 2 mg/ear for hyaluronidase and TPA induced mouse ear oedema bioassays respectively. The MLE extract exhibited the highest inhibition towards both hyaluronidase and TPA induced mouse ear oedema bioassays ($70.51 \pm 4.34\%$ and 73.00% , respectively). Thus, the MLE was selected for further fractionation. The chloroform fraction (CF) of the leaves showed higher inhibitory activity towards both hyaluronidase and TPA mouse ear oedema ($72.19 \pm 1.25\%$ and 90.0% , respectively) as compared to the petroleum ether (PF), ethyl acetate (EF) and water fraction (WF). Hence CF was subjected to further fractionation to afford sub-fractions C1, C2 and C3. Sub-fraction C3 revealed the highest inhibitory value for hyaluronidase activity ($74.26 \pm 3.79\%$) amongst all of the sub fractions. Purification of the compound from this subfraction led to the isolation of ursolic acid (**1**) in figure 1 [Chien-Ya et al., 2001]. This compound inhibited the hyaluronidase activity at $79.67 \pm 6.42\%$ and TPA mouse ear oedema at 73.00% which were comparable to those of the positive controls, apigenin ($88.97 \pm 7.93\%$) and indomethacine (72.00%), respectively.

Dose response study of ursolic acid (**1**) for the hyaluronidase inhibitory and TPA induced mouse ear oedema assays were carried out (Table 2). The IC_{50} values were $103.18\ \mu\text{M}$ and $0.42\ \text{mg/ear}$ for the two assays respectively. These values are comparable with those of the positive controls; apigenin ($214.74\ \mu\text{M}$) and indomethacin ($0.38\ \text{mg/ear}$) which indicated that ursolic acid (**1**) exhibited strong inhibitory effects against hyaluronidase and mouse ear oedema induced by TPA.

Subfraction C-2 which was the second most potent was also analysed for its compounds which gave $3\beta,19,23$ -trihydroxyurs-12-en-28-oic acid (barbinervic acid) (**2**), $3\beta,23$ -dihydroxyurs-12-en-28-oic acid (2α -hydroxy ursolic acid) (**3**) and 3β -hydroxyurs-11,12-epoxy-ursane-28,13-olide (**4**) (figure 1). Compounds **3** and **4** were not evaluated for both assays due to insufficient amount.

The bioassay-guided fractionation and isolation procedures carried out in this study proved that the active component responsible for the inhibition towards the hyaluronidase and TPA-induced inflammation of mice ear was ursolic acid (**1**). The inhibition of this compound was comparable with the positive controls, *i.e.* apigenin, and indomethacine, for both the bioassays. This result is in agreement



Table 1: Inhibitory activity of the methanol extracts of the leaves, stems and roots of *Prismatomeris tetrandra* and the chloroform extract of the leaves on TPA-induced inflammation in mice and Hyaluronidase inhibitory activity

Samples	Percentage inhibition towards hyaluronidase ^a	Inhibition of TPA – induced inflammation in mice, I.R. ^c (%)
Stem	64.31 ± 9.41 ^b	37.00 ^d
Leaves	70.51 ± 4.34^b	73.00^d
Roots	59.40 ± 6.91 ^b	25.00 ^d
<i>Leaf fractions:</i>		
Petroleum ether	60.47 ± 0.12	14.00 ^d
Chloroform	72.19 ± 1.25	90.00^d
Ethyl acetate	48.19 ± 2.41	7.00 ^d
Aqueous	60.19 ± 1.85	14.00 ^d
C1	61.26 ± 3.57	NT
C2	67.99 ± 10.23	NT
C3	74.26 ± 3.79	NT
ursolic acid 1	79.67 ± 6.42	73.00
Apigenin	88.97 ± 7.93	
Indomethacin		72.00

^aData representing the mean ± S.D of three independent experiments performed in triplicates.

% inhibition at 100 µg/ml concentration.

