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Research Article

Cytotoxic effect of tridham (TD) against human HepG2 Cell line: Isolation and characterization of 3,4,5-trihydroxybenzoic acid from aqueous extract of TD

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Abstract

Tridham (TD) is a combinational Siddha herbal drug consisting of three plant ingredients including seed coats of *Terminalia chebula* (family: Combretaceae) fruits of *Eleocarpus ganitrus* (family: Elaeocarpaceae) and leaves of *Prosopis cineraria* (family: Leguminosae). TD is used by Siddha practitioners, however, its active constituents have not been scientifically explored. The present study different TD crude extract was subjected to preliminary phytochemical screening using non polar to polar solvents. Total phenol and flavonoid contents were assayed. The redox potential of crude TD and its individual components were analyzed by Differential pulse voltammeter (DPV). The cytotoxic effect of TD on HepG2 cell line was evaluated. The inhibitory concentration (IC_{50}) of TD was found to be 80µg at the treatment period of 48 hour. DNA fragmentation analysis using agarose gel electrophoresis showed cytotoxic effects in TD treated groups. An active component was isolated from TD using column chromatography and identified by various spectroscopic and microscopy techniques. TD aqueous extract was found to exhibit high amount of phenolic and flavonoid contents. DPV showed that TD tends to contains easily oxidizable polyphenols compared to standard and individual ingredients. Systematic *in vitro* studies show that TD extract has growth inhibitory and cytotoxic effects on Hep G2 cell lines. The single crystal X-ray analysis of isolated crystals indicated that gallic acid crystallizes in monoclinic system with space group, P21/c. The structure was further established by elemental analysis, FT-IR, ^{1}H-NMR, ^{13}C-NMR and scanning electron microscope.

Key Words: *Terminalia chebula*, *Eleocarpus ganitrus*, *Prosopis cineraria*, Gallic acid, Spectroscopic techniques, HepG2 cell line.

Introduction

Medicinal products from plants have been recognized and utilized for centuries by ancient cultures for treating various human diseases. Presently it is estimated that about 80 % of people in developing countries still rely on traditional medicines based largely on various species of plants and animals for their primary health care.

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The popularity of herbal or complementary medicine is widespread and increasing across the world due to the expense and side effects of allopathic medicines. This has lead to a sudden upsurge in the number of herbal drug manufactures[1]. According to the World Health Organisation (WHO), around 25% of modern medicines are originated from traditional herbal medicines. In United States about 50% of pharmaceutical prescriptions contain at least one plant-derived ingredient[2]. In India, a vast repository of medicinal plants is used in traditional medical treatments[3]. The various indigenous systems such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat different ailments[4]. There are estimated to be around 25,000 effective plant-based formulations used in folk medicine and known to rural communities in India. It is well known that medicinal herbs are the richest sources of antioxidant compounds required for human health against free radical attrition[5]. Antioxidant molecules reduce the oxidative damage by reacting with free radicals and neutralize them by donating its own electron[6]. Antioxidants such as flavonoids contain numerous naturally occurring polyphenolic compounds[7]. The protective effects of polyphenol consumption against cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, and osteoporosis are widely reported.[8]

Many studies have shown that the plant derived drugs of present scenario such as paclitaxel, vincristine, vinblastine have come into use through the study of various indigenous medicines[9] . Apart from conventional individual herbal drugs, the mode of treating various diseases using combination therapy is currently becoming an important therapeutic approach. A preclinical model suggests that a combination of drugs has substantial activity compared to any of the constituents used as single drugs[10]. This is attributed to the improved treatment response or minimized development of resistance. Herbal concoctions have long been used in traditional medicine because of the synergistic action of various bioactive components present in the herbs. TD consists of three individual medicinal herbs such as *Terminalia chebula*, *Eleocarpus ganitrus* and *Prosopis cineraria*, in equal proportions that are used by traditional Indian Siddha practitioners for the treatment of cancer.

Terminalia chebula is an indigenous medicinal plant belongs to the Combretaceae family. It is a deciduous tree that can grow up to 30 m, with a trunk that can be as large as 1 m in diameter. Due to its medicinal

property, it has been used for the treatment of a number of diseases many centuries[11]. It has been well illustrated by Charaka in his text "Charaka Samhita[12]. The phytochemical analysis of *T. chebula* shows the presence of several phytocomponents namely, gallic acid, ellagic acid, tannic acid, ß-sitosterol, ethylgallate, chebulic acid, and mannitol. It is also one of the richest sources of ascorbic acid[12-13]. *T. chebula* is also known as chebulic myrobalan and plays a therapeutic role as homeostatic, antitussive, laxative, diuretic and cardio tonic activities [15-16]. It is also been used as a useful alternative for asthma[17], diabetes mellitus , renal disorders[18], gastric emptying[19] and gastric ulcers[20]. It has been reported as an active antioxidant[21], anticarcinogenic[21], and hepatoprotective agent[22-23]. *T. chebula* has also been found to exhibit anti-HIV[24]and antibacterial activity[25]. Lee *et al.*, [26] reported that chebulagic acid from immature seeds of *T. chebula* was found to suppress the onset and progression of collagen induced arthritis in mice.

