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GC-MS Profile and HPTLC Analysis of Leaf Extract of Canthium dicoccum (Gaertn.) Teijsm and Binn

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Abstract
Canthium dicoccum is a shrub belongs to the family Rubiaceae. Ethnomedicine has evident the medicinal properties of different parts of this plant. During the present investigation, qualitative phytochemical analysis was carried out on the leaf extract of *C. dicoccum* and the results revealed the presence of carbohydrates, glycosides, saponins, alkaloids, phenols, flavonoids, tannins, steroids and volatile oils. HPTLC analysis showed the presence of useful compound *i.e.*, Beta sitosterol. Further, investigation is been focused on the separation of phytochemical constituents by GC-MS technique using Perkin Elmer gas chromatogram and mass spectrometer. The mass spectra of the compounds found in three different extracts (Methanol, chloroform and petroleum ether) were matched with NIST 11 and Wiley 8 library. Sixteen compounds were identified in the methanolic extract, twenty four in petroleum ether and twenty in chloroform extract respectively. These compounds may be medicinally valuable and have wide applications in the pharmaceutical industry. The identified compound needs further research on toxicological aspects to develop safe drugs.

Keywords: *Canthium dicoccum*, leaf extracts, Phytochemicals, HPTLC, GC-MS analysis

1. Introduction
India is one of the richest mega biodiversity nations in the world. In India, many infectious diseases are still alarming and creating health problems. Variety of bacterial etiologic agents, such as pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Shigella* sp., and *Enterobacter* sp. are among the most common [11]. The medicinal plants are widely used by the traditional medical practitioner for curing various diseases in their day by day practice [4]. Plant derived medicine has made largest contribution to human health and well-being all over the world. Today, most of the world population depends upon plant based drugs for their primary health care needs. World Health Organization (WHO) estimates that 80% of the people living in developing countries almost extensively use traditional medicine. New ethno-pharmacological approach provides the way for inventing new drugs from the plant resources [10]. The basic medicinal properties of plants lies in due to the presence of chemical substances. These chemical substances produce a definite physiological action on human body which is generally known as phytochemicals [5]. *Canthium dicoccum* synonym *Psydrax dicoccos* commonly called 'Ceylon box wood' or 'malakafe', a species of angiosperm plant belongs to the family Rubiaceae. This plant is found in Deccan peninsula, Maharashtra, Assam, Bihar, Meghalaya and Western Ghats of India. It is an unarmed shrub or medium sized tree grows up to 10 mts. The plant possesses antipyretic activity and in India, bark powder boiled with sesame oil and used for treating rheumatic pains, febrifuge and also applied as plasters [3].
Rajarajeswari *et al.,* (2011) [7] reported the presence of nineteen bio-active compounds in the ethanolic leaf extract of *Canthium dicoccum* through GC-MS analysis. But the perusal of literature revealed that no references are available on *Canthium dicoccum* leaf extract except the work of Rajarajeswari *et al*. Hence the objective of this study was to identify and characterize the bioactive compounds using three different solvent extracts (methanol, chloroform and petroleum ether) of the leaf of *C. dicoccum* through phytochemical evaluation, HPTLC and GC-MS analysis.

2. Materials and Methods
2.1 Collection of plant material
Frequent field trips were undertaken to collect *Canthium dicoccum* from Sri Malai Mahadeshwara Hills, Kollegal taluk, Chamarajanagar district, Karnataka**.** The collected plant materials were brought to the laboratory, Department of Botany, Bangalore University, Bangalore and the material was identified with the help of Gamble flora [2] and authenticated by Shivanand S. Bhat, Govt. first grade college, Karwar. The leaves of *Canthium dicoccum* were removed off, washed with running tap water to remove the dust and other soil particles present on the surface. Later, they were dried under shade for 10-12 days and grounded into coarse fine powder for further analysis.

