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Identification of Flavonoids in Different Parts of *Terminalia catappa* L. Using LC-ESI-MS/MS and Investigation of Their Anticancer Effect in EAC Cell Line Model

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Abstract

In the present study, attempts are made to evaluate the *in vitro* cytotoxic potentials of aqueous extracts of *Terminalia catappa* L. bark, fruits and wood against Ehrlich Ascites Carcinoma (EAC) cell line and also to identify their phytochemical constituents using LC-ESI-MS/MS. Data of the results obtained from the present study revealed that the aqueous extract of fruit is more effective in controlling the growth of EAC cell lines (84.97%) when compared to bark (67.89%) and wood (37.34%) of *T. catappa*. LC-ESI-MS/MS analysis revealed the presence of Isorhamnetin in bark, fruit and wood, Rottlerin and Limocitrin in bark and wood, and Iristectorin-A in fruit and wood. Presence of various flavonoids such as Quercetin-3-Glucuronide, Quercetin-3,4'-O-di-beta-glucopyranoside, Geniposide, Rutin, Hesperetin, Flavanomarein, Kaempferol-7-neohesperidoside, Baccatin, Isorhamnetin, Peonidin, Iristectorin-A, Scoparin, Tricin, Cirsiliol and Isorhamnetin-3-Glucoside-4'-Glucoside in the fruit extract could be responsible for its higher level of anticancer effect when compared to bark or wood of *T. catappa*.

Keywords: *Terminalia catappa*; Aqueous extract; LC-MS; Flavonoids; EAC cell line; Anticancer; MTT assay.

INTRODUCTION

Cancer is one of the major health problems encountered in both developed as well as developing countries (Jemal *et al*., 2003). It is one of the most dreaded diseases of the 20th century and spreading further with continuance of increasing incidence in 21st century (Balachandran and Govindarajan, 2005). Chemoprevention is a rapidly growing field of oncology which aimed at preventing the cancer growth using natural or synthetic interventions (Sporn and Liby, 2005). Chemotherapy using synthetic drugs can produce severe toxic side effects, which resulted in restricted usage of the same (Ito *et al*., 1985). In recent years, a considerable attention has been paid to identify naturally occurring chemopreventive substances capable of inhibiting, retarding or reversing the process of carcinogenesis (Shukla and Kalra, 2007). Many chemical molecules isolated from plants and dietary sources have been reported to possess potentials to inhibit and delay the multistage process of tumour growth (Surh *et al*., 1998). The important advantages of plant based medicines are their safety, efficacy and affordability (Siddiqui, 1993).

Terminalia catappa L., a large spreading tree belonging to the family Combretaceae, is distributed throughout the tropics in coastal environments. Various extracts of leaves and bark of *T. catappa* have been reported to exhibit antibacterial (Neelavathi *et al*., 2013; Sangavi *et al*., 2015), anti-fungal (Parimala Gandhi *et al*., 2015), anti-inflammatory (Lin *et al*., 1999; Sivaranjani *et al*., 2015; Venkatalakshmi *et al*., 2015, antioxidant, anti-tumour (Venkatalakshmi *et al*., 2014), anti-HIV (Tan *et al*., 1991), hepato-protective (Chen *et al*., 1996) and anti-diabetic properties (Nagappa *et al*., 2003) besides being aphrodisiac (Ratnasooriya and Dharmasiri, 2000). The moderate consumption of the seed kernel is useful in treating sexual dysfunction among men, primarily for premature ejaculation (Ratnasooriya and Dharmasiri, 2000). The ethanol extract of the leaves of *T. catappa* inhibits osmotically-induced hemolysis of human erythrocytes in a dose-dependent manner (Chen *et al*., 1996). Punicalagin and punicalin isolated from the leaves are used to treat dermatitis and hepatitis as both have strong antioxidative activity (Lin *et al*., 1999).

In view of these therapeutic potentials of this plant, only the leaf material was extensively studied and other parts such as bark, wood and fruits are not investigated in detail. Hence, the present study has been taken up with an objective of assessing the *in vitro* cytotoxic potentials of bark, fruits and wood of *Terminalia catappa* against EAC cell line model in addition to analyzing their phytochemical profile using LC-MS.

