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Evaluation of *In vitro* Antioxidant Potential of *Aconitum napellus* Linn. Root Extract

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Research Article

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Abstract

Objective: The primary focus of the research work is to explore the antioxidant potential of ethanolic root extract of *Aconitum napellus* Linn. (EEAN).

Methods: Different methods like DPPH scavenging activity, hydroxyl radical scavenging activity, and nitric oxide scavenging activity was followed to evaluate the *in vitro* antioxidant profile. HPTLC fingerprint investigation was executed for qualitative determination of a promising number of constituents from the ethanolic extract. OECD guideline 423 was followed for the determination of acute toxicity in Wistar rat.

Results: The total yield was found 0.948% from the ethanolic root extract. The results revealed that the ethanolic root extract of *Aconitum napellus* Linn. possesses good antioxidant activity in a concentration-dependent manner. According to DPPH radical scavenging assay, a concentration of 494.50 ± 2.62 µg/ml of antioxidant material essential to rummage 50% of free radical. The ethanolic extract holds an IC_{50} value of 579.57 ± 1.85µg and 469.56 ± 2.16 µg as determined by hydroxyl radical scavenging assay and nitric Oxide scavenging assay respectively. The plant extract possesses significant dose-dependent (0.284 ± 0.0018 at 125 µg/ml and 0.923 ± 0.0003 at 500 µg/ml) reducing capacity. HPTLC fingerprint information supports numerous fundamental pieces of evidence like isolation, purification, quality assessment and standardization. *Aconitum napellus* Linn. was found to be highly toxic at a dose of 2000 mg/Kg which was confirmed by the death of the rat. Therefore, the LD_{50} of this ethanolic root extract was estimated to be ≥2000 mg/kg.

Conclusion: The *Aconitum napellus* ethanolic root extract in this study showed potential to be used as a natural antioxidant at a limited concentration. Root extracts from this plant might have potential applications in pharmaceutical and cosmeceutical formulations in the future.

Keywords: *Aconitum napellus* Linn.; Antioxidant study; HPTLC analysis; Acute toxicity

Abbreviations: AHRF: Asthagiri Herbal Research Foundation; DPPH: 2,2-diphenyl-1-picrylhydrazyl; HPTLC: *High-performance thin layer chromatography; OECD: The Organisation* for Economic Co-operation and Development; ROS: Reactive Oxygen Species; TEAC: Trolox Equivalent Antioxidant Capacity.

Introduction

Free radical can be characterized as an atom, molecule, or ion possesses one or more unpaired valence electrons that contribute to the development of several life-threading diseases counting aging [1]. Various endogenous (e.g. normal cellular metabolism) or may be exogenous (viz. irradiation) developments are responsible for the generation of free radical as a by-product. Free radicals can effortlessly react with reactive oxygen species (ROS) and make themselves active radical. Different activated forms of oxygen can be well-defined as ROS and they are of two distinctive types, free stimulated oxygen radicals (e.g. hydroxyl radical OH^-, superoxide anion radical O_2^-) and non-free stimulated oxygen radicals (viz. singlet oxygen 1O2, hydrogen peroxide H_2O_2). These activated ROS are responsible for approximately 10,000 oxidative hits to human cells in every second [2].

Free radicals are responsible for deteriorating various food items which result in off taste and diminished shelf-life. Antioxidants can capable to reduce the deterioration of foodstuffs by neutralizing functional free radicals, hence use as a preservative [3]. To reduce the degradation, some nutritional food merchandises contain a ubiquitous amount of flavonoids, a type of polyphenols possessing strong antioxidant activity [4].

Like Trolox Equivalent Antioxidant Capacity (TEAC) assay, various methods are available to isolate and categorize most promising antioxidants by paralleling antioxidant capability of numerous compounds.

In the field of chemistry, the method oxidation can be defined as a chemical reaction by which an electron is transferred to an oxidizing agent from any substance. An antioxidant, a complement of oxidant, can able to minimize the rate of an oxidation reaction in a certain extent and prevent the oxidative damage to the cells and other biochemicals. They can also inhibit the unrestrained production and assembly of ROS, protein degradation, and DNA strand break and guarantee sound health [5, 6]. Free radicals are highly responsible for development and progression of several diseases. In this connection, the involvement of LDL oxidation in cardiovascular disease can be produced as an example. This free radical-mediated LDL oxidation serves as a predecessor in different life-threatening diseases viz. cardiovascular disease, atherosclerosis, hypertension etc. by prompting many chemical or enzymatic pathways [7].

