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ScopeMed

Anthelmintic activity of a standardized extract from the rhizomes of *Acorus calamus* Linn. (Acoraceae) against experimentally induced cestodiasis in rats

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ABSTRACT

Background: The rhizomes of a herb *Acorus calamus* Linn. (Acoraceae) have been widely used as a traditional medicine to cure intestinal-helminthic infections in India and South Africa. **Aim:** This study was undertaken to investigate the *in vivo* anthelmintic activity of a standardized methanolic extract obtained from the rhizomes *A. calamus* in a rodent model. **Materials and Methods:** A methanolic extract obtained from rhizomes of *A. calamus* was characterized for active principle using nuclear magnetic resonance ^{1}H NMR, ^{13}C NMR, mass and infrared spectroscopy. The amount of active principle in rhizome isolated active fraction of plant was assayed using high-performance liquid chromatography (HPLC). Later, the standardized rhizome extract of plant and its active principle were tested for *in vivo* anthelmintic efficacy against experimentally induced *Hymenolepis diminuta*, a zoonotic cestode, infections in rats. **Results:** The study revealed that β-asarone is the active principle of plant. The HPLC analysis of local variety of *A. calamus* revealed that active fraction contains 83.54% (w/w) of β-asarone. The *in vivo* study revealed that treatment of *H. diminuta* infected rats by a single 800 mg/kg dose of rhizome extract for 5 days results into 62.30% reduction in eggs per gram of feces counts and 83.25% reduction in worm counts of animals. These findings compared well with the efficacy of a reference drug, praziquantel. The active principle β-asarone showed slightly better anthelmintic effects than crude extract. In acute toxicity assay, a single oral 2000 mg/kg dose of extract did not reveal any signs of toxicity or mortality in mice, and the LD50 of the extract was noted to be >2000 mg/kg. **Conclusion:** Taken together, the results of this study indicate that rhizomes of *A. calamus* bear significant dose-dependent effects against intestinal helminths. Further, the Indian variety of *A. calamus* contains high β-asarone content. Therefore, there exists a great potential to develop some suitable anthelmintic herbal products from this plant.

KEY WORDS: Anthelmintic, *Hymenolepis diminuta*, intestinal helminthe, soil-transmitted helminths, traditional medicine

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INTRODUCTION

Intestinal helminths affect more than 2 billion people worldwide and cause a range of adverse health problems, including anemia, diarrhea, and abdominal pain, impaired cognitive and physical development, particularly in developing countries [1]. Control is achieved by periodic deworming, and at present, two anthelmintics, i.e. albendazole and mebendazole are used in deworming programs in endemic regions. However, it has been recently reported that the global coverage of periodic deworming is still not sufficiently meeting target levels in all endemic regions [2]. As per the WHO latest data, in 2013, only 40% of children requiring treatment for intestinal helminths had access to anthelminthic medicines, and to the remaining 60% of children, these medicines were not accessible [3]. In many developing countries, people are still dependent on various herbal treatments to cure worm infections [4]. For example, in Asia, Africa, and some parts of Latin America, the herbal medicines, traditional treatments, and traditional practitioners constitute the main source of health care to treat various common ailments including intestinal worm infections [4]. Thus, these herbal medicines hold a great scope for not only new drug discoveries against parasitic diseases but also for further exploration for scientific evidence regarding the treatment and control of intestinal helminthiasis [5].

Acorus calamus Linn. (Araceae), commonly known as "sweet flag," is a perennial herb [Figure 1]. It is mostly found in swampy or marshy habitats in northern temperate and subtropical regions of Asia, North America, and Europe [6]. The rhizomes of this

