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Evaluation of antioxidant and antihemolytic activities of purified caulophyllumine-A from *Solanum mauritianum* Scop.

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Abstract
Currently, traditional plant-based medicines for primary health care are a challenge in the field of pharmacognosy. However, the majority of herbals have not yet undergone comprehensive chemical, pharmacological and toxicological studies to investigate their bioactive compounds. Bug weed (*Solanum mauritianum* Scop.) is an evergreen woody species of Solanaceae, native of South America. Ripe fruits are used by the local people as vegetable during famine periods and also the fruits and leaves are used to cure various ailments. In this scenario, the present investigation was undertaken to isolate the lead alkaloid molecule from *S. mauritianum* and to evaluate its antioxidant potentialities. In the first phase, the crude alkaloid was isolated, purified by column chromatography yielded a bluish coloured fraction-caulophyllumine-A and was further confirmed by NMR. Further, the antioxidant activity was assayed using the DPPH radical scavenging and other assays. The IC_{50} values ranged from 66.5 to 121 µg/ml. Protective effects of caulophyllumine-A, against H_2O_2 induced oxidative damage in plasmid pBR322 DNA was remarkable at the tested doses (µg/ml). Finally the antihemolytic potential of caulophyllumine-A was analyzed against human blood erythrocytes, whereby the % lysis of RBCs was found to be in the minimal range of 4.5 to 12.4% comparable with the control ascorbate.

Keywords: *Solanum mauritianum*; antioxidant; antihemolytic; NMR analysis

1. Introduction
Magical properties of herbals are mainly due to the presence of many secondary metabolites of different property. The herbals provide the crude drugs for the common man. The therapeutic potentiality of herbals lies in this phytochemicals that produce marked physiological action on the human body. Ethno pharmacological information is broadly considered an effective method in the discovery of new anti-infective molecules from plants. Many phytochemicals represent adaptive traits that have undergone radical changes during evolution in order to provide defense in plants against environmental [1] and biotic stress [2]. Defense strategy includes toxicity, antifeedant, microbicidal, antimetastatic and antinutrients.
There are many reports state that stress to cells may induce the production of reactive oxygen and nitrogen species (ROS/RNS), which trigger inflammation [3]. Antioxidant molecules scavenge these ROS/ free radicals and there by peroxidation or inflammation in many animal models [4]. Alkaloids and polyphenolics containing herbs have antioxidant and/or anti-inflammatory activity through quenching free radicals or chelate redox metals [5, 6]. ROSs also induces the ageing and related degenerative disorders. In addition, ROS are also involved in the initiation of heart diseases, chronic inflammation, and tumors [7]. Antioxidants function in both aqueous and/or membrane domains and effect gene expression in a positive manner. Endogenous antioxidants play optimal role in maintaining cellular integrity and thus balanced health. There are many reports supporting the interrelationship between ROS synthesis and altered activities of enzymatic and nonenzymatic antioxidants in diseases associated with aging or cancer.
Solanum species are used traditionally to treat various disorders such as pain, inflammation fever and enteric diseases. In addition, it shows much potential like antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic, antipyretic, microbicidal, cytotoxicity, anti-convulsant, antiulcerogenic and also against sexually transmitted diseases [8]. In Western countries, 80% of the people still apply traditional ethnic medicines obtained from natural herbals. In India, more than 75% of the indigenous people using medicinal plants as curative [8].

In this juncture, the present study was aimed at investigating the anti-hemolytic and antioxidant potential of purified caulophyllumine-A from *Solanum mauritianum*, in order to obtain data that might support the ethno-medical use.

2. Materials and methods

2.1 Plant Material

Fresh samples of *Solanum mauritianum* Scop., was collected from the wild habitat of Munnar hills, Idukki district, Kerala.