MATERIALS AND METHODS

Collection of plant material

The bark, fruits and wood of *Terminalia catappa* were collected from Mannargudi, Tamil Nadu. Plant materials were identified and authenticated in the department of CARISM, SASTRA University, Thirumalaisamudram, Tamil Nadu. Voucher Specimen was prepared and

deposited in Rabinat Herbarium, St. Joseph College, Trichy, TN, India. Collected materials were cleaned, shade dried and coarsely powdered in a lab mill to 1 mm particle size and used for further analysis.

Preparation of the extracts

Powdered samples of bark, wood and fruit of *T. catappa* L. were used for the preparation of aqueous extracts. The aqueous extract was prepared by taking 25 g of raw material in 250 ml of distilled water and kept at room temperature for 24 h. Then the content was filtered and the filtrate was frozen and then lyophilized. The residue was re-suspended in water at 1 mg/ml ratio and used for the experiment.

Anticancer activity

The EAC cells were obtained from Swiss mice after 15 days of induction in the Central Animal Facility, SASTRA University, Thanjavur. The peritoneal fluid containing EAC cells was collected aseptically using a 5 ml syringe and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) at 37°C. The cells were dispersed in a 96 well plate with a cell count of 9000 cells per well. Then the aqueous extracts of bark, wood and fruit of *T. catappa* was added at different concentrations (1000, 500, 250, 100, 50, 25 and 10 µg/ml) and then again incubated for 24 h in CO2 incubator with 5% CO2. The cells grown in medium without plant extract were considered as control. At the end, the medium was discarded, cells are washed with PBS and then 20 µl of MTT reagent (3,4,5-dimethyl thiazol-2-yl 2,5 di phenyl tetrazolium bromide) was added in each well and incubated for 6 h at 37°C in a water bath according to the method of Scudiero et al. (1988). Then 150 µl of acidic isopropanol was added and shaken for 30 min on a plate shaker under dark. The absorbance was measured at 540 nm in a micro-plate reader and the percentage growth inhibition was calculated using the following formula: (Absorbance of control - Absorbance of treated / Absorbance of control) x 100.

LC-MS analysis

The aqueous extract was filtered using 0.45 µm syringe filter and analyzed using liquid chromatography coupled to mass spectrometer (LC/ESI/MS/MS, MicroTOF-Q II, Bruker, Germany) to identify the phytochemical cosntituents. Solution (50 µl) was injected for liquid chromatography separations in a C18 reverse phase column (120 Å, 2.1 x 150 mm, 3.0 µm, Dionex, USA). UV detector was set arbitrarily at 330 nm. A discontinuous gradient elution at a flow rate of 0.2 ml/min was performed using mobile phase A represent acetonitrile and mobile phase B represent water (MilliQ) acidified with acetic acid (1%). The gradient started from 1% of A for 0.2 min and it was then brought to 75% A at 16th min and then reaching at 100% A at 19th min to 5% A at 21st min and was maintained at same condition till run ends at 30th min. Eluted compounds were then identified using MS and their respective MS/MS pattern. Mass spectrometer with ESI ionization at negative mode equipped with HyStar 3.2 software was optimized to detect the exact mass and mass fragmentation pattern of each eluted compound. TIC spectra were acquired and elaborated using the HyStar software Data Analysis module. MS/MS experiments were carried out by means of Auto scanning mode, where the mass spectrometer software made a choice in real time about the selection of ion to fragment based on the intensity of each peaks with a threshold set above 1500 absolute counts. Optimized parameters consisted in collision energy 10 eV, focusing potential of 350 voltage per peak, transfer time of 800 µs, pre-pulse storage of 5 µs the instrument was operated in the negative ion mode with a capillary voltage of 4.5 KV, capillary temperature was 270°C, sheath gas (N2) flow rate was 6 ml/min with 30.5 psi pressure and the data were acquired in the AutoMSn scanning modes with the scan range of 100 – 1500 m/z, Collision RF was 350.0 Vpp and Collision energy was set at 15.0 eV. The results of molecular mass were compared with mass bank data and the phytochemicals were identified.

