The Study of the Comparative Chemical Profile of Polar and Nonpolar Fractions of Cinnamomum Tamala Leaves by Chromatography and Spectroscopic Methods

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Abstract

Cinnamomum tamala (CT) is a member of the Lauraceae family, and its leaves are most popularly used as a spice in Indian food. Along with spice, it is associated with many medicinal values such as antioxidant, antidiabetic, antifungal, immunomodulation, etc. Here, we made a study about the qualitative analysis of different phytochemicals in different fractions of CT leaves, along with their TLC fingerprinting. Based on GC-MS analysis of polar and nonpolar fractions, Eugenol was reported as an active constituent that is present in excess in nonpolar fraction. Further, eugenol was analysed by High Performance Liquid Chromatography (HPLC) using a C-18 column. The results showed that the distribution of different phytochemicals varied among the different fractions, with the maximum amount of eugenol found in the non-polar fraction (Hexane fraction) and then in the total methanol fraction.

Keywords: Cinnamomum tamala, HPLC, GC-MS, Phytochemicals, Eugenol, Thin layer chromatography.

Introduction

Cinnamomum tamala (Lauraceae), is a component of Indian spices, used as medicine in Indian Medicine. The plant is grown especially in Himalaya region, Chemically, Terpenes and flavones are the main constituent of the plant leaves. According to previous report, Monoterpenes containing trans-sabinene hydrate, (Z)-β-ocimene, myrcene, α-pinene, β-sabinene and sesquiterpenes containing germacrene A, eugenol and α-gurjunene as major constituent (Mir et al., 2004) and flavones (Bhardwaj et al., 1983) such as Kaempferol, Quercetin and its derivatives. The reported flavones are Kaempferol and its derivatives such as Kaempferol-3-O- rhamnoside, Kaempferol-3-O-glucopyranoside, Kaempferol-3-O-sophoroside and Kaempferol 3,7-di-O-rhamnopyranoside. Other flavones are Quercetin, Quercetrin, Myricetin, 3,4,5,7-tetrahydroxy flavone and 3,3,4,5,7-pentahydroxyflavone.

The leaves are reported with antioxidant, antidiabetic (Chaurasia et al, 2011, 2013), antifungal (Ruberto et al., 2000), antidiarrheal (Rao et al., 2008), immunosuppressive property (Chaurasia et al., 2010). According to GC-MS report, Eugenol is main constituent of the CT leaves and all most property of the plant might be due to the component. Here, we have isolated different fractions of CT leaves and characterized them in terms of qualitative analysis of the

phytochemicals, their Thin Layer Chromatography (TLC) fingerprint and estimation of eugenol by HPLC methods.

Materials And Methods

Normal as well as HPLC grade solvents such as Hexane, Ethyl Acetate, Methanol, and Silica gels for TLC was purchased from Qualigens Fine Chemicals. Other chemicals are analytical grades. Eugenol was purchased from Sigma USA. The leaves of Cinnamomum tamala (CT) were purchased from local market and its authenticity was checked on pharmacogonostical parameters. It was dried on sun light and coarse powder was made. This powder was filled in a thimble of blotting paper and put in soxhlet for extraction. For the preparation of different extract the powder was extracted with hexane, ethyl acetate and methanol in successive manner in soxhlet extractor for 30 h and for the extraction in methanol, another CT powder in thimble was extracted in methanol for 30 hrs separately. The solutions thus obtained were distilled under reduced pressure and finally dried on water bath and desiccated until constant weight gain. Thus 4 types of solvent free extracts were made and abbreviated as: 1-Total methanolic fraction- (CTT), 2-Successive extracted hexane fraction- (CTH), 3-Successive ethyl acetate fraction (CTEA) and 4-Successive methanolic fraction (CTM). These extracts were dissolved in desired solvent @ 1 mg/ml and used as per requirements. For HPLC analysis, the extracts were dissolved in HPLC grade solvents diluted as per requirements.

Phytochemical analysis

Test of Phenols: Extract solutions were spotted on filter paper and phosphomolybdic acid is also added to the spot and exposed to ammonia vapor. Blue coloration of spot indicates the presence of phenols.

Test of steroids and terpenoids: One ml of the sample of drug was mixed with 1 ml chloroform, 2-3 ml acetic anhydride and 2 drop of conc. Sulfuric acid. Dark green color indicates the presence of steroids and dark pink color indicates the presence of terpenoids.