Figure 1: *Acorus calamus*. (a) whole plant, (b) local medicine men collecting the tubers of plant, (c) partly-processed rhizomes

plant possess numerous medicinal properties and have been very widely used against several diseases and ailments, particularly in Indian, Chinese, Korean, and Thai medicines [6,7]. In the Indian ayurvedic medicine, the rhizomes of A. *calamus* are used to treat mental ailments such as epilepsy, schizophrenia, and memory disorders, as well as diarrhea, bronchial catarrh, intermittent fevers, cough, and asthma [8]. In Indian folk medicines, A. *calamus* rhizomes are considered useful against diseases of nervous system, throat, and as an antitumor, antipyretic, and antitussive agent [6]. In Thai medicine, A. *calamus* rhizomes have been used for blood purification and as an antipyretic [9]. In addition, experimental studies have also shown that A. *calamus* rhizomes possess anticonvulsant [10], antidiarrheal [11], antimicrobial [12], antibacterial [13], and anti-inflammatory activities [6]. Similarly, McGaw *et al*. [13] have also reported that A. *calamus* rhizomes are used in traditional medicine in KwaZulu-Natal, South Africa as an anthelmintic. The ß-asarone isolated from the rhizome of A. *calamus* has shown significant *in vitro* anthelmintic effects against *Caenorhabditis elegans* [14]. In another *in vitro* study, the active principle of this plant also showed a fast acting paralytic effect that was followed by mortality in the larvae of dog roundworm, *Toxocara canis* [15]. It would thus appear from aforesaid account that although few studies have established the *in vitro* anthelmintic effects of this plant, except one, no report is available regarding the *in vivo* anthelmintic effects of this plant against human intestinal helminths. The only *in vivo* study on anthelmintic effects of this plant was undertaken by Mägi *et al*. in Estonia [16]. In this study, the dried rhizome powder of A. *calamus* was tested for its nematicidal effects against *Oesophagostomum* spp. infections in pigs, and was found to significantly lower down the worm burden and eggs per gram (EPG) of feces counts of animals [16].

One of the special features of A. *calamus* is that it exhibits a polyploidy nature and as many as four karyotypes of this plant (i.e. diploid, triploid, tetraploid, and hexaploid) are known which have been reported to follow geographical patterns of distribution in various regions of the world [17]. Interestingly, he karyotypes of A. *calamus* also vary greatly in their qualitative and quantitative composition of essential oil which contains β-asarone, its anthelmintic active principle. The content of β-asarone in calamus oil has been reported to be high, i.e., 70-96% in tetraploids, low around 5-19% in triploids plants, and zero in diploid plants [18]. Kumar *et al*. tested the essential oils from A. *calamus* collected from different locations in the Himalayan region of India and observed that all the oils differed in their qualitative and quantitative makeup, although β-asarone was the major constituent of all of them which revealed a potent *in vitro* activity on poultry roundworm, *Ascaridia galli* [19]. In India, A. *calamus* grows mostly in wild in Jammu and Kashmir, Himachal Pradesh, Manipur, Tripura states, etc. In recent years, a significant body of literature has emerged that presents contradictory findings about the ploidy status and contents of β-asarone in Indian A. *calamus* populations. For example, Ogra *et al*. reported that most of the accessions of A. *calamus* from India are triploids (except only few which are diploids) with β-asarone content in their oil varies from 82% to 89.4% [18]. Conversely, Ahlawat *et al*. opined that most of the A. *calamus* accessions in India are predominantly tetraploids with the β-asarone content in their oil ranging between 73% and 88%, and triploid varieties are rather rare with β-asarone content in their oils ranging between 6.92% and 8.0% [17]. Thus, it would appear that β-asarone content in A. *calamus* oil is of paramount importance as it can affect the perceived therapeutic efficacy of this plant from one geographical region to another. As is evident none of the previous studies have standardized the amount of β-asarone in the rhizome oil of A. *calamus* with regard to its *in vivo* anthelmintic effects. The present study was, therefore, undertaken to evaluate the *in vivo* anthelmintic efficacy of a standardized methanol extract from the rhizomes of A. *calamus*, using experimentally induced *Hymenolepis diminuta* (a zoonotic tapeworm species) infections in Wistar rats.

