ISOLATION AND CHARACTERIZATION OF ACTIVE COMPOUNDS PRESENT IN THE METHANOLIC FRUIT EXTRACT OF *TERMINALIA CHEBULA*

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Abstract

Consumption of *Terminalia chebula* fruit as food additive has become important in the promotion of human health, mainly due to their antimicrobial and other biological activities. Consequently, there has been a growing interest in identifying natural antioxidants and antimicrobials from these plants. This study aimed to characterize the phytochemical constituents of *T. chebula* which reveals the presence of alkaloids, terpenoids, flavonoids, tannins, glycosides, steroids and phenolic compounds. HPTLC profile showed the presence of five polyvalent phyto constituents, and the Rf value of the highest concentration of the phytoconstituents (36.56%) was found to be 0.89. Based on the purity and yield, three compounds were obtained with Rf values 0.57, 0.52 and 0.47. Compound 2 showed good inhibitory activity against the cariogenic organisms tested and was further selected for structure elucidation. The IUPAC name of compound 2 was found to be 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-cde]chromene-5,10-dione. The chemical formula was $C_{16}H_{10}O_8$ and the exact mass was found to be 330.04, thus the molecular weight was 330.04. The present finding indicates the presence of new derivative of ellagic acid in the methanolic fruit extract of *T. chebula* and it might be a promising compound for the development of antimicrobial agent against oral pathogens in humans.

Keywords: *T. chebula*, cariogenic organisms, phytochemicals, metabolite profiling, structure elucidation.

Introduction

Terminalia chebula is one among the most important medicinal plants, which are widely used in the traditional system of medicine [1]. The plant contains chebulinc acid, tannic acid, gallic acid, resin, anthroquinone and sennoside. It also contains glycosides, sugar, triterpenoids, steroids and small quantity of phosphoric acid and these compounds were demonstrated to exhibit anti-bacterial, anti-fungal, anti-viral and anti-carcinogenic activities [2].

The World Health Organization has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards [3]. Therefore, in recent years, advancements in chromatographic and spectral fingerprints have played an important role in quality control of complex herbal medicines [4].

For the estimation of chemical and biochemical markers, fingerprint analysis by high performance thin layer chromatography (HPTLC) has become an effective and powerful tool [5, 6, 7]. It also offers a better resolution of active constituents with reasonable accuracy in a short time. Since column chromatography and TLC techniques purifies larger samples and also use normal phase systems, i.e. a polar stationary phase (silica) eluted with organic solvents of increasing polarities, they are suitable for sample purification, qualitative assays and preliminary estimates of the compounds in plant extracts [8]. The liquid dilution method is followed as it gives precise, reproducible results and requires just a small volume of compound to determine the minimum inhibitory concentration (MIC) for isolated compounds against cariogenic organisms [9].

Compounds derived from natural products are mostly identified using techniques such as infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) that provides information leading to the complete structural determination of natural products. Structural elucidation based on these techniques has been the most successful for determining both simple and complex structures [10]. MS can be used to identify the molecular weight and confirm the structure of the isolated compounds or natural products [11]. Hence, the present study aims at the following analysis: Phytochemical analysis to determine the phytochemicals present in methanolic fruit extract of *T. chebula*; HPTLC fingerprinting, Column chromatography and TLC to isolate the active compounds; MIC to check the effect of active compounds against the cariogenic organisms; IR, ^{1}H NMR, ^{13}C NMR and MS to determine the structure of active compound isolated from the methanolic fruit extract of *T. chebula.*

Materials and Methods

Collection and preparation of plant extract

Terminalia chebula fruits were purchased from the local drug store in Nagercoil, Tamil Nadu and were washed with distilled water, left dry at room temperature before they were crushed and ground prior to the extraction. The methanol extract was prepared as referred by Rani *et al*. [12].

Phytochemical studies

The concentrated residues from the methanolic fruit extract of *T. chebula* was used to detect the secondary plant metabolites including alkaloids, terpenoids, saponins, flavonoids, tannins, glycosides, steroids, anthraquinone and phenolics using standard methods with some modifications [13, 14, 15, 16, 17, 18].

Metabolite profiling by HPTLC

The profile of methanolic fruit extract of *T. chebula* was studied using high performance thin layer chromatography [19]. It was performed on silica gel plate 60 F254 (20x20cm, 0.5mm Merck and Co. Inc.). 20µl of the extract was taken in the CAMAG syringe. The extract in the syringe was applied to the precoated silica gel plate of 12cm height and 3cm width using automatic CAMAG applicator. The sample loaded plate was kept in TLC twin trough developing chamber after saturation with the solvent vapor and the plate was developed using ethyl acetate : hexane : formic acid (7.0:3.0:0.5) solvent system. The developed plate was dried by hot air to evaporate solvents from the plate and immersed in vanillin and 10% sulphuric acid to view the bands under visible light. The plate was fixed and scanning was done by the scanner with a deuterium lamp of wave length 200-400nm. Chromatographic fingerprint was developed utilizing upgraded WINCATS software for detection of phyto-constituents present in the methanol extract and Rf values were tabulated.

Isolation of active compounds

Fractionation of compounds using column chromatography

The method of Selowa *et al*. [20] with some modifications was utilized to elute the fractions during column chromatography. An overnight dried mixture of 5.1gm methanolic fruit extract of *T. chebula* and 11gm of silica gel in hexane solvent was thinly spread on the top of the column. The extract was covered with cotton wool and column was eluted with increasing solvent polarity from hexane to ethyl acetate. A volume of 500ml of 100% (100:0) hexane was initially used to elute the column, followed by 900ml of hexane : ethyl acetate (95:5), 700 ml of hexane : ethyl acetate (90:10), 700ml of hexane : ethyl acetate (85:15) and 600 ml of hexane : ethyl acetate (80:20) , 600ml of hexane : ethyl acetate (75:25) , 800 ml of hexane : ethyl acetate (70:30) and 200 ml of ethyl acetate (0:100 v/v). The fractions were gathered and concentrated by using the rotary evaporator.