Test of Flavonoids (Sinoda test): A piece of mg ribbon was added to 1 ml extract solution and added 1 ml concentrated hydrochloric acid. Pink or red color indicates the presence of flavonoids.

Test of Alkaloids:

Dissolved the extract in 2% HCl solution and 2-3 drop of mayer's reagent was added. White or yellow brown precipitate indicates the presence of alkaloids. A drop of methanolic extract was spotted on pre-coated TLC plate and sprayed by modified Dragondroff's reagent. Orange color of the spot indicates the presence of alkaloids.

Test of Anthraquinone: One ml of solution of different extracts of CT leaves were mixed with 10 % FeCl3 solution, 1 ml of HCl solution, heated and filtered. Filtrate was shaken with diethyl ether and it as further extracted by strong ammonia. Pink or red deep color indicates the presence of Anthraquinone.

Test of reducing sugar: The extract solution was mixed with Fehling solution and heated for 5 minutes. Appearance of red color indicates the presence of reducing sugar.

Test of Tannin: In one ml of extract solution, 10% alcoholic ferric chloride solution was added. Dark blue or greenish color indicates the presence of tannin. Test of saponin: The extract solution was vigorously shaken with distilled water. Formation of froth indicates the presence of saponin.

Thin Layer Chromatography (TLC): TLC is an important technique for separation and identification of different class of natural product. This technique allows the separation of different components by the differential migration of solute between two phages, a stationary and mobile phage. The adsorbent (silica) acts as stationary phage and solvent systems were used as mobile phage. Polar compound have less affinity for solute, stick to it and move slowly up as mobile phage moves. The compounds will comparatively move quickly up the plates, so Rf values will be more, mixture of compounds will be separate according to polarities.

Preparation of plates: Slurry of silica gel was prepared in distilled water as the ratio of 1:2 (W/V) and poured into TLC applicator which was adjusted to 25 mm thickness. The slurry was spread on glass plates of variable size according to need. The coated plates were air dried and then heated at 105°C for 1h in oven. It was cooled and protected from moisture. Before use, it was re-heated at 110°C for 10 min.

Separation of compounds

The test samples were dissolved in suitable solvents and spotted by using fine capillary on TLC plates 1 cm above from the bottom of the plates. After air drying, the plate was transferred to saturated TLC chamber (saturated by desired solvent systems) and allowed to rise to solvents (mobile phage) up to 1 cm below of coating. The plate was removed and dried in air for 10 min to remove solvents and placed in iodine chamber. After 5 min spots were observed on the plate whose Rf values were calculated as the distance traveled by spot/distance traveled by solvents.

GC-MS Analysis:

The CTH and CTT were characterized on gas chromatography-mass spectrum (GC-MS), at Bhagawati Analytical Labs Limited, Hyderabad, India. It was carried out at 70 eV at 250°C at a flow rate of 2 mL/min in a fused silica capillary GC column (50 m × 0.25 mm, film thickness 0.25 μm). The carrier gas was helium, and the initial temperature was 100°C for 5 min, which then raised at a rate of 5°C/min to 250°C.

HPLC analysis of Eugenol:

The HPLC system consisted of a LC-10-AT pump (Shimadzu, Kyoto, Japan), a SIL-10A auto-injector (Shimadzu, koyoto, Japan), ODS C-18 RP-18 Merck column (Intersile 250 x 4.6 mm I.D., 5 μm) and a SPD-10A UV-VIS detector (Shimadzu, Koyoto, Japan). The different concentration of eugenol was prepared in HPLC grade methanol and then different concentration of eugenol was prepared (Table 3). Twenty micro litre of different concentration of eugenol was injected in HPLC column. It was eluted at the rate of 1ml/min and the detection was carried at 280 nm by UV detector at room temperature. The mobile phase was Methanol and acetonitrile (80:20) which was filtered through 0.45micron millipore membrane filter paper. Similarly, different extract was dissolved in HPLC methanol and their different concentration was prepared and filtered before use. The concentration of eugenol in different

fractions of CT leaves was calculated with the help of their AUC at a particular retention time (RT). (Yun et al., 2010).