MATERIALS AND METHODS

Plant Material

The rhizomes of A. *calamus* [Figure 1] were collected from North Tripura district of Tripura (24° 36' N latitude and 92° 19' E longitude) in October 2010. The plant specimen was identified by a Curator in the Department of Botany, North-Eastern Hill University (NEHU), Shillong, and a voucher specimen (No. AKY-11883) has been retained in the Department of Zoology, NEHU. The rhizomes were dried under shade and ground into fine powder form. Plant material was extracted with different solvents, n-hexane, n-butanol, chloroform, ethyl acetate, acetone, methanol, and water using Soxhlet extractor at 40°C for 4-5 h. The process was repeated thrice with fresh solvent. The ratio of sample to solvent was 1:10 (m/v). Each extract was subsequently filtered, and the filtrates were concentrated under reduced pressure in a vacuum rotary evaporator. The crude extracts were subjected to thin layer chromatography (TLC) plate, and the separation pattern of the extract was monitored in chloroform and methanol (9:1), which showed distinct separated spots. The methanol extract gave maximum number of spots in TLC, and therefore, it was selected for *in vivo* testing. The final yield of methanol extract was 15% (w/w).

Characterization of Active Component

Sample preparation for column chromatography was done by adsorption of extract on activated (105°C, 30 min) silica gel (100-200 mesh) with ratio 1:10, respectively. Extract was kept for drying in a rotary evaporator, and finally, lyophilized until free flowing material was formed. The dried free flowing prepared sample was subjected to column chromatography using a 20 cm × 2.5 cm glass column filled with silica gel (mesh size: 100-200, SRL) in n-hexane. Prepared sample of methanol extract was added to the free volume at the head of the column. After settling down of the material, fractionation was conducted over silica gel with n-hexane/chloroform (98:2-90:10) solvent system. Fractions were collected, and the solvent was removed to reduce volume of fraction by evaporation in vacuum at 40°C. Dried fractions were suspended in chloroform:methanol (3:1) and diluted when necessary. In each solvent preparation, five fractions were collected and monitored by TLC method (n-hexane:chloroform [9:1]). Separation pattern of fractions on TLC plate was observed by iodine vapor. After concentrating and left standing for overnight, purity of the entire fraction was tested on TLC plate. Fraction no. 3-8 showed single spot on TLC plate. A pale yellow liquid of R_f value 0.42 was obtained in n-hexane:chloroform (9:1) on precoated TLC plate (Aluchrosep Silica Gel 60/UV254, SD fine, size 5 cm × 20 cm and 0.2 mm thickness). To confirm the purity of isolated compound, parallel spot was run on TLC plate with standard β-asarone in n-hexane/chloroform (9:1). The purity of this compound, yellow oil, was confirmed by TLC using various solvent systems and ^{1}H NMR.

The chemical structure of the calamus oil isolated fraction was predicted through detailed spectroscopic procedures- ^{1}H NMR, ^{13}C NMR, mass and infrared (IR) spectroscopy. IR spectrum was recorded in chloroform on a Fourier transform IR spectrophotometer (Nicolet Impact 1-410) calibrated against the polystyrene absorption at 1601 cm^{-1}. Mass spectrum was recorded by liquid chromatography (LC) - mass spectrometry using waters ZQ-4000 mass spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded using $CDCl_3$ as solvent in a Bruker Avance II-400 NMR machine, considering tetramethylsilane (TMS) as an initial standard and chemical shift values were in δ ppm values. Spectra were referenced to TMS (^{1}H) or solvent (^{13}C) signals. Fraction containing active compound was further applied to reverse-phase high-performance LC (RP-HPLC) system (SHIMADZU, LCIOAT, Kyoto, Japan) equipped with Shimadzu SPD-10A ultraviolet-visible detector. RP-HPLC analysis was carried out in isocratic conditions using C_{18} reverse phase column with a particle size of 5 μm, 250 mm × 4.6 mm. Samples were filtered through 0.45 μm ultra-membrane filters (Millipore, Germany). Running conditions included: Injection volume, 20 μl; mobile phase, methanol:0.5% acetic acid in water (75:25 v/v); flow rate, 1 ml/min; and detection wavelength at 210 nm. The calibration curves of β-asarone were linear from 0.01~100 μg/ml (r = 0.994, n = 6). The percentage of β-asarone in isolated fraction was determined by calculating the peak area of HPLC chromatograms.

Experimental Animals

Wistar rats of either sex, weighing 180-200 g, were used. Animals were acclimatized for 15 days in the laboratory and had *ad libitum* access to standard rodent food and water. *H. diminuta* infection in rats was maintained by inoculating the cysticercoids obtained from experimentally infected flour beetle *Tribolium confusum*, the intermediate host [20]. All the experiments on rats were conducted after due approval by the Institutional Ethics Committee (animal models), NEHU, Shillong.