Eleocarpus ganitrus commonly known as Rudrakasha belongs to the Elaeocarpaceae family. Rudraksha is a large evergreen broad leaved tree that is found to grow naturally and abundantly in tropical and subtropical areas. It finds a prominent place in Ayurveda, an ancient system of Indian medicine[27]. The fruit is reported to possess several phytoconstituents such as alkaloids, steroids, triterpenoids tannins, flavonoids, carbohydrates and cardiac glycosides[28]. *E.ganitrus* has been reported to yield a potentially anti-cancer agents such as gallic acid, and ellagic acid[29-30]. The phytochemical investigation of *E.ganitrus* fruit has shown the presence of elaeocarpidine, elaeocarpine, rudrakine, quercetin, palmitic acid, isopalmitic acid and linoleic acid[34,29]. *E.ganitrus* fruit was employed for treating various diseases such as hysteria, coma, bronchitis and depression[35]. According to the Ayurvedic medical system, wearing of Rudraksha can have a positive effect on heart and nerves[36]. The fleshy part of the fruit is used for treating various diseases such as epilepsy and mental illness[37]. Singh *et al.*, [38] reported a significant amount of phytocomponents along with ellagitannins that are used in multi targeted therapy of cancer[39]. The other pharmacological activities includes hypoglycemic[40] antihypertensive, anti aging and anti arthritic properties. This herb is also effective in hysteria, cough, asthma[41] hepatic diseases peptic ulcer [42].

Prosopis cineraria is a tanniferous plant, which belongs to the family Leguminosae. It grows extensively in dry parts of tropical regions. *Prosopis* species has been widely used to cure basic ailments in the traditional system of medicine[43]. Several bioactive compounds such as flavonoids, alkaloids, diketones, phenolic contents and vitamins have been isolated from various parts of the plant [44-45]. The bark is considered antihelminthic and is used to treat various ailments, including asthma, bronchitis, and leprosy [46]. The aqueous extract of tree bark was also found to possess anti-inflammatory and anti cancer activity[47]. The smoke of the leaves was used for the treatment of eye ailments. Leaf paste of *P. cineraria* is applied on boils and blisters, mouth ulcers in livestock and on open sores of the skin[48]. *P.cineraria* is also considered to have various medicinal values such as antibacterial, antifungal, antiviral, anti diabetic with many other pharmacological properties; Sharma *et al.*, [44]. The study on phytochemicals in the leaves of *P. cineraria* have showed the presence of alkaloids (spicigerine), steroids (campesterol; cholesterol; sitosterol; stigmasterol), ketones and alkane (hentriacontane).

Literature survey shows that the three components of TD have their proven medicinal value individually. Amalgamation of these is expected to increase the antioxidant activity due to the synergistic effect. Recently, the anticancer activity of TD against aflatoxin B_1 induced experimental hepatocellular carcinoma (HCC) has been reported by Ravinayagam *et al.*, [49]. Cell lines derived from human cancers play a critical role in understanding the molecular pathophysiology of cancer and its treatment. In the present study, TD was qualitatively analyzed for its total phenol and flavonoid content. The cell viability effect of TD on HepG2 cell line was evaluated and one of the active components, gallic acid was separated and characterized using various physico-chemical techniques.

Material and Methods

Chemicals and reagents

The used solvents (ethanol, methanol, ethyl acetate, hexane) were purchased from Sigma-Aldrich and used as-such.

TD preparation with plant materials

TD is an amalgamation of *Terminalia chebula* seed coats, dry seeds of *Elaeocarpus ganitrus* and *Prosopis cineraria* leaves. The three plants were collected and submitted for botanical authentication to the Department of Centre for Advance Study (CAS) in Botany, University of Madras, Guindy Campus, Chennai, India. The authenticated herbarium numbers was, CASBH-16 for *Terminalia chebula*, CASBH-17 for *Elaeocarpus ganitrus*, and CASBH-18 for *Prosopis cineraria*.

Extract preparation

The constituents were washed, air dried in shade and then finely ground. The components were then mixed in equal proportions (on weight basis) to get TD. The finely ground ingredients were mixed in equal proportion as used in traditional medicine. The drug was successively extracted using polarity of solvents (aqueous, ethanol, methanol, ethyl acetate, hexane and Hydro-methanol). The extract of TD was prepared in 3:1 ratio by adding 30 ml of distilled water to 10 grams of combined TD and mixed well. The extract was prepared by the method of Thirunavukkarasu *et al.*, [50] with slight modifications and then concentrated by lyophilization under vacuum pressure to powder consistency. The extract was stored in airtight containers in a dry place.

Preliminary phytochemical screening

A successive extraction of TD was carried out with different solvents such as hexane, ethyl acetate, methanol, hydromethanol and water (non-polar to polar). The extract was subjected to preliminary phytochemical screening of various plant constituents according to Kokate[51]. The total flavonoid content of TD was determined according to colorimetric method as described by Zou *et al*., [52] . The total phenolic content of the extract was determined by Folin-Ciocalteu method[53].

Cell culture

Human hepatocellular carcinoma cell line (Hep G2) was obtained from Cell Repository at National Centre for Cell Science, Pune. The cells were maintained in Centre for Biotechnology, Anna University, Taramani Campus, Chennai, India. Hep G2 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin–streptomycin, and were maintained at 37ºC and 5 % CO2. All cells (1 × 10 4) per well were seeded in 96- well plates. For routine passage, cells were split at 1:4 to 1:6 ratios when they reached confluence, generally every 4 days.

Cell viability effect of TD using dose and time determination studies

MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

The cell viability effect of TD was determined by the MTT assay (Mosmann, [54]. Ten µl of MTT solution (5 mg/ml in PBS) was added to each well. The plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. After the incubation, 180 µl DMSO was added to each well. The plates were kept at dark for one night. Absorbances were measured at 570 nm using a microplate reader (Sirios, Seac Radim Group, Italy). The percent of viable cells was determined by comparing the average absorbance in drug treated cells with control cells exposed to vehicle alone.