Thin layer chromatography of eluted fractions

TLC of eluted fractions was carried out on TLC glass plates (20 × 20cm) coated with 0.2mm thickness silica gel. Plates were activated at 105ºC for 30 minutes in a hot air oven. An aliquot of all the concentrated fractions was loaded on the activated silica gel TLC plates with equal distance with the help of a micropipette. The plates were kept in a chromatographic chamber after drying. The solvent system consists of hexane and ethyl acetate in the ratio of 80:20(v/v) and the plate was developed. The plate was removed from the chamber, when the solvent front had reached the predetermined height and the solvent front was marked precisely with pencil. Then the plates were dried and the spots were located by exposing the plate to vanillin and UV light of 254nm. Fractions having the same number of spots with similar Rf values on the TLC plate were pooled to get pure fractions/compounds. The compounds showing purity and maximum yield were taken for further study.

Selection of active compound by broth dilution method

The isolated compounds were tested for antimicrobial activity against cariogenic organisms. Assay was performed in 96 well microtitre plates. 1mg of purified and crystallized compounds was dissolved in 1ml of DMSO (stock solution). The stock solutions were diluted in the concentrations of 1µg to 0.016µg. The broth dilution method was carried out according to the procedure described by Yadav *et al*. [21].

Structure elucidation of active compound

The dried, purified bioactive metabolite was dissolved in ethyl acetate and filtered to eliminate impurities. After leaving this solution overnight at -20ºC, the semisolids obtained were collected. The semisolids were again re suspended in a minimum quantity of NMR solvent (DMSO) and analyzed by nuclear magnetic resonance spectroscopy (BRUKER/NMR-400MHz), Infra red spectroscopy (BRUKER, alpha-E) and Mass spectroscopy (SHIMADZU -EIMS) to identify the compounds. The chemical structure was drawn using ChemBioDraw Ultra 13.0.

Results

Phytochemical studies

The methanolic fruit extract of *T. chebula* was subjected to several tests to find out the presence or absence of its chemical constituents. The result (Table 1) reveals the presence of alkaloids, terpenoids, flavonoids, tannins, glycosides, steroids and phenolic compounds. Saponin and anthraquinones were absent.

Table 1: Phytochemical analysis in methanolic fruit extract of *T. chebula*

Sl. No	Constituents	Methanol extract
1	Alkaloids	+
2	Terpenoids	+
3	Saponin	-
4	Flavonoids	+
5	Tannins	+
6	Glycosides	+
7	Steroids	+
8	Anthraquinones	-
9	Phenolic compounds	+

+ present; - absent

Metabolite profiling by HPTLC

The HPTLC study revealed that the methanolic fruit extract of *T. chebula* showed best results in Ethyl Acetate: Hexane: Formic acid (7.0:3.0:0.5) solvent system. After scanning and visualizing the plates in absorbance mode at visible light range (400-600nm), best results were shown at 400nm. On derivatizing the HPTLC plates with vanillin sulphuric acid, purple, pink, yellowish orange and brown spots were observed. HPTLC images shown in fig.1 indicate that the sample constituents were clearly separated without any tailing and diffuseness. The results from HPTLC finger print scanned at wavelength 400 nm for methanolic fruit extract of *T. chebula* revealed the presence of five polyvalent phytoconstituents (Table 2). The Rf values ranged from 0.5 to 0.89. The highest concentration of the phytoconstituents was found to be 36.56% and its corresponding Rf value was found to be 0.89 with top coordinate 533 and bottom coordinate 823.

Table 2: HPTLC profile of methanolic fruit extract of *T. chebula*

Lane Data for Plate

ID	X Coordinate	Y Coordinate	Width	Height	Number of Anchors	Number of Bands	
1	2197	400	253	2616	1	5	
Band Data for Plate							
ID	Rf	Top Coordinate	Bottom Coordinate	Area	Volume (Scaled)	Volume (Real)	Area %
1	0.89	533	823	73370	2706.9	270689001	36.56
2	0.658	1192	1357	41745	116.92	11691636	29.80
3	0.574	1417	1568	38203	882.71	88271447	19.04
4	0.54	1577	1657	20240	366.08	36607582	10.08
5	0.5	1677	1784	27071	181.03	18102656	13.49

