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Anesthetic, Antioxidant and Antibacterial Activities of *Anacyclus pyrethrum* Root Extract – An *In vitro* and *In vivo* Animal study

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ABSTRACT

Anacyclus pyrethrum commonly referred to as 'Akarkara' is widely recognized in ayurvedic Indian medicine for its therapeutic benefits. It's hard, compact, fusiform root was reported to be responsible for its medicinal properties. Various phytochemical and biological assessments have been reported in the literature for the importance of A. pyrethrum. The present study was made to exploit the medicinal properties including antimicrobial effect, antioxidant and anesthetic effect of the root extract of Anacyclus pyrethrum. The results showed effective antioxidant activity against the stable radical DPPH. The antibacterial activity of the root extract is not efficient compared to the standard antibiotics, and the anaesthetic effect of the plant was evaluated against live fish models (cyprinous carpio) with extract of concentrations ranging from 150-400 mg/l. The anesthetized fishes showed a better result at 400 mg/l dose of plant extract, with induction and recovery time between 3 to 5 minutes.

KEYWORDS

Anacyclus pyrethrum, Anesthesia, Antimicrobial, Antioxidant, Fish Anesthesia

Greentree Group

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INTRODUCTION

Anacyclus pyrethrum from Asteraceae family is a native plant of India with many therapeutic benefits. The root of Anacyclus pyrethrum is fusiform with hard and compact structure. The plant roots and leaves have been reported to have good medicinal values in traditional system of Indian medicine. It is referred to as Akarkara in Ayurvedic texts, and is commonly known for its aphrodisiac property.

In folk remedies it was used for stimulating salivary glands and found to be useful in treatment of toothache, paralysis of the tongue and muscles of throat as well as neuralgic affections of the teeth. They have anti-inflammatory (Annalakshmi R et al 2012, Rimbau V et al 1999)[1,2], antibacterial, antioxidant and insecticidal properties (Zaidi SM et al., 2013)[3]. Their medicinal properties are due to the presence of the compound called flavonoid (Harald 1978, Benitez et al 2010)[4,5]. The plant root contains essential oils and an alkaloid known as pellitorine. The pellitorine of the root also exhibits local anesthetic activity, which was found to be effective in several animal studies. Fishes have been widely used for experimental procedures to detect the effect of new drugs. The chemical anesthetics used in fish have changed gradually (Stuart NC 1981) and shifted towards green anesthesia for reduced risk[6]. Hence plant extract have been used to immobilize fishes. These plant extracts are added to water in various forms and its efficacy varies greatly from light sedation to complete loss of sensation and death if exposed for longer time.

AIMS AND OBJECTIVES

The aim of the study was to analyse (chromatographic analysis) the presence of pyrethrum compound in prepared root extract and substantiate the anesthetic effect of root extract in live fish model, and to check the antimicrobial and antioxidant property of the root extract in vitro.

MATERIALS AND METHODS

Preparation of plant extract

The roots of the plant Anacyclus pyrethrum was obtained from a standard supplier from the hilly regions of Pathanamthitta district of Kerala. They were dried, powdered and were subjected to cold extraction method using methanol (HPLC grade, High performance liquid chromatography) as solvent. After a period of 72 hours in the shaker, the active ingredients from plant root were dissolved in methanol. This solvent was then treated under rotary evaporator, to separate the alcohol (methanol) and to

obtain the plant extract. The plant extract obtained was volatile in nature. Once the required quantity of extract was obtained (25 gram), they were subjected to Freeze dryer-Lypolyser at -50^0c to obtain a more solid form of the extract. To analyse the anesthetic activity, 1gram of the prepared extract was dissolved in 10 ml of distilled water. The extracts were divided into 6 groups of different concentrations (ranging from 150mg of extract in 1 litre of water to 400mg in 1 litre). For in vitro antibacterial activity, 2% of the extract was prepared. They were then stored in a refrigerator for further use.

In vitro antibacterial activity

Disc diffusion method was used to test the antibacterial effect of the extracts of Anacyclus pyrethrum. The pathogens used were Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, and Salmonella typhi. Sterilized nutrient agar were poured in the petri plates and inoculated with the test organism. After solidification, the plant extract was placed in centre position of disc. The plates diffused for half an hour and were then incubated for 24 hours. After incubation, the zones of inhibition of plant extract against the given test organisms were observed. The tests were done with plant extract diluted with water in four concentrations 20%, 40%, 60%, and 80%, and compared with the control distilled water. Then 2% methanol extract was tested in replicates of four and the effective zone of inhibition was obtained by comparing with the control used (methanol). Considering the zone obtained by plant extract as A, and control as B, the effective zone of inhibition was recorded by calculating the values of A-B. Antibiotic ciprofloxacin was used as the standard drug to compare the antimicrobial activity.

