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Phytochemical investigation of *Hybanthus enneaspermus* and its cell culture

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Abstract
Hybanthus enneaspermus popularly known as Rathanapurushor, is known for its aphrodisiac, hypocholesterolemic, antiinflammatory, hepatoprotective, antiarthritic, antioxidant, cardioprotective, antiepileptic and other therapeutic activities. *Hybanthus* conserved in natural repositories is under severe intimidation because of its intensive use in local market for medicinal purposes. In view of its ethnomedicinal importance, novel technologies are explored for the production of secondary metabolites. In this present study, dried plant and cell cultures were investigated for presence of phytochemicals of therapeutic significance by GC-MS analysis. The results revealed the presence of 57 different phytochemicals. Of these, only six compounds were similar in both dried plant and cell culture. The major compound was stigmasterol and desulphosinigrin in dried plant and cell culture respectively. However dried plant showed the presence of more number of phytochemicals of therapeutic significance than the cell culture.

Keywords: *Hybanthus enneaspermus*, rathanapurushor, cell culture, phytochemicals, GC-MS

Introduction
Hybanthus enneaspermus (L.) F. Muell. popularly known as Rathanapurushor, Pursharathna (Sanskrit, Hindi) and Orithal Thamarai (Tamil) is known for its unique medicinal properties. The plant has a mention in Siddha, Ayurvedic and other traditional medicinal systems for its immense medicinal potential. The preparations made from the leaves and tender stalks of the plant are used in herbal medicine for its aphrodisiac and tonic properties (Yoganarasimhan, 2000) [22]. The root is diuretic and administrated as an infusion in gonorrhea and urinary infections (The Wealth of India, 1959; Nagaraju and Rao, 1996) [8, 19]. The roots also possess marked nephroprotective activity and could have promising role in the treatment of acute renal injury (Pushpangadan and Atal, 1984) [10]. The fruits and leaves are used as antidotes for scorpion stings and cobra bites by the Yanadi tribes (Reddy *et al.*, 1989; Sudarsanam and Sivaprasad, 1995) [17, 12].

Increase in use of medicinal plants led to sudden rise in market demand that resulted over exploitation and ultimate decline in natural habitat. Medicinal plants all across the globe are getting endangered because of their ruinous harvesting for the production of medicines as most of them grow as wild under natural conditions. *H. enneaspermus* conserved in natural repositories is under severe intimidation because of its intensive use in local market for medicinal purposes and not being cultivated commercially to fulfill its markets requirements. In view of its ethnomedicinal importance, there is a need to conserve the wild stock of *H. enneaspermus* and to explore novel technologies for the production of secondary metabolites which is of therapeutic importance. Large-scale plant cell and tissue culture is an attractive and alternative approach to traditional methods of plantation and drug development as it offers controlled supply of biochemicals, irrespective of the availability of plant materials. In this present study phytochemicals present in dried plant and cell cultures of Hybanthus was investigated to understand the scope of its exploitation in therapeutics.

Materials and Methods
Dried plant material and seeds were received from SKM Siddha and Ayurvedha Company (India) Limited, Saminathapuram, Modakkurichi, Erode, Tamil Nadu. Dried plant material was used for phytochemical analysis. Seeds were used to raise plants in green house of Department of Plant Biotechnology, CPMB & B, TNAU, Coimbatore. The leaves of these plants were used as explants for initiation of callus. Friable callus was used for the establishment of cell suspension cultures and cell extract was used for GC-MS analysis.

Preliminary phytochemical analysis

Dried plant material of *H. enneaspermus* was used for phytochemical studies. The dried plants were finely ground using a blender. The samples were transferred to conical flasks and extracted using five different solvents namely methanol, ethanol, water, petroleum ether and hexane (1:10 w/v of sample: solvent) and kept in orbitary shaker for 24 h at 100 rpm. The extract was allowed to settle and then filtered through a Whatmann filter paper No: 42 (125 mm) and the solvent layer was allowed to evaporate and the filtrate was screened for the presence of alkaloids, carbohydrates, proteins, saponins, flavonoids, phenols, tannins, glycosides etc as reported by Tiwari *et al.* (2011) [20], Iyengar (1995) [5], Siddiqui and Ali (1997) [15] and Hossain *et al.* (2013) [4].