2.2 Extract preparation
10 g of powdered material was extracted with 50 ml each of methanol, chloroform and petroleum ether and kept in water bath for 4 h at 50° C and filtered after cooling with Whatmann filter paper. The filtrate containing crude extract was transferred to Eppendorf's vials and subjected for phytochemical evaluation, HPTLC and GC-MS analysis.

2.3 HPTLC Analysis
INSTRUMENT: CAMAG High Performance Thin Layer Chromatography System
Comprising of: Applicator **–** Linomat 5, Digistore – 2, Multiwavelength Scanner, Transparent Chromatographic Tank and HPTLC pre-coated silica plate, Silica Gel 60 F254, 10 X 10 cm (Merck**)**

Sample Preparation
1g of leaf extract of Sample of *Canthium dicoccum* was mixed with 50 ml HPTLC grade Methanol and kept in water bath at 70º-80ºC for 30 min after cooling to room temperature the mixture was filtered and concentrated to 5 ml before proceeding to spotting.

Standard Preparation
10mg of Beta-Sitosterol reference standard mixed with 20 ml of Methanol and kept in water bath for 30 min at 70º-80ºC then filtered and concentrated to 5 ml and proceed for spotting.
Mobile phase: Use Upper phase of mixture of Butyl alcohol: Water: Acetic acid [5: 4: 1]

Chromatography
10 ml of (Use Upper phase) mixture of Butyl alcohol: Water: Acetic acid [5: 4: 1] was transferred to chromatographic tank and allowed to saturate for 30 minutes. Later 5 µl of sample(s) and 5 µl Standard (as 10 mm bands separated by a distance of 15 mm; at 10mm from the base) was applied on a HPTLC silica plate using a Linomat HPTLC applicator. The plate was allowed to be in fume hood to let the solvent to evaporate. Thereafter, the plate was placed in the tank as near vertical as possible ensuring that the line of application is well above the solvent level. The lid should be replaced tightly and allowed the solvent to ascent to 1.5cm below the top of the plate. The plate was removed from the tank and let it to air dry in fume hood. Retention factor (Rf) values were calculated by using the following formula.

$$Rf = \frac{\text{Distance travelled by the solvent}}{\text{Distance travelled by the sample}}$$

HPTLC was performed on TLC plate pre-coated with silica gel 60 F254 of thickness 0.2 mm (Merck) of size 10 × 10 cm. Beta sitosterol (1 ml) and samples (5 ml) were applied on the plate as band of 8.0 mm width using Hamilton syringe and CAMAG Linomat V sample applicator. The plate was developed to a distance of 8.0 cm in a CAMAG twin trough chamber previously saturated with mobile phase Butyl alcohol: Water: Acetic acid in the ratio [5: 4: 1] for 30 min. After development, the plate was dried at room temperature and densitometric evaluation was performed at 366 nm in CAMAG TLC scanner 3 linked to WINCATS software. The image of the plate was captured at short range in UV chamber.

2.4 GC –MS ANALYSIS
Instruments and Chromatographic conditions
For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 1 µl of methanolic, chloroform and pet. ether extracts were employed separately. The injector temperature was maintained at 260° C, the ion source temperature 200°C, the oven temperature was programmed for 80°C (isothermal for 4 min.) with an increase of 10°C/min to 200° C, then 5° C/min to 280° C, ending with a 6 min isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 sec. and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 50 min. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

Identification of components
The interpretation on mass spectrum of GC-MS was done using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The relative retention times (Rt) and mass spectra of the extract components were compared with those of authentic samples. The spectra of the compounds are matched with WILEY 8.0 and NIST 11 libraries. The name, area, retention time, area and its percentage, height percentage and base of the components of test materials were ascertained.

