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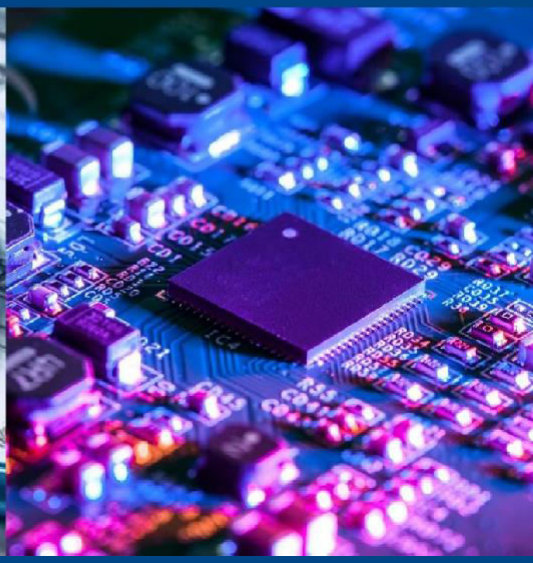

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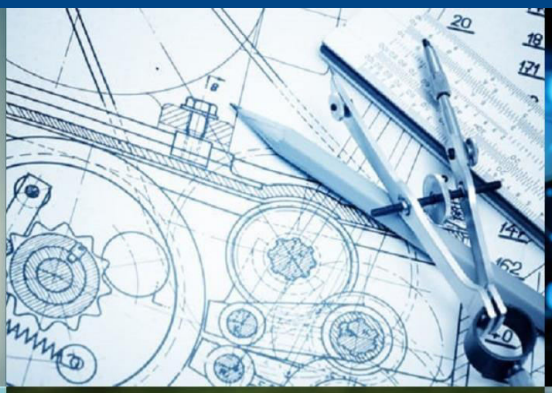

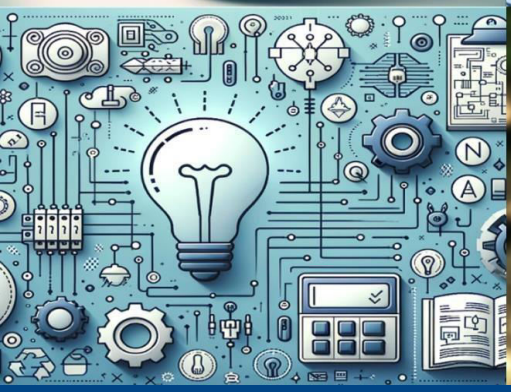

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Preliminary GC-MS Profiling and Concentration-Dependent in Vitro Anthelmintic Activity of Aqueous Leaf Extract of Annona Squamosa L. Against Pheretima Posthuma

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ABSTRACT: Helminth infections remain a major neglected public-health concern in tropical and subtropical regions, and the search for plant-derived anthelmintic leads remains scientifically relevant. The present short communication reports the preliminary GC-MS profile and *in vitro* anthelmintic activity of an aqueous leaf extract of *Annona squamosa* L. collected from Poovachal, Kerala, India. GC-MS non-target library screening of the aqueous extract recorded several high-match tentative constituents, including N,N-dimethylaminoethanol, 1,3-bis(1,1-dimethylethyl)benzene, 2,4-di-tert-butylphenol, hexadecanoic acid methyl ester and methyl stearate. Anthelmintic activity was evaluated against adult Indian earthworms, *Pheretima posthuma*, using extract concentrations of 25, 50, 100 and 200 mg/5 mL prepared in distilled water. Albendazole (1 mg/mL) served as the reference drug and distilled water as the vehicle control. The extract showed a concentration-dependent decrease in both time to paralysis and time to death. At 25 mg/5 mL, paralysis and death occurred at 70.55 ± 0.3 and 128.30 ± 1.8 min, respectively, whereas at 200 mg/5 mL the corresponding times declined to 18.14 ± 0.3 and 45.02 ± 0.7 min. The 100 mg/5 mL concentration produced a paralysis time close to that of albendazole, while the 200 mg/5 mL concentration produced shorter operational paralysis and death times than the reference drug under the assay conditions. These findings indicate promising preliminary vermicidal activity of the aqueous *A. squamosa* leaf extract and justify further work using authenticated voucher material, standardised extract preparation, bioactivity-guided fractionation, parasite-specific helminth models and toxicity profiling.