Gas chromatography-mass spectrometry (GC-MS) analysis

Preparation of samples

i. Dried plant material

For GC-MS analysis, plant powder was subjected to solvent extraction using methanol for 72 h in a shaker. The extracts were concentrated to remove the solvent and filtered with Whatman No.1 filter paper. Clear extract was used for secondary metabolite analysis by GC- MS.

ii. Cell extract from suspension culture

The cell extract was prepared by centifuging the cell suspension at 5000 rpm for 20 min. The pelleted cells were subjected to solvent extraction using methanol for 72 h in a shaker. The extracts were concentrated to remove the solvent and filtered with Whattman No.1 filter paper. Clear extract was used for secondary metabolite analysis by GC- MS.

Analysis of samples

GC-MS analysis was carried out on a Thermo GC-Trace Ultra Ver.5.0 Thermo MS DSQ II System. The chromatography was performed using the DB5-MS capillary standard non-polar column. Helium gas was used as a carrier as well as eluent at a constant flow of 1 ml/min. About 1 µl methanol extract was injected using a micro syringe. Injection temperature was set at 260 °C. The oven temperature was programmed from 70 °C with an increase of 6 °C/min. raised to 260 °C, 1 min. isocratic and cooled to 70 °C, followed by the additional 5 min. delay. The ion trace integration was done using the mass lab finds target method for the characteristic fragment of assigned peaks. Total GC running time was 34 min. Spectra of the unknown components were compared with the spectra of known components stored in the National Institute Standard and Technology (NIST) and Wiley Spectra Libraries having more than 62,000 patterns. The molecular weight, molecular formula and the number of hits used to identify the name of the compound from NIST and Wiley Spectra Libraries were recorded. Prediction of biological activities of each compound was based on Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA.

Results and Discussion

The dried plant material of *Hybanthus* extracted with different solvents *viz*., methanol, ethanol, water, petroleum ether and hexane revealed the presence of alkaloids, carbohydrates, proteins, flavanoids, phenols, diterpenoids, sterols, tannins and glycosides (Table1). Methanolic extract showed the presence of more number of secondary metabolites among all the five solvents extracts used. Methanolic extract showed the presence of diterpenes, tannins, flavanoids, alkaloids, phenols, glycosides, phytosterols, diterpenes, steroids, carbohydrates and proteins followed by ethanolic extract, which showed the presence of alkaloids, phenols, glycosides, phytosterols, steroids, carbohydrates and proteins. The results are on par with the earlier works and presence of similar compounds was observed in methanolic extract of *Hybanthus enneaspermus* (Priya *et al.*, 2011[9]; Krishnan *et al.*, 2012 [7]; Krishnamoorthy *et al.*, 2014 [6]; Anand and Gokulakrishnan, 2012 [1]; Singh *et al.*, 2015) [16]. Aqueous extract, recorded the presence of flavanoids, alkaloids, glycosides, phytosterols, diterpenes, carbohydrates and proteins while petroleum ether extract, showed the presence of alkaloids, glycosides, phytosterols and proteins and the results are in agreement with the result obtained by Raveendra and Britto (2007) [11], Hemashenpagam and Praveena (2010) [3] and Krishnamoorthy *et al* (2014) [6]. Hexane extract showed positive result for very few secondary metabolites namely alkaloids, phenols, steroids, carbohydrates and proteins.

Table 1: Phytochemical screening of whole dried plant

Sl. No	Phyto-chemicals	Qualitative tests	Ethanol	Methanol	Water	Petroleum ether	Hexane
1.	Alkaloid	Dragendorff"s test	+	+	-	+	-
2.	Carbohydrates	Benedict"s test	+	+	+	+	+
3.	Protein	Millon's test	+	+	+	+	-
4.	Saponins	Foam test	-	-	-	-	-
5.	Flavonoids	Alkaline regeant test	-	+	+	-	-
6.	Phenols	Ferric chloride test	+	+	-	-	+
7	Tannins	Lead acetate test	-	+	-	-	-
8	Glycoside	Legal's test	+	+	+	+	+
9	Phytosterols	Salkowski test	-	+	+	+	-
10.	Diterpenes	Copper acetate test	-	+	-	+	+
11.	Steroids	Sulphuric acid Test	+	+	-	-	-