^bMean for percentage inhibition were significantly different (one- way analysis of variance, p < 0.05)

^cI.R.: inhibitory ratio at 2.0 mg/ear for extracts and fractions, 0.5 mg for ursolic acid 1 (n=7)

^dMean for percentage inhibition were not significantly different (Duncan's multiple range test, p=0.05)

with the reported findings for ursolic acid (**1**) as a potent anti-inflammatory agent. Not only does it inhibit human leucocyte elastase (HLE), but also 5-lipoxygenase and cyclooxygenase activities [Safayahi et al., 1997; Najid et al., 1992; Khota et al., 2000; Ho et al., 2001; Liu, 1995]. Hirota, Mori, Yoshida, and Iriye in 1990 [Hirota et al., 1990] reported that ursolic acid (**1**) inhibited 12-*O*-hexadecanoyl-16-hydroxyphorbol-13-acetate (HPPA)-induced inflammation. It also inhibited concanavalin A induced histamine release which can cause severe inflammation [Tsuruga et al., 1991]. However, to the knowledge of the authors, this is the first report on the inhibitory activity of ursolic acid **1** towards hyaluronidase.

The strong inhibition of the leaf extract towards hyaluronidase and TPA-induced inflammation owing to the presence of ursolic acid (**1**) as the major compound is a valid justification for the use of *P. tetrandra* as an ingredient in dermatological preparations.

Mouse ear oedema has been used as an *in vivo* model for inflammation studies. The methanol leaf extract and chloroform leaf fraction prevent the ear thickness or the ear weight from increasing after the TPA application, whereas the vehicle control shows no effect or very minimal. It was determined by examining the ear thickness and weight. It showed that the extract and fraction as well as the compound *i.e.* ursolic acid (**1**) protect the skin from the infiltration of monocytes, one of the steps in the early events of skin inflammation.



Table 2: Hyaluronidase inhibitory activity at the concentration of 100-2000 μM and effect on TPA induced mouse ear oedema of selected isolated compounds.

	IC ₅₀ inhibition towards hyaluronidase ^a (μM)	IC ₅₀ inhibition of TPA-induced inflammation in mice ^b (mg/ear)
Ursolic acid 1	103.18	0.42
Barbinervic acid 2	286.95	NT
Apigenin	214.74	
Indomethacin		0.38

^aNA-Inhibitory activity < 20% at concentration up to $50 \times 10^2 \mu\text{M}$; Positive control-Apigenin; Values were presented as the mean of three independent experiments performed in triplicates.

^bNT-Not tested; Positive control-Indomethacin (n=7).

In the *in vitro* study using hyaluronidase inhibitory assay, the inhibition of the enzyme was indicated by the amount of hyaluronic acid remaining. The extracts, fractions and compounds which inhibited the hyaluronidase would give a highly white precipitate solution. The hyaluronic acid which has not been hydrolyzed by the hyaluronidase will react with the serum albumin to produce the white precipitate. Hyaluronic acid is known to participate in many biological processes such as cell migration, wound healing, malignant transformation and tissue turnover [Deepa et al., 2006]. Hyaluronidase is however the enzyme that degrades these multifunctional high molecular polysaccharide. It is thus the extract of *P. tetrandra* and ursolic acid (**1**) that has inhibited hyaluronidase which in turn helped in reducing the hyaluronic acid degradation and therefore reducing the disease progression and spreading of venom/toxins and bacterial pathogens. They could also serve as contraceptives against anti-tumour, anti-bacterial and anti-venom/toxins activities.

Conclusion

As a conclusion, the stems, roots and leaves of *P. tetrandra* showed interesting anti-inflammatory activities for both assays conducted in this study. Our finding also justifies the claims of the usage of this plant to treat wounds and snakebites. This is due to the fact that compounds which have been used to treat snake bites or to promote wound healing are known to possess anti-hyaluronidase activity and thus delay or stop the diffusion of toxins after the envenomation. Furthermore, this is the first communication on the occurrence of compounds **2-4** in this plant. It is also the first report on the anti-inflammatory properties of ursolic acid (**1**) based on the hyaluronidase inhibitory activity.

Acknowledgment

The authors would like to acknowledge the Ministry of Science Technology and Innovation (MOSTI) and the Malaysia Toray Science Foundation (MTSF) for their financial support for this study, Dr.