RESULTS AND DISCUSSION

Cancer is often associated with increased risk of death and the toxic side effects caused by the modern medicine, many cancer patients seek alternative and complementary methods of treatment such as usage of phytomedicine (Kim and Park, 2002). At present chemotherapy is considered as the most efficient approach for cancer treatment. Even though it significantly improves symptoms and the quality of life of cancer patients, only modest increase in survival rate can be achieved. As a palliative care, many cancer patients use herbal therapies. Medicinal plants are well known for their immunomodulatory and antioxidant activities by enhancing both non specific and specific immunity (Lin *et al*., 1996; Agarwal *et al*., 2001). Plants contain phytochemicals with strong antioxidant activities which may prevent and control cancer and other diseases by protecting the cells from the deleterious effects of the 'free radicals'. Now-a-days researchers are focusing their research towards the development of an ecofriendly anti caner drug from plant sources, which resulted in newer chemotherapeutic agents such as paclitaxel, vincristine, podophyllotoxin and camptothecin.

In the present study a common plant *T. catappa* was selected and aqueous extracts of bark, fruits and wood were screened for anticancer potential against Ehrlich Ascites Carcinoma cell lines employing MTT assay with a view to develop a natural and safe anticancer drug. Aqueous extract of bark produced cytotoxicity up to 68% at a 1000 µg/ml (Figure 1). Even at a concentration of 250 µg/ml, cytotoxicity percentage was found to be more than 50%. Aqueous extract of fruits has shown maximum cytotoxicity against EAC cell lines (85% at 1000 µg/ml). Cytotoxicity was found to be maximum even at a very low concentration (56% at 50 µg/ml). The death of the cells caused by the test drug might be due to the loss of mitochondria which is one of the hallmarks of the apoptosis pathway (Christopher, 1992). Aqueous extract of wood

exhibited only minimum cytotoxicity when compared to other extracts. From the MTT assay, it is evident that the cytotoxicity of the extracts was dose dependent. Based on the results obtained from the present study, the aqueous extract of fruit was found to be more effective in

controlling the growth of EAC cell lines when compared to bark and wood extracts of *T. catappa*. Hence, *Terminalia catappa* fruit extract could be considered as a source of potential anticancer drug and further *in vivo* models must be used to establish its anticancer efficacy.

LC-MS analysis of aqueous extract of different parts of *T. catappa* revealed the presence of various high polar compounds (Table 1, Figure 2, Supplementary file 1). Bark contains Isorhamnetin, Chlorogenic acid, Rottlerin, Chrysoeriol, Morin, Limocitrin, Peltatoside, Thermopsoside and Iridin (Figure 3). Quercetin-3-Glucuronide, Quercetin-3,4'-O-di-beta-glucopyranoside, Geniposide, Rutin, Hesperetin, Flavanomarein, Kaempferol-7-neohesperidoside, Baccatin, Isorhamnetin, Peonidin, Iristectorin-A, Scoparin, Tricin, Cirsiliol and Isorhamnetin-3-Glucoside-4'-Glucoside were present in fruit extract of *T. catappa* (Figure 4). The *T. catappa* wood extract possess the phytochemicals such as Mucic acid, Quercetin-7-O-rhamnoside, Limocitrin, Rottlerin, Sophoricoside, Hesperetin, Quercetin-3-D-xyloside, Isorhamnetin, Iristectorin-A, Kaempferol-3-O-alpha-L-arabinoside and Myricetin-3-Galactoside (Figure 5).

Isorhamnetin was found to present in bark, fruit and wood of *T. catappa*, which was reported to exhibit antioxidant and anti-inflammatory effects with inhibition of COX-2 expression (Seo *et al*., 2014). Chemopreventive action of isorhamnetin was proved against different cancer cell types (Kim *et al*., 2011; Saud *et al*., 2013; Li *et al*., 2014). Rottlerin and Limocitrin were found to occur in both bark and wood of *T. catappa*. Anticancer effect of rottlerin and limocitrin has been reported from previous studies (Guthrie *et al*., 2000; Kim *et al*., 2005; Jane *et al*., 2006; Kurosu *et al*., 2007). Iristectorin-A in found in both fruit and wood. Zhu *et al*. (2014) reported the anticancer role of Iristectorin-A compound in the rhizome of *Belamcanda chinensis*.