2.2 Isolation and purification of caulophyllumine-A

40 gram of shade dried fruit sample was first subjected to continuous soxhlet extraction non polar to polar solvents (petroleum ether, chloroform, ethyl acetate, ethanol and water). As the chloroform (or ethyl acetate) fraction was found to contain more alkaloid by dragendorff's reagent test, it was lyophilized and subjected to fractionation by column chromatography using silica of mesh size 60/120. 100% chloroform yielded a bluish coloured fraction. This fraction was eluted with ethyl acetate. It was lyophilised and again subjected to column chromatography for purification using petroleum ether and chloroform in the ratio 4:1. The purified fraction was dried and subjected to proton NMR [9].

2.3 Antioxidant activity

2.3.1. 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging was carried by employing the protocol of Villano *et al*., [10]. Pure samples of 25-150 µg/ml) were added to methanol solution of DPPH (100 µM). Absorbance was read at 517 nm after 15 min incubating at room temperature. The values were mean ± SD. Vitamin C and quercetin was used as synthetic antioxidants.

2.3.2 Reducing power

Reducing power ability of the sample was measured using the protocol of Ferreira *et al.,* [11]. 2.5 ml of sample (25-150 µg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and were incubated for 20 min at 50 °C. Further, 2.5 ml of 10% trichloroacetic acid was mixed to the sample to inhibit the reaction. Reaction mixture was the centrifuged for 10 min at 2000 g. The supernatant of the reaction mixture (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. Finally, OD the sample was recorded at 700 nm.

2.3.3 Metal chelating

Metal chelating of the sample was examined with the volume 25-300 µg/ml, 1 ml. Add 0.05 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine to the mixture. After 10 min incubation at room temperature, OD of the sample was recorded at 562 nm [12].

2.3.4. Nitric oxide scavenging

Kumaran and Karunakaran, [13] methodology was adapted for measuring nitric oxide scavenging. 25-150 µg/ml samples was mixed with 10 mM sodium nitroprusside and incubated for 150 min at 25 °C. Add 0.5 ml of Griess reagent (sulfanilamide- 1%, phosphoric acid- 2% and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) to the reaction mixture. Finally, OD of the sample was recorded at 546 nm.

2.3.5 Hydrogen peroxide scavenging

Gulcin *et al.,* [14] protocol was used to evaluate H_2O_2 scavenging potential. 2 ml, 50 -300 µg/ml extract was mixed with 0.6 ml of hydrogen peroxide solution (40 mM) in pH 7.4 phosphate buffer. The OD of the reaction mixture was recorded at 230 nm against blank.

2.4. Hemoglobin-induced linoleic acid assay

2 ml reaction mixture containing 50-500 µg/ml sample, 40 mmol/l, phosphate buffer with pH 6.5, 0.0016% haemoglobin suspension and 1 mmol/l linoleic acid emulsion [15]. Reaction mixture was incubated for 45 min at 37 °C. Add 2.5 ml of ethanolic solution of hydrochloric acid (0.6%) to the sample for arresting lipid peroxidation. Quantity of peroxidation was measureed via thiocyanate method by recording the OD at 480 nm after adding 100 µl of $FeCl_2$ (0.02 mol/l) and 50 µl of ammonium thiocyanate (0.3 g/ml).

2.5. Preparation of human erythrocytes

Blood samples were collected from consenting volunteers who visited the Medical College, by veni-puncture into lithium heparinized sterile tubes. The blood samples were stored at about 4 ^oC. The collected blood was mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm and packed cell were washed with isosaline (0.85%, pH 7.2) and a 10% (v/v) suspension was made with isosaline.

2.6. Protective role of extracts against H_2O_2 induced hemolysis

Stability role of the alkaloids against H_2O_2 induced hemolysis was evaluated according to the method of Prakash Yoganandam *et al*., [16]. (2010). Different concentrations of the alkaloids (0.5 ml, 50,100,150 µg/ml) were mixed with 2 ml of erythrocyte suspension (4%) and the volume of reaction mixture was made up to 5 ml with phosphate buffered saline. After 5 min incubation at 25 °C, 0.5 ml of H_2O_2 solution was added to the reaction mixtures. After 240 min incubation at 25 °C, reaction mixtures were centrifuged (2500 g, 10 min). Absorbance of the reaction mixture was recorded at 540 nm.

3. Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance (p<0.05) and the means separated by Duncan's multiple range tests. The IC_{50} values were calculated from linear regression analysis.

4. Results and Discussion

Herbal drugs secured significance in recent years because of their genuine efficacy as phytomedicines. The lead molecule or principles present in these herbal products act either as templates or precursor for synthetic drugs. The spectrum of alkaloids isolated from herbals depends on many factors, such as the nature of tissue, the extraction period, temperature, and

the polarity of solvent system. Alkaloids have been proved to have multiple biological effects, including antioxidant potential.

4.1 Caulophyllumine-A

The bluish coloured purified fraction was identified as Caulophyllumine-A with the molecular formula $C_{15}H_{21}NO_4$ with molecular weight 291.331 g/mol (Fig.1a & b).

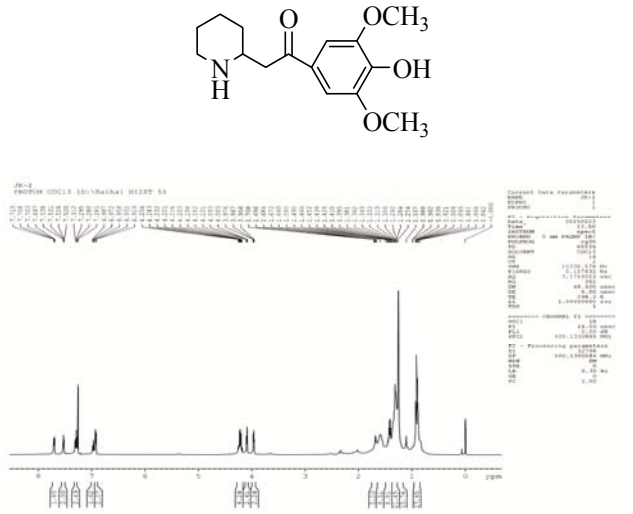

Fig 1 a & b: Structure of Caulophyllumine-A and its proton NMR spectrum

4.2. Antioxidant efficacy of Caulophyllumine-A

Caulophyllumine-A showed remarkable DPPH radical scavenging activity (IC_{50} = 66.5 ± 0. 76 µg/ml) and was comparable with synthetic antioxidants, while in Fe^{2+} chelating activity, the alkaloid displayed a moderate activity (IC_{50} = 115.2 ± 0.56 µg/ml) (Table 1 & 3). The percentage of inhibition of nitric oxide radical enhanced at par with the dose. In this assay, the alkaloid showed the highest nitric oxide-scavenging (IC_{50} = 183.7 ± 0.88 µg/ml vs. quercetin 20 ± 0.01 µg/ml). Quercetin showed good NO radical scavenging activity but it has tumor inducing role, [17] therefore demand in terms of novel biomolecule for NO quenching has been received attention. Similarly, caulophyllumine-A was potential for scavenging H_2O_2 in dose dependent manner (Table 1). Vitamin C showed more significant activity than the alkaloid. In reducing power assay, increasing OD of reaction mixture at 700 nm suggests an increase in reductive ability. Table 4 shows the dose dependent reducing power of caulophyllumine-A, with significance at 5% level ($p < 0.05$).

Caulophyllumine-A showed moderate activity in haemoglobin-induced linoleic acid assay (Table 3). Significance at 5% level (p <0.05). Further, caulophyllumine-A did not show any side effects on erythrocytes (Table 6).

The antioxidant (AOX) activity may be due to varied mechanisms such as inhibition of chain initiation, scavenging of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts. It is therefore important that for validating the effectiveness of AOXs, several biochemical protocols and various substrates should be used. Hemolysis is usually connected with free radical damage and its inhibition by antioxidants is an effective measure. The protective activity can be explained by the scavenging effect by caulophyllumine-A against free radicals induced membrane lipid peroxidation. Many researchers provided evidence that secondary metabolites provide stability in erythrocyte membranes via scavenging the free radicals and ROSs or as source of membrane stabilization. In fact, a positive correlation has been noticed between phytochemicals and antioxidant activity in many plant species.

