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PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF *HOMALIUM ZEYLANICUM* BENTH.

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ABSTRACT

Homalium zeylanicum is a rare and phytochemically less explored tropical tree. Chemical examination of bark and leaf of this species yielded phenolic compounds 3-Phenyl coumarin (1) and 4'-O-Methyl-Epigallo catechin (2). The structures of the compounds were established on the basis of various NMR spectral data. The extracts of bark, leaf and their isolated compounds showed both antioxidant and anticancer activities in *in vitro* mode. The acetate derivative of compound 2 showed significant anticancer activity against human cancer cell line IMR-32 (IC_{50} 6.26 µg/ml) in the MTT assay.

KEYWORDS: ***Homalium zeylanicum,*** **Antioxidant activity, Anticancer activity, Phenolic compounds.**

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INTRODUCTION

There are about 93 genera and about 1300 species in the family Flacourtiaceae, mainly distributed in the tropics. The genus *Homalium* consists of about 180 species distributed all over the world. *Homalium zeylanicum* Benth. is a rare tree growing up to 25 meters tall and mostly found in the Western Ghats of India and Srilanka. In our continuing activity of phytochemical exploration and their biological activity screening, leaf and bark of *H. zeylanicum* was studied. Previous phytochemical studies on this species reported phytochemicals like coumarins,[1-4] alkaloids[5], Triterpenoids[6] etc. In continuation to our phytochemical exploration of medicinal plants of Deccan region[7,8] we report the isolation and identification of a coumarin 1 and epigallocatechin 2 from the leaves and bark of *H. zeylanicum* for the first time and also report the antioxidant and anticancer profiles of various extracts and isolated compounds.

MATERIALS AND METHODS

(i) General procedure

NMR spectra were recorded on Burker Avance 300 MHz in $CDCl_3$ and Pyridine-*d5* using TMS as internal standard. Electron impact (EI) and chemical ionization mass spectra were recorded on a VG Micro mass model 7070H instrument. All the fractions were monitored on silica gel precoated TLC plates of Merck and spots were visualization observed under UV (254 nm) and also by Anisaldehyde-sulphuric acid spray reagent heating at 110^0C. Silica gel (100-200 mesh) used for column chromatography was procured from Merck.

(ii) Collection plant material

The fresh leaf and bark of *H. zeylanicum* were collected at Tirumala hills of Chittoor district in Andhra Pradesh, India during February 2012 and identified by Dr. K. Madhava Chetty, Plant Taxonomist at Department of Botany, S.V. University, Tirupati, Andhra Pradesh, India. A voucher specimen (No. CIMAP-HZ/2/12) has been deposited at CIMAP, Research Centre, Hyderabad. India.

(iii) Extraction and isolation

Air dried leaves and bark of *H. zeylanicum*, 1.0 Kg each, were powdered and successively extracted with solvents hexane, ethylacetate and methanol by using soxhlet apparatus. All the leaf extracts were concentrated at reduced pressure to get crude residues of 10 gm in hexane, 40 gm in ethyl acetate and 150 gm in methanol respectively. Similarly all the bark extracts were concentrated at reduced pressure to get crude residues of 12 gm in hexane, 50 gm in ethyl acetate and 170 gm in methanol respectively. Leaf extracts were subjected to phytochemical separations by column chromatography using silica gel 100-200 mesh size. Ethylacetate extract yielded Stigmasterol (60 mg) in 30% ethylacetate in hexane where as methanol extract yielded 3-phenyl coumarin (1, 60 mg) in 20% ethylacetate in hexane respectively. Similarly Bark extracts were subjected to phytochemical separations by column chromatography using silica gel 100-200 mesh size. Ethylacetate extract yielded Stigmasterol (80 mg) in 30% ethylacetate in hexane and 4'-O-methyl epigallocatechin (2, 100 mg) in 50% ethylacetate in hexane respectively. Compound 2 was acetylated using pyridine/acetic anhydride to get a penta acetate derivative 3. Structures of the isolated compounds elucidated on the basis of spectroscopy.

Acetylation of Compund 2

4'-O-methyl epigallocatechin (20 mg) was dissolved in pyridine and to this acetic anhydride was added slowly while stirring at room temperature under nitrogen atmosphere for 12 h. Later, the solvent was removed under reduced pressure and the residue was diluted with distilled water and extracted thrice with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to get the final product. The crude products were purified by column chromatography with ethyl acetate in

hexane gave a pure compound (10 mg) 3 as a colorless amorphous powder.