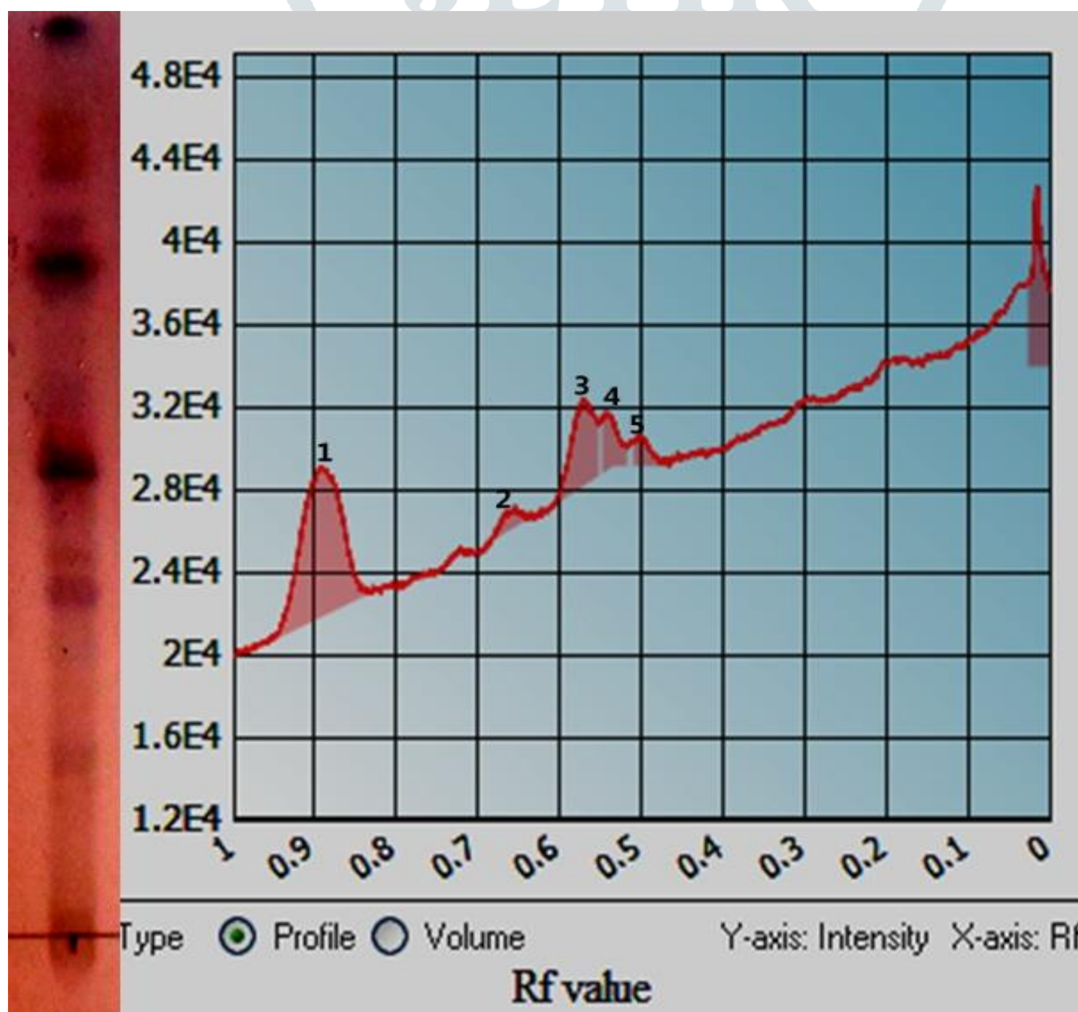


Fig.1: HPLTC plate and chromatogram of methanolic fruit extract of *T. chebula*

Isolation of active compounds

About 331 fractions were eluted from column and concentrated in rotary evaporator. After spraying with vanillin, three distinct fractions/ compounds were obtained by pooling the fractions having similar Rf

values (Table 3 and fig. 2). Fractions 18-23 were pooled together and evaporated in rotary evaporator to yield 30mg of compound 1 and the Rf value was 0.57. Fractions 144-161 contained same Rf value (0.52) on TLC chromatogram and the resultant pure compound 2 yield 150mg. 115mg of compound 3 was obtained which was pooled from the fractions 235-247 having the same Rf value 0.47. All the three compounds obtained were checked for antimicrobial activity against cariogenic organisms.

Table 3: Solvent system and yield of compounds isolated from column chromatography

Sl. No	Compound	Solvent system	Yield (mg)
1	1	100:0 / Hexane : ethyl acetate	30
2	2	85:15 / Hexane : ethyl acetate	150
3	3	75:25 / Hexane : ethyl acetate	115

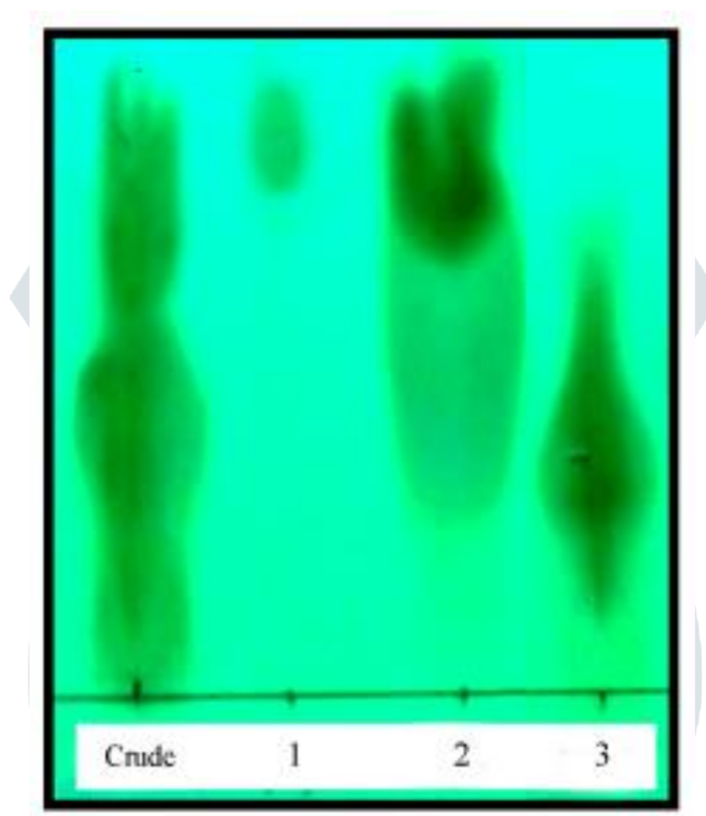


Fig. 2: TLC chromatogram showing crude extract and isolated compounds under UV light of 254nm (Rf: compound 1 = 0.57, compound 2 = 0.52, compound 3 = 0.47)

Selection of active compound by broth dilution method

There was no sign of inhibition observed on the microtitre plate loaded with different concentrations of isolated compound 1 indicating that compound 1 does not have any inhibitory activity against the cariogenic organisms tested. Among the other two compounds isolated, compound 2 showed good inhibitory activity against the cariogenic organisms tested and was further selected for structure elucidation (Table 4 and fig. 3).