Acute Toxicity Study

The rhizome extract was subjected to acute toxicity study according to the OECD guidelines [21]. The extract was tested at a limit dose of 2000 mg/kg by oral route using five female Swiss albino mice. Each animal was dosed individually with 2000 mg/kg dose of extract and observed for any adverse toxicity or mortality for 2 weeks. The LD50 was predicted to be above 2000 mg/kg if three or more animal survived in this experiment.

Evaluation of *In Vivo* Anthelmintic Activity

The anthelmintic effects of extract were tested on adult *H. diminuta* infections in rats. Animals were divided into five groups, each comprising of 6 rats. Each animal was then orally infected with four cysticercoids and maintained in a separate cage. Group I animals served as the untreated controls and received water with a few drops of 1% dimethyl sulfoxide (vehicle), daily on days 21-25 post-inoculation (p.i.) of cysticercoids. Groups II, III, and IV of animals were treated with 200, 400, and 800 mg/kg, respectively, doses of plant extract on days 21-25 p.i. of cysticercoids. Group V of animals served as the positive controls and was given 5 mg/kg of praziquantel (distocide®), the reference drug for the same duration. The efficacy of extract was determined by percentage reduction in EPG counts and percentage reduction in worm counts during pre-and post-treatment periods [22]. Herein, the EPG counts of animals were estimated for 3 days (days 18-20 p.i.) before treatment and 3 days (days 26-28) after treatment. Finally, all the animals were sacrificed on day 39 p.i., and the worms in their intestine were recovered to work out the percentage reduction in worm counts.

Statistical Analysis

Data from experiments are expressed as mean ± standard error of mean. The level of significance between treatment and control was analyzed by Student's *t*-test. The $P < 0.05$ was considered to be statistically significant.

RESULTS

The TLC profile of methanolic rhizome extract of *A. calamus* showed six spots [Figure 2]. The R_f value of isolated purified compound was recorded to be 0.42. The chemical structure of the calamus oil isolated fraction was predicted through ^{1}H NMR, ^{13}C NMR, mass and IR spectroscopy and found related

to β-asarone. The HPLC chromatogram of the isolated fraction, showing β-asarone peak, is depicted in Figure 3. The structure of isolated compound, β-asarone was confirmed with the literature [12,14] and showed similar spectral patterns.

Administration of 2000 mg/kg single limit dose of A. *calamus* rhizome extract to five mice did not reveal any signs of toxicity or mortality in any animals. All the treated animals were found to be healthy and normal in their behavior, breathing, posture, food and water consumption, etc., during the observation period of 14 days.

As monitored by EPG counts and percentage reduction in worm counts, A. *calamus* rhizome extract showed dose-dependent efficacy ($P < 0.001$) against adult *H. diminuta* infections in rats [Table 1]. Treatment of *H. diminuta* infected rats by a single 800 mg/kg dose of extract for 5 days (days 21-25 p.i. of cysticercoids) resulted into 62.30% reduction in EPG counts and 83.25% reduction in worm counts at necropsy of rats on day 39. This was well comparable with the effects of reference drug praziquantel which caused 85% reduction in EPG counts and 81% reduction in worm burden of animals. Herein, the control animals maintained an almost uniform trend in their EPG counts during the pre- and post-treatment periods. In a similar manner, the active principle of plant, β-asarone also revealed dose-dependent anthelmintic effects ($P < 0.001$). The treatment of animals by 40 mg/kg dose of β-asarone resulted in comparatively better efficacy and caused up to 92% of worm reductions and 79% EPG reductions in experimental animals [Table 2].