In vitro antioxidant activity

Determination of DPPH (1-1-Diphenyl 2-Picryl Hydroxyl)

The free radical scavenging or antioxidant activity of Anacyclus pyrethrum methanolic root extract was done by measuring the hydrogen donating or radical scavenging ability of the plant against the stable radical DPPH (Hasan et al 2006)[7]. DPPH solution (0.1 mM) was prepared in methanol, and 1ml of this solution was added to 3ml of the extract solution at different concentration ranging from (25-400ug/ml). The mixture was shaken vigorously and left undisturbed at room temperature for 30 minutes. The absorbance was then measured at 517 nm by using a UV-visible spectrophotometer (Genesys 10 UV). The control or reference

compound used was ascorbic acid. Lower absorbance value of reaction mixture indicated a higher free-radical-scavenging activity. The capability to scavenge the DPPH radical was calculated using the equation, DPPH scavenging effect (%) = $[(A_0-A_1)/A_0) \times 100]$,

where A_0 is the absorbance of the control reaction (Ascorbic acid), and A_1 is the absorbance of presence of the extract sample and standard (DPPH).

Chromatographic analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of methanol extract of Anacyclus pyrethrum was carried out in order to characterize the extract.

The Clarus 680 GC was used for the analysis and employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. Then 1μL of extract sample was injected into the instrument. The oven temperature was maintained as follows: 60°C for 2 minutes; increased to 300°C at the rate of 10°C per minute and maintained at 300°C fpr 6 minutes. The mass detector conditions were monitored accordingly. The spectrums of the components obtained were compared with the database of spectrum of known components stored in the GC-MS NIST (National Institute of Standards and Technology 2008) library.

In vivo anesthetic activity in live Fish models

The protocol for experimentation was approved by Institutional Animal Ethics Committee (KSR College of Technology) and was in accordance with international standard on the care and use of experimental animals (Ref no: 1826/Po/EreBi/S/15/CPCSEA). The live fish models used were 2-3 months old common carp (Cyprinus carpio). Fishes of intermediate sex was used for the experiment. Before the trial the fishes were kept in an outdoor cement tank with regular monitoring of temperature, water circulation, lighting and were provided standard food pellets. A pre-trial was conducted to establish the effective dose range of plant extract. The fishes (n=36) were divided into 6 groups based on the extract concentration, with 6 fishes in each group.

The experiment was conducted in two glass tanks. A recovery tank of 45 L X 25 W X 29.5 H in centimetre was filled till 18 cm

with fresh tap water, so that the tank contains 20 litre of water. The fish to be anesthetized was kept in this tank under regulated water circulation and temperature for a period of 30 minutes before experiment. It was then transferred to a test tank containing 1 litre of water maintained under same temperature. A measured volume of plant extract was added to water in test tank and mixed thoroughly for 20 seconds. Then a fish was introduced into the tank and was subjected to immersion anesthesia. The time taken to reach the pre-determined stages of anesthesia were monitored (Ramanayaka JC 2006)[8]. The Stages of anesthesia and their characters were observed as modified by Stuart 1985.

S1 - Loss of equilibrium, but fish responded to tactile stimuli.

S2 - Reduced response, partial loss of equilibrium, fish can hold on hand, reduced operculum activity.

S3 - Loss of response, can pull by posterior fin.

S4 - Total loss of equilibrium, fish lay down.

As soon as the fish reached the S4 stage of anesthesia, it was transferred to the recovery tank. This recovery tank had a normal circuit of aerator for oxygen perfusion which could help the fish recover from anesthesia. The time taken to gain the complete equilibrium was taken as the recovery time from anesthesia. Each concentration of plant extract was replicated in 6 fishes, and no fish was reused. The fishes were then monitored for a period of 15 days for any mortality due to the experiment.

RESULTS

Antimicrobial activity

The effect of diluted and undiluted 2% methanol extract of Anacyclus pyrethrum were tested against five different microorganisms. The extracts were tested against the broad spectrum antibiotic Ciprofloxacin. The diluted extract tested under four different concentrations (20%, 40%, 60% and 80%) exhibited an effective zone of inhibition at 80 % concentration, with 9 mm inhibition zone in B. Subtilis, followed by 10 mm in E. coli and K. Pneumonia, 12mm in S. typhi and 11 mm in S. aureus. The undiluted 2% methanol extract showed highest inhibition zone of about 15 mm in B. Subtilis, 13 mm in S. aureus, 12 mm in E. coli and S. typhi and 11 mm in K.Pneumonia. The standard antibiotic ciprofloxacin showed an inhibition zone ranging from 20-23 against the pathogens used. The results indicate that Anacyclus pyrethrum root extract showed minimal antibacterial action against the

microbes used, and when compared with the standard ciprofloxacin. The results are tabulated in table 1.