The GC-MS result of the dried plant and cell culture revealed the presence of 57 different phytochemicals. Of these 57 compounds, only six compounds were similar in both dried plant and cell culture (Table 2). The major compound in dried plant was stigmasterol which showed the percentage peak area of 39.61 at the retention time of 32.33 (Fig 1). Stigmasterol is the precursor for the synthesis of progesterone and it is also an intermediate in the biosynthesis of androgens, estrogens, corticoids and in the synthesis of vitamin D_3. Stigmasterol is one of the fundamental compound from which numerous synthetic and semi-synthetic compounds of pharmaceutical importance are produced. The other compounds present in dried plant sample were rhodopin, carotene, milbemycin, lycoxanthin, astaxanthin etc. Velayuthum *et al.* (2015) [21] and Suman *et al*. (2016) [18] also recorded similar compounds through GC-MS except for

stigmasterol. Analysis of the cell extract through GC-MS, showed the presence of desulphosinigrin and tetraacetyl-d-xylonic nitrile (percentage peak area: 13.03) as major compounds. Desulphosinigrin possesses antiepileptic and anticancerous properties (Saravanan *et al.*, 2014) [13]. Other compounds identified in cell extract were 2-myristynoyl pantetheine, carotene, rhodopin, digitoxin, morphinan derivative etc (Table 2 and Fig 2).

Table 2: Analysis of phytochemicals in dried plant and cell culture using GC-MS

S. No.	RT	Name of Compound	Percentage of peak area	
			Dried plant	cell culture
1.	1.88	2-Myristynoyl pantetheine	_	2.03
2.	2.24	9,12,15-Octadecatrienoic acid	0.36	0.87
3.	2.63	Pseudosolasodine diacetate	0.78	-
4.	3.10	9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol	0.18	-
5.	3.18	1-Monolinoleoylglycerol trimethylsilyl ether	-	0.18
6.	3.55	Ethyl iso-allocholate	0.19	-
7.	3.79	Morphinan-4,5-epoxy-3,6-di-ol, 6-[7-nitrobenzofurazan-4-yl]amino	-	0.35
8.	3.89	Rhodopin	0.23	-
9.	4.73	Cinnamic acid	0.36	-
10.	5.12	Pregn-4-ene-3	0.40	-
11.	5.62	Carbamic acid	0.44	-
12.	6.03	Rhodopin	-	0.43
13.	6.60	6,9,12,15-Docosatetraenoic acid,	0.19	-
14.	7.09	1-Monolinoleoylglycerol trimethylsilyl ether	0.19	0.75
15.	7.54	Digitoxin	-	2.39
16.	7.54	Ethyl iso-allocholate	-	2.39
17.	7.54	Acetamide,	0.31	0.14
18.	8.76	9,12,15-Octadecatrienoic acid,	0.30	0.44
19.	9.72	Carotene	0.28	2.05
20.	10.54	Tetraacetyl-d-xylonic nitrile	-	13.03
21.	10.54	Desulphosinigrin	-	13.03
22.	10.6	Benz[e]azulene-3,8-dione,	0.50	-
23.	11.33	Myristynoyl pantetheine	0.29	-
24.	11.33	Morphinan-4,5-epoxy-3,6-di-ol,	0.29	-
25.	11.98	Hexadecenoic acid,	0.43	0.14
26.	12.72	Milbemycin b,	0.46	-
27.	13.39	1H-Indole	0.21	-
28.	13.49	Monolinoleoylglycerol trimethylsilyl ether	0.56	2.49
29.	14.37	9,10-Secocholesta-	-	0.14
30.	14.53	Spirost-8-en-11-one, 3-hydroxy-,	0.23	-
31.	14.79	1,4-Benzenediol,	0.54	-
32.	14.81	Demecolcine	-	1.37
33.	15.34	Pyridazine	-	0.25
34.	15.57	0-Bisnorhopane	-	0.47
35.	15.94	Butanoic acid,	0.18	-
36.	16.42	Cyclopropanebutanoic acid,	-	3.37
37.	16.71	Lycoxanthin	0.42	-
38.	17.16	Propanoic acid,	-	5.73
39.	17.3	4-dinitrophenylhydrazine	0.39	-
40.	17.69	Glycine	0.30	-
41.	17.79	4-bis butylboronate	-	2.36
42.	18.32	Cyclobarbital	0.19	-
43.	18.68	Cyclopropanenonanoic acid,	0.56	-
44.	19.36	Spirost-8-en-11-one	0.21	-
45.	19.48	2,3-dihydroxypropyl ester	-	2.3
46.	20.05	tetradecamethyl-	2.38	-
47.	20.31	9,10-Secocholesta	0.43	-
48.	21.53	4-Piperidineacetic acid,	0.21	-
49.	22.84	Astaxanthin	0.52	-
50.	22.92	2,3-Dihydroxypropyl elaidate	0.44	-
51.	23.55	adecahydrocyclopenta[a]phenanthren-2-on	0.19	-
52.	24.4	Docosanoic acid,	0.39	-
53.	26.24	3-methoxy-7,11,18-triacetoxy-	0.23	-
54.	26.67	Androstane-11,17-dione,	0.11	-
55.	26.67	Phthalic acid	-	4.36
56.	27.97	cyclic methylboronate	0.51	-
57.	32.33	Stigmasterol	39.61	-
		Total	41	24