3. Results and Discussion
Canthium dicoccum is a moderately growing tree belongs to the family Rubiaceae. Leaves are simple, opposite deccusate, interpetiolar linear stipules with broad base, glabrous beneath, mid rib raised above, secondary nerves 4 – 7 pairs, tertiary nerves obscure, extremely variable, ovate to elliptic, ovate or somewhat rounded, 5 to 15 cm long, 1.5 to 8 cm wide, and usually pointed at both ends, apex acuminate, base cuneate, margin entire coriaceous, shining above. Flowers are white borne on inflorescence axillary cymes with very slender stalks, 5 to 10 mm long, and borne in compressed, short-stalked cymes. Calyx is cut off at the end or obscurely toothed. Corolla is bell-shaped, with a 4 to 6 mm tube, and five somewhat pointed lobes. Fruit is a drupe, ovoid, pedicel 1.5 cm long, rounded, 6 to 10 mm long, slightly flattened and obscurely 2-lobed.
Qualitative phytochemical analysis clearly revealed the presence of eleven different phytoconstituents such as carbohydrates, proteins, glycosides, alkaloids, phenols, flavonoids, tannins, terpenoids, saponins, sterols and volatile oils in the methanolic extract and ten in chloroform extracts and seven in petroleum ether extracts respectively (Table 1). However gums, mucilage and non-volatile oils are absent in methanolic extract while proteins, terpenoids and non-volatile oils are absent in chloroform and in petroleum ether extract six phytoconstituents such as saponins, tannins, terpenoids, volatile oils, non-volatile oils and gums and mucilage were found to be absent. Glycosides, phenols and flavonoids were abundantly found in all the three extracts whereas non-volatile oils were found to be absent.
During the present investigation, HPTLC analysis was carried out for the isolation of useful compounds using β-sitosterol as standard. The chromatogram bands clearly showed the presence of Beta sitosterol. This is recorded for the first time from methanolic leaf extract of *Canthium dicoccum.* The result of the HPTLC analysis for the methanolic leaf extract showed that Beta sitosterol in the extract which was quantified by comparing the total peak area of Beta sitosterol band in standard solution with that of the tested sample (Figure 1).
GC-MS analysis of all the three leaf extracts of *C. dicoccum* revealed the presence of sixteen compounds in methanol, twenty in chloroform and twenty four in petroleum ether respectively were characterized and identified (Table 2, 3 & 4). The retention time (RT) are in minutes and the

chromatogram along with the peak are represented in figures 2, 3 & 4. The most common chemical compound identified in all the three extracts was Decanoic acid. However alpha, delta and gamma Tocopherol were found in methanol and petroleum ether extracts. The derivatives of decanoic acid such as tri, hexa, hepta and octadecanoic acids were recorded in chloroform extract, while hepta and dodecanoic acid in the extract of petroleum ether and tetra decanoic acid in methanolic extract.

Vinnarasi *et al.,* (2018) [11] recorded the presence of same phytoconstituents such as saponins, tannins, flavonoids, alkaloids, carbohydrates, phenolic compounds. Sathyanarayana (2017) [9] conducted preliminary phytochemical screening and antioxidant activity in *Canthium dicoccum.* Their studies revealed that all the three extracts such as petroleum ether, Ethyl acetate and aqueous extract showed the presence of carbohydrates, glycosides, saponins, alkaloids, proteins, amino acids, phyto-sterols, triterpenoids, phenolic compounds and tannins. They further concluded that this plant has good bioactive compounds hence it can be used as potential source of natural antioxidants and pharmaceuticals. During the present investigation the methanolic leaf extract of *Canthium dicoccum* showed the presence of carbohydrates, proteins, glycosides, alkaloids, phenols, flavonoids, tannins, steroids and volatile oils. Our results are in conformity with the work of Sathyanarayana. Therefore this plant can be used for various herbal drug formulations**.**

Rajarajeswari *et al.,* (2011) [7] reported the occurrence of ninteen phytoconstituents in the ethanolic leaf extract of *C. dicoccum* whereas in our work the three different solvents (methanol, chloroform and petroleum ether) of leaf extracts revealed the presence of sixteen, twenty and twenty four compounds respectively.