Table 1 Antibacterial Effect of Anacyclus pyrethrum Extract

Test organisms	Diluted plant extract in mm (A)				Control water (B)	Effective zone of Inhibition (A-B)				Ciprofloxacin (standard antibiotic used)
	20%	40%	60%	80%		20%	40%	60%	80%	
B. Subtilis	10	11	12	14	5 mm	5	6	7	9	22
E. Coli	10	12	13	15	5 mm	5	7	8	10	21
S. typhi	8	11	13	17	5 mm	3	6	8	12	20
S. aureus	11	13	14	16	5 mm	6	8	9	11	22
K. pneumonia	12	12	14	15	5 mm	7	7	9	10	23
	Undiluted alcoholic extract replicates in mm					**Control alcohol (B)**		**Effective zone of Inhibition (A-B)**		
	1	2	3	4	Mean (A)	5 mm				
B. Subtilis	20	18	21	21	20	5 mm		15		
E. Coli	16	18	16	16	17	5 mm		12		
S. typhi	17	15	20	16	17	5 mm		12		
S. aureus	16	18	17	21	18	5 mm		13		
K. pneumonia	14	16	17	19	16	5 mm		11		

Antioxidant activity

DPPH Scavenging activity

Reduction of DPPH radicals by the scavenging action of plant extract can be observed by decrease at 517nm. The IC_{50} value of plant extract and ascorbic acid was found to be 55.83±1.92 µg/ml, 10.38±0.52 respectively. The results are expressed in mean ± Standard error of mean (SEM) of 3 replicates in table 2.

Table 2 DPPH free radical scavenging activity

S. No.	Concentration µg /ml	Inhibition (%)	IC 50 Value µg/ml
1	25	31.71±0.2	55.83±1.92
2	50	45.5±0.31	
3	100	58.2±0.39	
4	200	69.63±0.58	
5	Ascorbic acid		10.38±0.52

The values are Mean ± SEM of 3 replicates.

Chromatographic Analysis

The GC-MS analysis of methanolic extract of Anacyclus pyrethrum, showed presence of compounds with different molecular weights 223, 246, 251, 266, 270 and 280. The fragmentations matched with pyrethrin. The results are represented in the figure 1, were Peak I has been identified as cinerin I,

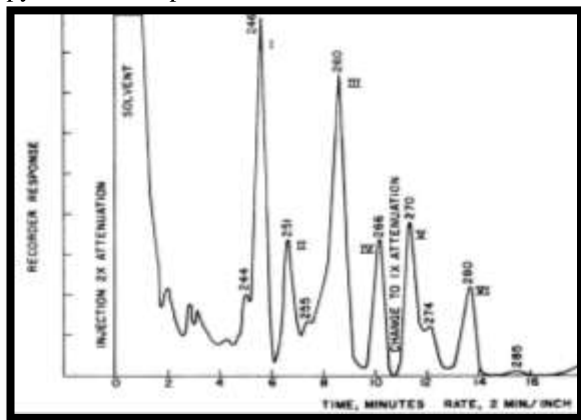
peak II as a cinerin-type compound, peak III as pyrethrin I, peak IV as a cinerin-type compound, peak V as cinerin II and peak VI as pyrethrin II.

Table 3 Time taken to reach different stages of anesthesia and its recovery in fish models

Concentration (mg/l)	Time taken in minutes				Recovery (minutes)
	S1*	**S2***	**S3***	**S4***	
Group I (150)	9	12	13.50	15	1
Group II (200)	6.2	8	11	13.20	2
Group III (250)	5	6.15	7	8.20	2
Group IV (300)	3.50	4.20	5.45	6.20	3
Group V (350)	2	2.45	4.20	5	3
Group VI (400)	1.30	2.25	3.15	3.45	4

* S1, S2, S3 and S4 are Stages of anesthesia

Figure 1 Chromatographic response from pyrethrum concentrate showing the numbered peaks of pyrethrum compounds.



Anesthetic activity in live fish models

The time of different stages of anesthesia and recovery time have been tabulated in table 3.There was a significant decrease in the time taken for the induction of S1 anesthesia as the extract dosage increased. In Group I (150 mg of extract used) the time to achieve S1 was 9 minutes. It gradually reduced with increasing dosage and it was 1 minute 30 seconds in Group VI (450mg of extract used). Similarly the stage 4 (S4) was achieved at 15 minutes in Group I and with increased dosage it was reduced to 3 minutes 45 seconds in Group VI.