KEYWORDS: *Annona squamosa*; aqueous leaf extract; GC-MS; anthelmintic activity; *Pheretima posthuma*; albendazole; medicinal plants

I. INTRODUCTION

Soil-transmitted helminth infections are among the most common parasitic infections worldwide and remain highly relevant to tropical and subtropical public health. The World Health Organization estimates that nearly 1.5 billion people are infected globally, particularly in communities with inadequate sanitation, unsafe water and poor hygiene conditions [1]. Although periodic deworming, sanitation and health education remain central to control programmes, continued investigation of new anthelmintic leads is justified because plant-derived metabolites may provide structurally diverse antiparasitic scaffolds.

Medicinal plants are commonly screened for anthelmintic activity because secondary metabolites such as tannins, phenolics, flavonoids, alkaloids and saponins may interfere with helminth motility, tegumental integrity, energy metabolism or neuromuscular coordination [2,6,7]. In vitro screening with *Pheretima posthuma* is widely used for preliminary observation of paralysis and mortality endpoints, particularly in pharmacognostic studies, although such assays must be followed by parasite-specific assays and in vivo validation before therapeutic interpretation [4,5].

Annona squamosa L. (Annonaceae), commonly known as custard apple or sugar apple, is a tropical medicinal plant of considerable pharmacognostic interest. Leaves of this species contain diverse nutritional and phytochemical constituents and have been investigated for antioxidant, antidiabetic, antimicrobial, anti-inflammatory, anticancer and other biological activities [2]. Previous reports have also described anthelmintic activity of *A. squamosa* leaf extracts against earthworm models [3,8].

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The present short communication reports two connected laboratory observations on an aqueous *A. squamosa* leaf extract: preliminary GC-MS non-target library profiling and concentration-dependent *in vitro* anthelmintic activity against *P. posthuma*. The aim was to provide a scientifically cautious and publication-oriented report of the available findings while clearly identifying methodological limits that should be addressed in subsequent standardised studies.

II. MATERIALS AND METHODS

2.1 Plant Material and Sample Description

Fresh mature leaves of *Annona squamosa* L. were collected from the premises of Government L.P. School, Poovachal, Thiruvananthapuram District, Kerala, India. The material used for laboratory testing was recorded as an aqueous leaf extract, with the anthelmintic assay sample coded ASL H2O and the GC-MS sample recorded as 1834 Sample Code 2 Aqueous Extract. Formal herbarium voucher deposition and extractive yield were not available in the analytical reports used for the present short communication; this limitation is explicitly considered in the interpretation of the results.

2.2 GC-MS Non-Target Library Screening

The aqueous extract was subjected to GC-MS non-target library screening. The analytical report identified the sample as 1834 Sample Code 2 Aqueous Extract, with data acquisition recorded on 03 May 2025 and analysis generated on 05 May 2025 using instrument CH-GCMSMS-02. Tentative compound identification was based on library search matching. Since the report was a non-target library-search output, the identified compounds were interpreted as tentative hits and not as confirmed standards-based quantifications. For manuscript presentation, selected high-abundance or high-match hits were summarised descriptively rather than treated as definitive quantitative phytochemical concentrations.

2.3 Experimental Organism for Anthelmintic Assay

Adult Indian earthworms (*Pheretima posthuma*) were procured from Kerala Agricultural University, Vellayani, Thiruvananthapuram, Kerala, India. The worms were washed with tap water to remove adhering soil and debris. Worms measuring approximately 5-10 cm were used, and worms of approximately comparable size were selected for each treatment.