Trypan blue assay

Trypan blue assay was performed to assess the cell viability effect of the drug according to the method of Kugawa *et al*., [55]. Trypan blue (0.4%) was dissolved in PBS to be used for cell counting. 0.2 ml of trypsinized cell solution was placed in a tube and 0.3 ml medium was added and then mixed following addition of 0.5 ml 0.4% trypan blue. After 5 min, cells were counted in a hemocytometer. Number of cells not stained with trypan blue was counted as viable cells.

$$\text{Percentage of viability} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} 100$$

DNA fragmentation analysis by agarose gel electrophoresis

Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation by the method of Yokozawa and Dong [56]. The DNA from HepG2 control cells and treated cells were isolated following the manufacturer's instructions provided by the kit (Bangalore Geni) and dissolved in TE buffer. The DNA samples (1mg) were electrophoresed on 1.8 % Agarose gel using TBE buffer at 40 V for 3 h. Then, the gel was stained with Ethidium bromide (EtBr) and viewed under UV –Transilluminator and photographed.

Isolation of phytoconstituent

TLC is a widely employed chromatography technique for the identification of herbal constituents [57]. Based on the TLC pattern, an appropriate chloroform/methanol solvent mixture was selected for the column chromatography. The isolation of pure compound was carried out using column chromatography over silica gel (60–120 mesh) using varying proportion of chloroform: methanol (95:5, 90:10, 85:15,80:20, 75:25, 70:30, 65:35, 60:40 v/v) as eluent.

Characterization of pure compounds

Elemental analysis

The elemental composition of the pure compound was determined using Perkin Elmer A Analyst 100 atomic absorption spectroscopy.

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR is used to obtain and facilitate the identification of bioactive compounds [58]. The mid-infrared spectrum of sample was recorded on a Nicolet 6700 instrument using a KBr pellet technique. About 4 mg of sample was ground with 200 mg of spectral grade KBr to form a mixture and then made into a pellet using a hydraulic press under the pressure of 5 ton/cm^2 into a self-supported wafer of 13 mm diameter. This pellet was used for recording the infra-red spectra in the region 400-4000 cm^{-1}.

Differential pulse voltammeter (DPV)

DPV has been applied to characterize a range of antioxidants, including phenolic acids and flavonoids [59-61]. DPV technique is helpful for correlation between redox potentials and antioxidant properties [62, 63]. Differential pulse voltammetric study was performed based on the method of Naik *et al*., [64] on Ecochemie Auto Lab, PGSTAT 20 Model and by using a conventional three electrode system of glassy carbon working electrode, an auxiliary electrode (platinum wire) and a reference electrode (Ag–AgCl). Glassy carbon electrode was resurfaced with alumina. The electrochemical cell containing aqueous solution of 100 mg/ml extract and 0.1 M KCl buffer (pH 7.4) was kept under thermostatic condition at 25 ± 0.1˚C. The cyclic voltammograms were recorded in differential pulse voltammeter at the voltage range of -0.25 to 1.3 V at a scan rate of 100 mV/s.

Single crystal x-ray diffraction

Single crystal of gallic acid monohydrate with dimensions 0.35 x 0.30 x 0.25 mm was glued on glass needle with araldite. This needle was mounted on a sample holder, fixed and aligned in the x-ray goniometer. The data were collected at 293 K on a Bruker-Axs Smart Apex diffractometer using graphite monochromatized Mo Ka radiation (k = 0.71073 Å). The dimension of the crystal

were measured and the x-ray diffraction pattern collected using SHLEXTL (5.1 V) All structures were solved by direct methods and subsequent Fourier difference techniques and refined anisotropically for all non-hydrogen atoms by full-matrix least squares calculations on F2 using the SHELXTL package.

Nuclear magnetic resonance analysis (NMR)

The identification of gallic acid was carried out using ^{1}H-NMR, and ^{13}C-NMR spectroscopy analysis. The ^{1}H-NMR spectrum was recorded by Varian Unity Inova 400MHz NMR spectrometer operating at the frequency of 400 MHz in deuterated $CDCl_3$ solution with tetramethylsiloxance (TMS) as internal standard. NMR data were acquired for 64 scans at a temperature of 30 ^{o}C. The ^{13}C-NMR measurement was made with coherent off-resonance ^{1}H decoupling or with broad-band ^{1}H decoupling at the frequency of 100.6 MHz.

Scanning electron microscopy (SEM)

SEM image was recorded using a JEOL, JSM-5500LV scanning electron microscope. The samples were suspended in methanol and the specimen stub was dipped into the liquid, and evaporated. This specimen was coated with gold using ion sputter coater then pictures were developed on thin photographic paper.

Results and discussion

Phytochemical analysis

Phytoconstituents are important source of antioxidant and are capable to terminate the free radical chain reactions [65]. The secondary plant metabolites constituting several unknown pharmacological activities have been investigated as a potential source of therapeutic agents through preliminary phytochemical analysis. The phytochemical analysis of TD crude extract was performed to assess the presence of secondary metabolites. Successive isolation of phytochemical components from plant material is largely dependent on the type of solvent used in the extraction procedure. Phytochemical analysis of TD was carried out in polar and non polar solvents. The extracts used were water, hydromethanol, methanol, ethyl acetate and hexane.

Table 1 shows the presence of various components in the different extracts which were analyzed qualitatively. Analysis of the extracts revealed the presence of secondary metabolites such as alkaloids, flavanoids, tannins, phenols, steroids, carbohydrates, saponins, terpenoids and protein. Comparatively, the aqueous extract of TD was found to possess the maximum number of phytochemicals and this was used for further investigations. There has been great interest in the use of such natural antioxidants for the prevention or therapy of many diseases caused by the implication of oxidation stress[66].