Results

Phytochemical analysis of different fractions of CT leaves

Successive Hexane fraction (CTH), Ethyl acetate fraction (CTEA), Methanol fraction (CTM) and total methanol fraction isolated separately (CTT) from CT leaves were dissolved in suitable solvents and analyzed for the presence of different phytochemicals such as Phenols, Tannins, terpenoids, flavonoids and saponins along with it the estimation of proteases, reducing sugar and endotoxins as described in method section. Different fractions of CT leaves showed different distribution of these phytochemicals as given below in table 1.

Fingerprinting of different fractions of CT leaves in different solvent system by Thin Layer Chromatography (TLC): Different fractions of CT leaves (CTH-Successive hexane fraction, CTEA-Successive ethyl acetate fraction, CTM-Successive methanol fraction and CTT-total methanol fraction) were dissolved in suitable solvent and spotted on silica gel pre-coated TLC plate and allowed to rise in different solvent systems in saturated TLC chamber. Their Rf values were recorded as the ratio of distance traveled by spots to the distance traveled by solvent system as described in method section. TLC fingerprinting showed that the availability of spots in almost solvent systems is 2 only a few systems showed 3 and 5 spots as listed in table 2.

Characterization of extracts

Different fractions of CT leaves were characterised by quantitative analysis and GC-MS. CT leaves were found to be rich in eugenol, which was in the highest concentration in CTH. The total phenolic content of CTH was also higher than CTT. Interestingly, other fractions were rich in flavones but poor in eugenol (Table 1A and 1B).

Characterization of different fractions of CT leaves in terms of Eugenol: Eugenol was analysed in different fractions of CT leaves (successively extracted hexane fraction-CTH), and total alcoholic fraction –CTT) by comparing their chromatographic appearance of the peak at retention time 6.43±0.10 min. The resolution was carried out in solvent system of 80% methanol in water (HPLC grade) and the absorbance was recorded at 280 nm. The quantity of Eugenol was calculated by their Area under Curve (AUC). CTH was found to be rich in eugenol followed by CTT whereas other either in Trace in Eugenol content (< 2 %). Further Eugenol as well as other volatile compounds were analysed on GC-MS as described in method section and found that CTH was rich in Eugenol which is 71 % by weight and CTT have 60 % Eugenol by weight. (Table 3, 4 and Figures)

Discussion

The biological activity of natural product or herbal preparations depends upon the presence of different phytomolecules such as terpenes, flavones, tannins, alkaloid, anthaquinones etc but the fractions/compounds having more phenolic group were associated with different biological activity especially related with free radicals (Ferguson., 2001). Flavones are most popular phytomolecules that associated with many biological activities which were associated with the presence of polyphenolic groups (Bravo, 1998, Ross & Kasum,

2002, Manach et al., 2004). Poly phenols are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging (Manach et al., 2004). Cinnamomum tamala leaves are associated with many phytomolecules such as terpenes, flavones, saponins, phenols etc. Kaempferol and its derivatives such as Kaempferol-3-O- rhamnoside, Kaempferol-3-O-glucopyranoside, Kaempferol-3-O-sophoroside and Kaempferol 3,7-di-O-rhamnopyranoside were reported by other authors (Bharadwaj et al., 1983). Other flavones are Quercetin, Quercetrin, Myricetin, 3, 4, 5, 7-tetrahydroxy flavone and 3, 3, 4, 5, 7 pentahydroxy flavone were also reported. These flavones are potent free radical scavengers, anti-inflammatory property and many shows immunomodulation (almost immunosupression) (Li et al, 1991, Namgoong et al., 1994, Johnson et al., 2003).

CT leaves are also rich in volatile oil in which many terpenes along with eugenol (71 %) were reported by our GC-MS report of its hexane (CTH) as well as total methanol fraction (CTT). Eugenol is associated with single hydroxyl group on benzene ring due to which it shows free radical scavenging and anti-inflammatory property (Yogalakshmi et al., 2010). On the basis of qualitative analysis of total phenols and total flavones, it was found that successive ethyl acetate fraction of CT leaves are rich in flavones whereas total phenolic content is high in its hexane fraction and showed enhanced biological activity which could be due to the presence of Eugenol. CT leaves are rich in monoterpenes and sesquiterpenes (Mir et al., 2004) which are associated with immunosuppressive property (Juergens et al., 1998). Thus, it was concluded that the trend the biological activity of different fractions of CT leaves are CTH>CTT> CTEA>CTM. Same order was found with the distribution of eugenol on the basis of GC-MS as well as HPLC analysis. It means, most probably the biological activity of CT leaves is may be due to the presence of eugenol.

