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Qualitative phytochemical analysis of bioactive constituents in acetone extract of seeds of *Hydnocarpus pentandra*

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Abstract

Ethnoveterinary medicine focuses on the practical use of community or area based traditional and indigenous knowledge including the collection, preparation and administration of medicinal plants or plant parts for curing diseases of animals. Hydnocarpus pentandra is a plant endemic to South India which is traditionally known for the antileprotic activity of its seed oil. Diverse pharmacological activities of the plant extracts depend on the presence of its secondary metabolites. The present research was conducted to explore the phytochemical constituents present in the acetone solvent extract of the seeds of H. pentandra. Preliminary phytochemical screening of the extract using colorimetric reactions revealed the presence of steroids, triterpenes, diterpenes, flavonoids, alkaloids and carbohydrates. Gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of different compounds which included paromomycin, D-streptamine, carnegine, 4-propyl acridine and R-limonene. The acetone extract could be considered as a potent source of new molecules with different pharmacological actions.

Keywords*: Hydnocarpus pentandra, phytochemical screening, secondary metabolites*

Hydnocarpus pentandra (Malayalam-*Marotti*) belonging to family Flacourtiacea is endemic to the moist deciduous and semi-evergreen forests of Western Ghats of India. Seed oil and leaves of the plant possess medicinal properties as per published scientific reports (Sahoo *et al*., 2014; Deepa *et al*., 2016). Seed oil known as chaulmoogra oil has anti-inflammatory activity and is topically applied in the treatment of leprosy, leukoderma, sprains, rheumatism and skin diseases. Jacobsen and Levy (1973) proposed that the *in vitro* antileprotic activity of *H. pentandra* was due to cyclopentenyl fatty acids especially hydnocarpic acid and chaulmoogric acid which are the major constituents of the chaulmoogra oil. Formulations prepared from the seed also had action against scabies, wound healing, obesity and skin disorders (Sahoo *et al*., 2014). Climatic and seasonal variations during the collection of plant materials, solvents used and the extraction procedures could duly influence the biological activity of the plant because of variation in the composition of plants at various stages of growth and due to the difference in growing conditions. The concentration of various molecules in plants during the stages of growth cycle also varies (Webster *et al*., 2008). Hence, development and standardisation of traditional medicines should be conducted in a systematic way for identification of the biological activity, better reproducibility and quality assurance.

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Materials and methods

Collection and authentication of plant

Fruits of the plant *H. pentandra* were collected from the households of Vaikom taluk of Kottayam district, Kerala. The seeds (Fig. 1) separated were taxonomically identified and authenticated by Mr. Shine G., Department of Silviculture and Agroforestry, College of Forestry, Vellanikkara, Thrissur, Kerala. The voucher specimen was deposited in the herbarium of the Department of Veterinary Clinical Medicine, Ethics and Jurisprudence (KVASU/CM/1/2022).

Preparation of the extract

The seeds were separated from the pods and dried. The seeds were later crushed in grinder and hundred grams of the crushed seed were transferred to clean thimble for extraction. Acetone extraction was conducted in soxhlet extraction apparatus at 56 ºC until the solvent became clear. The extract was then filtered with Whatman filter paper 1 and dried using rotary flash evaporator at Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy and the yield was calculated. The dried extract was transferred to air tight containers, labelled and stored under refrigeration conditions for further evaluations.

Determination of the yield

The yield of evaporated dried extract on dry weight basis was calculated using the equation,

Yield (per cent) = (W1/W2) x 100

W1: weight of extract after evaporation of solvent

W2: dry weight of the sample

Preliminary phytochemical analyses

Preliminary phytochemical analyses of acetone extract of the seed were performed to find out the presence or absence of primary and secondary metabolites as per standard procedures (Harborne, 1998) in the Department of Veterinary Pharmacology, CVAS, Mannuthy and the analytical laboratory of CARe Keralam Limited, Koratty, Kerala.

Test for detection of alkaloids

One gram of the extract was mixed with five millilitres of ammonia and then extracted with an equal volume of chloroform. To this extract, five millilitre of dilute hydrochloric acid was added. The acid layer obtained was further tested with the following reagents for the presence of alkaloids.

