



Antitumor Activity of *Turnera subulata* Sm. (Turneraceae) in Hep G2 Cancer Cell Line

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Authors' contributions

This work was carried out in collaboration among all authors. Author PSBJKS interpreted the data, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MPK managed the analyses of the study, managed the literature searches, assisted in preparation of manuscript. Author SP designed the study, supervised and managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i29A31578

Editor(s):

(1) Dr. Jongwha Chang, University of Texas, USA.

Reviewers:

- (1) Irineu Ferreira da Silva Neto, Faculty of Medicine of Juazeiro do Norte, Brazil.
(2) Reyad-UI-Ferdous, Shandong Provincial Hospital, Shandong University, China.
(3) Antonio Carlos Pereira de Menezes Filho, Goiano Federal Institute, Brazil.
Complete Peer review History: <http://www.sdiarticle4.com/review-history/67323>

Original Research Article

Received 01 February 2021

Accepted 06 April 2021

Published 21 May 2021

ABSTRACT

Turnera subulata are bright common garden flowers that are grown in Asian regions. Mostly flowers of several kinds have medicinal properties and applications. This is as one of a kind that contains compounds that has medicinal application. The present studies are targeted to investigate the phytochemical composition through GC-MS technique, antimicrobial activity and antitumor activity via MTT assay against Hep G2 (or HepG2), a human liver cancer cell line. Ethanol (EtOH) extracts has reasonable antitumor activity against Hep G2 for period of 24 h and 48 h and the aqueous part was non-reactive. From gas chromatography-mass spectrometry (GC-MS), a sum of 27 identified natural compounds exhibiting against cancer cell. Some traces of flavouring agents and antifungal agent with very low GC-MS peak lengths are too observed furaneol (2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one), benzeneacetaldehyde, benzoic acid and undecanoic acid. Hence, the result that indicates the flavouring agents including flavonoids along with phenolic and other acidic compounds have important characteristic property in reducing and treating against cancer cells.

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Keywords: *Turnera* genus; phytochemicals; antitumor; cancer cell; Hep G2; GC-MS; MTT assay.

1. INTRODUCTION

Naturally there are thousands of flower varieties of impressive properties and applications. Mostly *Turnera* species has wide medicinal application, among them *Turnera subulata* that are grown in tropical America including south-east Asia and it is easily available. It is also used as an herbal medicine for treating respiratory, reproductive system, digestion in the human body. Yellow alder flower like *Turnera ulmifolia* having antimicrobial, antioxidant and anticancer activities [1-3].

Likewise *Turnera subulata* is white alder or dark-eyed *Turnera* flower that has excellent treatment for bronchitis, cough, amenorrhoea and dysmenorrhoea, this is according to the previous studies by using the entire plant and root syrups for the treatments [4]. Recent study on *Turnera diffusa* a *Turnera* species, through methanolic extraction it was investigated that it has cytotoxic effect on breast cancer cells [5]. *T. subulata* has also proved that is capable of both scavenging free radicals and reducing oxidants [6]. Other than *Turnera* there are other species like *Gundelia tournefortii* that has anticancer properties [7]. As from the literature survey and study the *T. subulata* was not experimented on anticancer so far, the present study done for antitumor activity against Hep G2 human liver cancer cell line.

UV-Vis spectroscopy between 200 to 800 nm ranges kept to determining the absorbance in MTT assay procedure, GC-MS analysis were performed to obtain phytochemical composition that are potentially against the cancerous cell. About 27 identified compounds are present in flower extract notably as phenolic, aldehyde and acids like ascorbic acid, undecanoic acid responsible for the medicinal property.

This study will provide the benefits of the composition and the results of analysis of *Turnera subulata* composition, this will be the first to report on antitumor activity, instead of examining the whole plant our paper is targeted to profile only flower via ethanol extraction.

2. MATERIALS AND METHODS

2.1 Reagents and Equipments

Ethanol (Sigma-Aldrich, ACS reagents,99.5% purity), sodium sulphate anhydrous powder

(Sigma-Aldrich, ACS reagents,99% purity). Dimethyl sulfoxide (DMSO) (Calbiochem-Sigma-Aldrich, ACS reagent,99% purity), MTT reagent (Sigma-Aldrich, culture grade, 97.5% purity), GC-MS(Agilent-7890 A GC), Distillation setup, Whatman (Merck- Cytiva, Grade 42) filter paper and distilled water.