Any food or dietary supplements containing antioxidant can competent to minimize the free radicals induced cell damage. Plants are a rich source of various organic antioxidant chemicals and a systematic diet of antioxidants from plants is very crucial sustaining sound health [8]. A sufficient diet of antioxidant-rich food can able to slow down or inhibit certain diseases like aging. In this connection, different antioxidant rich (viz. vitamin E, resveratrol, carotene, selenium, vitamin C etc.) dietary supplements are marketed by many nutraceutical companies.

Mother Nature provides different ailments to prevent or cure various diseases and among them, several plants possess numbers of medicinal properties [9-12]. Traditional healers use their knowledge to cure or treat different diseases with the help of medicinal plants [13-15]. Based on their ethnomedicinal properties, one can accelerate design and development of rational drugs or plant-derived physiologically active molecules after being fused with synthetic chemistry, QSAR and nanotechnology to combat various deadly diseases like diabetes and cancer [16-20].

The present objective of this research work is to find out the antioxidant potential of EEAN i.e. ethanolic root extract of *Aconitum napellus* Linn.

Plant Introduction

Aconitum napellus is an herbaceous perennial plant growing to 2-5 feet tall, with hairless stems and leaves. The flowers are violet in colour in mid-summer. Although the poison from this plant was used as an arrow toxin, it was first introduced in homeopathic medicine in 1805 [21]. *Aconitum napellus* is also known as monk's hood in English, vatsanabha in Sanskrit, bachnag in Hindi and kathvish in Bengali. It has been used to relieve stress, anxiety, fever due to typhoid, measeles etc.

But high dose for a longer period can cause swollen throat, sneezing and cough.

Traditional Uses of *Aconitum napellus* Linn.

This plant is used to prepare homeopathic preparation from long time [22]. The extracts obtained from *Aconitum* species are usually employed in the traditional Chinese and Japanese medicine as analgesics, anti-rheumatics and also for the treatment of neurological disorders [23]. It is also used as an anti-microbial agent [24].

Phytochemical Evaluation

From literature survey, it was proved that *Aconitum napellus* Linn. possesses Quercetin3-O-(6-transcaffeoyl)-β-glucopyranosyl-(1→2)-β-glucopyranosyl-7-O-α-rhamnopyranoside and quercetin-3-sophoroside-7-rhamnopyranoside [25]. Apart from this, it also possesses alkaloid, carbohydrate, glycosides, protein, flavonoid, tannin but it is devoid of terpene, gum & mucilage and steroid.

Materials and Methods

Collection of Plant

The plant material was collected from Kulasekharam of Kanyakumari district and authorized by Prof. P. Jeyaraman Chief Botanist PARC, Chennai, India.

Preparation of Plant Extracts [26]

Air-dried roots (1.5 kg) were extracted by hot maceration procedure with absolute ethanol (3 L) at a temperature not exceeding 55°C for 4 days and filtered. This filtrate was concentrated by rotary evaporator. This concentrated material was used for next study.

Source of Chemicals

All the chemicals were of analytical reagent grade and purchased from Loba Chemie, ACROS Organics, Merck lab, S.D. Fine chemicals, Fluke.

In vitro antioxidant Evaluation

DPPH Method: Free radical scavenging assay was measured by DPPH method [27].
DPPH percentage inhibition (%) = Abs (control) - Abs (sample) / Abs (control) X 100

Hydroxyl Radical Scavenging Assay: The free radical scavenging capacity of EEAN was measured by hydroxyl radical Scavenging method [28].
Hydroxyl scavenging activity (%) = Abs (control) - Abs (sample) / Abs (control) X 100

Nitric Oxide Scavenging Assay: Nitric oxide scavenging technique was used to find out the antioxidant activity of ethanolic root extract [29].
Nitric oxide scavenging assay (%) = Abs (control) - Abs (sample) / Abs (control) X 100