The work of Prabhu *et al.,* (2013) [6] on the ethanolic leaf extract of *Canthium parviflorum* recorded twenty two compounds through GC-MS analysis. Phytol is one of the most important phytoconstituent having highest peak and it is in conformity with the present work while gamma-stigmasterol was second peak constituent in their work and in our work alpha, delta and gamma Tocopherol were recorded for the first time.

The methanolic leaf extract of *B. montanum* was analyzed by GC-MS and the chromatogram showed the presence of fifteen compounds [1] while Rajanna and Santhosh kumar (2018) [8] reported the presence of eighteen compounds of medicinal value through GC-MS profile of bark extract of *S. auriculata*.

Table 1: Qualitative phytochemical analysis of leaves extract of *C. dicoccum*

Sl. No.	Phytoconstituents	Methanol	Chloroform	Petroleum ether
1	Carbohydrates	+	++	+
2	Proteins	+	-	+
3	Glycosides	+++	++	++
4	Saponins	+	+	-
5	Alkaloids	+	++	+
6	Phenols	++	++	+
7	Flavanoids	+++	+++	+++
8	Terpenoids	+	-	-
9	Tannins	++	+	-
10	Steroids	++	+	+
11	Volatile Oils	++	+	-
12	Non Volatile Oils	-	-	-
13	Gums and Mucilage	-	+	-

+: low or sparingly; ++: moderate; +++: high

A – Beta Sitostreol Ref STD, B – Sample-*C. dicocum*.