Estimation of total phenol and flavonoids content of TD

Fig.1 depicts the total phenolic content of TD aqueous extracts. The phenolic content was calculated as gallic acid (mg/g) equivalents. Several plant products, having distinctive phenolics that possess antioxidant activity are known to be mainly polyphenolic substances[67]. There was a positive correlation between the total phenolic content and antioxidant capacity. The phenolics, particularly polyphenols exhibit a wide variety of beneficial biological activities in mammals, including antiviral, antibacterial, immunostimulant, antiallergic, hepatoprotective, antiinflammatory and anticarcinogenic effects[68]

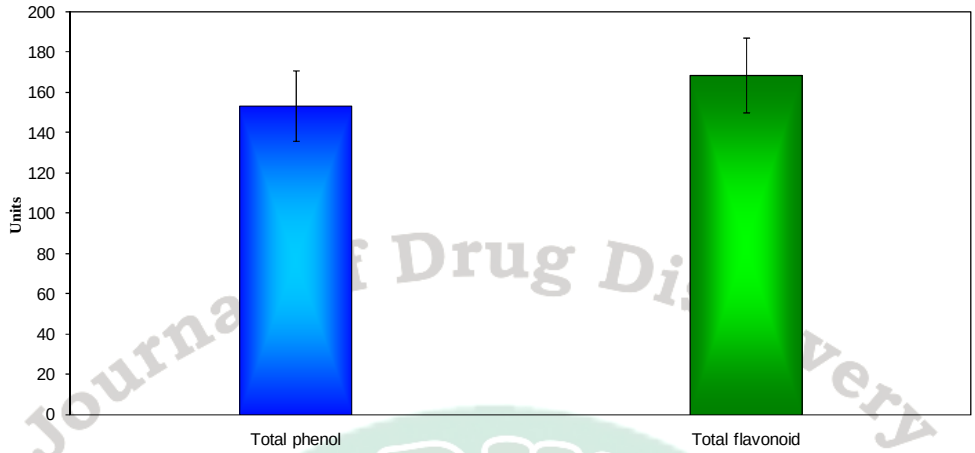
Flavonoids contain certain health effects and their antioxidant, radical scavenging, anti-mutagenic and anti-carcinogenic properties are well known[69]. Fig.1 depicts the total flavonoid content of TD aqueous extracts. The flavonoids content was calculated as rutin (mg/g) equivalents. The therapeutic applications of flavonoids are important in the human body system. Biochemical investigations on the mechanism of action of flavonoids have shown that these compounds can inhibit a wide variety of enzymes and protect the cell[70].

FT-IR spectroscopy analysis of crude TD

FT-IR provides valuable information regarding the identification of compounds or functional groups present in an unknown mixture of plant extracts[71-1-72]. Fig.2 shows the FT-IR analysis of the crude TD. The appearance of strong absorption bands in the region of 3339 to 3332 cm^{-1} usually comes from stretching vibrations of the O-H and N-H stretching frequencies.

Hydrogen bonding has a significant influence on the peak shape and intensity, causing peak broadening and shifts in absorption to lower frequencies. The CH stretching bands of alkene and aromatic C-H stretch vibrations are clearly seen at 2973 to 1447 cm^{-1}. Almost all the carbonyl C=O stretching bands are strong and occur at 1870 to 1550 cm^{-1}, whereas ketones, aldehydes, carboxylic acids, amides and esters showed IR absorption at 1709 to 1610 cm^{-1}. Conjugation, ring size, hydrogen bonding, steric and electronic effects often result in significant shifts in absorption frequencies. C-N bending alkaloids have absorption bands at 1447-1206 cm^{-1}. Aromatic compounds contain delocalized p electrons from the resonance-stabilized double bonds, showing skeletal vibrations (including C-C stretchings within the ring) in the region between 1650 to 1400 cm^{-1} and weak combination and overtone bands in the region between 2000 to 1650 cm^{-1}. This result shows that TD contains various compounds such as aldehydes, ketones carboxylic acids, amides, esters and alkaloids.

Fig. 1. Total phenol and flavonoid content of TD extract.



Values are the average of duplicate experiments and represented as mean ± SD
Units: Total phenol - mg/g, Gallic acid equivalents, Total flavonoid - mg/g, Rutin equivalents

Fig. 2. Analysis of crude TD aqueous extract by Fourier Transform- Infra Red (FT-IR) spectroscopy

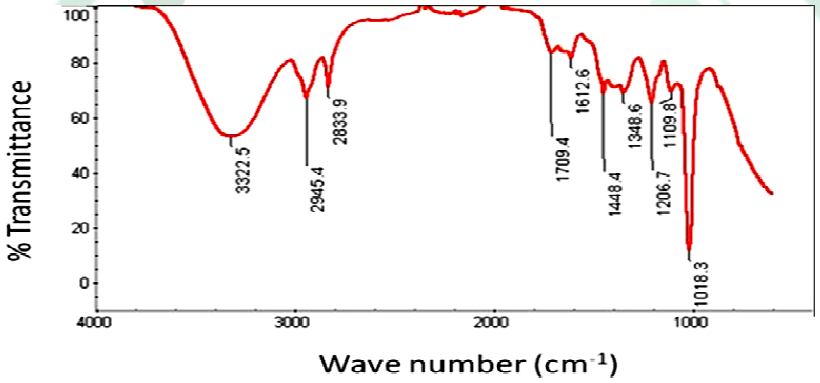
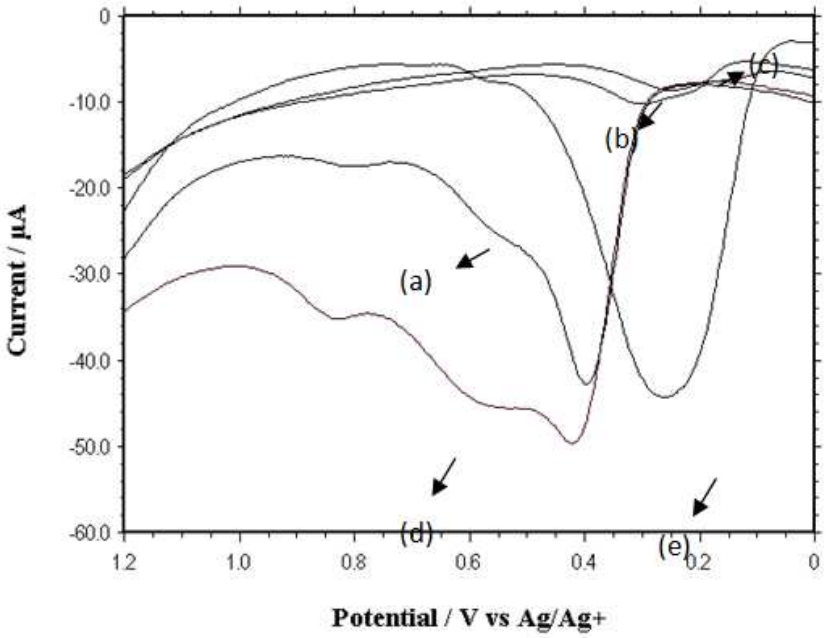