Total Reducing Ability: The reducing power of the ethanolic root extract was determined by various concentrations of the ethanolic extract, phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%), trichloroacetic acid (10%), ferric chloride (1%) [30].
HPTLC Analysis: HPTLC of the EEAN sample was identified by AHRF method. The sample applicator used for the procedure was CAMAG Linomat IV. The applied sample was scanned by CAMAG TLC Scanner II.
Volume of sample loaded- 10µl
Mobile phase- Diethylamine:Ethylacetate:Toluene (10:20:70)
λ- max: 254nm
Lamp: Deuterium

Determination of Toxicity

Experimental Animals: Adult male Wistar rats (150-180 g) were used for this study. Standard laboratory diet and tap water were used to feed all the animals. The care and use of the animals were strictly followed in accordance to the guidance of the Institutional Ethical Committee (constituted under the Guidelines Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. - 1237/PO/C/2008/CPCSEA). After one week of habituation, animals were used for the experiments. Each animal was tested only once. All efforts were made to reduce animal suffering.

Acute Oral Toxicity Study (OECD^{423}): OECD guidelines 423 were followed for the oral toxicity study. It is a stepwise practice with three animals of a single sex per stage. Based on the mortality and/or morbidity of the animals a few stages may be compulsory to evaluate the toxicity of the test ingredient. This method has a benefit over other procedures because of marginal usage of animals while permitting for satisfactory data [31-33]. The technique uses well-defined doses (2000mg/kg body weight) and the outcomes permit an ingredient to be ranked and categorized according to the globally synchronized system. The concentrated EEAN extract was dissolved in distilled water for oral administration. The initial dose of the EEAN extract was 2000mg/kg bodyweight (p.o). The dose was administered to the mice which were fasted overnight with water *ad libitum* and examined for signs of toxicity symptoms like change in diarrhea, convulsions, salivation, tremors, sleep, skin color and also respiratory, autonomic and CNS effects.

Statistical analysis: Results were shown as mean ± S.D. for each group (where, number of each *in-vitro* antioxidant experiment, n=3; the number of experimental animals, n=6). SPSS 9.0 for Windows (Chicago, IL, USA) software was used for statistical analysis. For multiple comparisons, one-way analysis of variance (ANOVA) was performed. In cases where ANOVA indicated noteworthy differences, Tukey test was performed. $P < 0.01$ was considered to be statistically important.

Results

Percentage Yield Study

The percentage yield of the EEAN was found to be 0.948%.

DPPH Radical Scavenging Assay

Figure 1 shows the antiradical activity of EEAN. The scavenging activity was increased with the increasing concentrations (25-800 µg). IC_{50} value (the amount of antioxidant material required to scavenge 50% of free radical in the assay system) of EEAN was found to be 494.50 ± 2.62 µg/ml.

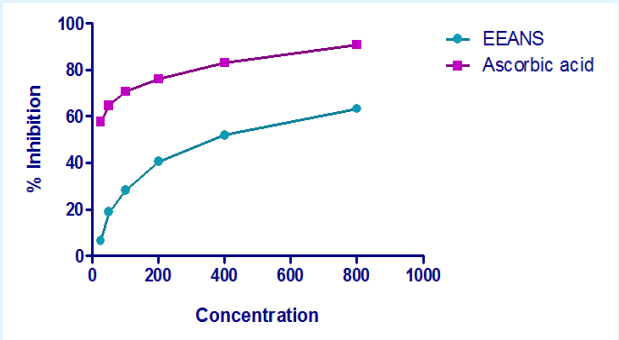

Figure 1: Free radical scavenging activity of EEAN by DPPH method.

Hydroxyl Radical Scavenging Assay

Figure 2 shows the antioxidant capacity of EEAN by scavenging the hydroxyl radical. The antioxidant activity was increased from 6.35 ± 0.32% at 25 µg/ml to 60.47 ± 0.456 at 800 µg/ml concentration. The IC_{50} value of the extract was found to be 579.57 ± 1.85µg.

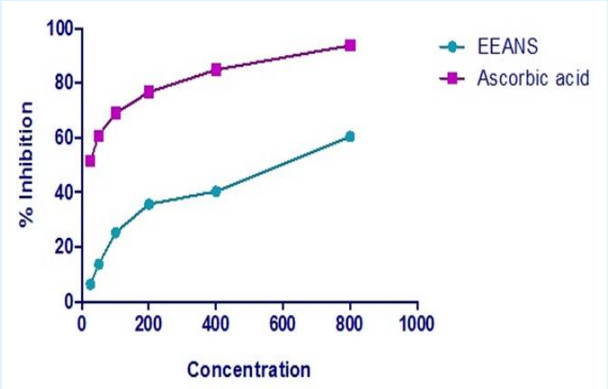

Figure 2: Free radical scavenging activity by Hydroxyl radical scavenging method.