Table 4: Minimum Inhibitory Concentration (MIC) of isolated compounds (1, 2 and 3) by broth dilution method

Sl. No	Cariogenic organisms	Compound 1 (µg/ml)	Compound 2 (µg/ml)	Compound 3 (µg/ml)
1	*Enterobacter hormaechei* strain A1	>1	0.125	0.25
2	*Enterobacter* sp.A2(2016)	>1	0.031	0.125
3	*Micrococcus luteus* strain A3	>1	0.063	>1
4	*Klebsiella pneumoniae* strain A4	>1	0.125	0.5
5	*Exiguobacterium* sp.A5(2016)	>1	≤0.016	>1
6	*Staphylococcus sciuri* strain A6	>1	0.063	>1
7	*Acinetobacter radioresistens* strain A7	>1	>1	>1
8	*Weissella confusa* strain A8	>1	0.125	0.25
9	*Bacillus cereus* strain A9	>1	0.063	0.063
10	*Bacillus subtilis* strain A10	>1	≤0.016	>1
11	*S. aureus* MTCC 740	>1	0.063	0.031
12	*P. aeruginosa* MTCC 424	>1	0.5	0.125
13	*E. faecalis* MTCC 439	>1	0.125	0.125
14	*S. mutans* MTCC 497	>1	0.031	0.5
15	*L. acidophilus* MTCC 10307	>1	0.063	0.5
16	*C. albicans* MTCC 227	>1	0.5	0.5
17	*S. cerevisiae* MTCC 170	>1	0.5	0.125

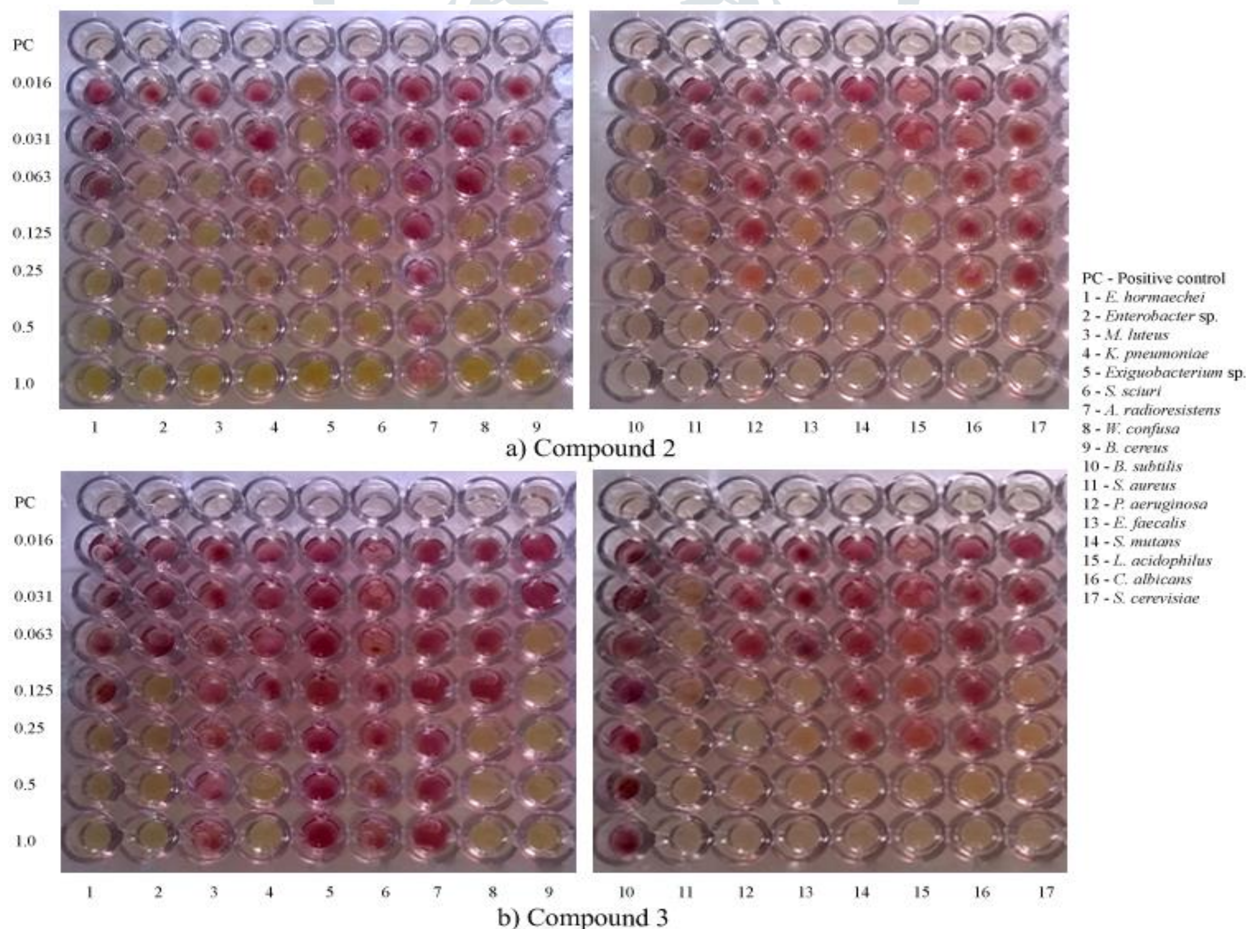


Fig. 3: Minimum Inhibitory Concentration (MIC) of isolated compounds

Structure elucidation of active compound

The isolated compound 2 of methanolic extract of *T. chebula* has been characterized by spectral techniques such as IR, ^{1}H NMR and ^{13}C NMR.