Fig 1: HPTLC chromatogram of *Canthium dicoccum* methanolic leaf extract

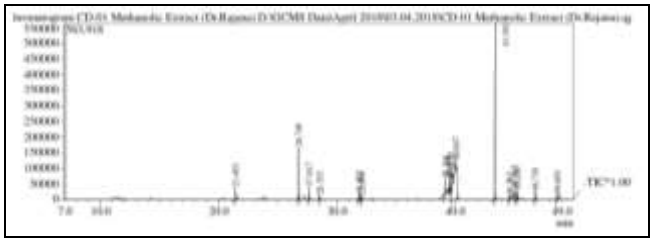

Fig 2: GC-MS chromatogram of methanolic leaf extract of *C. dicoccum*

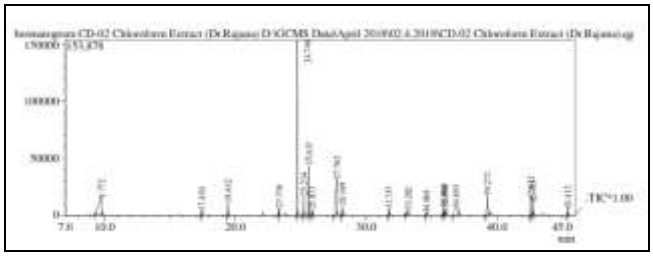

Fig 3: GC-MS chromatogram of chloroform leaf extract of *C. dicoccum*

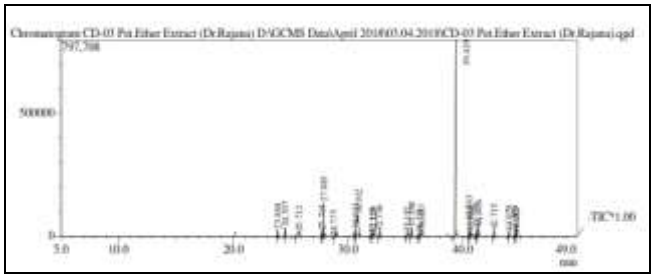

Fig 4: GC-MS chromatogram of petroleum ether leaf extract of *C. dicoccum*

Table 2: Phytochemical compounds found in the Methanolic extract

Sl. No.	R. Time	Area	Area%	Height	Height%	Name	Base m/z
1	21.403	159688	2.59	41059	3.30	Cyclopenta[C]pyran-4-carboxylic acid, 7-methyl-, methyl ester	190.00
2	26.749	395696	6.41	169783	13.64	Neophytadiene	68.05
3	27.617	87816	1.42	44031	3.54	6-OCTEN-1-OL, 3,7-DIMETHYL-, PROPANOATE	82.10
4	28.535	35877	0.58	16949	1.36	TETRADECANOI ACID, 12-METHYL-, METHYL ESTER	74.00
5	31.852	48150	0.78	20091	1.61	6(E),9(Z), 13(E)-PENDECTRIENE	79.05
6	32.084	31402	0.51	13959	1.12	(E)-PHYTOL	71.05
7	39.168	193821	3.14	50588	4.07	1,2-BENZENEDICARBOXYLIC ACID	149.00
8	39.550	153237	2.19	43050	3.46	19-D-TORULOSOL	55.05
9	39.617	113287	1.84	36332	2.92	alpha.-Amyrin	149.15
10	40.047	2634977	42.72	125035	10.05	Lupeol	95.10
11	43.381	1913610	31.02	563918	45.32	Squalene	69.10
12	44.563	42461	0.69	14197	1.14	1-(2-HYDROXYETHOXY)TRIDECANE	57.05
13	45.015	126892	2.06	28673	2.30	SPIRO[BICYCLO[5.1.0]OCTANE-3,2'-OXIRAN]-2-ONE,8-ACETYL-6,6,7-TRIMETHYL-	69.05
14	45.243	88947	1.44	31701	2.55	DELTA.-TOCOPHEROL	137.05
15	46.738	89182	1.45	26655	2.14	gamma.-Tocopherol	151.10
16	48.603	71477	1.16	18377	1.48	(+)-.ALPHA._TOCOPHEROL	165.10
		6168520	100.00	1244398	100.00		

Table 3: Phytochemical compounds found in the Chloroform extract

Sl. No.	R time	Area	Area %	Height	H %	Name	Base m/z
1	9.722	229687	13.94	14957	3.94	Be%&o'ami%e//6()4*(ami%o(2*(meth+,(5*(p+rimi i%+,.(3(/e%zo+,thio(5(&orm+,(4(meth+,(5(aza(3(he0e%+, -ih+-roge%ephosphate	105.05
2	17.430	12115	0.74	4503	1.19	2(TERT(BUTYL(4((1,1,3,3(TETRAMETHYLBUTYL)PHENOL 191.10	191.10
3	19.432	30783	1.87	11178	2.94	C+c,ope%ta)c.p+ra%(4(car/o0+,ic aci-, 7(meth+,(, meth+, ester	190.00
4	23.336	17302	1.05	6716	1.77	TRIDECANOIC ACID 11	73.00
5	24.746	389573	23.64	153878	40.53	Neoph+ta-ie%e	68.05
6	25.234	29552	1.79	17467	4.60	Bic+c,o) 4.1.0.hepta%e, 2 (meth+,(	81.05
7	25.613	94267	5.72	43950	11.58	1(HEXADECYNE	82.05
8	25.873	10969	0.67	5467	1.44	2,7(OCTADIENIOL ACETATE	79.05
9	27.762	280967	17.05	31823	8.38	HEXADECANOIC ACID	73.00
10	28.169	29496	1.79	8788	2.31	HEPTADECANOIC ACID, ETHYL ESTER	88.05
11	31.737	21448	1.30	5657	1.49	3(HYDROXY(4,4(DIMETHYLDIHYDRO(2(13C)FURAN(2(ONE	71.05
12	33.202	43965	2.67	4592	1.21	OXACYCLOHEXADEC(11(YNE(2,5(DIONE	79.10
13	34.604	8039	0.49	2439	0.64	N(ALLYLOXYMETHYLACRYLAMIDE	55.05
14	35.960	26334	1.60	6879	1.81	1((BETA(HYDROXYETHOXY)(2(BUTYNE	71.05
15	36.008	31882	1.93	5996	1.58	EICOSYL ACETATE	55.05
16	36.893	123075	7.47	6771	1.78	4,8(DIMETHYL(3(E),7(NONADIENYL THIOACETATE	69.05
17	39.272	104634	6.35	17570	4.63	CYCLOHEXANOL, FORMATE	82.10
18	42.612	113749	6.90	14217	3.74	2(OCTYLDODECAN(1(OL	57.05
19	42.755	33478	2.03	10780	2.84	1(METHYL(2(CARBOXALDEHYDE(3(1(CARBOXALDEHYDE)(ETHENYL(CYCLOPENTANE	55.05
20	45.412	16403	1.00	6065	1.60	1((2(HYDROXYETHOXY)TRIDECANE	57.05
		1647718	100.00	379693	100.00		