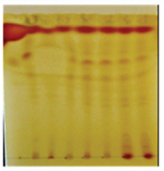
Plant	Solvents	TLC profile	No. of spots
Acorus calamus	Chloroform and methanol (9:1)		n-hex: 3 n-but: 1 Chloroform: 4 Ethyl acetate: 5 Acetone: 5 Methanol: 6 Aquoeus: 5
Beta-asarone	Chloroform and methanol (9:1)		R_f value of β- asarone: 0.42 Methanol extract: 6 Standard beta-asarone: 1 Isolated beta-asarone: 1

Figure 2: Thin layer chromatography of rhizome extract of *Acorus calamus*, standard β-asarone and isolated compound, β-asarone

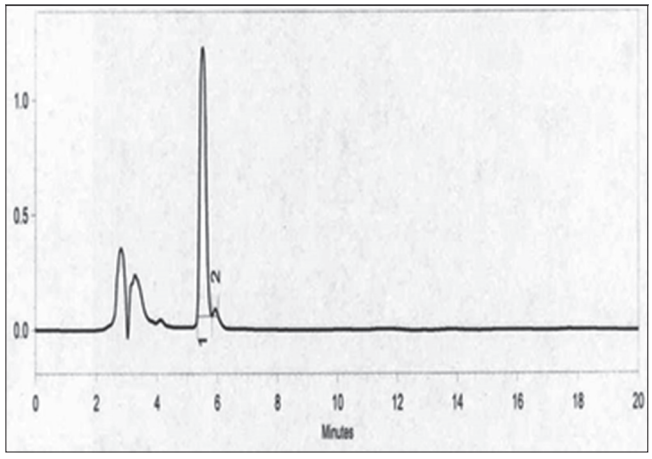


Figure 3: High-performance liquid chromatography chromatogram of *Acorus calamus* rhizome active fraction showing β-asarone peak (peak 1)

DISCUSSION

India is considered as one of the most affected countries by intestinal helminths. According to latest available data, more than two-thirds of children aged 1-14 are in need of deworming in India [3]. As the anthelmintic drugs are yet to reach all endemic regions in the required quantities, traditional herbal medicines hold a great scope for the treatment of intestinal helminths.

During our on-going studies on documentation and scientific validation of traditional anthelmintic plants of India, it came to our notice that rhizomes of A. *calamus* have long been used by natives to cure intestinal worms. Although some previous workers have demonstrated that A. *calamus* rhizomes bear significant *in vitro* anthelmintic activity [14,15], so far no sufficient efforts have been made to systematically evaluate the *in vivo* anthelmintic activity of this plant. As it is well established, this plant exhibits a ploidy nature [17], and different varieties of this plant vary in its contents of active principle β-asarone [18], therefore, an evaluation of its *in vivo* anthelmintic effects with regard to the quantity of β-asarone present in its rhizomes seems desirable. Hence, this study was undertaken to evaluate the *in vivo* anthelmintic effects of a standardized methanol extract of A. *calamus* rhizomes against experimentally induced cestodiasis in rats.

The present study revealed that both the A. *calamus* rhizome extracts as well as its active principle, β-asarone possess dose-dependent effects ($P < 0.001$) against adult *H. diminuta* infections in rats. The study also indicated that treatment of rats by a single 800 mg/kg dose of extract for 5 days resulted into 62.30% reduction in EPG counts and 83.25% reduction in worm counts at necropsy of animals, which compared well with the efficacy of reference drug praziquantel. In recent years, researchers have shown increasing interest in studying the anthelmintic potentials of medicinal plants [5,23,24]. Several other studies have also employed *H. diminuta* - rat experimental model to validate the anthelmintic effects of various traditional anthelmintic plants [5,25]. These studies have mostly ascertained the anthelmintic potentials of various medicinal plants on adult or larval stages of *H. diminuta* and discussed their findings by reductions in EPG counts and worm burdens of experimental animals. The findings of our study are in agreement with the findings of two previous studies by Yadav and Tangpu and Yadav *et al*., wherein the treatment of *H. diminuta* infected rats by *Solanum myriacanthum* fruit extract revealed 60.49% reduction in the EPG counts and 56.60% reduction in worm counts, and by *Gynura angulosa* leaf extract showed 78.60% reduction in EPG counts and 70.75% reduction in worm counts of animals [25,26].