2.4 Preparation of Test and Control Solutions

Four concentrations of the aqueous *A. squamosa* leaf extract were prepared freshly in distilled water: 25, 50, 100 and 200 mg/5 mL, equivalent to 5, 10, 20 and 40 mg/mL, respectively. Albendazole (1 mg/mL) was used as the reference standard, and distilled water served as the vehicle or negative control. Test and standard solutions were prepared freshly before the assay.

2.5 In Vitro Anthelmintic Assay

Three earthworms of approximately similar size were placed in each treatment solution. Time to paralysis was recorded when no movement of any sort was observed except when worms were vigorously shaken. Time to death was recorded after confirming that the worms neither moved when shaken vigorously nor responded when dipped in warm water at 50 degrees C. A maximum observation period of 180 min was maintained for recording paralysis and death endpoints.

2.6 Data Handling

Anthelmintic results are presented as mean ± reported variation as supplied in the laboratory result sheet. Because individual worm-level raw observations and the exact identity of the error term were not available, inferential statistics were not performed. Percentage reduction from the lowest to the highest extract concentration was calculated for both endpoints. GC-MS results are reported descriptively as tentative library hits using retention time, component area and match factor.

III. RESULTS

3.1 GC-MS Profile of the Aqueous Leaf Extract

The GC-MS non-target library-search output showed multiple tentative components distributed across the chromatographic run. The most prominent and/or higher-match entries included N,N-dimethylaminoethanol, 1,3-bis(1,1-dimethylethyl)benzene, 2,4-di-tert-butylphenol, hexadecanoic acid methyl ester and methyl stearate. The high component areas observed for fatty acid methyl esters and phenolic/aromatic hits are consistent with the presence of volatile or

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derivatised semi-volatile constituents in the aqueous extract. However, because no authentic standards, retention indices or targeted calibration were used, the GC-MS findings should be interpreted as preliminary chemical profiling.

Table 1. Selected tentative GC-MS library hits recorded for the aqueous Annona squamosa leaf extract

Retention time (min)	Tentative compound	Component area	Match factor	Comment
3.4475	N,N-Dimethylaminoethanol	34,733,063.9	93.1	High-area amine hit; interpret cautiously
12.8406	Benzene, 1,3-bis(1,1-dimethylethyl)-	9,009,672.9	97.0	High-match aromatic hydrocarbon hit
13.5344	Dodecane, 4,6-dimethyl-	2,095,329.1	90.7	Branched alkane hit
19.1700	2,4-Di-tert-butylphenol	3,551,370.7	93.9	Phenolic antioxidant-type library hit
21.9685	Heneicosane	2,982,360.3	89.2	Long-chain hydrocarbon hit
22.4972	Hexadecane, 2,6,10,14-tetramethyl-	1,663,382.3	85.1	Branched hydrocarbon hit
24.4195	Hexadecanoic acid, methyl ester	26,314,952.9	97.1	Fatty acid methyl ester hit
24.8056	2,6,10-Trimethyltridecane	1,567,048.0	83.3	Branched hydrocarbon hit
26.5923	Methyl stearate	17,754,971.5	97.2	Fatty acid methyl ester hit

Note. The table reports selected library-search hits only. The entries should be treated as tentative because confirmation with authentic standards, retention indices and targeted calibration was not performed.

3.2 Anthelmintic Activity

The vehicle control did not produce recorded paralysis or death within the reported observation format. Albendazole produced paralysis at 31.26 ± 0.8 min and death at 86.00 ± 1.2 min. The aqueous *A. squamosa* leaf extract showed a clear concentration-dependent decrease in both endpoints. At 25 mg/5 mL, the extract produced paralysis and death at 70.55 ± 0.3 and 128.30 ± 1.8 min, respectively. At 200 mg/5 mL, these endpoints decreased to 18.14 ± 0.3 and 45.02 ± 0.7 min, respectively. Relative to the lowest tested concentration, the highest concentration reduced time to paralysis by 74.3% and time to death by 64.9%.