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Different fractions of CT leaves	Solubility	Phenolic Content(mg/L of gallic acid equivalents (GAE)	Total Flavone content (%)	% Yield	Available phytomolecules
CTH	DMSO and $CHCl_3$	1.93±0.50	20.90±2.54	5.25±1.5	**Phenols Tannins Terpenoids Flavones**
CTEA	Methanol	1.32±0.65	43.26±2.66	3.5±1.6	**Phenols Flavones**
CTE	Methanol	1.09±0.52	30.60±4.52	11.9±0.8	**Phenols Flavones**
CTT	Methanol	1.53±0.66	27.14±3.84	27.2±1.2	**Phenols Tannins Terpenoids Flavones**

S.No.	**CT Fraction**	**Solvent System**	**Solvent Ratio**	**No. of Spot**	**Rf Value**
1	Hexane Fraction (CTH)	Hexane+Benzene	9:1	3	0.25, 0.38, 0.5
		Benzene+Hexane	9:1	2	0.48, 0.66
		Ethyl Acetate+Benzene	8:2	2	0.43, 0.63
2	Ethyl Acetate Fraction (CTEA)	Ethyl Acetate+Benzene	8:2	2	0.40, 0.80
		Ethyl Acetate+Methanol	9:1	5	0.63, 0.71, 0.75, 0.82, 0.87
3	Successive Methanol Fraction (CTE)	Ethyl Acetate+Benzene	8:2	2	0.41, 0.77
		Ethyl Acetate+Methanol	9:1	3	0.31, 0.57, 0.78
4		Benzene+Hexane	9:1	2	0.34, 0.58

	Total Alcoholic Fraction	Ethyl Acetate+Benzene	8:2	2	0.47, 0.54
		Ethyl Acetate+Methanol	9:1	3	0.33, 0.61, 0.81

Table 2.5A: Standard curve of Eugenol in HPLC:

Concentration of eugenol in µM	**AUC**	**Retention time (RT)**
133.37	4958.1	6:51
100.03	3023.3	6:55
66.68	2388:4	6:27
33.34	1052.2	6:40
16.34	541.9	7.09

Fig 2.5A: Regression line drawn between concentration of Eugenol and area under curve (AUC) of a peak at 6.43±0.10 retention time in RP -18 HPLC column