Nitric Oxide Scavenging Assay

Figure 3 shows the appreciable antioxidant activity by scavenging the nitric oxide. The value of the antioxidant assay increased from 7.62 ± 0.24% at 25 µg/ml to 63.64 ± 0.10% at 800 µg/ml. The IC_{50} value was found to be 469.56 ± 2.16 µg.

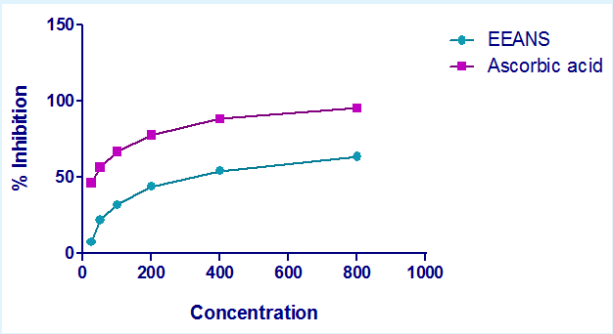

Figure 3: Nitric Oxide scavenging activity of EEAN.

Total Reducing Ability

The result shows that the EEAN possesses significant antioxidant properties which could react with free radicals to stabilize and terminate radical chain reactions. The reducing powers of ethanolic root extract of *Aconitum napellus* Linn. was 0.284 at 125 µg/ml and increased to 0.923 at 500 µg/ml (Figure 4). The standard drug ascorbic acid shows the reducing power 1.63 at 500 µg/ml.

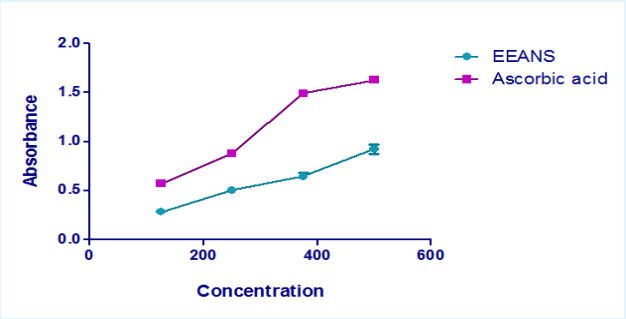

Figure 4: Determination of Reducing Power of EEAN.

HPTLC analysis

The chromatographic profiles of the ethanolic root extract of EEAN were performed on silica gel 60F254. The plate using Diethylamine: Ethylacetate: Toluene (10:20:70) as mobile phase was given in Figure 5. Thin layer chromatography profiles revealed 2 distinct spots under UV (254 nm). This was again confirmed in HPTLC

chromatogram (Figure 6) where The *Rf* values and the peak area percentage were observed and given in Table 1.

Figure 5: TLC Chromatogram of EEAN.

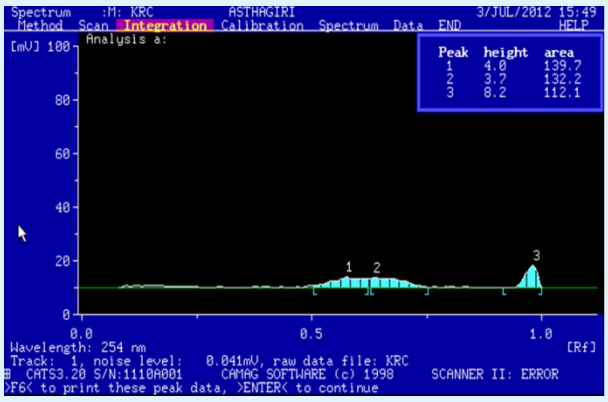

Figure 6: HPTLC Chromatogram of EEAN.

Peak	Rf	Lambda max	Height	Area in mV
3	1	254	8.2	112.1

Table 1: HPTLC fingerprint of ethanolic extract of EEAN.

Discussion

Three different radical scavenging assay has been performed to find the antioxidant potential of EEAN.