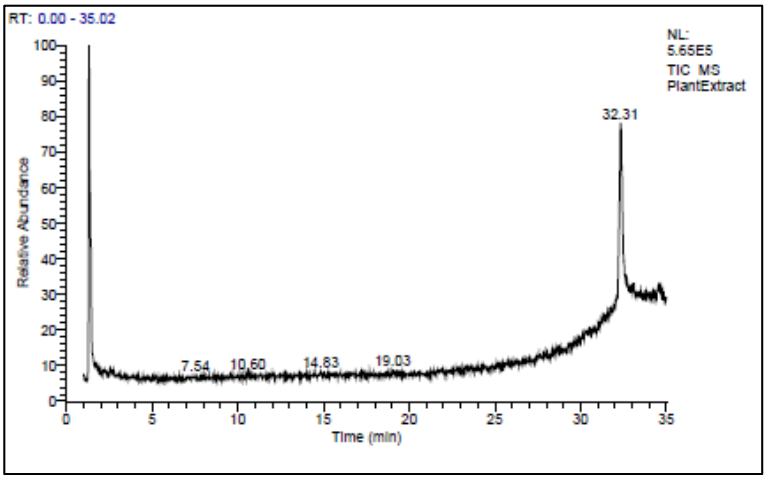

Fig 1: GC-MS analysis of methanolic extract of dried plant

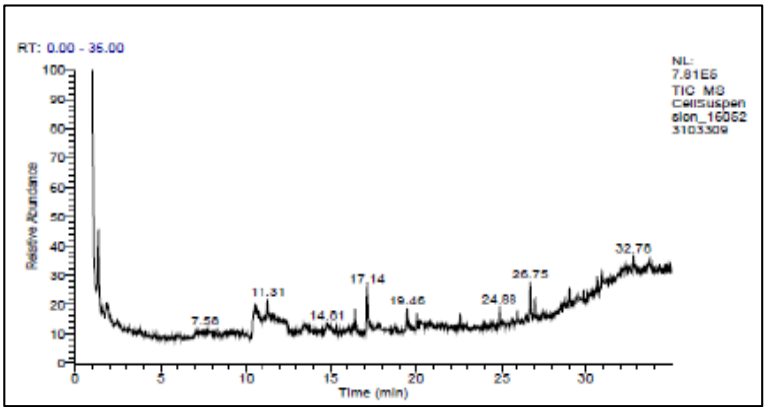

Fig 2: GC-MS analysis of methanolic extract of cell culture

Biological significance of the compounds in dried plant and cell extract is given in Table 3. Wide array of therapeutic compounds *viz*., hypocholesterolemic, antiinflammotory, hepatoprotective, antiarthritic, antooxidant, cardioprotective, antiepileptic, antidiabetic, analgesic etc., are present in dried plant. However in cell extract the number of compounds identified was comparatively lower (24 nos.) than the dried plant (41 nos.) and major therapeutic compound like stigmasterol was documented only in dried plant. Similarly, the therapeutically significant compounds *viz*., desulphosinigrin, digitoxin and demecolcine were documented only in cell culture. Variations in phtochemicals identified through GC-MS under *in vivo* and *in vitro* conditions in Hybanthus were documented by Velayuthum *et al.* (2015) [21].