Dragendorff's test

Eight drops of Dragendorff's reagent were added to one millilitre of acid extract. Development of reddish-brown precipitate indicated presence of alkaloids.

Mayer's test

To one millilitre of the acid layer, eight drops of Mayer's reagent were added. Presence of alkaloids was indicated by development of a cream-coloured precipitate.

Wagner's test

One millilitre of Wagner's reagent was added to one millilitre of the acid layer and checked for the presence of a reddish-brown precipitate.

Test for detection of steroids: Salkowski's test

Fifty milligrams of the extract were dissolved in three millilitres of chloroform. Few drops of concentrated sulphuric acid were added and the solution was allowed to stand. Development of red colour indicated presence of steroids.

Test for detection of flavonoids

Shinoda test

Shinoda test was performed to detect the presence of flavonoids. To two milligrams of the extract, ten drops of dilute hydrochloric acid followed by a piece of magnesium were added. Formation of pink, reddish or brown colour indicated the presence of flavonoids.

Fig. 1. Seeds of *H. pentandra*

Fig. 2a Test for alkaloids

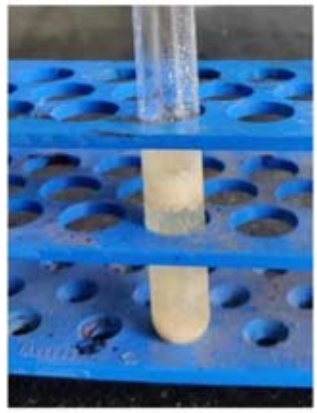

Fig. 2b Test for flavanoids

Fig. 2c Test for triterpenes

Fig. 2d Test for steroids

Fig. 2d Test for steroids

Fig. 2e Test for triterpenes

Fig. 2. Preliminary phytochemical analysis of acetone extract

Lead Acetate test

In lead acetate test three millilitres of 10 per cent lead acetate solution were added to plant extract. Development of yellow residue confirmed the presence of flavonoids.

Test for detection of glycosides

Picric acid test

Presence of glycosides was detected using picric acid test. Two millilitres of picric acid were added to the test solution. Formation of yellow precipitate indicated presence of glycosides.

Benedict's test

Fifty milligrams of the extract were mixed with one millilitre of water and then five millilitres of Benedict's reagent were added. Formation of brownish red precipitate indicated presence of reducing sugars.

Test for detection of phenolic compounds

Folin Ciocalteu reagent test

One millilitre of the sample was mixed with four millilitre of 20 per cent sodium carbonate solution and added one millilitre Folin ciocalteu phenol reagent. Development of a greenish or blue colour indicated presence of phenol.

Ferric chloride test

Five milligrams of the extract were dissolved in one millilitre of water and five drops of 10 per cent ferric chloride were added. Formation of a bluish black colour indicated presence of phenols.

Test for detection of saponins

Foam test/ froth test

Two hundred grams of the extract were shaken with five millilitres of water. Development of stable foam that persisted for 10 minutes indicated the presence of saponins.

Test for detection of tannins

Lead acetate test

To one millilitre of the filtrate three drops of lead sub acetate solution were added. A creamy gelatinous