Phytochemicals like alkaloids can donate hydrogen or electron leads to change DPPH colour from violet to yellow and thus evaluated as DPPH radical scavenging antioxidants [18]. Chapova *et al*. [19] reported potent DPPH radical scavenging activity for *Hyssopus officinalis* and was remarkable than *H. angustifolius*. They reported that the change in activity may be associated with the phytochemical content. Reducing power assay is related to the electron donating ability leads to reduction of Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} complex formed was read at 700 nm [11]. The table 4 reveals the concentration and OD correlation in terms of reducing power of the molecule. Interestingly, IC_{50} value was 115.2 ± and was comparable to the vitamin C ($p < 0.05$). In iron ion chelating model, the alkaloid competes with ferrozine in trapping iron ion, and thus inhibited ferrozine-iron complex synthesis. Excessive nitric oxide radical production has potential role in the pathogenesis of many diseases like sepsis and renal failure and therefore scavenging of NO was considered as medicinal [20]. In addition, NO scavengers block chain reactions initiated by these radicals that are deleterious for human health. $H2O_2$ is not much reactive but leads to cytotoxicity through synthesis of hydroxyl radicals in the cell system. Therefore, $H2O_2$ scavenging can help to maintain human health from derailments [21]. Membrane lipids are high in unsaturated fatty acids that are most sensitive to oxidative events, mainly the linolenic acids and arachidonic acids, which are the pivotal molecules for lipid peroxidation [22]. Erythrocytic cells are target components of oxidative burst due to the presence of membrane polyunsaturated fatty acids and also coupled with oxygen transport to haemoglobin molecules. Antihemolytic activity by phytochemicals has been reported and in the present study the remarkable IC50 value suggests the potentiality of the plant against hemolysis of RBC [21].

Rizwan *et al*., [7] screened the phytochemicals and evaluated antioxidant activity of *Solanum indicum* fruits. AOX potentiality of the extract was less than the present results. Yoshino *et al.,* [4] correlated antioxidant and antiinflmatory activities of oregano extract. Gutierrez *et al.,* [8] compared selected *Solanum* species in terms of anti-inflammatory and antioxidant activities of crude methanol extracts of alkaloid. Similarly, alkaloids with AOX potential were evaluated by Pronob Gogoi and Islam [23] among *Solanum nigrum* and *S. myriacanthus.* Alkaloids of *Haloxylon* and soybean were isolated, identified and evaluated for antioxidant, antibacterial and anticancer activities [24].

Table 1: IC_{50} value of antioxidant potential of caulophyllumine-A using different scavenging assays. (P<0.01).

	Caulophyllumine-A	Vitamin C	Quercetin
DPPH free radical scavenging	66.5 ± 0. 76	24.8 ± 0.33	33.3 ±1.55
Nitric oxide scavenging	121 ± 0.88	89 ±2.4	25 ±0.01
H_2O_2 scavenging	69.8 ±1.2	20 ±0.97	38.9 ±2.5
Fe^{2+} chelating potential	115.2 ± 0.56	18.3 ±2.1	44.6 ±1.7

Table 2: DPPH and Nitric oxide scavenging assay of the alkaloid caulophyllumine-A. Values are mean ± SD. P < 0.05%

		DPPH assay (%)		NO (%)		
Concentration (µg/ml)	Caulophyllumine-A	Ascorbate	Quercetin	Caulophyllumine-A	Quercetin	Ascorbate
25	19.3 ± 0.89	28± 0.04	17.5 ± 0. 06	25.4 ± 0.15	50 ± 0.01	14.5 ± 0. 08
50	38.6± 1.22	44 ± 0.46	27 ± 0. 02	34.4± 0.35	57 ± 0.06	19 ± 0. 75
75	55.2±0.55	59 ± 0. 05	38.6 ± 0.86	40.8± 0.25	60 ± 0. 07	31 ± 0.04
100	60.7 ± 0.14	66 ± 0.09	50 ± 0. 01	46.7 ±0.54	69 ± 1.33	42 ± 0. 05
125	64± 0.1	72 ± 0.8	57 ± 0.9	52±0.91	80 ± 0.38	54 ± 0.09
150	67 ± 1. 7	73 ± 0.4	58 ± 0.28	60 ± 0.63	82 ± 0.88	52 ± 0.09

