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Inhibitory potential of important phytochemicals from *Pergularia daemia* (Forsk.) chiov., on snake venom (*Naja naja*)

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KEYWORDS

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Abstract *Pergularia daemia* (Forsk.) chiov., is a milk weed of Asclepiadaceae family. In the present study β-sitosterol, β-amyrin, α-amyrin and lupeol were identified in the leaf by GC–MS. Molecular docking studies were performed to evaluate their activities on phospholipase A2 (PLA2) and L-amino acid oxidase enzymes which constituted a rich source in snake venoms (*Naja naja*). Snake venom Phospholipase A2 with PDB code 1A3D devoid of co-crystallized ligand was extracted from Protein Data Bank. Using Molegro Virtual Docker two cavities are formed by cocrystallization. L-Amino acid oxidase (PDB code 4E0V) was a receptor model with a co-crystallized ligand FAD. Among the phytochemicals analysed, β-sitosterol displayed high affinity of binding to the active site regions of phospholipase A2 and L-amino acid oxidase, respectively. The affinity of binding was −125.939 and −157.521 kcal/mole identified by gold scores. α-Amyrin and β-amyrin had two hydrogen bond interactions with PLA2. Hence this study suggests that β-sitosterol identified in *P. daemia* can antagonize PLA2 and LAAO activities and forms a theoretical basis for the folk use of the plant against snake venom.

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1. Introduction

Pergularia daemia is also called as Yugmaphala a perennial, small, twining herb with major pharmacological activities being reported on different parts of the plant. Arial shoots are known to possess anti-emetic and expectorant properties [1]. Singh et al. reported the probable use of plant parts as anti-asthmatic, rheumatic swellings and anti snake venin [2]. The plant also acts as an Anthelmintic and antiseptic [3–5]. Dried leaves are Anti rheumatic, anti bronchitic, have wound healing properties, reduce body pains, infantile diarrhoea, amenorrhoea and dysmenorrhoea [1,6–8]. Fresh root is used as abortifacient and can treat gonorrhoea [10]. Shoots treat whooping cough [9,11], stem bark acts against malaria, and twig as an antipyretic and appetizer [12,13]. Moreover, Aqueous, ethanolic and petroleum ether extracts of *P. daemia*

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leaves exhibited significant analgesic, antipyretic activities and antibacterial properties [14,15]. Phytochemicals like glucosides and cardenolides (calotoxin, calotropagenin, dihydrocalotropagenin, calotropin, uscharidin) in seed, coroglaucigenin, corotoxigenin, uscharidin and uzarigenin in stem are identified [16]. Roots were reported to contain β-sitosterol, lupeol, lupeol acetate, α, β-amyrin and its acetate [17–19]. Apart from this, Jalalpure et al. reported leupol-3-β-transcrotonate along with the acetate of α-amyrin, β-amyrin, oleanolic acid and β-sitosterol from the hexane extract of whole plant [15]. Organic esters, fatty acids and phenolic compounds were identified by GC MS analysis of the ethanolic extract of the plant [20].

Though the literature shows the folk use of the plant to treat snake bite, there exists no theoretical evidence. The present work, reports the isolation and structural elucidation of β-sitosterol, β-amyrin, α-amyrin and lupeol simultaneously from the leaf powder of *P. daemia* using GC–MS for the first time. Further computer-aided analysis was initiated to evaluate the activity of these compounds against snake bite after their *in vitro* studies thus aiming at developing a theoretical evidence for folk use of the plant.

2. Material and methods

2.1. General experimental procedure

All the chemicals in the study were purchased from Sigma and Merck Pvt. Ltd and are used without further purification. The venom was purchased from Calcutta Snake Park, Calcutta, India, in its pure form. GC–MS, column, protein data, docking was performed in Ohmlina Centre for Molecular Research, India. The whole plant of *P. daemia* (Forsk) chiov., was collected in the month of August, 2010, from Krishna district, India. The sample was identified using trnL-trnF intergenic spacer sequence based phylogenetic tree (Fig. 2) and a voucher specimen No. 02 was deposited in the herbarium of Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, India (Fig. 1).

Figure 1 Twig of *Pergularia daemia* bearing flowers and fruit. The leaves are almost glabrous above and velvety below. The flower corolla forms a greenish-yellow or dull white tube. The fruits release ovate seeds covered with velvety hairs.

2.2. Extraction, isolation and analysis by GC–MS

As the plant was selected based on the traditional use, the initial extract was prepared as described by the traditional healer. 10 gms of the leaf powder was extracted using 200 MilliQ-water at 50 °C for 4h and cooled to room temperature, filtered and treated with methylene chloride (3 × 100 ml) to extract more lipophilic components. The Leaf residue (remained on filter paper) was dissolved in 200 mL of n-Hexane and sonicated for 30 min to dissolve any lipophilic substance that is not removed from the leaf powder [21,22]. Methylene chloride and n-Hexane extracts are combined and passed through a sodium sulphate plug. The combined extract was concentrated in a rotary evaporator up to 10 mL and then further upto 1 mL using nitrogen concentrator and was analysed using GC–MS (5975C Inert MSD) system equipped with a splitless capillary injection port using HP-5MS, 0.25 mm × 0.25 μm × 30 m column [23]. Helium (99.999%) was used as a carrier gas at a constant flow of 1 mL/min and an injection volume of 0.5 EI was employed with an injector volume of 1 μL under splitless mode. Ion source temperature was maintained at 280 °C. The oven temperature was programed from an initial temperature of 70 °C hold for 5.0 min with an increase of 10 °C/min to 320 °C for a total run time of 40 min. Mass spectra were taken at 70 eV, a scan interval of 0.5 s and with MS scan mode from 25 to 550 m/z. The chromatograms thus obtained were recorded (Fig. 4).

2.3. In-vitro tests for the inhibition of PLA2 activity

PLA2 activity of *N. naja* venom was performed using an indirect haemolytic assay on agarose–erythrocyte–egg yolk gel plates [24]. Increasing doses of the venom were added to the wells in the agarose gels with 1.2% sheep erythrocytes, 1.2% egg yolk as a source lecithin and 10 mM $CaCl_2$, incubated at 37 °C overnight and haemolytic haloes were measured (Fig. 3). Control wells contain 15 μL of saline. The minimum indirect haemolytic dose (MIHD) corresponds to the dose that induced a haemolytic halo of 20 mm diameter. 1 MIHD of *N. naja* venom was used. Test solutions and dose of venom (0.005 mL each) were pre-incubated for 1hour at 37 °C. After centrifugation at 10,000 rpm for 10 min, the supernatant (20 μL) was tested for PLA2 activity by incubating the plates at 37 °C for 20 h. Results were expressed as percentage inhibition of enzymatic activity (Table 1 and Fig. 7), where 100% inhibition produced no clear zone. The total phenol and flavonoid content of the extract was estimated using tannic acid and quercetin standard graphs (Figs. 5 and 6).

2.4. In vitro tests for the inhibition of protease activity of the venom

Venom Proteolytic activity was assayed as described by Ibrahim et al. [25] with a slight modification. 1 mL of *N. naja* (10–2500 μg) with 1 mL of 1% casein in 0.1 M Sorensen's phosphate buffer of pH 7.6 was incubated for 30 min at 37 °C. Undigested casein was precipitated and the reaction was terminated by adding 3 ml of 5% trichloroacetic acid

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