Highest anticancer potential was observed in the fruit extract of *T. catappa* when compared to bark and wood (Figure 1), which be due to the presence of a large number of phytochemicals such as Quercetin-3-Glucuronide, Quercetin-3,4'-O-di-beta-glucopyranoside, Geniposide, Rutin, Hesperetin, Flavanomarein, Kaempferol-7-neohesperidoside, Baccatin, Isorhamnetin, Peonidin, Iristectorin-A, Scoparin, Tricin, Cirsiliol and Isorhamnetin-3-Glucoside-4'-Glucoside in the fruit extract of *T. catappa*. Because, the anticancer activity was reported for most of these compounds such as Quercetin-3-glucuronide (Moon *et al*., 2001), Rutin (Metodiewa *et al*., 1997; Guardia *et al*., 2001), Hesperetin (Zarebczan *et al*., 2011), Peonidin (Ho *et al*., 2010) and Tricin (Cai *et al*., 2005). Thus, presence of such active principles contributed the high anticancer activity shown by the *T. catappa* fruit extract.

S. No.	Bark	Fruit	Wood
1.	Isorhamnetin (MW 316, RT 8.9-9.3)	Quercetin-3-Glucuronide (MW 478, RT 2.9-3.2)	Mucic acid (MW 210, RT 3.5)
2.	Chlorogenic acid (MW 354, RT 9.7-9.8)	Quercetin-3,4'-O-di-beta-glucopyranoside (MW 626, RT 6.1-6.2)	Quercetin-7-O-rhamnoside (MW 448, RT 6.8-6.9)
3.	Rottlerin (MW 516, RT 9.9-10.3)	Geniposide (MW 388, RT 6.5-6.6)	Limocitrin (MW 346, RT 7.5-7.6)
4.	Chrysoeriol (MW 300, RT 11.4-11.6)	Rutin (MW 610, RT 8.5-8.6)	Rottlerin (MW 516, RT 8.0)
5.	Morin (MW 302, RT 17.1-17.2)	Hesperetin (MW 302, RT 8.5-8.7)	Sophoricoside (MW 432, RT 8.5-8.9)
6.	Limocitrin (MW 364, RT 17.9-18.0)	Flavanomarein (MW 450, RT 8.7-8.8)	Hesperetin (MW 302, RT 8.8-8.9)
7.	Peltatoside (MW 596, RT 18.3)	Kaempferol-7-neohesperidoside (MW 594, RT 9.2-9.3)	Quercetin-3-D-xyloside (MW 434, RT 9.8-9.9)
8.	Thermopsoside (MW 534, RT 20.8-21.0)	Baccatin (MW 586, RT 9.7)	Isorhamnetin (MW 316, RT 9.9-10.2)
9	Iridin (MW 521, RT 23.0)	Isorhamnetin (MW 316, RT 10.0-10.2)	Iristectorin-A (MW 492, RT 12.2)
10	--	Peonidin (MW 301, RT 10.1-10.2)	Kaempferol-3-O-alpha-L-arabinoside (MW 418, RT 13.5-13.9)
11	--	Iristectorin-A (MW 492, RT 11.3-11.4)	Myricetin-3-Galactoside (MW 480, RT 14.9-15.0)
12	--	Scoparin (MW 462, RT 11.7-11.8)	--
13	--	Tricin (MW 330, RT 11.6-11.9)	--
14	--	Cirsiliol (MW 330, RT 12.5-12.6)	--
15	--	Isorhamnetin-3-Glucoside-4'-Glucoside (MW 640, RT 13.4)	--

Table 1 LC-MS/MS data on aqueous extract of *T. catappa* bark, wood and fruit

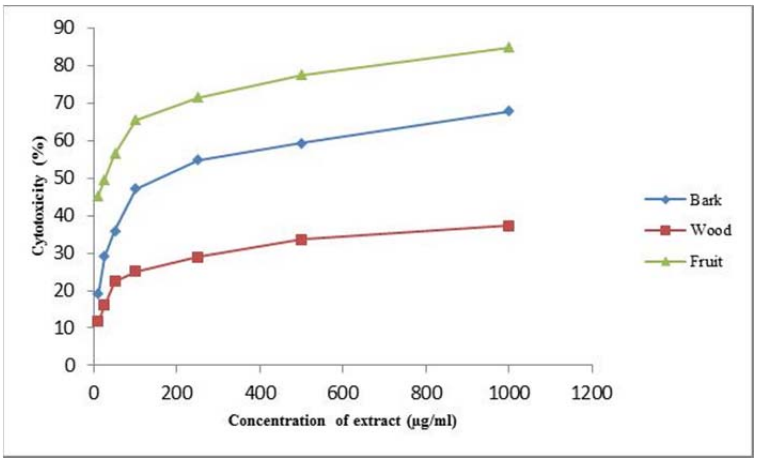


Figure 1: ***In vitro*** **cytotoxic activity of aqueous extracts of different parts of** ***Terminalia catappa*** **L. in EAC cell line model**