Table 3: Biological activities of the compounds identified using GC-MS

S. No	RT	Name of Compound	Biological activities	References
1	1.88	2-Myristynoyl pantetheine	Hypocholesterolemic,Cosmetic, Flavour, Lubricant	Sasikala and Mohan (2014) [14]
2	2.24	9,12,15-Octadecatrienoic acid	Antiinflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge Antihistaminic, Antiarthritic, Anticoronary, Antieczemic Antiacne, Antiandrogenic	Anand and Gokulakrishnan (2012) [1]; Velayuthum *et al.* (2015) [21]
3	3.18	1-Monolinoleoylglycerol trimethylsilyl ether	Antiacne, Antiandrogenic	Velayuthum *et al.* (2015) [21]
4	3.79	Morphinan-4,5-epoxy-3,6-di-ol, 6-[7-nitrobenzofurazan- 4-yl]amino	Antiageing, Analgesic, Antidiabetic.	Saravanan *et al.* (2014) [13]
5	6.60	6,9,12,15-Docosatetraenoic acid,	Antiinflammatory, Insectifuge, Hypocholesterolemic, Cancer preventive, Nematicide, Hepatoprotective, Insecticide	Anand and Gokulakrishnan (2012) [1]; Velayuthum *et al.* (2015) [21]
6	7.54	Digitoxin	Antioxidant, Cardioprotective.	Anand and Gokulakrishnan (2012) [1]; Brintha *et al*., (2017) [2]; Sasikala and Mohan (2014) [14]
7	8.76	9,12,15-Octadecatrienoic acid,	Hypocholesterolemic, Cosmetic, Flavour, Lubricant	Anand and Gokulakrishnan (2012) [1]; Velayuthum *et al.* (2015) [21]
8	9.72	Carotene	Antibacterial, Antiplasmodial, Antileishmanial, Antiplasmodial.	Sasikala and Mohan.(2014) [14]; Saravanan *et al.* (2014) [13]
9	10.54	Desulphosinigrin	Antiepileptic, Anticancerous.	Sasikala and Mohan (2014) [14]; Saravanan *et al.* (2014) [13]

10	11.33	Morphinan-4,5-epoxy-3,6-di-ol,	Antiageing, Analgesic, Antidiabatic	Anand and Gokulakrishnan 2012) [1]
11	11.98	Hexadecenoic acid,	Antioxidant, Hypocholesterolemic Nematicide,Pesticide, Lubricant, Antiandrogenic, Flavor, Hemolytic	Anand and Gokulakrishnan (2012) [1]; Brintha *et al*., (2017) [2]; Velayuthum *et al.* (2015) [21]
12	12.72	Milbemycin b	Antimicrobial.	Saravanan *et al.* (2014) [13]
13	14.37	9,10-Secocholesta-	Anticancer, Cocaine analogue	Sasikala and Mohan (2014) [14]
14	16.71	Lycoxanthin	Antibronchitic, Anticoronary, antioxidant	Saravanan *et al.* (2014) [13]
15	18.68	Cyclopropanenonanoic acid	Anticephalopathic, Antihepatotic, Sweetener	Velayuthum *et al.* (2015) [21]
16	24.4	Docosanoic acid	Pesticide, Herbicide, Insecticide, Pheromone Antibacterial, Antioxidant, Antiviral, Candidcide	Anand and Gokulakrishnan (2012) [1]; Velayuthum *et al.* (2015) [21]
17	32.33	Stigmasterol	Antidiabetic, anticancerous.	Saravanan *et al.* (2014) [13]

Conclusion

Phytochemicals produced by *Hybanthus* and its cell culture under *in vitro* conditions shows compositional variation. GC-MS analysis of dried plant and the cell extract revealed that both the samples produced therapeutically important compounds; however dried plant revealed the presence of more number of phytochemicals of therapeutic significance than the cell extract. Stigmasterol which is the precursor or an intermeidate in the synthesis of reproductive hormones is present only in dried plant. The presence of stigmasterol in higher concentration may contribute towards the aphrodisiac properties of Hybanthus.

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