Antioxidants are responsible for the reduction of DPPH into its hydrazine form and make it into a diamagnetic molecule by electron donation. λ_{max} of DPPH, a stable free radical is at 517 nm. Diminution in absorption at 517nm is one of the distinctive indication of DPPH reduction. In this study, the result was determined as a ratio between the percentage of absorbance decline of DPPH radical in existence of EEAN to the absorbance of DPPH radical alone at 517nm. According to this study, it may be assumed that DPPH gets reduced to its resultant hydrazine form by EEAN. This reduction clues to the color conversion of DPPH from purple to yellow based on a number of electrons taken up [12, 34-35].

Various types of ROS viz. hydroxyl radical and singlet oxygen are produced in the biochemical system due to the reaction between H_2O_2 and superoxide radical. They are the master regulator for numerous life-threading diseases like cytotoxicity, aging by modifying or damaging DNA, and carcinogenesis etc. From this analysis, it may be hypothesized that EEAN may capable to quench hydroxyl radical and scavenge ROS, and can check the detrimental effects of ROS at the cellular level.

Nitric oxide is lipophilic in nature. At biological pH, it produces nitrogen dioxide once it reacts with oxygen. The various physiological roles of nitric oxide are governing signal transmission, inflammatory response, and vasodilation [36]. Scavengers of nitric oxide race with oxygen and hinder the conversion of nitric oxide [37]. Our study reveals that EEAN may act as an antioxidant by obstructing the conversion of nitric oxide.

An examination of reducing power can explore the antioxidant profile of polyphenols. The reducing reaction is directed by the existence of reductones, which display antioxidant property by providing hydrogen atom and collapsing free radical chain reaction.

Depending on the reducing power capacity of antioxidants present in EEAN, the ferric ion is gets reduced into a ferrous ion, which determined by color change [38].

Appropriate documentation and quality control of the presence of active constituent can be done by HPTLC fingerprint analysis. Different chromatographic record (like HPLC, HPTLC, LC-MS/MS) can offer several elementary pieces of information like chemical compounds for identification, isolation, purification, metabolic safety, permeability, and pharmacokinetic profile of a particular plant species in the animal model also [39, 40].

In HPTLC due to solvent, we observe first 2 peaks. Peak 3 confirms the presence of active principles in the extract. Although the further experiment is mandatory to identify and categorize those compounds. This HPTLC fingerprint

analysis profile of *Aconitum napellus* Linn. may be used for quality evaluation and standardization.

In acute toxicity studies, mortality was observed in the groups treated with *Aconitum napellus* Linn. at the dose of 2000mg/kg (Tables 2 & 3). So, the dose must need to optimize before experiment and treatment.

Parameters observed	I hr	II hr	III hr	IV hr
Piloerection	-	-	-	
Edema	-	-	-	
Urine stains	-	-	-	
Alopecia	-	-	-	
Loss of writing Reflex	-	-	-	
Circling	+	+	+	D
Nasal sniffing	+	+	+	E
Lacrimation				A
Seizures				D
Righting reflex				
Grip strength				
Eye closure at touch				
Rearing				
Straub tail				

+: Presence; -: Absence

Table 2: Acute toxicity dose 2000 mg/kg (P.O). Initial observation (Day-I).n= Three animals of a single sex.

Parameters observed	Day-1	Day-2	Day-3	Day-4
Piloerection				
Edema				
Urine stains				
Alopecia				
Loss of writing reflex				
Circling	D	D	D	D
Nasal sniffing	E	E	E	E
Lacrimation	A	A	A	A
Seizures	D	D	D	D
Righting reflex				
Grip strength				
Eye closure at touch				
Rearing				
Straub tail				

Mortality was observed in the groups treated with EEAN at the dose of 2000mg/kg.

Table 3: Acute toxicity-Daily observation. n= Three animals of a single sex.

Conclusion

EEAN showed significant antioxidant profile. This study offers experimental support for the traditional medicinal plants.

To confirm therapeutic effectiveness and quality control of the drug along with its identification, this HPTLC data will serve as the reference standard for scientists engaged. So along with the antioxidant property of this plant, HPTLC fingerprint data of root extract of *Aconitum napellus* Linn. can be used as diagnostic tool for the correct identification of the plant and also used to estimate genetic variability in their population.

Based on the toxicity study, *Aconitum napellus* Linn. was found to be toxic at a dose of 2000mg/kg.
Still, molecular studies of the above-mentioned plant may open up new hope in drug discovery and research.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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