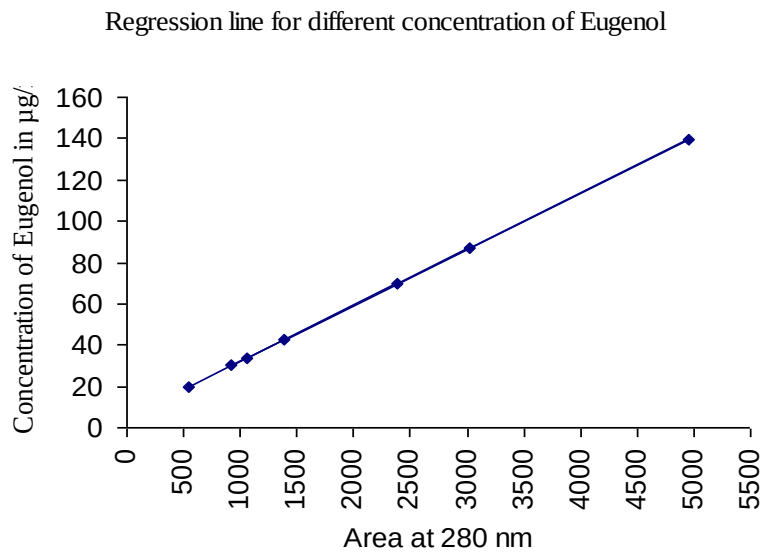

Fig 2.5B: HPLC Chromatogram of Eugenol (33.34 µg/ml at 280 nm) in RP-18 column

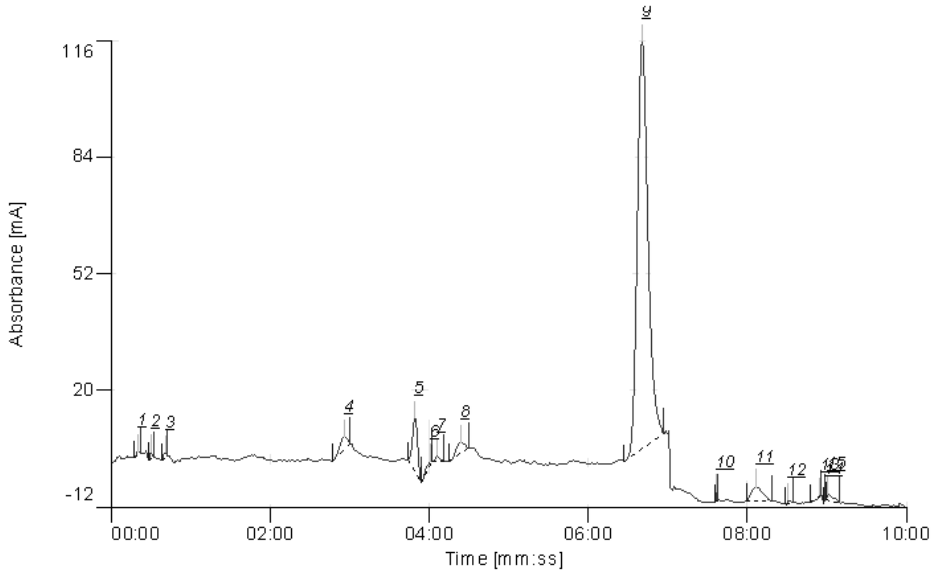
116
84
52
20
-12
Absorbance [mA]
1
2
3
4
5
6
7
8
9
10
11
12
00:00
02:00
04:00
06:00
08:00
10:00
Time [mm:ss]