IR

A broad signal at 3424cm^{-1} confirmed the presence of –OH functionality. A peak at 2924cm^{-1} indicated the vibrational frequency of C-H (Sp^3 hybridized R carbon). A peak at 1762 cm^{-1} indicated the presence of an ester functional group. A well defined peak at 1399cm^{-1} in fingerprint region confirmed the O-C functionality (ether). A frequency 1627cm^{-1} indicated vibrational frequency C=C in the benzene ring (fig. 4).

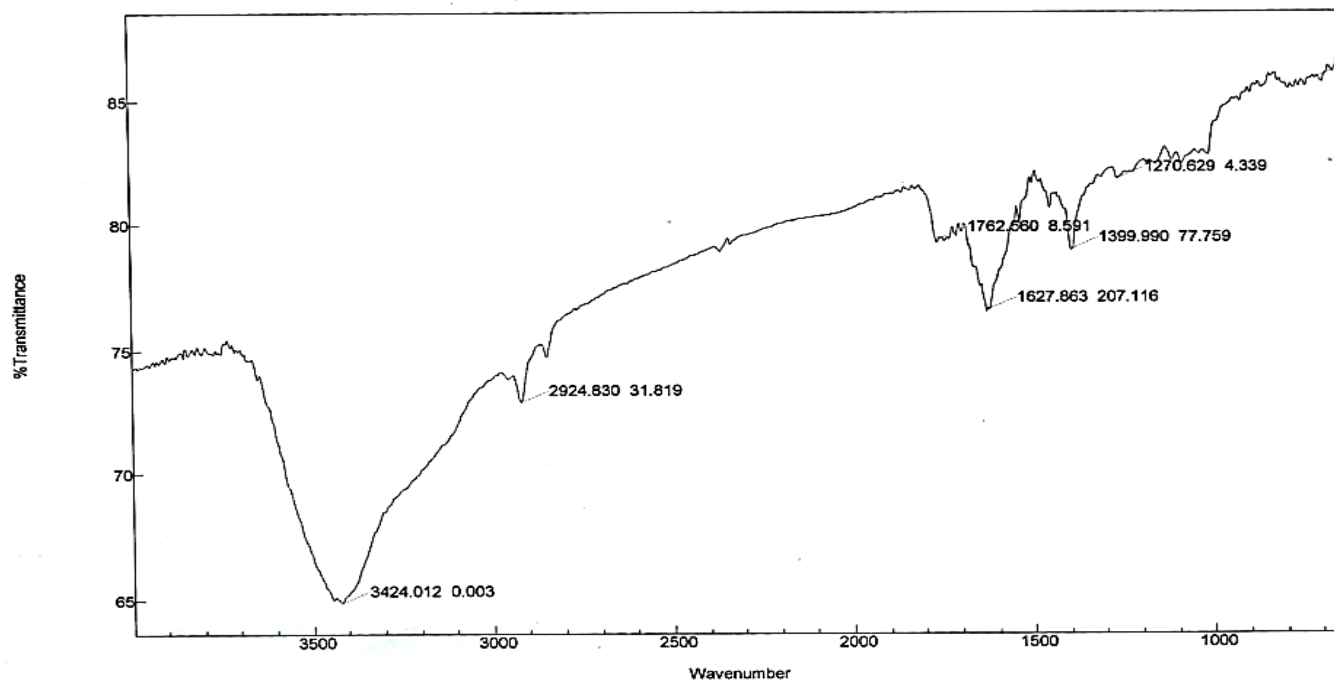


Fig.4: Infra Red spectrum of compound 2

^{1}H NMR

δ: 2.518 (singlet DMSO exchange) shows the presence of very weak acidity of the proton in the isolated compound. δ: 3.518 indicate the presence of methyl group protons attached to oxygen. δ: 6.8 (singlet) indicate the presence of benzene ring protons. The three independent signals confirm the 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione (fig. 5).