(iv) Determination of Total phenolics

The total phenolic content of all extracts were determined as per the reported Folin–Ciocalteu method[9]. An aliquot (1 ml) of extracts and standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to a 25 ml volumetric flask, containing 9 ml of distilled deionised water. Then, 1 ml of Folin-Ciocalteu reagent was added to the mixture. After 5 min, 10 ml of 7% Na_2CO_3 was added. The volume of the solution was adjusted with deionised water. The reaction mixture was incubated at 24°C for 90 min and absorbance of mixtures was measured at 750 nm against blank. The values of total phenolics in all extracts were estimated by comparing the absorbance of each with those of a standard response curve generated with gallic acid and the total phenolic content was expressed as gallic acid equivalents per mg of extract.

(v) Determination of Total flavonoids

Total flavonoid content was measured in all extracts by the aluminium chloride colorimetric assay[9]. An aliquot of extracts and standard solution of quercetin (20, 40, 60, 80 and 100 mg/L) was added to a 10 ml volumetric flask, containing 4 ml of distilled deionised water. To this flask 0.3 ml of 5% sodium nitrate ($NaNO_2$) was added and after 5 min, 0.3 ml of 10% aluminium chloride was added. At 6^{th} min, 2 ml of 1 M NaOH was added and total volume adjusted to 10 ml with distilled deionised water. The solution was mixed well and the absorbance was measured at 510 nm against blank. The values of total flavonoids in all extracts were estimated by comparing the absorbance of each with those of a standard response curve generated with quercetin and the total flavonoid content was expressed as quercetin equivalents per mg of extract.

(vi) Biological activities

Cancer is an uncontrolled cell proliferation and it is a serious disease to the human today. Free radicals generated in the body are one of the important reasons that cause cancer. hence researches on the anticancer and antioxidant compounds from natural sources are getting importance in the drug discovery point of view.

Antioxidant activity

Radical scavenging on DPPH reduction[10] and hydrogen peroxide assay[11] methods were employed to evaluate antioxidant activity of all the extracts and isolated compounds. The antioxidant values of the extracts as well as isolated compounds are summarized in Table 2 & 3.

Anticancer activity

In addition to radical scavenging activity, cytotoxic activity was also tested for the extracts and isolated compounds using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay[12]. Three human cancer cell lines IMR-32 (neuroblastoma cell line), MCF-7 (breast adinocarcinoma cell line) and Hep-G2 (hepatoma cell lines) were used. The assay was dependent on the reduction of the tetrazolium salt MTT (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product dissolved in DMSO and measured at 570 nm. The percentage cell inhibition was determined using the reported method.

RESULTS AND DISCUSSION

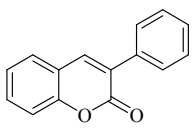
(i) Chemistry

Chromatographic partition of the ethyl acetate extract (40 g) of dry leaf (1.0 kg) *H. zeylanicum* on silica gel using n-hexane/ethylacetate mixtures of increasing polarity, led to the isolation of the 3-phenyl coumarin (1). Similarly Chromatographic partition of the ethyl acetate extract (50 g) of dry bark (1.0 kg) *H. zeylanicum* on silica gel using n-hexane/ethylacetate mixtures of increasing polarity, led to the isolation of the stigmasterol and the 4'-O-methyl epigallocatechin (2). Compound 2 was acetylated using pyridine/acetic anhydride to get a penta acetate derivative (3). The structure of the isolated compounds established on the basis of spectroscopic analysis and by comparison with previously reported data. Based on the physical, chemical and spectroscopic properties and

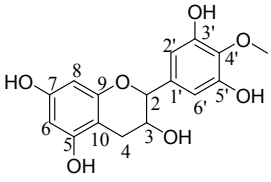
previous reported literature, compound 1 is a coumarin, was confirmed as 3-Phenyl coumarin. Compound-2 was obtained white amorphous powder. The compound showed a molecular ion peak at *m/z* 321 [M+H]+ in ESI-MS spectrum. ^{13}C – NMR spectrum measured in Deuterated CD_3OD displayed 16 carbons. Based on DEPT spectrum it has the presence of eight quaternary, six tertiary, one secondary and one primary carbon. In addition to ^{1}H and ^{13}C NMR (Table 1) a cross peak between C-4' and the methyl protons were observed in the heteroneuclear multiple bond connectivity experiment. This compound can easily be identified by the characteristic signals arising from H-3 (3.80-5.20 ppm) and H-4 (2.50-3.10 ppm) of the heterocyclic ring, which have cross correlated in Cosy spectra. The only cross peak associated with C-3' is between C-3' and H-2'. This further confirmed that the methyl group is connected to C-4' but not C-3'. These relations were confirmed by the HMBC spectral correlation. According to COSY and HMBC correlations, the structure was confirmed as 4'-O-Methyl-Epigallo catechin. Compounds 1 and 2 were isolated for the first time from this plant.The structure of the acetate derivative 3 was elucidated on the basis of 1H-NMR as 4'-O-methyl epigallocatechin penta acetate. The spectrum displayed a bunch of five methyl signals resonating between δ2.1 to δ2.4 ppm, integrating for 15 protons, indicates the presence of five hydroxyls in the parent compound 2. The Structures are shown in figure 1.