Table 1. Phytochemical analysis of Tridham (TD) in different extracts

S.No	Name of the test	Hexane Extract	Ethylacetate Extract	Methanol Extract	Aqueous Extract	Hydro Methonolic Extract
1	Alkaloids	-	-	-	+	+
2	Flavonoids	-	-	+	+	+
3	Tannin	+	+	+	+	+
4	Steroid	-	-	-	+	-
5	Sugar	+	+	+	+	+
6	Terpenoids	+	+	+	+	-
7	Saponin	+	-	-	+	-
8	Protein	-	+	+	+	+

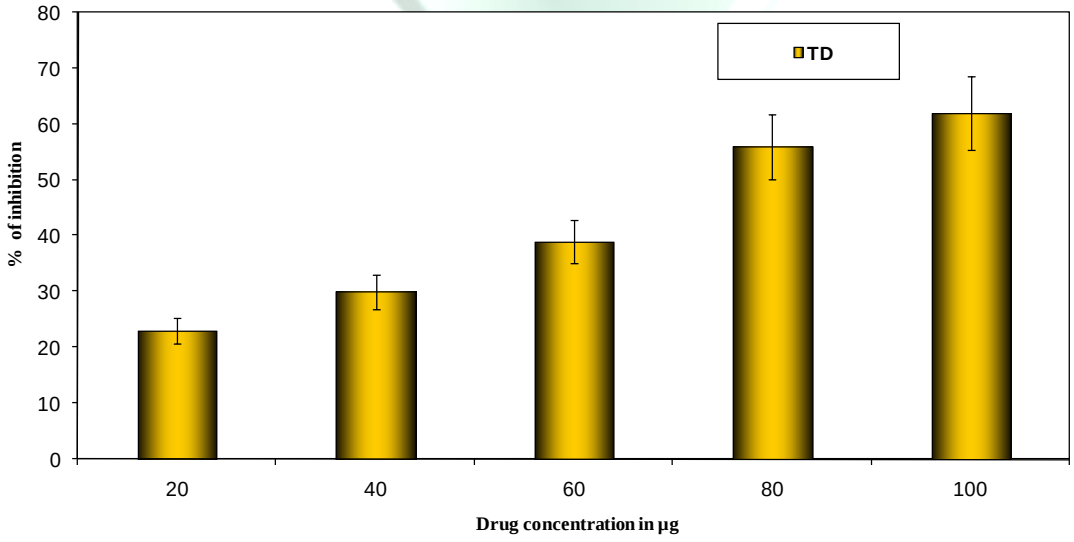
+ Indicate presence, – Indicate absence

Fig. 3. Redox potential of individual ingredients



a *Terminalia chebula*
b *Elaeocarpus ganitrus,*
c *Prosopis cineraria,*
d combined ingredients TD aqueous extract and
e standard (ascorbic acid) using differential pulse voltammeter.

Fig.4. Cell viability effect of TD on HepG2 cells by MTT assay.



Values are expressed as mean ± SD for three independent experiments **Redox potential of crude TD using differential pulse voltammeter**

Electrochemical techniques are commonly used to obtain the redox potential of polyphenols to estimate the reducing power of an antioxidant[73]. This technique in principle is based on the electrochemical properties of the sample[74]. Among the electrochemical methods, DPV technique is a rapid and effective analysis that provides higher sensitivity and better peak resolution than CV for studying the electrochemical behavior of biological systems[75]. Fig. 3 shows the voltagrams for aqueous extract of individual ingredients (a) *Terminalia chebula, (b) Elaeocarpus ganitrus, (c) Prosopis cineraria,* (d) TD and (e) Standard (ascorbic acid). In the present study ascorbic acid was taken as a standard for analysis of aqueous extracts of TD. For ascorbic acid the reduction potential is at 0.25 V, whereas for TD the reduction potential is at 0.42 V. Differential pulse voltammeter confirms the presence of *Terminalia chebula, Elaeocarpus ganitrus* and *Prosopis cineraria*, The three aqueous extracts have peak maxima at low reduction potentials of 0.4 V, 0.32 V, 0.27 V and, these values are lower than TD reduction potential. These results show that TD contains easily oxidizable polyphenols compared to standard and individual ingredients that might help in arresting the free radical nature of several oxidants via the electron transfer reaction.

Cell viability effect of TD on HepG2 Cell lines

Hep G2 cells were treated with TD to study the cytotoxic effect (Fig. 4). As the concentration increased from 20 to100 µg/ml, the percentage inhibition of HepG2 cells increased with increasing dosage of TD treatment. The growth was inhibited by 50% in cells (IC_{50}) exposed to 80µg/ml. TD mediated growth inhibition might be exerted through a cytotoxic effect. Treatment with TD caused a dose-dependent reduction in cancer cell survival.