Table 2. In vitro anthelmintic activity of aqueous Annona squamosa leaf extract against Pheretima posthuma

Treatment	Concentration (mg/5 mL)	Equivalent concentration (mg/mL)	Time to paralysis (min)	Time to death (min)
Distilled water	-	-	Not recorded	Not recorded
Albendazole	1 mg/mL standard	1	31.26 ± 0.8	86.00 ± 1.2
ASL H2O	25	5	70.55 ± 0.3	128.30 ± 1.8
ASL H2O	50	10	46.09 ± 1.5	92.37 ± 0.4
ASL H2O	100	20	31.48 ± 0.6	77.24 ± 0.2
ASL H2O	200	40	18.14 ± 0.3	45.02 ± 0.7

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Note. Values are mean ± reported variation from the laboratory result sheet. ASL H2O = aqueous *Annona squamosa* leaf extract/sample. The crude extract concentrations are not directly comparable with the mass concentration of pure albendazole.

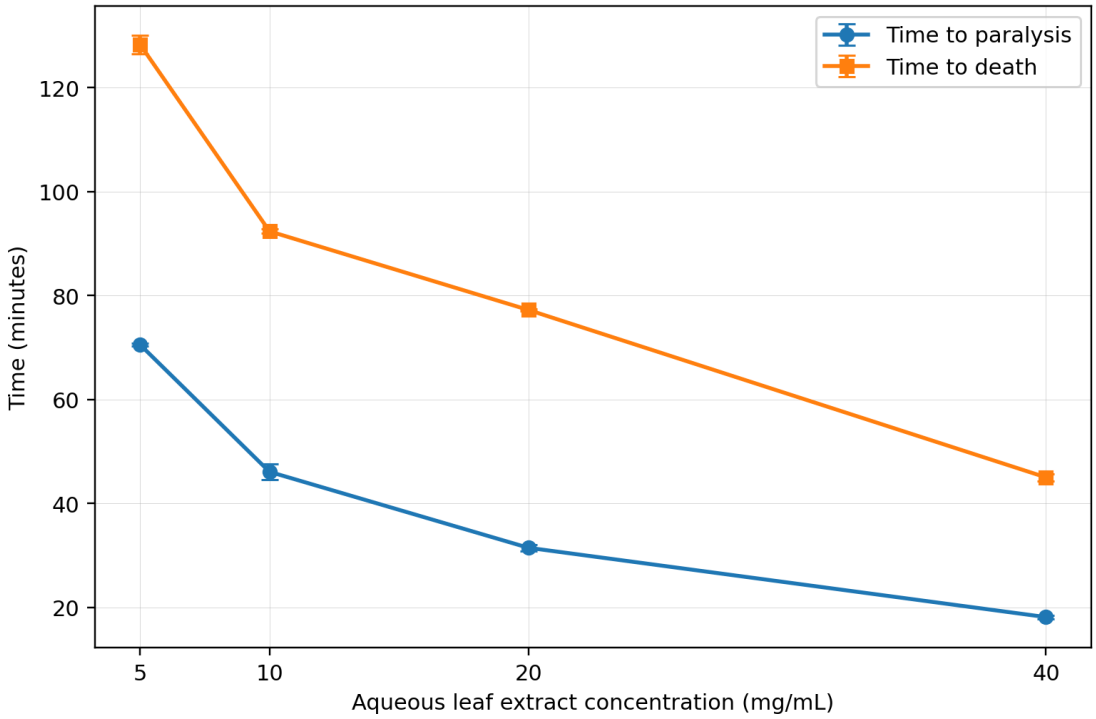

Figure 1. Concentration-dependent reduction in time to paralysis and time to death of Pheretima posthuma exposed to aqueous Annona squamosa leaf extract. Error bars represent the reported variation in the laboratory result sheet.