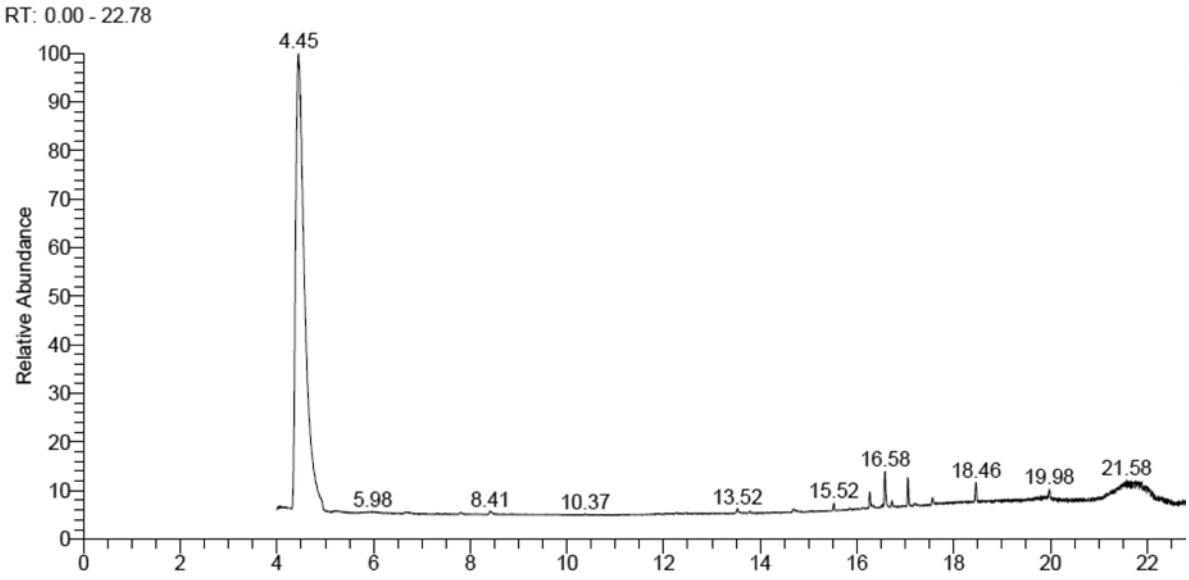

Fig. 3. GC-MS spectrum of the acetone extract of seeds of *H. pentandra*

Table 1. Preliminary phytochemical analysis findings of acetone extract

Sl. No	Phytochemical screened	Test performed	Result
1	Saponins	Foam test	-
2	Glycosides	Benedict's test	-
		Picric acid test	-
3	Steroids	Salkowski's test	+
4	Triterpenes	Liebermann Burchardt test	+
5	Diterpenes	Copper acetate test	+
6	Flavanoids	Lead acetate test	+
		Shinoda test	+
7	Tannins	Lead Acetate test	-
		Gelatin test	-
8	Phenolic compound	Ferric chloride test	-
		Folin ciocalteu reagent test	-
9	Alkaloids	Dragendorff's reagent test	+
		Mayer's test	+
		Wagner's test	+
10	Carbohydrates	Molisch's test	+

precipitate indicated presence of tannins.

Gelatin test

One gram of the extract was mixed with a few drops of one per cent solution of gelatin containing 10 per cent sodium chloride. Development of a whitish precipitate indicated presence of tannins

Test for detection of carbohydrates

Molisch's test

Crude extract was mixed with two millilitres of Molisch's reagent and the mixture was shaken properly. After that, two millilitres of concentrated sulphuric acid were poured carefully along the side of the test tube. Appearance of a violet ring at the interphase was indicative of the presence of carbohydrates.

Test for detection of triterpenes

Liebermann Burchardt test

Three milligrams of the extract were mixed with three millilitres chloroform in a test tube. To this added five drops of acetic anhydride and one millilitre of concentrated sulphuric acid along the sides of the test tube. Development of a deep red ring at the junction of two layers confirmed the presence of triterpenes.