In group I with low extract concentration the recovery time was rapid at 1 minute, while it increased upto 4 minutes at higher dosage of 400 mg.

DISCUSSION

The result of the study showed that the methanol root extract of Anacyclus pyrethrum has antibacterial, antioxidant and anesthetic effect. The presence of the compound pyethrin in the plant root has been previously confirmed in many literatures. In an earlier study done by Beckman and Allen 1966, isolated six different components called pyrethrin I, cinerin I, jasmolin I and pyrethrin II, cinerin II and jasmolin II, from this plant extract[9]. In the present study the GC-MS analysis showed traces of compounds from the prepared root extract like cinerin I and II,

and pyrethrin I and II, which are the major compounds responsible for its medicinal value. Pyrethrin was reported to be responsible for its local anesthetic activity in previous animal studies. Gopalakrishna et al (1987) and Muralikrishnan K et al (2016) reported the local anesthetic action of alcoholic root extract and compared the effect with lignocaine[10,11]. In this present study in order to substantiate the local anesthetic activity, a live fish model was used. Cyprinus caprio commonly known as common carp was used to check the anesthetic activity. The fishes were subjected to immersion anesthesia, by dissolving measured quantity of drug in water (Neiffer DL and Stamper MA 2009) similar to inhalational anesthesia as in terrestials[12].

Marking and Mayer (1985) recommended the desirable induction and recovery time of anesthesia to a fish as 3 and 5 minutes, respectively[13]. In present study the tested plant extract demonstrated this desirable effect in Group V and VI where 350 mg and 400 mg of extract was used. In a similar study Stuart et al (1981) quoted that the effectiveness of anesthesia was decided based on the time taken to reach S4 stage and its recovery time. Anacyclus pyrethrum at 400mg extract concentration in Group VI had initiation of S1 at 1 minute and 30 seconds and S4 stage at 3 minutes and 45 seconds. The recovery time was around 4 minutes due to the increase in drug dosage.

The experimental fishes were observed for 15 days after completion of the study. No anesthetized fish died during the follow up period.They also showed normal activity indicating that the plant extract had no lingering adverse effects.

In the present study, the extracts exhibited minimal antibacterial effect. There was no significant difference between the inhibition zones created by 80% diluted extract and undiluted extract used. This could be due to the lower concentration of extract (2%) used. Naderi N et al 2012 showed minimal antibacterial effect against Staphylococcus sanguis and Staphylococcus aureus. No antibacterial effect was seen against Streptococcus mutans and Pseudomonas aeruginosa, and this was attributed to the reduced dose of the extract used[14]. Kumar A et al (2012) showed similar results, with lower antibacterial activity when compared to the standard ciprofloxacin antibiotic[15].

The antioxidant activity was determined using radical-scavenging activity against the radical DPPH. It is a widely used stable radical model, and provides a quick method for the evaluation of free radical scavenging

activity. DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The effects of antioxidants on DPPH radical scavenging is due to their hydrogen-donating ability. The absorption maximum of this radical in methanol was measured at 517 nm. The decrease in absorbance of DPPH radical can be seen due to presence of antioxidants in test sample. This reaction between antioxidant molecules and the free radical is attributed to the scavenging activity of DPPH by hydrogen donation. Hence, DPPH is usually used as a substrate to evaluate the antioxidant property of any compound (Fatimah ZI 1998)[16]. It was visually noticeable as a change in colour from purple to yellow. This bleaching of the DPPH radical by change in colour, is representative of the capacity of the test compound (A pyrethrum) to scavenge free radical (Hu et al 2000)[17]. In the present study, the change in colour was seen in concentrations of 100 µg /ml and 200 µg /ml.

Based on the data obtained from this study, the root extracts have effective free radical or scavenger activity. This may limit free radical damage occurring in the human body. A gradual increase in the scavenging ability with increasing concentration ranging from 25 µg/ml to 200 µg/ml of extract was seen.

CONCLUSION

1. The phytochemical analysis of Anacyclus pyrethrum root showed presence of the compound pyrethrin and its fragments.
2. The extract showed antioxidant activity against stable radical DPPH with IC 50 value of 55.83 ± 1.92.
3. The anesthetic activity of pyrethrin in live fish models subjected to immersion anesthesia was effective with dose ranging from 300-400mg/l of plant extract.
4. The antimicrobial activity was lesser compared to standard ciprofloxacin drug due to the lower concentration (2%) of the extract used.

Hence this study provides a justification for antioxidant, antibacterial and anesthetic activity of the extract of Anacyclus pyrethrum.

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