Figure 2. Representative assay photograph showing the positive control, negative control and aqueous Annona squamosa leaf extract concentrations used in the in vitro anthelmintic activity experiment.

IV. DISCUSSION

The aqueous leaf extract of *A. squamosa* displayed appreciable *in vitro* anthelmintic activity against *P. posthuma*, as shown by a consistent reduction in paralysis and death times with increasing extract concentration. The response was biologically coherent: higher extract concentrations produced faster immobilisation and mortality, and the maximum

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tested concentration produced the strongest effect. At 200 mg/5 mL, death occurred at 45.02 min compared with 128.30 min at 25 mg/5 mL, indicating a marked concentration-dependent response.

The activity profile agrees with earlier reports that *A. squamosa* leaf extracts possess anthelmintic potential [3,8]. The GC-MS profile provides preliminary chemical context for this activity. Fatty acid methyl esters, phenolic/aromatic compounds and hydrocarbon-type constituents were among the prominent tentative hits. In particular, the presence of a phenolic library hit such as 2,4-di-tert-butylphenol is notable because phenolic compounds are frequently associated with membrane disruption, oxidative stress modulation and antimicrobial or antiparasitic bioactivity. However, it would be scientifically inappropriate to attribute the observed anthelmintic effect to any single compound at this stage because the GC-MS profile was non-targeted and the extract was not fractionated.

Several classes of phytochemicals previously reported in *A. squamosa* leaves may plausibly contribute to anthelmintic effects. Tannins may bind to proteins on the helminth cuticle or digestive tract and interfere with nutrient availability or tegumental integrity; saponins may alter membrane permeability; and alkaloids, phenolics and flavonoids may influence neuromuscular function, oxidative balance and metabolic processes [2,6,7]. These mechanisms should be treated as plausible explanations rather than conclusions, since the present work did not include enzyme assays, tegumental microscopy, metabolomics, receptor-level testing or bioactivity-guided isolation.

The comparison with albendazole must be interpreted cautiously. At 100 mg/5 mL, the extract produced a paralysis time nearly identical to albendazole, and at 200 mg/5 mL it produced shorter paralysis and death times than the standard under the operational conditions of the assay. Nevertheless, albendazole is a purified drug used at 1 mg/mL, whereas the test material was a crude aqueous extract at much higher mass concentrations. Therefore, these results demonstrate promising *in vitro* activity but do not establish equal or superior pharmacological potency.

The major limitation of the present short communication is that the available analytical reports did not include a formal taxonomic authentication certificate, herbarium voucher number, detailed extraction procedure, extractive yield, individual raw replicate values or confirmatory GC-MS standards. These omissions should be corrected in the full thesis and in any extended journal article. Despite these limitations, the study provides useful preliminary evidence that aqueous *A. squamosa* leaf extract contains GC-MS-detectable constituents and exhibits concentration-dependent vermicidal activity in the *P. posthuma* model.

V. CONCLUSION

The aqueous leaf extract of *Annona squamosa* L. collected from Poovachal, Kerala, showed preliminary GC-MS-detectable phytochemical constituents and concentration-dependent *in vitro* anthelmintic activity against *Pheretima posthuma*. The highest tested concentration, 200 mg/5 mL, produced the fastest paralysis and death times, indicating strong preliminary vermicidal activity under the assay conditions. The findings support further investigation of *A. squamosa* leaves through authenticated plant collection, standardised aqueous extraction with yield reporting, targeted phytochemical confirmation, bioactivity-guided fractionation, parasite-specific helminth assays and toxicity evaluation.

Declarations

Ethical Statement

The study used invertebrate earthworms only and did not involve human participants or vertebrate animals. Biological material was handled according to laboratory practice for invertebrate screening assays.

Funding

No external funding was reported for this work.

Conflict of Interest

The author declares no conflict of interest.

Data Availability

The data supporting this short communication are included in the article. Additional raw laboratory records, if available, may be provided by the corresponding author on reasonable request.

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VI. ACKNOWLEDGEMENTS

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