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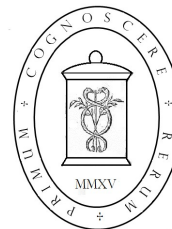
Rasadah Mat Ali for her moral support and the staff of Natural Products Division involved in giving their technical support for this study (Siti Asha Abu Bakar, Mohd. Faisal Iskandar Sukhairi, Salbiah Man and Mohd. Faridz Zol Fatah).

Declaration of Conflict of Interest

No conflict of interest associated with this work.

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Figure 1: -*Prismatomeris tetrandra*

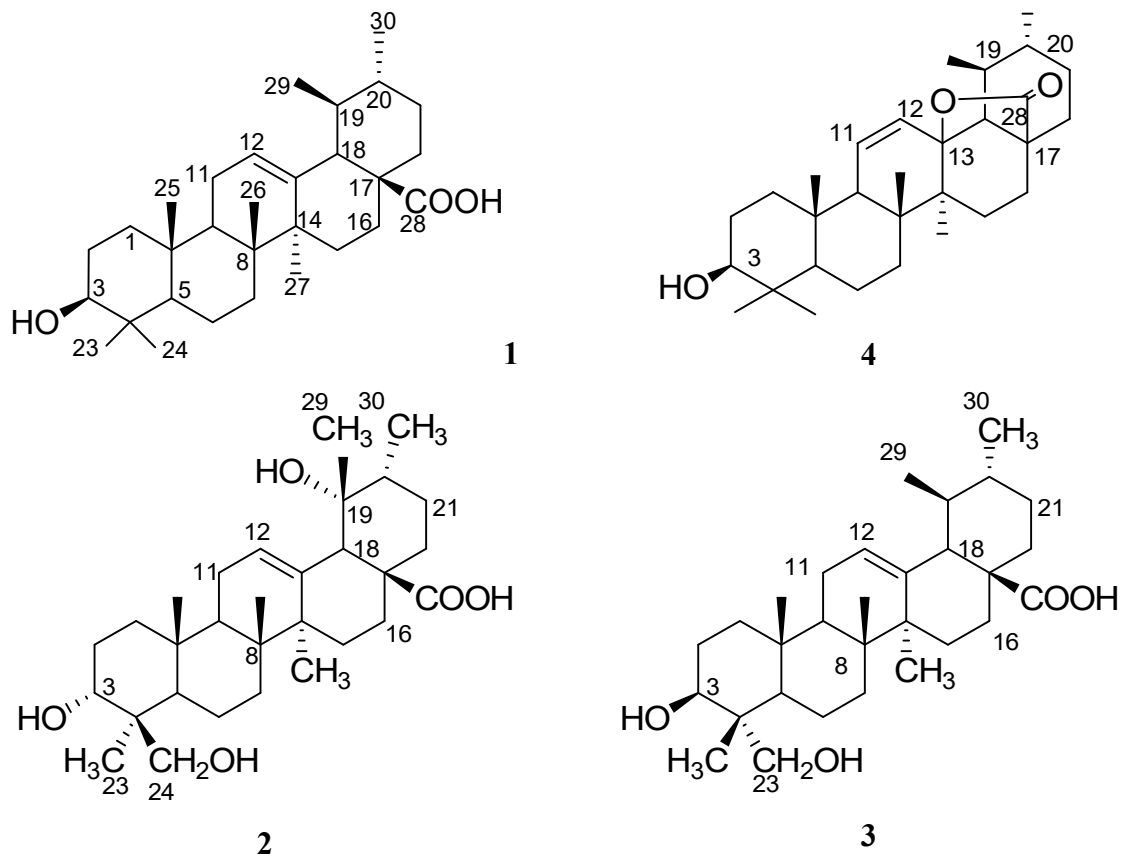
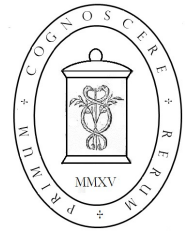


Figure 2: Isolated triterpenes from *Prismatomeris tetrandra*