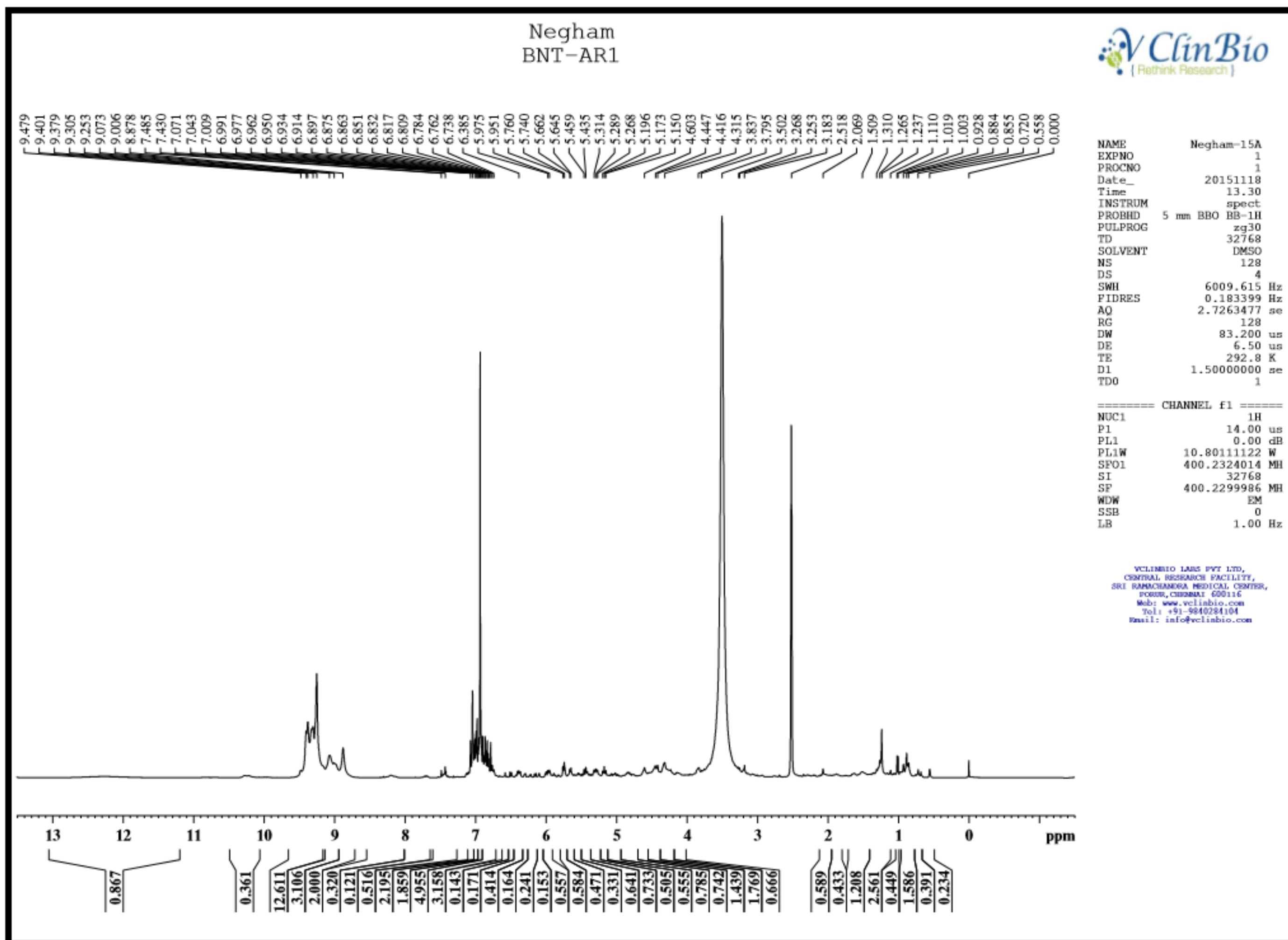


Fig. 5: ^{1}H NMR of compound 2

^{13}C NMR

δ: 170 ppm (singlet) confirms the ester group carbon. δ: 40 ppm singlet confirms the CH_3 carbon attached to hetero element oxygen. The presence of δ: 145,139, 120, 110 etc. confirms the ^{13}C values of benzene carbon. This may be supported by earlier [1]NMR and IR spectra (fig.6).