2.2 Plant Material

The *T. subulata* was collected in the Garden of college campus (10.8148° N, 78.6735° E) (Bishop Heber College, Tiruchirappalli, Tamilnadu, India) in the month of December, 2017. Flowers were collected in both dry and wet condition, at the time atmospheric temperature was at 33°C and pressure of 1019 mBar according to the geographical data. The flowers were cut into small pieces using cleaned scissors; the same were transferred to polythene cover and taken to the laboratory for further studies (Taxonomist: Dr. Anand Gideon, Bishop Heber College).

2.3 Ethanol Extraction

Flowers (*T. subulata*) were collected in the afternoon, cleaned thoroughly in tap water and then in distilled water. A 10 g of flowers were put into a brown bottle which contains 250 mL of 82% ethanol solution and shaken well, kept at room temperature (25°C) for a period of 72 hours and then, the coagulant mixture was filtered out and transferred into distillation setup with water bath underneath. The distillation was continued until the temperature was reached at 70°C, this process was continued 4 hours and then the distillation was stopped after obtaining homogeneous mixture. From the process, 15mL of pure sample *T. subulata* flower extracts was collected and subsequently, the alcohol which was present in the mixture was evaporated in rotary evaporator with negative -750 mmHg. After collection of extract, sodium sulphate powder was added into the extract to remove water content. Before the addition, the sodium sulphate powder was maintained at 100°C for 3 hours in a hot air oven to remove moisture/water content, thereafter a few minutes later the extract was filtered using Whatman-42 (Pore size of 2.5 µm) quantitative ashless filter paper. The pure form of ethanolic extract was filtered out. Then the collected sample was used for carrying out further studies and the remaining was stored in refrigerator.

2.4 Antimicrobial Test

Disc diffusion method was utilized to determine the antimicrobial activity, before proceeding with MTT assay Fig. 1(B). The four microorganisms that were procured from National Chemical Laboratory (NCL) Pune investigated with EtOH extract are *Escherichia coli* (NCIM 2065), *Candida albicans* (NCIM 3102), *Staphylococcus aureus* (NCIM 2079) and *Aspergillus niger* (NCIM 105) Fig. 1(A). Maintained sub culture on Nutrient Agar (NA- Frasco, Kasvi) and Sabouraud Dextrose Agar medium (SDA-Frasco, Kasvi) for bacteria and fungi respectively. The inhibition was compared with the effect exhibited from the control samples namely standard Ciprofloxacin (5 µg disc) for bacteria; Nystatin (100 µg disc) for fungi. The standard disc was obtained from Periyar College of Pharmaceutical Sciences, Trichy. After obtaining the positive results from the antimicrobial studies the sample was continued for MTT assay. Table 4 denotes the zone of inhibition (in mm) 18, 14, 12 and 15 mm respectively.

2.5 Treatment Description and MTT Assay

MTT assay-Antitumor screening: For antitumor assessment, test was disintegrated in Dimethyl sulfoxide (DMSO), reduced the concentration in culture medium and used to treat the picked cell line (Hep G2) over an sample concentration four unique obsessions of 1, 10 25 and 50 µL mL⁻¹ (1 - 50 µL mL⁻¹) for a period of 24 hours and 48 hours. The cell line was acquired from National Centre for Cell Science (NCCS). The DMSO was used as the dissolvable control. A downsized appropriate measure using 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-2H-tetra-zolium bromide (MTT) was completed by the strategy portrayed by standard procedure (Mosmann, 1983). To each well, 20 µL of 5 mg mL⁻¹ MTT in phosphate-buffer saline (PBS) was incorporated and covered with aluminium foil, and agonized for 4 h at 37°C. The purple formazan was broken down by expansion of 100 µL of 100% DMSO to each well. The absorbance was seen at 570 nm (assessed) and 630 nm (reference) using a 96 well plate per user (Bio-Rad, Hercules, CA, USA). Data was assembled for four imitates. Each value used to ascertain the mean qualities. The level of hindrance was determined, from this data, using the equation. The inhibition concentration (IC₅₀ µL mL⁻¹) value was resolved as the concentration of the complex that is

needed to decrease the absorbance to a large portion of that of the control.

Equation:

$$\frac{\text{Mean absorbance of pure cells (control)} - \text{mean absorbance of treated cell (test)}}{\text{Mean absorbance of pure cells (control)}} \times 100$$

2.6 Gas Chromatography-Mass (GC-MS) Method

Distinguishing the proof of phytochemical components was directed via utilizing the information base of National Institute Standard and Technology MS library (NIST-MS library). The extricate from the *T. subulata* by means of ethanol was investigated with GC MS utilizing Agilent-7890 A GC instrument combined with MS-5975 inactive MSD and triple hub mass selective ion detector. The DB-5MS segment with measurements of 30 m x 0.2 mm fine section was utilized for the examination. The temperature limits up to 300°C and initial temperature was kept at 150°C. 1 µL of test was infused with split mode at the ratio 10:1. Helium gas at the flow pace of 0.8 mL min⁻¹ was applied as transporter gas for a run time of 22 minutes.