Table 3: Metal chelating and H_2O_2 scavenging assay of the alkaloid caulophyllumine-A. Values are mean ± SD. P < 0.01%

		Metal chelating (%)		H_2O_2 scavenging (%)		
Concentration (µg/ml)	Caulophyllumine-A	Ascorbate	Quercetin	Caulophyllumine-A	Ascorbate	Quercetin
50	22 ± 0.42	32± 0.01	16 ± 0. 04	30 ± 0.05	37 ± 0.6	13.5 ± 1. 7
100	40± 0.8	44 ± 0.48	22 ± 0. 035	36± 0.05	50 ± 4.6	22± 0. 05
150	61±1.25	65 ± 0. 07	36 ± 0.09	50± 0.01	62 ± 0. 57	28 ± 0.39
200	65.7 ± 0.14	72 ± 0.27	45 ± 0. 03	61 ±0.6	71 ± 1.8	36 ± 0.067
250	71± 0.06	80 ± 0.3	50 ± 1	70±0.57	80 ± 0.15	50 ± 0.49
300	73 ± 0.7	81 ± 1.4	58 ± 0.01	73 ± 0.63	83 ± 0.08	51 ± 0.64

Table 4: Reducing power of the alkaloid caulophyllumine-A. Values are mean ± SD. P < 0.05%

		Reducing power (OD)	
Concentration (µg/ml)	Caulophyllumine- A	Ascorbate	Quercetin
25	0.38 ± 0.01	0.55± 0.02	0.22 ± 0. 04
50	0.45± 0.02	0.80 ± 0.06	0.31 ± 0. 05
75	0.84±0.02	1.37± 0. 03	0.46 ± 0.06
100	1.4 ± 0.01	1.61 ± 0.05	0.89 ± 0. 01
125	1.7± 0.03	1.7 ± 0.06	1.1 ± 0.04
150	1.8 ± 0.04	2.06 ± 0.01	1.3 ± 0.08

Table 5: Haemoglobin-induced linoleic acid assay of the alkaloid caulophyllumine-A. Values are mean ± SD. P < 0.05%

Concentration (µg/ml)	Caulophyllumine-A	Ascorbate	Quercetin
50	9 ± 0.03	20 ± 0.01	5 ± 0.6
100	16 ± 0.23	32 ± 0.02	11 ± 0.1
200	37 ± 0.01	45 ± 0.02	14 ± 0.3
300	48 ± 0.64	57 ± 0.05	27 ± 0.5
400	66 ± 0.41	68.25 ± 0.7	35 ± 0.2
500	70 ± 0.55	71 ± 0.4	44 ± 0.61

Table 6: Antihemolytic assay of the alkaloid caulophyllumine-A. Values are mean ± SD. P < 0.05%

	% hemolysis
RBC + H_2O_2	100
Caulophyllumine-A (50 µg/ml) + RBC	4.5 ± 0.05
Caulophyllumine-A (50 µg/ml) + RBC +H_2O_2	8.1 ± 0.08
Caulophyllumine-A (100 µg/ml) + RBC + H_2O_2	11 ± 0.04
Caulophyllumine-A (150 µg/ml) + RBC + H_2O_2	12.4 ± 0.033
Ascorbate (100 µg/ml) + RBC	3.6 ± 0.01
Ascorbate (100 µg/ml) + RBC + H_2O_2	7.4 ± 0.04

5. Conclusion

In conclusion, caulophyllumine-A from *Solanum mauritianum* can be used as potential herbal antioxidant. The results support the medicinal usage of the plant. Excessive production of reactive oxygen species have important role in the pathogenesis and other life style diseases like ageing, diabetes, cancer, neurodegenerative disorders and others. The healthy way to scavenge free radicals which cause the oxidative burst is with the help of antioxidant phytochemicals. The obtained data appear as promising in the search of potent antioxidant molecule and may be novel sources of drugs. Further studies are warranted to substantiate using *in vivo* animal models.

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