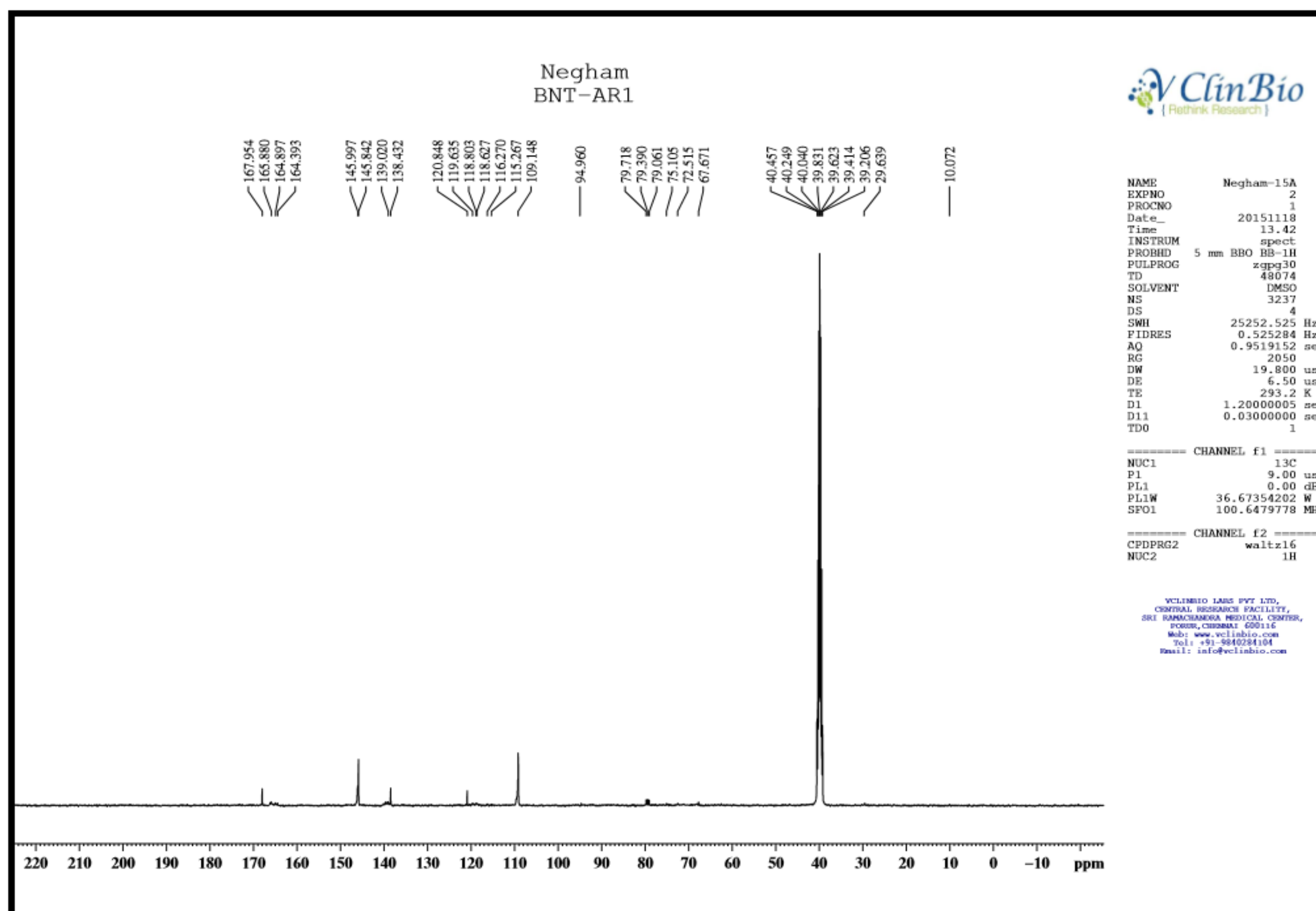


Fig. 6: ^{13}C NMR of compound 2

Mass Spectrum

A m/z value 330.04 indicates molecular ion peak for the derivative of ellagic acid. A fragmented various species are known at m/z values 74.0, 87.0, 109.0, 147.0 etc. The base peak at 74.0 confirms highly concentrated positively charged ions in a mass spectrometer (fig. 7).

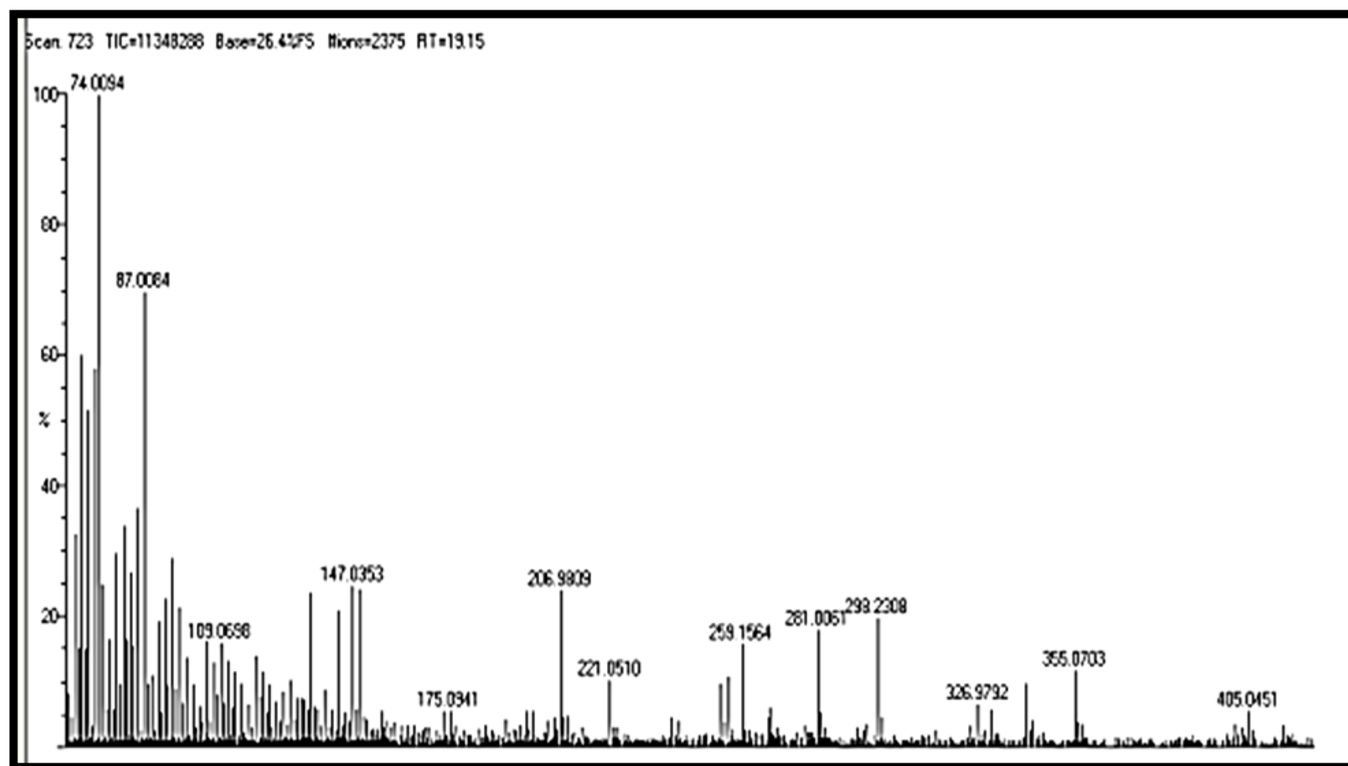
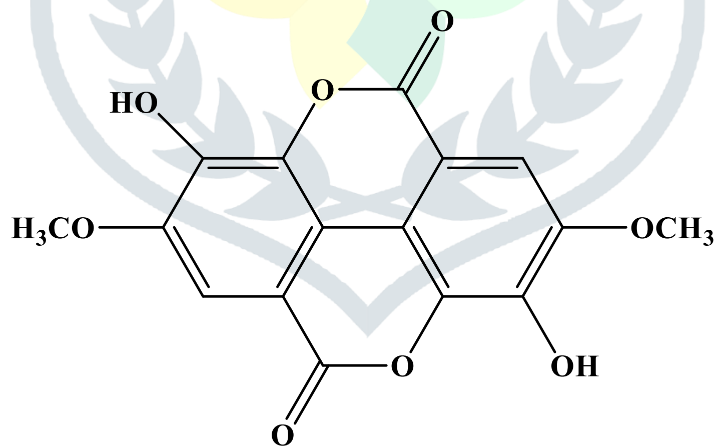


Fig. 7: Mass spectrum of compound 2

Structure analysis

The structure of compound 2 was drawn and analyzed utilizing ChemBioDraw Ultra 13.0. The IUPAC name of compound 2 was found to be 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione. The chemical formula was $C_{16}H_{10}O_8$ and the exact mass was found to be 330.04, thus the molecular weight was 330.04. The structure reveals the molecular ion peak as m/z 330.04 (100.0%), 331.04 (17.7%) and 332.04 (3.0%). The elemental analysis of compound 2 was found to be C, 58.19; H, 3.05 and O, 38.76. (fig. 8).