Cell viability effect of TD on Hep G2 cells by Trypan blue assay.

Fig. 5 shows the results of Trypan blue assay. The Hep G2 cells were treated with the TD for 24h, 48h and 72h. Therein the total cell count was determined using a hemocytometer. Based on their time kinetics, the 48 h treatment was more effective than 24 and 72 h. So, in the present study, 48h treatment period was used. Chronological concentrations of drug and time incubation showed significant growth inhibition of Hep G2 cells.

DNA fragmentation analysis

DNA fragmentation is a hallmark of apoptosis[76]. Apoptosis is cell autonomous and is accompanied by the shrinkage and fragmentation of both cells and their nuclei, loss of microvilli and extensive degradation of chromosomal DNA[77]. Fig.6 depicts the agarose gel electrophoresis of DNA from HepG2 cells treated with TD. The HepG2 cells showed DNA fragments on incubation with TD (Group II). Whereas the untreated HepG2 cells did not show any DNA fragmentation. These studies support the possibility that the cytotoxicity of TD was through apoptosis.

The cytotoxic effects observed in the present study may be attributed to the potential effect of the three components present in TD. DNA fragmentation in the TD treated groups may be attributed to the presence of the components of the drug which possess anti tumour activity. Kamei *et al*., 1999 has reported *Terminalia* to exhibit growth suppression against HTC-15 and HepG2 cells. *Terminalia chebula* extract has gallic acid and chebulinic acid that is reported to have growth inhibitory and cytotoxic effects on several human cancer cell lines [78, 79]. Elaeocarpus species has been found to have cytotoxic activity towards nasopharynx carcinoma cell lines[80]. Therefore the present cytotoxicity might be due to the synergistic effect of the constituents of TD that possess antioxidants, phenols, tannins, flavanoids and polyphenol. Thus, the observed decrease in cell viability and reduction in cell count on incubation with the drug might have been brought about by flavonoid arbitrated cytotoxicity. Numerous recognized phenolic compounds have been reported to induce apoptosis[81] which may be a primary mechanism of their anti-cancer activity. The results of the present study reveal that TD induces active growth inhibition in HepG2 cells. It is therefore certain that many components present in TD are known to induce apoptosis in cancer cells. Therefore, isolation of active natural component from TD may provide the possibility of an application for cancer prevention and therapy.

Isolation and identification of gallic acid in TD

The isolation of pure compound was carried out by analyzing TLC and using column chromatography. The asymmetric unit of title compound, $C_7H_6O_5.H_2O$, consists of one 3,4,5-trihydroxybenzoic acid molecule (gallic acid) and one water molecule (Fig. 7). The compound crystallizes in monoclinic system with space group, P21/c. The gallic acid molecule is essentially planar. In the crystal, the components are linked by O—H---O hydrogen bonds. An intramolecular O—H---O hydrogen bond occurs in the gallic acid molecule, which is linked to the water molecule by a further O—H---O hydrogen bond. The crystal structure has two intramolecular hydrogen bonds and four intermolecular hydrogen bonds. The C—C bond distances range from 1.3789(18) Å to 1.4704(18) Å. The longest C—C bond distance is between C6 and C7 with 1.4704(18) Å. The C—O bond distance are range from 1.2283(17) Å to 1.3637(17) Å. The shortest C—O bond distance is between C7 and O4 with 1.2283(17) Å. The C—O bond distance for different crystal structure was given 1.359 (2) Å by Genç *et al.*, [82]. The torsion angles at ring belong to gallic acid molecule are range from 0.0 (3)° to 0.28 (19)°. Therefore, the gallic acid molecule close to planar. The torsion angles for the C(6)-C(1)-C(2)-C(3) -0.9(2) and for C(6)-C(1)-C(2)-O(1) is 179.54(14). Up to now, the different crystal structure of gallic acid monohidrate form have been reported by Jiang *et al.*, and

Okabe *et al.*,. The unit-cell parameters belong to these crystal structures have given in Table 2. [83,84]

FT-IR spectroscopy analysis of pure gallic acid

The FT-IR spectra of pure isolated gallic acid showed typical hydroxyl stretching frequencies due to phenolic and carboxyl acid groups in the region 3220-3390 cm^{-1}, overlapped with aromatic C-H stretch at 3020 cm^{-1} (Fig. 8). The characteristic bands of COOH is elucidated with the presence of band near 1700 cm^{-1} due to carbonyl C=O stretch and a band near 1450 cm^{-1} appears due to C-O single bond. The C=C double bonds of aromatic appear at 1620 cm^{-1}. The spectral feature in the range 1240 – 1330 cm^{-1} was attributed to the phenolic stretch. The weaker bands in the range 1020-694 cm^{-1} are for the out of plane CH bending of aromatic ring. The reported framework bands clearly coincide with earlier report suggested for gallic acid [85].

Spectral and elemental analysis

The purified compound appear as brown crystals: The chemical shift values of ^{1}H-NMR and ^{13}C-NMR spectra are given as follows. ^{1}H-NMR (300MHz, $CDCl_3$) δ: 3.98 (d, 4H, OH), 6.9 (s, 2H, Ar^{CH}), 11.19 (s, 1H, COOH). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 109.5, 121.2, 138.8, 145.9, 168.6. The composition of the crystal was deduced from the elemental analysis. Anal. Cal. for $C_{32}H_{44}N_2O$ C, 81.31; H, 9.38; N, 5.93. Found: C, 81.33; H, 9.34; N, 5.95.