Table 1
^{1}H and ^{13}C- NMR data of compounds 1 and 2

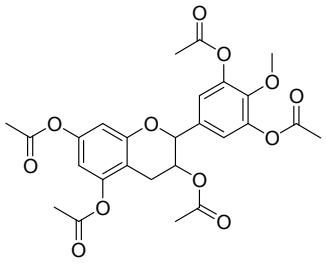
Compound-1				Compound-2			
Position	**^{1}H ppm**	**^{13}C NMR**	**Nature of carbon**	**Position**	**^{1}H ppm**	**^{13}C NMR**	**Nature of carbon**
2	-------	162.70	-C-	2	4.74	79.68	CH
3	-------	154.09	-C-	3	4.10	67.40	CH
4	6.89	102.22	CH	4	2.81	29.15	CH_2
5	-------	137.95	-C-	5	-------	157.15	-C-
6	8.23	130.10	CH	6	5.86	95.92	CH
7	7.38	135.27	CH	7	-------	157.95	-C-
8	7.46	128.56	CH	8	5.83	96.49	CH
9	8.26	130.38	CH	9	-------	157.67	-C-
10	-------	121.00	-C-	10	-------	100.10	-C-
11	-------	132.40	-C-	1'	-------	136.11	-C-
12	7.83	125.68	CH	2'	6.44	107.21	CH
13	7.41	129.24	CH	3'	-------	151.34	-C-
14	7.44	126.37	CH	4'	-------	136.56	-C-
15	7.65	129.24	CH	5'	--------	151.34	-C-
16	7.81	125.68	CH	6'	-------	107.21	CH
				$-OCH_3$	3.70	60.77	CH_3



3-Phenyl Coumarin **1**



4'-O-Methyl-Epigallo catechin **2**



4'-O-methyl epigallocatechin penta acetate **3**

Figure 1
Chemical structures of compounds 1, 2 and 3

(ii) Determination of Total phenolics

The total phenolic content in the leaf extracts of hexane, ethylacetate and methanol (1 mg) were equivalent to 17.21, 49.03 and 45.32 µg/ml and that of bark extracts were 39.95, 70.23 and 90.01 µg/ml of gallic acid respectively.

(iii) Determination of Total flavonoids

The total flavonoid content in the leaf extracts of hexane, ethylacetate and methanol (1 mg) were equivalent to 2.23, 7.01 and 9.10 µg/ml and that of bark extracts were 3.62, 9.32 and 9.95 µg/ml of quercetin respectively.

(iv) Biological activities

Antioxidant activity

By using DPPH radical scavenging assay, methanol extract of bark, compound 2 and ethylacetate extract of leaf were showed to be the most active. By using hydrogen peroxide assay methanol extract of bark and ethylacetate extract of leaf were the most active respectively (Tab-2 & 3).

Table 2

Effect of H. zeylanicum Bark extracts and isolated compounds in the antioxidant assay

Conc. (µg/ml)	% Inhibition by DPPH				% Inhibition by H_2O_2			
	AA	EA	Me	2	AA	EA	Me	2
5	75.27 ± 0.66	75.57 ± 0.79	77.54 ± 0.94	36.26 ± 1.20	73.51 ± 0.06	20.12 ± 0.92	40.25 ± 1.32	32.68 ± 0.84
10	75.60 ± 0.81	77.68 ± 0.80	78.56 ± 1.16	38.39 ± 0.76	78.60 ± 0.07	36.54 ± 1.02	45.56 ± 0.58	48.69 ± 1.23
25	76.67 ± 0.94	78.14 ± 0.76	79.92 ± 0.35	55.49 ± 0.87	79.35 ± 0.86	70.58 ± 0.62	71.95 ± 1.34	69.59 ± 1.56
50	79.30 ± 0.85	78.28 ± 0.35	80.53 ± 0.89	68.51 ± 0.67	80.26 ± 0.83	76.24 ± 1.23	72.86 ± 0.83	70.58 ± 0.89
125	81.38 ± 1.07	79.37 ± 0.36	82.21 ± 0.37	76.91 ± 1.38	82.51 ± 1.07	78.59 ± 1.43	78.97 ± 0.87	75.91 ± 1.23
250	81.76 ± 1.16	80.6 ± 0.35	83.26 ± 0.34	77.63 ± 1.00	84.18 ± 1.13	80.12 ± 1.48	81.58 ± 1.21	79.68 ± 1.54
IC_{50} µg/ml	8.13	8.56	8.129	6.01	31.25	27.53	26.83	43.62

AA- Ascorbic Acid, He-Hexane; EA- Ethyl acetate; Me-Methanol.