Fig 2.5C: HPLC Chromatogram of CTH (50 µg/ml) in RP-18 column at 280 nm

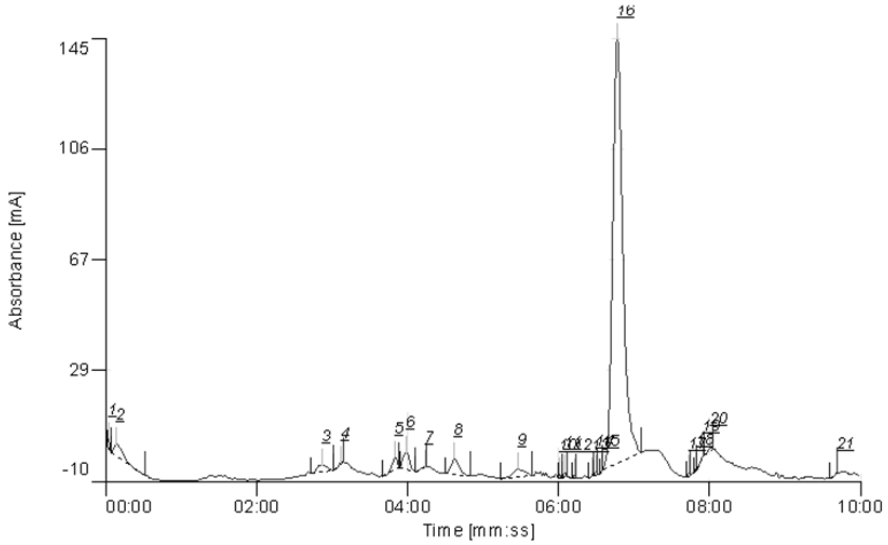

Fig 2.5D: HPLC Chromatogram of CTT (50 µg/ml) in RP-18 column at 280 nm

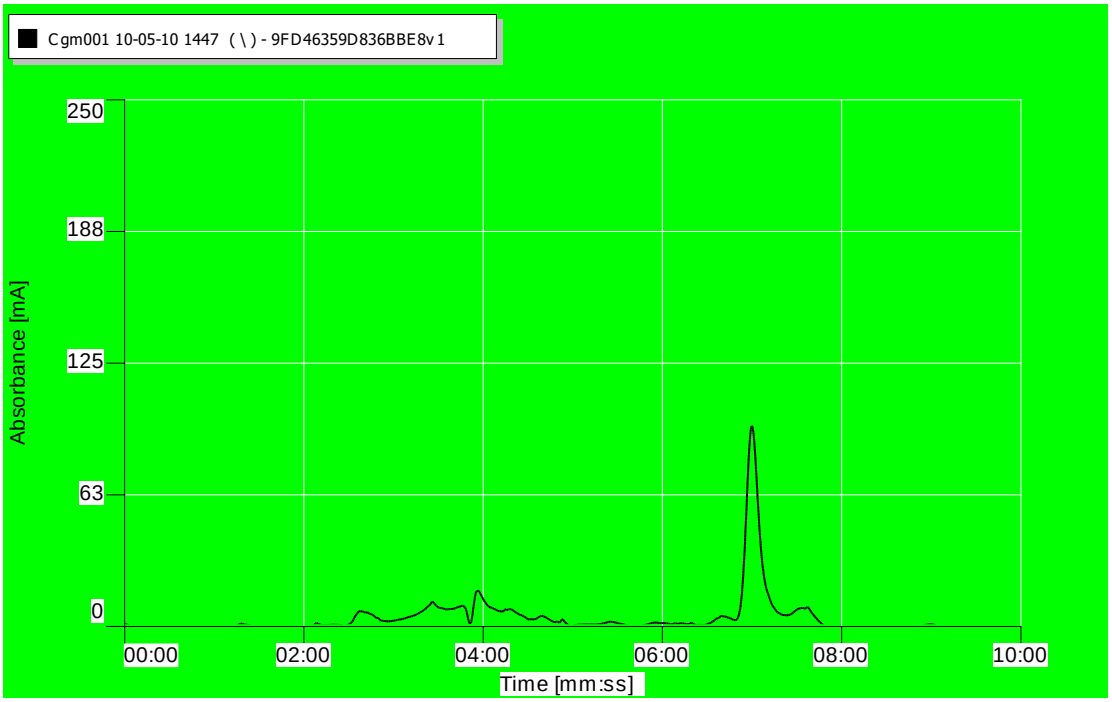

Table 2.5B: Concentration of eugenol in different fractions of CT leaves:

CT Fraction	Conc. Of eugenol in µg/mg extract
CTH	83.0±3.60
CTEA	Trace (<2 %)
CTE	Trace (<2 %)
CTT	68.2±2.68

Table 2.5C: Composition of hexane fraction of CT leaves (CTH) as analyzed by GC-MS.

RT	Compound	AUC	%
3.15	3,5-Diathiahexanol 5,5-dioxide	23917338	0.809
3.53	Glycerin	32888850	1.112
3.71	2,2'-Dioxirane	4066673	1.38
4.36	3-Ethoxy-1,2-propandiol	1584643	0.054
4.44	O-Butylisourea	2739172	0.093
4.78	2-Furanmethanol	5125417	0.173
6.74	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2292087	0.078
8.58	Clindamycin	6695567	0.226
9.85	4H-Pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl	12329972	0.417
10.28	Isobornyl acetate	8478483	0.287
10.72	p-Menth-1-en-8-ol	6315206	0.214
10.93	Bicyclo[3,1,0]hexan-3-ol,4-methylene-1-(1-methylethyl-(1α, 3α, 5α)	5479404	0.185
12.63	Ascaridole epoxide	4673234	0.158
12.88	3,5-Heptadienal-2-ethylidene-6-methyl	3483852	0.118
12.97	Bicyclo[3,1,1]heptan-2,3-diol,2,6,6-trimethyl	10517869	0.356
13.68	Eugenol	2095466379	70.858
14.33	Benzene,1,2dimethoxy-4-(2-propenyl)	24671601	0.834
14.73	Caryophyllene	16888577	0.571
15.04	Naphthalene, 1,2,3,5,6,7,8,8α-octahydro-1-8α-dimethyl-7-(1-methylethynyl)	6933469	0.234
15.59	Cedrene	16084627	0.544
15.91	1H-Cycloprop[e]azulene 1α2,3,5,6,7,7, α,7b octahydro-1,1,4,7-tetramethyl-[1Ar]	32890432	1.112
16.26	Phenol,2-methoxy-4-(2-propenyl)-acetate	79496819	2.688
16.87	2-Propen-1-ol,3-(2,6,6-trimethyl-1-cyclohexen-1-yl)	12573958	0.425
17.18	(-)-Spathulenol	73531530	2.486
17.28	Caryophyllene oxide	20482298	0.693
17.42	Phenol,2,6-dimethoxy-4-(2-propenyl)	35403862	1.197
18.29	5β,7βH,10α-Eudesm-11-en-1α-ol	41385782	1.399
18.90	2-Cyclohexene-1-one 4-(3-hydroxybutyl)-3,5,5-trimethyl	15791306	0.534
19.19	Isoaromadendrene epoxide	19468795	0.658
19.37	4-((1E)3-hydroxy-1-propenyl)2-methoxy phenol	19405130	0.656
19.52	Estra,1,3,5(10)-trien-17β-ol	17785505	0.601
20.07	2-Cyclohexene-1-one 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butanyl)	32195101	1.089
22.83	1-Propyl,3,6-diaxahomoadamantan-9-ol	13865646	0.469