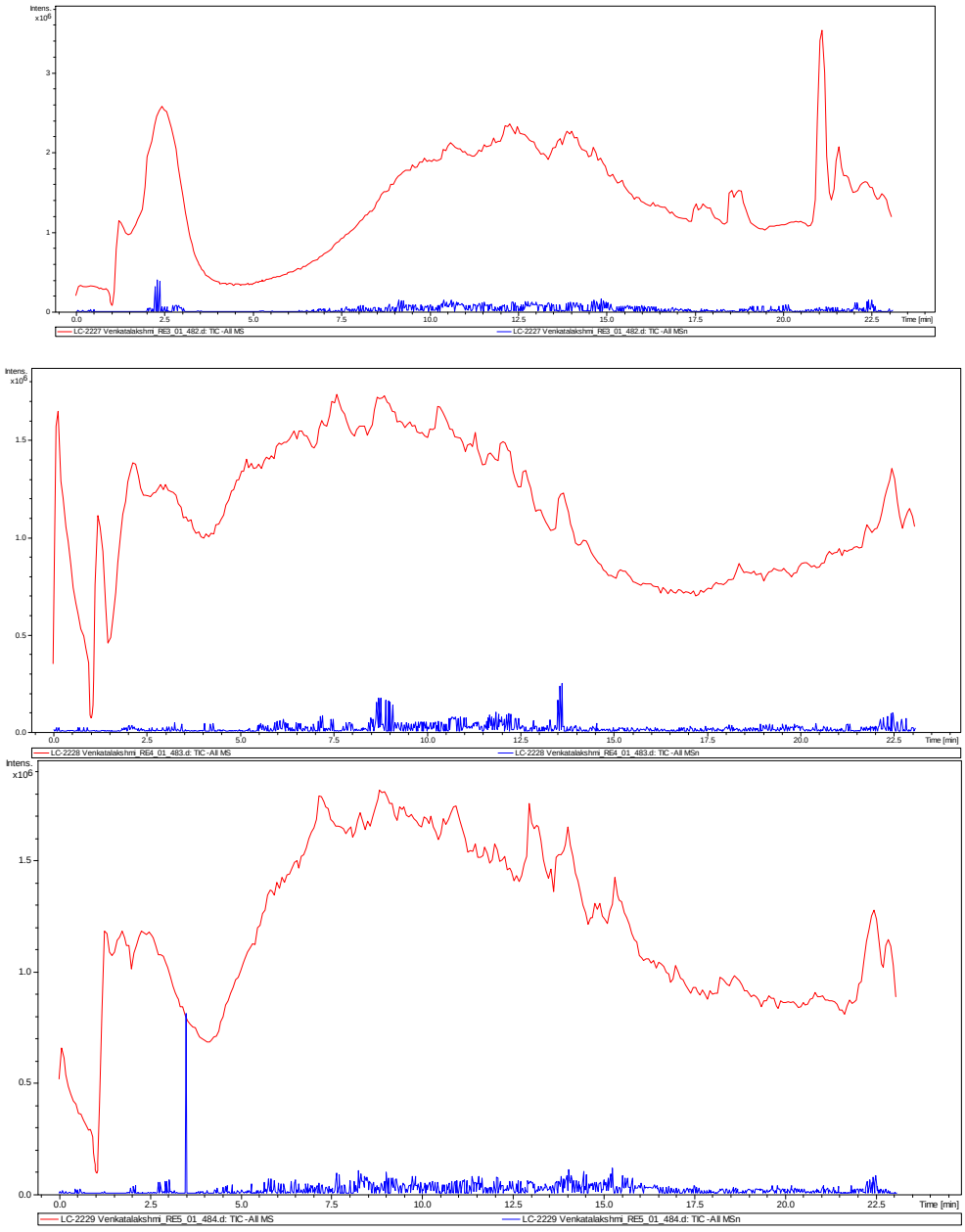
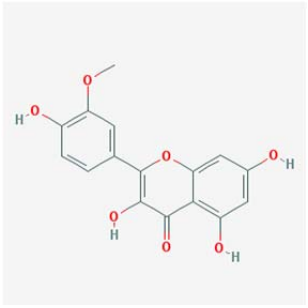
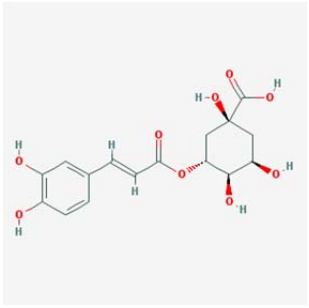
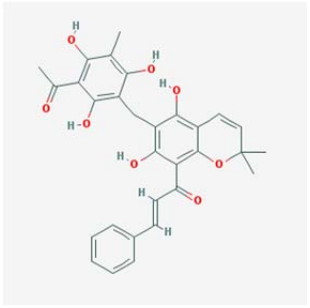
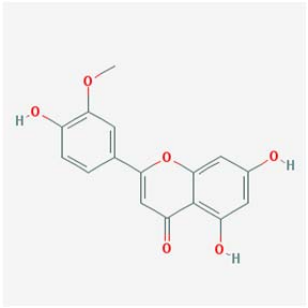