Table 2. Phytochemicals detected in acetone extract on GC-MS analysis

Sl. No.	Compound	Mol.wt.	Retention time (min.)	Peak area (%)	Probablity (%)
1	1,4 Cyclohexanediol	116	4.44	81.33	11.37
2	4-Methylpiperazine-2-carboxylic acid	144	4.61	0.12	14.09
3	Acetamide, N(4hydroxycyclohexyl)	157	4.68	0.03	28.95
4	Carbamic acid, (1-cyano2-hydroxyethyl), 1,1dimethylethyl ester	186	4.93	0.18	10.70
5	13-Heptadecyn-1-ol	252	7.79	0.04	12.51
6	Paromomycin	615	14.68	0.16	43.62
7	N-[2-[[2-Pyridylmethyl] amino] ethyl] aziridine	177	14.73	0.01	15.83
8	Paromomycin	615	14.76	0.01	27.66
9	D Streptamine	482	15.52	0.26	34.63
10	7-Heptadecyne, 1-chloro	270	15.85	0.05	10.42
11	Octanal, (2,4dinitrophenyl) hydrazone	308	16.33	0.14	25.37
12	Penta-2,4-dien-1-one, 5-dimethylamino-1-[5-(4-dimethylamino) Buta-1,3-dienyl-2-thienyl]	302	16.52	0.07	25.37
13	Pyrimidine, 2--fluor5-chloro 4-amino	147	16.58	1.73	16.25
14	2,6-Dimethyl-3,4bis (Trimethylsilyloxy methyl) pyridine	311	17.05	1.16	11.72
15	Paromomycin	615	17.19	0.09	43.09
16	1H-1,3-Disilaindene, 2,3-dihydro	150	17.57	0.46	38.05
17	1,3,2-Dioxaphosphorinane, 2-(3-indolyl) (4-methoxyphenylamino) methyl, 2-oxide	372	17.72	0.03	19.20
18	9,12,15-Octadecatrienoic acid, 2,3- dihydroxypropyl ester, (Z,Z,Z).	352	18.01	0.02	13.74
19	Paromomycin	615	18.20	0.02	79.59
20	Paromomycin	615	18.32	0.03	86.51
21	9,12,15-Octadecatrienoic acid, 2,3 dihydroxypropyl ester, (Z,Z,Z)	352	18.40	0.02	21.19
22	4-Propylacridine	221	18.46	0.97	69.39
23	7-Hydroxy-6, 9a-dimethyl-3-methylene-decahydro- azuleno[4,5-b] furan-2,9-dione	264	18.57	0.03	23.60
24	Paromomycin	615	18.75	0.11	58.55
25	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	352	18.78	0.04	18.23
26	Penta2,4dien-1-one	302	18.80	0.02	34.45
27	4-Methoxy-6-methyl-5-nitroisobenzofuran-1,3-dione	237	18.86	0.03	16.51
28	Paromomycin	615	18.90	0.05	60.94
29	R-Limonene	184	18.96	0.08	13.77
30	Carnegine	221	19.02	0.02	21.06
31	1,3-Dimethyl-3-hydroxy-5-methoxyoxindole	207	19.12	0.05	15.43
32	1-Heptatriacotanol	536	19.18	0.02	20.42
33	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	352	19.20	0.03	20.86
34	12-Hydroxy-14-methyl-oxa-cyclotetradec-6-en-2-one	240	19.25	0.03	28.45
35	R-Limonene	184	19.43	0.07	22.84
36	7-Azabicyclo[4.1.0]heptane, 1-methyl-4-(1-methylethyl)	153	19.80	0.04	42.30
37	Paromomycin	615	19.82	0.03	27.13
38	4-Propylacridine	221	19.98	0.71	55.65
39	Carnegine	221	20.10	0.04	28.38
40	R-Limonene	184	20.17	0.04	48.92
41	13-Heptadecyn-1-ol	252	20.19	0.10	21.28
42	1-Heptatriacotanol	536	20.53	0.03	30.09
43	Methyl 15-hydroxy-9,12-octadecadienoate	310	20.62	0.08	18.88
44	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	352	20.64	0.03	19.16
45	Carnegine	221	20.98	0.03	30.69
46	Methyl 3á-hydroxyolean-18-en-28-oate	470	21.68	0.19	64.30
47	Methyl 2á,3á-hydroxyolean-12-en-28-oate	486	22.09	0.03	82.05
48	Methyl 3á-hydroxyolean-18-en-28-oate	470	22.16	0.08	76.88

Test for detection of diterpenes

Copper acetate test

About five milligrams extract were mixed with three millilitres of five per cent copper acetate solution. Development of green colour indicated the presence of diterpenes.

Gas chromatography -mass spectrometry (GC- MS) analysis

Recognition of the compounds in the acetone extract was carried out by a Triple Quadruple GCMSMS, Thermo Fisher Scientific model at Central Instruments Laboratory, College of Veterinary Animal Sciences, Mannuthy. Components were separated using gas chromatography (Trace 1300) and constituents were analysed using TSQ 8000 MS/MS. The components of the extract were separated with a DB-5MS capillary column 30 metre length, 0.25 mm diameter and 0.25 µm film thickness. The oven temperature program was an initial temperature of 80 °C for 2 min. which was increased to 250 °C for 5 min. at a rate of 15 °C per minute, then to 150 °C per min. The total time taken was 22.78 min. The ionising energy used was 70 eV and the carrier gas used was Helium at flow rate of 1mL/min. Full-scan mass spectra were collected within the scan range 50-500 atomic mass units (amu). Compounds were identified with the National Institute of Standards and Technology MS Search 2.0 library.