3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione
Chemical Formula: $C_{16}H_{10}O_8$
Exact Mass: 330.04
Molecular Weight: 330.25
m/z: 330.04 (100.0%), 331.04 (17.7%), 332.04 (3.0%)
Elemental Analysis: C, 58.19; H, 3.05; O, 38.76

Fig. 8: Structure analysis of 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione using ChemBioDraw Ultra 13.0

Discussion

Plant phenols are groups of natural products with variable structure that are well known for their beneficial effects on health possess significant antimicrobial and antioxidant activities [22, 23]. Earlier work revealed the presence of several constituents like tannins, flavonoids, sterols, amino acids, fructose, resin, fixed oils, and also contains compounds like anthraquinones, 4, 2, 4 chebulyl-dglucopyranose, terpinenes and terpinenols in *T. chebula* plant [24]. This study reveal the presence of chemical constituents like alkaloids, tannins, steroids, phenolic compounds, flavonoids and terpenoids in the methanolic fruit extract of *T. chebula.*

HPTLC fingerprint studies confirmed the results of phytochemical screening by the presence of various coloured bands at different wavelengths symbolizing the presence of particular phytocompounds. Sujogya *et al.* [25] reported the presence of alkaloids, flavonoids, triterpenoids, carbohydrates, glycosides, saponins, protein and amino acid in methanolic leaf extracts of *C. fistula*. The present results are in accordance with Sujogya *et al.* [25] by showing the presence of flavonoids, alkaloids, tannins, steroids, glycosides, phenolic compounds, terpenoids in the methanolic fruit extract of leaves of *T. chebula* by phytochemical analysis. The HPTLC finger print scanned at wavelength 400nm for methanolic fruit extract of *T. chebula* showed five peaks with Rf values ranging from 0.5 to 0.89. Thomas *et al*. [26] also reported 5 peaks by conventional extraction of *T. chebula* with one of the phytoconstituents have Rf value 0.49. Parameswari *et al*. [27] targeted on two compounds with Rf values 0.56 and 0.9 from 9 peaks obtained from methanolic fruit extract of *T. chebula*. Thus, HPTLC fingerprint profile along with their Rf values was recorded, which would serve as a reference standard for the scientist engaged in research on the medicinal properties of plants.

Isolation of phytocompound was done by column chromatography using hexane: ethyl acetate as the mobile phase. The fractions which are eluted from the 100:0, 85:15 and 75:25v/v mobile phases showed different components. Fractions 18-23 eluted from 100:0v/v mobile phase which exhibited similar bands and similar Rf value of 0.57 were pooled together to get compound 1. Similarly, fractions 144-161 eluted from 85:15v/v has similar Rf value 0.52 and fractions 235-247 eluted from 75: 25v/v mobile phase was with similar Rf value 0.47. The fractions with similar Rf value were pooled together to get the compounds 2 and 3 respectively. Parameswari *et al*. [27] used column chromatography and TLC techniques to isolate active compounds present in *T. chebula*. Mahlo [28] also isolated phytochemicals from *Breonadia salicina* using column chromatography and have isolated 4 compounds with four different Rf values. Mahlo [28] utilized microplate dilution assay and bioautography to determine the antimicrobial activity of isolated compounds. In the present study, microplate dilution assay was used to check the efficacy of isolated compounds and found that, compound 1 has no activity against the cariogenic organisms tested. The active compound 2 showed minimal inhibitory activity against all the cariogenic organisms tested and were further selected for structure elucidation.

The active compound was analyzed by IR, NMR and MS for identification. IR studies showed the presence of –OH stretching, C-H vibrational frequency, ester functional group, finger print region (O-C) which confirms the ether and C=C benzene ring. Based on ^{1}H NMR spectra, the presence of very weak acidity of proton, methyl group protons attached to oxygen, benzene ring protons and the presence ester group carbon, CH_3 carbon attached to hetero element oxygen and benzene carbon in ^{13}C NMR spectra of compound 2 clearly evidences the formation of a new derivative of ellagic acid and the spectral data is in agreement with the literature of Kemp [29] and Silverstein *et al*. [30]. The mass spectral data of the present study also showed that the compound 2 had a molecular ion peak at m/z 330.04 and the base peak at 74.0 confirms highly concentrated positive charge. The IUPAC name of the compound 2 was identified as 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione and its molecular formula was $C_{16}H_{10}O_8$.

The main component of ellagic acid is tannin, which is a general descriptive name for a group of polymeric phenolic substances [31]. Plant polyphenols have been shown to have many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions [32]. One of tannin's molecular actions is to complex with proteins through so called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation [33]. Thus, antimicrobial action of ellagic acid may be related to its ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and complex with polysaccharide.

Several ellagic acid rhamnosides were isolated from the stem bark [34] and wood [35]. A new ellagic acid derivative was also isolated from the fruits of *Eucalyptus globulus* Labill by Guo and Yang [36]. The

present study describes the isolation and structural elucidation of a new ellagic acid with the IUPAC name of 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione.

Conclusion

A new ellagic acid derivative 3,8-dihydroxy-2,7-dimethoxychromeno[5,4,3-*cde*]chromene-5,10-dione inhibited all the cariogenic organisms tested, hence it might be a promising compound for the development of antimicrobial agent against oral pathogens in humans.

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