Table 1: Anthelmintic effects of *Acorus calamus* rhizome extract on adult *H. diminuta* worms in rats as assessed by reduction in EPG and worm counts ($n=6$)

Groups	EPG (mean±SEM)		Percentage difference in EPG counts (A-B)	Number of worms recovered/ rat (mean±SEM)	Percentage reduction in worm counts
	Pre-treatment (days 18-20) (A)	Post-treatment (days 26-28) (B)			
Control	15.645±320	15.728±334	0.53	4.00±0.00	0.00
Plant extract (mg/kg)					
200	15.677±333	10.277±226*	−34.44	2.17±0.30**	45.75
400	15.683±354	8.661±413*	−44.77	1.33±0.21**	66.75
800	15.766±319	5.944±241*	−62.30	0.67±0.21**	83.25
Praziquantel (mg/kg)					
5	15.883±352	2.370±24*	−85.08	0.75±0.34**	81.25

Plant extract and praziquantel were administered on days 21-25 p.i. with four cysticercoids per rat. *$P<0.001$ versus pre-treatment value, Student's *t*-test; **$P<0.001$ versus control, Student's *t*-test, EPG: Egg per gram, SEM: Standard error of mean, *H. diminuta*: *Hymenolepis diminuta*, p.i.: Post-inoculation

Table 2: Anthelmintic effects of active principle, beta-asarone on adult *H. diminuta* worms in rats as assessed by reduction in EPG and worm counts ($n=6$)

Groups	EPG (mean±SEM)		Percentage difference in EPG counts (A-B)	Number of worms recovered/ rat (mean±SEM)	Percentage reduction in worm counts
	Pre-treatment (days 18-20) (A)	Post-treatment (days 26-28) (B)			
Control	15.622±320	15.716±334	0.60	4.00±0.00	0.00
Beta-asarone (mg/kg)					
10	15.953±157	8.753±559*	−45.13	1.67±0.33**	58.25
20	15.640±161	7.733±127*	−50.56	1.00±0.26**	75.00
40	15.493±125	3.260±79*	−78.96	0.33±0.21**	91.75
Praziquantel (mg/kg)					
5	15.892±352	2.380±24*	−85.02	0.70±0.22**	82.50

Beta-asarone and praziquantel administered on days 21-25 p.i. with four cysticercoids per rat. *$P<0.001$ versus pre-treatment value, Student's *t*-test; **$P<0.001$ versus control, Student's *t*-test, EPG: Egg per gram, SEM: Standard error of mean, *H. diminuta*: *Hymenolepis diminuta*, p.i.: Post-inoculation

In this study, the anthelmintic effects of plant active principle, β-asarone were noted to be slightly better than crude extract and it caused up to 92% of worm reductions in experimental animals. In a related testing of root tuber, extract of *Carex baccans* (a traditional anthelmintic plant of India) and its active principle resveratrol revealed slightly lower anthelmintic efficacy [27]. This study revealed that treatment of rats by extract of *C. baccans* and resveratrol results into 56.01% and 46.05% EPG reductions, and 44.28% and 31.03% decrease in worm burden, respectively. In the study by Magi *et al*., the rhizome extract of *A. calamus* tested at 5 g/kg dose against pig-nodular worm *Oesophagostomum* spp. also showed 98% reduction of worm burden in pigs [16]. It thus appears from these studies that *A. calamus* rhizomes do possess potent *in vivo* anthelmintic effects.

In the current study, the chemical structure of isolated purified compound was predicted using various spectroscopic analytical procedures and found related to β-asarone. The spectral data of isolated purified when compared with the existing literature [12,14] showed similar spectral patterns and hence was identified as β-asarone. The yield of β-asarone was calculated to be 83.54% (w/w) in the isolated fraction, which indicates that local variety of *A. calamus* in this area is tetraploid in nature.

In acute toxicity assay, treatment of mice by a single oral dose of 2000 mg/kg of plant extract did not reveal any signs of toxicity or mortality within the 2-week observation period, and therefore, the LD50 of the extract was interpreted to be >2000 mg/kg. According to the globally harmonized system of classification and labeling of chemicals, substances having an LD50 value >2000 mg/kg are considered as relatively safe [28]. Therefore, it can be suggested that rhizome extract of *A. calamus* is practically devoid of any acute oral toxic effects in experimental animals.

CONCLUSIONS

Taken together, the results of this study show that the rhizomes of *A. calamus* bear significant dose-dependent effects against intestinal helminths, and the local Indian variety of this plant contains high β-asarone content. Therefore, there exists a great potential to develop some suitable anthelmintic herbal products from this plant.

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