24.03	Phytol	30351952	1.026
24.29	Oleic acid	68311550	2.310
26.21	Ethyl iso-allocholate	8372358	0.283
26.44	1-Heptatriacotanol	3687423	0.125
27.40	1-Phenenthrenecarboxylic acid,tetradecahydro-7-(2-methoxy-2-oxoethylidene)	9386172	0.317
27.63	2,7-Diphenyl,1,6-dioxopyridazino[4,5:2',3']pyrrolo[4,5-α]pyridazine	15577382	0.527
28.02	Ethyl iso-allocholate	7673666	0.259
28.28	Vitamin E	40736431	1.377
28.40	Ethyl iso-allocholate	4770901	0.161
29.03	2-Butenoic acid,2-methyl-2-(acetyloxy)1-1α,2,3,4,6,7,10,11,11α-decahydro	7406374	0.250
29.79	Ethyl iso-allocholate	19154761	0.648
29.90	Card-20(22)-enolide,3-[(6-oxy-3,4-O-methylenehexopyranos-2-ulos-1-yl)oxy	18369754	0.621
30.09	Cholestane-3,6,7-triol,(3β,5α,6β 7α)	18579748	0.628

Table 2.5D: Composition of CTT as analyzed by GC-MS

3.49	Acetic acid, 1-methyl ethyl ester	32028463	1.154
4.42	O-Butylisourea	3742446	0.135
8.57	4(1H)-Pyrimidinone,2,6 diamino-5-nitroso	4294888	0.155
9.85	4H-Pyran 4-one, 2,3-dihydro-3,5-dihydrox-6-methyl	7756914	0.280
10.72	3-Cyclohexene,1-methanol,α, α,4-trimethyl-(S)-	4469628	0.161
10.94	Bicyclo [3,1,0]hexane 3-ol,4-methylene 1-(1-methylethyl)- acetate	2566999	0.093
12.64	Ascaridole epoxide	3079235	0.111
12.96	Methyl 6-oxoheptanoate	6904285	0.249
13.68	Eugenol	1668855247	60.139
14.33	1,2-dimethoxy-4-(2-propenyl)-	20421808	0.736
14.73	Caryophyllene	15578171	0.561
15.04	Patchoulene	9047339	0.326
15.91	1H-Cycloprop[e]azulene 1α2,3,5,6,7,7, α,7b octahydro-1,1,4,7-tetramethyl	14047295	0.506
16.26	Phenol,2-methoxy-4-(2-propenyl-acetate	65694021	2.367
17.18	(-)-Spathulenol	62220240	2.242
17.42	Phenol,2,6-dimethoxy-4-(2-propenyl)-	31145852	1.122

19.17	Isoaromadendrene epoxide	15909562	0.573
19.42	Diepicedrene-1-oxide	33237883	1.198
19.53	Estra-1,3,5 (10)-trien-17 β-ol (Mestranol)	15105790	0.544
20.08	2-Cyclohexene-1-one 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butanyl)	18355665	0.661
20.64	3,7,11,15-Tetramethyl-2-hexadecene-1-ol	20626887	0.743
21.28	[1,1'Bicyclopropyl]-2-octanoic acid,2'-hexyl-methyl ester	6478647	0.233
24.03	Phytol	22324844	0.804
24.29	Oleic acid	68073691	2.453
26.21	Ethyl iso-allocholate	5729238	0.206
27.40	Pregn-5-en-20-one,(acetyloxy)16,17-epoxy-6-methyl-(3β, 16α]	6956871	0.251
29.03	2-[4-methyl-6-(4,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	7865305	0.283
29.90	Card-20(22)-enolide,3-[(6-oxy-3,4-O-methylenehexopyranos-2-ulos-1-yl)oxy	11857840	0.427