Table 3

Antioxidant Activity of H. zeylanicum Leaf

Conc. (µg/ml)	% Inhibition by DPPH			% Inhibition by H_2O_2		
	AA	EA	Me	AA	EA	Me
5	75.27 ± 0.66	22.27 ± 0.82	40.56 ± 1.29	73.51 ± 0.06	23.23 ± 0.93	41.65 ± 1.32
10	75.60 ± 0.81	34.86 ± 1.00	59.57 ± 0.78	78.60 ± 0.07	38.54 ± 1.02	56.28 ± 0.58
25	76.67 ± 0.94	72.35 ± 0.78	72.55 ± 1.38	79.35 ± 0.86	72.69 ± 0.62	73.89 ± 1.34
50	79.30 ± 0.85	75.50 ± 1.20	73.16 ± 0.92	80.26 ± 0.83	75.58 ± 1.23	75.61 ± 0.83
125	81.38 ± 1.07	76.57 ± 1.08	74.63 ± 0.95	82.51 ± 1.07	79.96 ± 1.43	78.24 ± 0.87
250	81.76 ± 1.16	78.23 ± 1.01	75.53 ± 1.20	84.18 ± 1.13	81.28 ± 1.48	81.96 ± 1.21
IC_{50}	8.13	6.01	8.25	31.25	30.85	39.12

AA- Ascorbic Acid, He-Hexane; EA- Ethyl acetate; Me-Methanol.

Anticancer activity

Although the extracts and their isolated compounds of bark and leaf of *H. zeylanicum* showed *in vitro* anticancer activity but, Ethyl acetate extract of leaf and Hexane extract of bark showed

potential cytotoxicity against IMR-32 cell lines (IC_{50} 1.76 & 4.21 µg/ml). Similarly, compound **3** showed potential cytotoxicity against IMR-32 cell lines (IC_{50} 6.26 µg/ml) when compared with its parent compound 2 (IC_{50} 32.2 µg/m) (Tab-4).

Table 4
Anticancer activity of extracts of leaf, bark and their isolated compounds

Cell lines	Leaf Extracts IC_{50} µg/ml			Bark Extracts IC_{50} µg/ml			Compounds IC_{50} µg/ml			Doxorubicin **µM/ml**
	He	EA	Me	He	EA	Me	1	2	3	
MCF-7	39.23	16.14	60.27	17.25	99.34	64.22	87.50	18.41	16.39	0.84
IMR-32	52.06	1.76	245.42	4.21	72.36	78.3	17.46	32.2	6.26	0.80
Hep-G2	34.23	17.18	89.21	33.46	66.16	102.32	59.43	45.36	25.10	0.60

He-Hexane; EA- Ethyl acetate; Me-Methanol.

SUMMARY AND CONCLUSION

Chromatographic fractionation of *H. zeylanicum* leaf and bark resulted 2 compounds; compounds 1 and 2 are first time isolated from this plant along with well known compound stigmasterol. The n-hexane and ethyl acetate extracts and its isolated compounds screened for total phenolic content, total flavonoid content, antioxidant and anticancer activities. The leaf ethyl acetate extract and bark methanol extracts showed more total phenolic content and methanol extracts of both parts of the plant showed good total flavonoid content. Methanol extract of bark, compound 2 and ethylacetate extract of leaf were showed to be the most antioxidant activity in DPPH radical scavenging assay. By using hydrogen peroxide assay methanol extract of bark and ethylacetate extract of leaf were the most active. Although the extracts and their isolated compounds showed *in vitro* anticancer activity but, Ethyl acetate extract of leaf and Hexane extract of bark showed potential cytotoxicity against IMR-32 cell lines (IC_{50} 1.76 & 4.21 µg/ml). Similarly, compound 3 showed potential cytotoxicity against IMR-32 cell lines (IC_{50} 6.26 µg/ml) when compared with its parent compound 2 (IC_{50} 32.2 µg/m) which may have further therapeutic value.

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