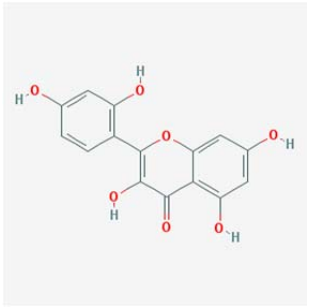
Figure 2 LC-MS/MS results of *Terminalia catappa* bark, fruit and wood

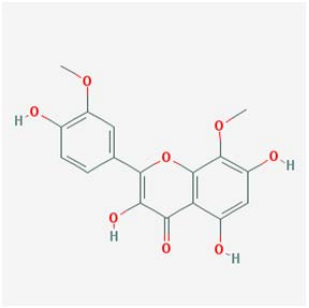

Isorhamnetin

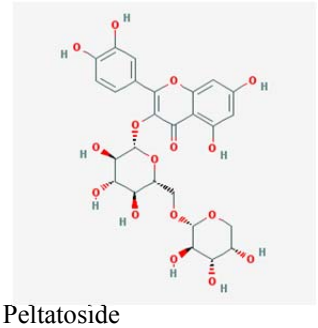

Chlorogenic acid

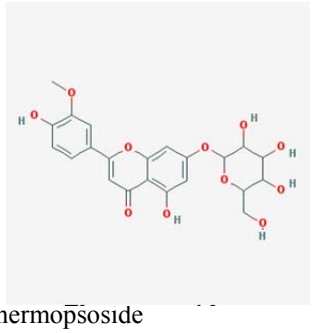

Rottlerin


Chrysoeriol


Morin


Limocitrin


Peltatoside


Thermopsoside

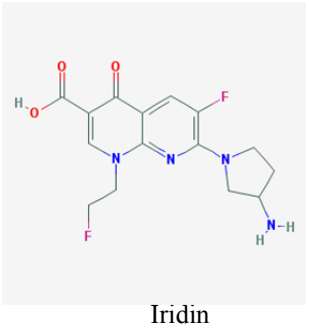
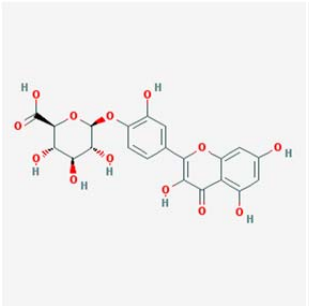
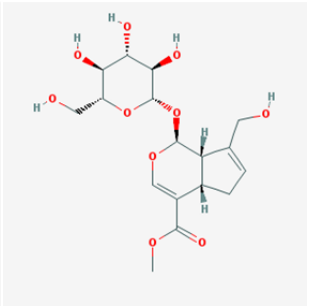
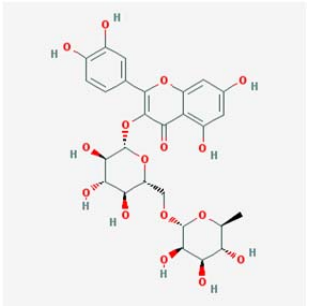
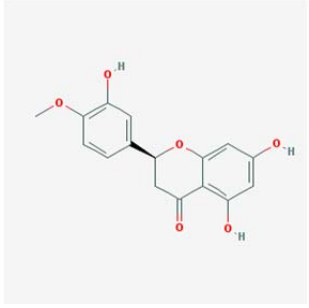