SEM images

The scanning electron microscopy technique is a powerful method for investigation of surface structures of herbal medicines namely leaves, pollen grains and seeds[86]. The particle morphology of the crude aqueous extract of TD and therein purified sample gallic acid was investigated using scanning electron microscopy (Fig. 9). SEM not only produces images that are analogous to those from an optical microscope, but it also can produce images whose contrast is based on compositional variations of specimens. The SEM images of crude TD and purified gallic acid with different morphologies for crude and purified samples are compared at 1, 5 and 10µm, respectively. The SEM image of crude shows an irregular morphology with a mixture of uneven crystals with no obvious inter crystalline space (Fig. 9 (a,b,c)). The presence of a distinct rod like morphology was observed for purified sample, with twisted, pilling of fibrils (Fig. 9 (a_1,b_1,c_1)). At a similar magnification of 10 µm, the observed features of purified gallic acid coincide with the SEM images of original gallic acid which was obtained through recrystallization using the batch supercritical anti-solvent process[87].

In conclusion, the present study shows the preliminary qualitative analysis of TD in non polar and polar solvents. Comparatively, the aqueous extract of TD was found to possess bioactive components such as flavonoids, tannins, phenols, steroids, carbohydrates, saponins, terpenoids and proteins. The presence of several phytoconsituents was identified by spectroscopy analysis such as FT-IR. Assay of electrochemical analysis by DPV revealed that TD contains easily oxidizable polyphenols compared to standard and individual ingredients. One of the natural antioxidants, gallic acid was isolated with high purity from TD. The structure was identified on the basis of several spectroscopic and microscopic techniques. Gallic acid is a phenolic antioxidant compound that helps to protect cells against oxidative damage[88,89]. The presence of gallic acid in TD makes it a potential therapeutic agent. It also needs to be mentioned that further isolation of several unknown antioxidant compounds present in TD is underway, to fully understand the activity of TD for the development of the therapeutic drug.

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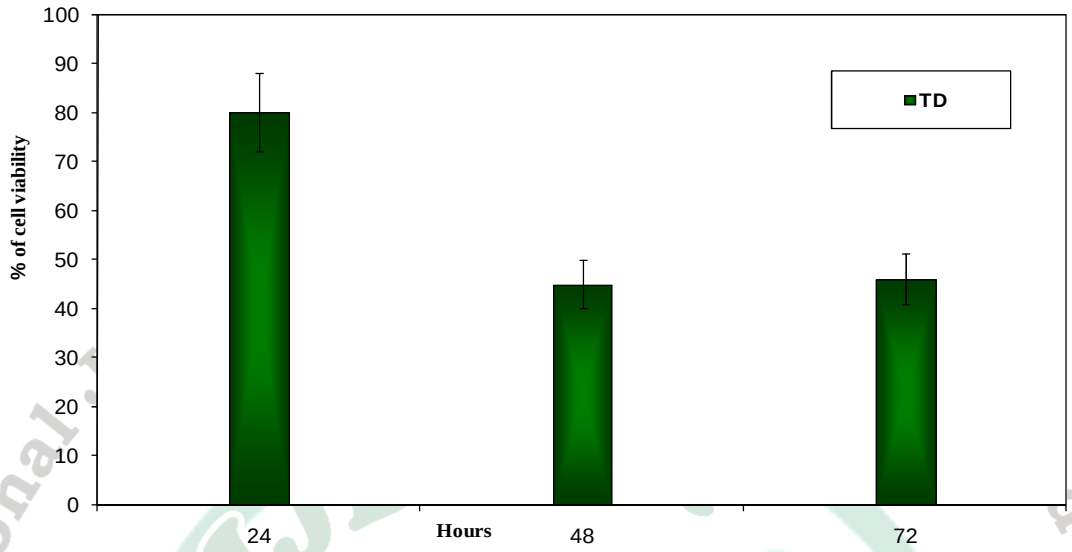
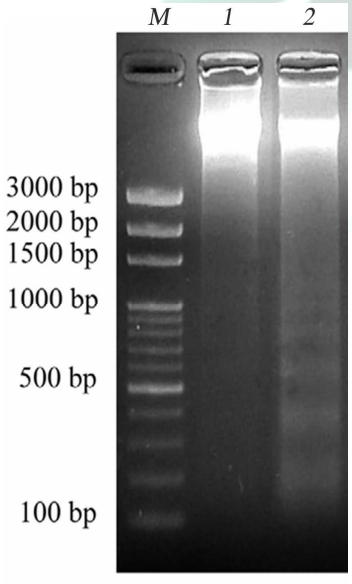
Fig.5. Cell viability effect of TD on Hep G2 cells by Trypan blue assay.



Values are expressed as mean ± SD for three independent experiments

Fig.6. DNA fragmentation analysis by agarose gel electrophoresis



Lane M-Marker

Lane 1-Hep G2 cells

Lane 2-Hep G2 cells treated with TD

Fig.7. The crystal structure of the title compound, $C_7H_6O_5.H_2O$. The crystal structure has been deposited at the Cambridge Crystallographic DataCentre and allocated the deposition number CCDC 853620.

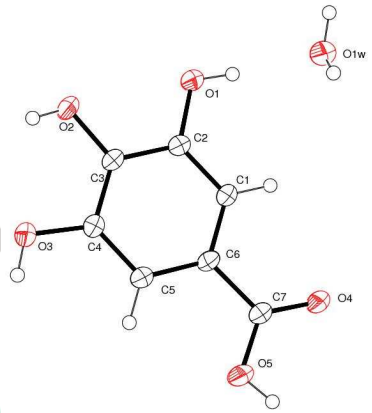


Fig. 8. Analysis of gallic acid isolated from TD aqueous extract using Fourier Transform- Infra Red (FT-IR) spectroscopy