Results and discussion

The fruits of *H. pentandra* were collected from Vaikom taluk situated in the north west of Kottayam district, Kerala state (9° 46' 5.11" N and 76° 25' 4.68" E). The fruits of the plant were globose and woody in nature. The yield of acetone extract obtained was 3.59 %.

Preliminary qualitative phytochemical analysis

The preliminary qualitative screening of the acetone extract of seeds using colorimetric reactions revealed the presence of steroids, triterpenes, diterpenes, flavonoids, alkaloids and carbohydrates. Presence of steroids was detected by development of a red colour in the reaction mixture in Salkowski's test. Development of a red ring in Liebermann Burchardt test confirmed the presence of triterpenes. Green colour formation in copper acetate test indicative of presence of diterpenes was observed in the acetone seed extract. Flavonoids were detected by development of yellow residue in lead acetate test and a brown colour in Shinoda test. A reddish-brown precipitate in Dragendorff's test, cream coloured precipitate in Mayer's test and a reddish-brown precipitate in Wagner's test confirmed the presence of alkaloids. Presence of the primary metabolite carbohydrate was detected by the presence of a violet ring in Molisch's test (Table 1, Fig. 2).

Pharmacological activities attributed to the seed oil of *H. pentandra* might be due to the presence of these primary and secondary metabolites. However, records were scarce on the qualitative analysis of acetone extract of *H. pentandra* seeds. Suhas and Vilas (2014) had reported the presence of alkaloids, saponins, tannins and flavonoids in the dried seed hull powder of *H. pentandra.* Gebka *et al*. (2022) reported that flavonoids in plants possessed photoprotective and antioxidant properties which prevented the development of skin diseases and the ability of flavonoids to stimulate the circulation aided in the healing of lesions. Antifungal, antibacterial and antiviral activities of natural plant alkaloids were observed by Thawabteh *et al*. (2019). Similarly, Patel and Savjani (2015) had reviewed about the potential anti-inflammatory activity of plant steroids. Thus, various secondary metabolites imparted pharmacological activities either alone or in combination. This might be the reason for the indigenous medicinal properties assigned for the seed oil of *H. pentandra*. Since the acetone extract had several of these primary and secondary metabolites, the extract could be a potential drug candidate.

Gas chromatography-mass spectrometry

Gas chromatography mass spectrometry analysis of the acetone extract is depicted in Fig. 3 with X axis showing the retention time and Y axis showing the intensity or the quantity of the component present. About 48 phytoconstituents were identified from the GC-MS spectrum of which the major ones were paromomycin, D-streptamine, carnegine, 4-propyl acridine and R-limonene (Table 2).

Paromomycin identified in the acetone extract was found to have activities analogous to neomycin and has antibacterial activity as well as action against intestinal amoebiasis (Vardanyan and Hruby, 2006). Anti-leishmania activity of paromomycin was identified by Iqbal *et al*. (2023). D-streptamine containing antibiotic kanamycin is used in the treatment of infected wounds, sepsis, meningitis and peritonitis according to Vardanyan and Hruby (2006). Streptomycin as reported by Swayze *et al*. (2007) has a streptamine core and is considered as an antibiotic antifungal drug and is used in the treatment of mycobacterial infections. Alihosseini and Sun (2011) reported antibacterial activity of naturally derived acridine in addition to its colourant activity. Similarly, each of the molecules identified by GC-MS analysis has different pharmacological activities. Each of these compounds can be identified, isolated and systematically studied for its useful effects and toxic effects making acetone extract of seeds a rich source of biologically active molecules.

Conclusion

Hydnocarpus pentandra is a plant native of south India which is traditionally known for its antileprotic activity. The acetone extract of the seeds of the plant was found

to have presence of carbohydrates and the secondary metabolites including steroids, flavonoids, alkaloids, diterpenes and triterpenes. Gas chromatography- mass spectrometric spectrum of the extract was identified to have about 48 molecules bestowed with wide range of pharmacological activities, the presence of which makes it a probable drug source. The pharmacological activities of each of these compounds once identified can make the acetone extract a novel drug source.

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Conflict of interest

Authors do not have any conflict of interest

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