Iridin

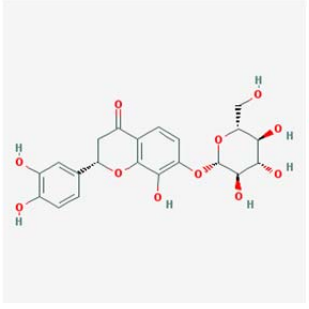
Figure 3: Phytoconstituents of *Terminalia catappa* bark

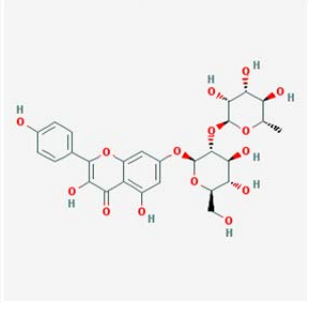

Quercetin 3-glucuronide

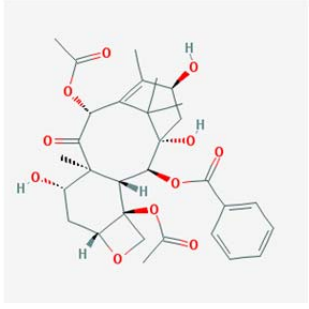

Geniposide

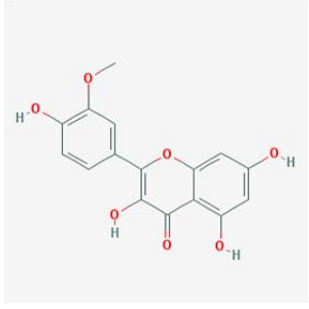

Rutin

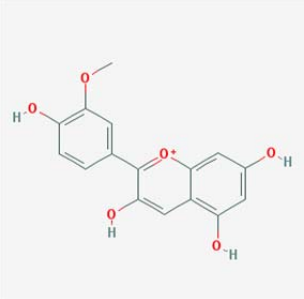

Hesperetin


Flavanomarein

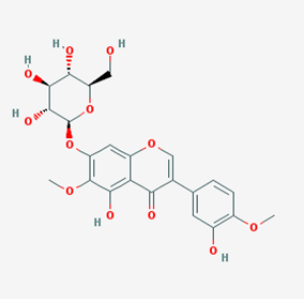

kaempferol-7-neohesperidoside


Baccatin

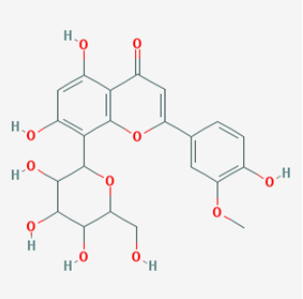

Isorhamnetin



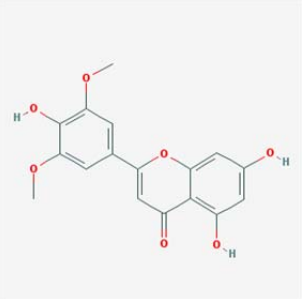
Peonidin



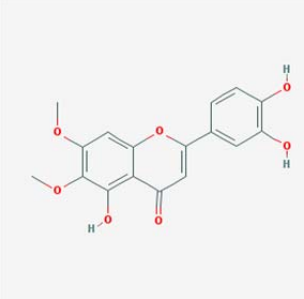
Iristectorin-A



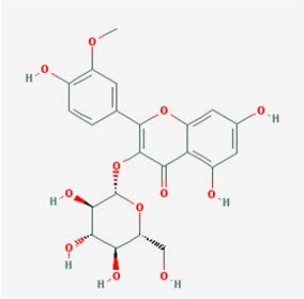
Scoparin



Tricin

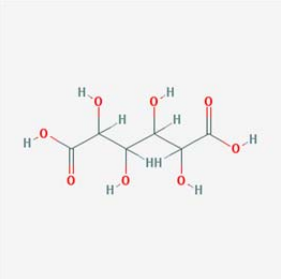


Cirsiliol

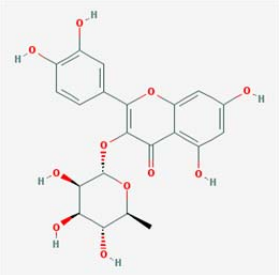


Isorhamnetin-3-glucoside

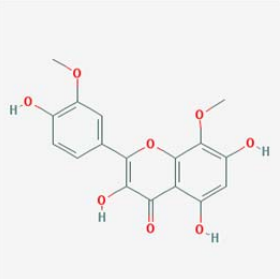
Figure 4: Phytoconstituents of *Terminalia catappa* fruit



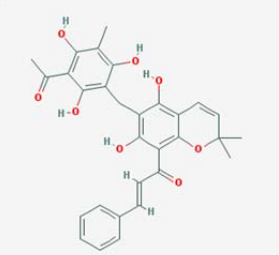
Mucic acid



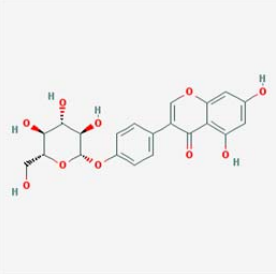
Quercetin 7-O-rhamnoside



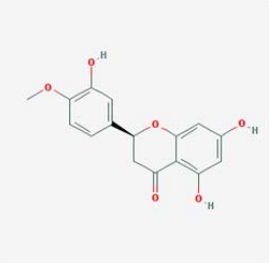
Limocitrin



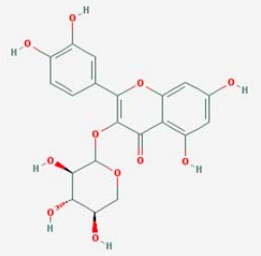
Rottlerin



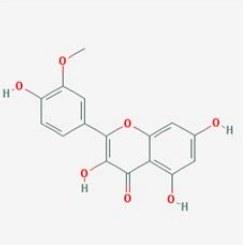
Sophoricoside



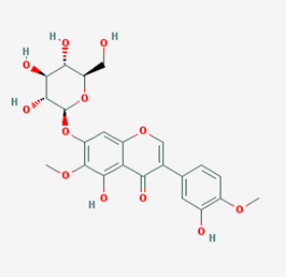
Hesperetin



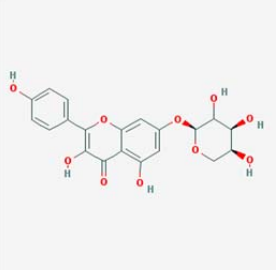