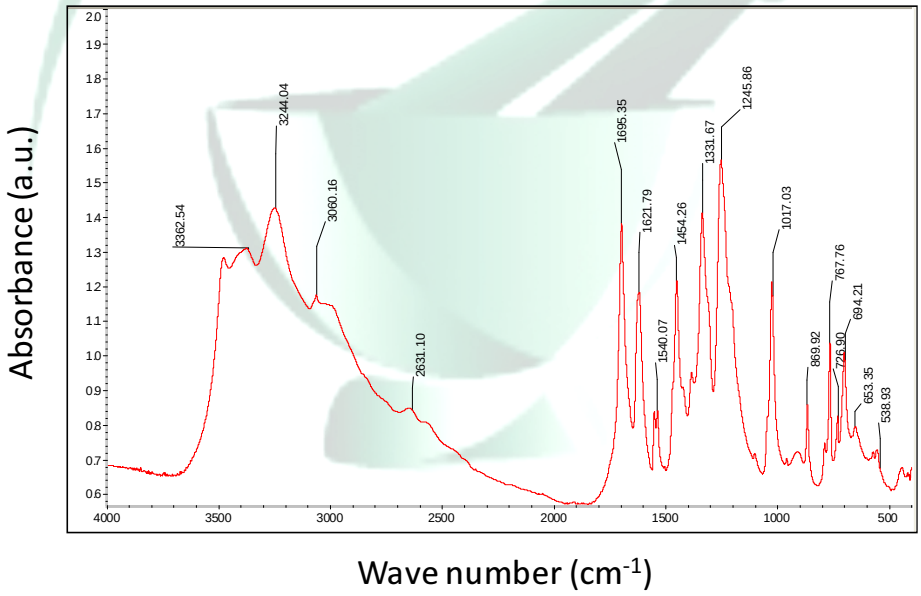


Fig. 9. Scanning electron microscopy (SEM) images showing the surface view of crude and isolated pure compound gallic acid (Scale bar = 1, 5, 10 µm).

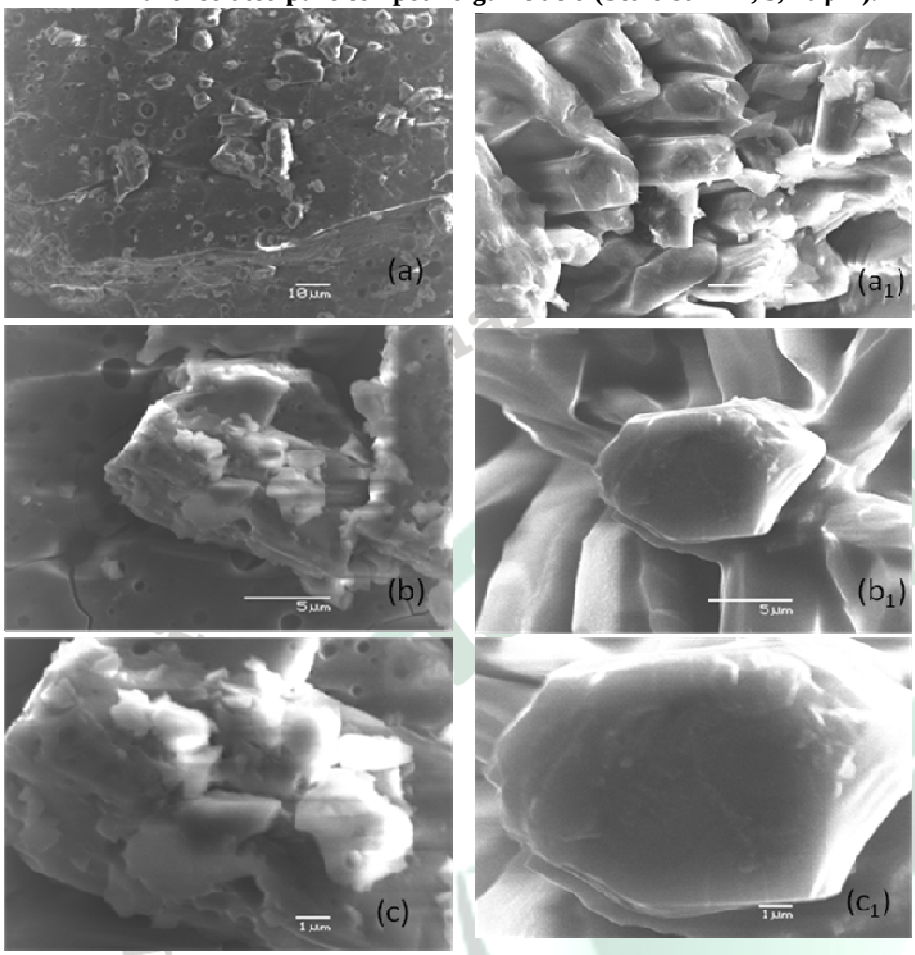


Figure 9a: Crude aqueous TD at 10 µm
Figure 9b: Crude aqueous TD at 5 µm
Figure 9c: Crude aqueous TD at 1 µm
Figure $9a_1$: Isolated Gallic acid at 10 µm
Figure $9b_1$: Isolated Gallic acid at 5 µm
Figure $9c_1$: Isolated Gallic acid at 1 µm

Table 2. Comparative crystal structures of gallic acid monohydrate (Å,°).

Unit-cell parameters		
Jiang *et al.*, (2000)	**Okabe *et al.*, (2001)**	**Gallic acid from TD**
Monoclinic	Monoclinic	Monoclinic
Space group: P21/c	Space group: P2/n	Space group: P21/c
a = 5.794 (4)	a = 14.15 (1)	5.7774(2) A
b = 4.719 (5)	b = 3.622 (9)	4.70540(10) A
c = 28.688 (5)	c = 15.028 (10)	c = 28.6586(10)
β = 95.08 (3)	β = 97.52 (7)	beta = 95.214(2) deg
V = 781.4 (3)	V = 764 (1)	775.86(4) A^3

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