Table 4: Phytochemical compounds found in the Petroleum ether extract

Sl. No.	R time	Area	Area %	Height	H %	Name	Base m/z
1	23.888	64173	0.77	30716	1.99	METHYL 8,9-EPOXYEICOSAN-5(Z),11(Z),14(Z)-TRIENOATE	79.05
2	24.537	82811	1.00	40098	2.59	HEPTADECANOIC ACID, METHYL ESTER	74.05
3	25.712	179784	2.17	19826	1.28	DECANOIC ACID	60.00
4	27.745	50744	0.61	23647	1.53	1-UNDECYNE	67.05
5	27.885	362597	4.37	140622	9.10	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER, (Z,Z,Z)-	79.05
6	28.775	76629	0.92	2304	0.15	3-CHLOROBICYCLO[2.2.0]HEX-2-ONE	55.05
7	30.633	119180	1.44	25571	1.65	UNKNOWN TERPENE IN GRAPES	69.05
8	30.932	1177330	14.19	89033	5.76	(5Z)-2,6,10-TRIMETHYL-1,5,9-UNDECATRIENE #	69.05
9	32.110	100905	1.22	14137	0.91	16-Heptadecenal	68.05
10	32.223	93752	1.13	12366	0.80	NITRIC ACID, NONYL ESTER	57.05
11	32.736	66088	0.80	21551	1.39	Dodecanoic acid, 2-hexen-1-yl ester	82.10
12	35.152	94457	1.14	16665	1.08	1-(2-HYDROXYETHOXY)TRIDECANE	57.05
13	35.548	92604	1.12	36428	2.36	6(E),9(Z),13(E)-PENDECTRIENE	55.05
14	36.191	57293	0.69	12187	0.79	.delta.-Tocopherol, O-acetyl-	137.10
15	36.447	244825	2.95	25895	1.68	(+)-.gamma.-Tocopherol, O-methyl-	165.10
16	39.429	4515164	54.42	797708	51.61	Squalene	69.10
17	40.563	175890	2.12	61528	3.98	TETRACOSANE	57.05
18	40.668	74248	0.89	20640	1.34	2-OCTYLDODECAN-1-OL	57.05
19	41.227	105095	1.27	32867	2.13	DELTA.-TOCOPHEROL	137.10
20	41.376	257137	3.10	43077	2.79	14-Heptadecenal	57.05
21	42.715	92599	1.12	28117	1.82	.gamma.-Tocopherol	151.10
22	44.029	75565	0.91	20152	1.30	DOCOSANE	57.05
23	44.569	96020	1.16	20211	1.31	(+)-.ALPHA.-TOCOPHEROL	165.10
24	44.682	42093	0.51	10441	0.68	15-Oxabicyclo[12.1.0]pentadeca-6,10-diene-7-methanol,5-hydroxy-1,11-dimethyl-4-(1-methylethenyl)-	81.10
		8296983	100.00	1545787	100.00		

4. Conclusion

During the present investigation, GC-MS analysis clearly revealed the presence of sixteen compounds in the methanolic extract, twenty four in petroleum ether and twenty in chloroform extract respectively. HPTLC results confirmed the presence of Beta sitosterol in the methanolic leaf extract. These compounds may be medicinally valuable and have wide applications in the pharmaceutical industry. The identified compound needs further research on toxicological aspects to develop safe drugs.

5. Acknowledgment

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