Quercetin 3-D-Xyloside



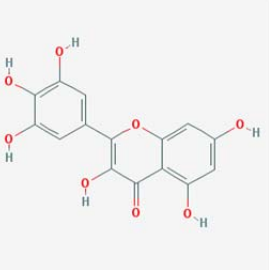
Isorhamnetin



Iristectorin



Kaempferol-3-o-alpha-L-arabinoside



Myricetin

Figure 5: Phytoconstituents of *Terminalia catappa* wood

CONCLUSIONS

Evaluation of anticancer effect of aqueous extracts revealed that the fruit sample has higher level of cytotoxicity against EAC cell line. The results of the present study suggested the presence of various high polar compounds in different parts of *Terminalia catappa*. The bark, fruit and wood extracts possess Isorhamnetin, while bark and wood alone contain Rottlerin and Limocitrin, whereas the fruit and wood shows the presence of Iristectorin-A. In general, the number of high polar compounds (flavonoids) was found to be high in fruit extract when compared to bark and wood and it could be reason for its high anticancer potential. Hence, a potent herbal anticancer drug can be developed from *Terminalia catappa* after conducting further in-depth studies. Further, in the fruit itself, the outer fleshy skin, middle shell and inner kernel could be investigated separately and if the byproducts (skin and shell) possess significant activity, it could be used as drug while the kernel serves as nutritious nuts.

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