3. RESULTS AND DISCUSSION

In vitro antitumor activity antitumor activity was performed for *T. subulata* extract through *in vitro*. The effects of *T. subulata* extract on Hep G2 a human liver cancer cell line were determined using MTT assay. The Hep G2 cell line was exposed to *T. subulata* ethanol extracts ranges of 0.1; 10; 25; 50 µL for 24 hours and 48 hours (Fig. 1).

As a result, the antitumor activity increases in increase in the amount of extract exposed amount of 0.1 to 50 µL leads to increase to inhibition range from 0.21 to 10.38% after 24 hours and 0.32 to 11.52% after 48 hours in cancerous cell line and the plotted graph illustrate the 24 hours and 48 hours exposure (Table 1; Fig. 2).

GC-MS Phytochemical analysis of *T. subulata*. The GC-MS analysis revealed 27 compounds (Fig. 3), among those 7 major compounds (Fig. 4) have antitumor activity. These 7 compounds are first time found in *T. subulata* as per our knowledge and literature survey. The compounds are phenol, 1,1,3-triethoxypropane, 2,3-dihydro-3,5-dihydroxy-6-

methyl-4H-pyran-4-one, 5-hydroxymethylfurfural, xanthosine, tetradecanoic acid, and l-(+)-ascorbic acid-2,6-dihexadecanoate (Tables 2, 3).

Phenol (PubChem CID: 996) a phytochemical which is commonly available in most of the plant as phenolic agent. In *T. subulata* ethanol extracts it was identified and weighed ~6.28% and trace of phenol, 2,4-bis(1,1-dimethylethyl) (PubChem CID: 528937) was too found. As far as by the

survey, ten phenolic compounds including eugenol, hesperidin, carvacrol, thymol, and kaempferol were identified in *Crozophora tinctoria* accessions by high-performance liquid chromatography (HPLC) technique has best anticancer activity against HeLa cell lines [8]. Studies through *in vitro* preclinical and epidemiologically plant phenolic acid can retard the cancer growth and has potent in anticancer activity [9] (Table 3).

Table 1. Antitumor activity

S. no	Concentration of the sample (µL)	Percentage of cell death 24 h	Percentage of cell death 48 h
1	0.1	0.21	0.32
2	10	4.36	5.27
3	25	7.45	8.84
4	50	10.38	11.52

Table 2. Phytochemicals of *Turnera subulata* from the ethanol extract by Gas Chromatography-Mass Spectrometry

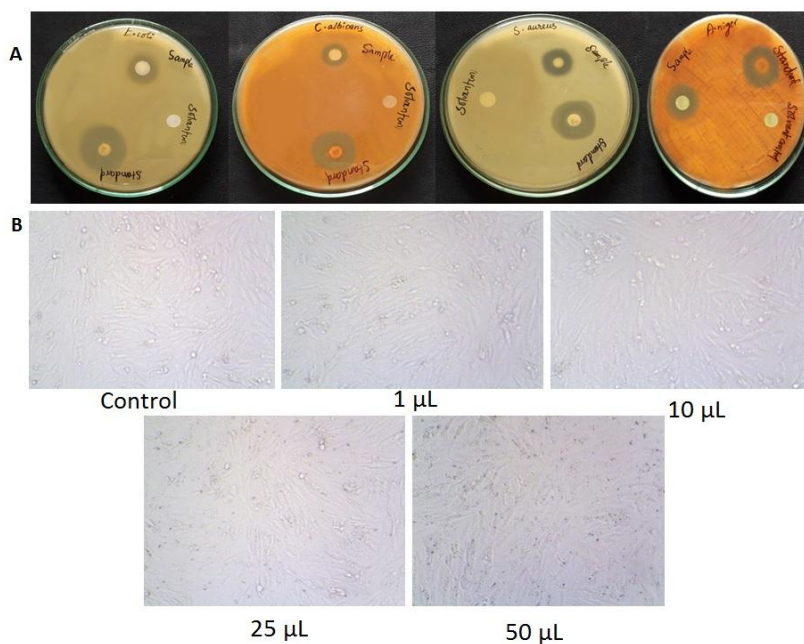
S. no	Ret. time	Name	Molecular formula	Molecular weight
1.	4.455	2(5H)-furanone	C ₄ H ₄ O ₂	84
2.	4.59	2-cyclopenten-1-one, 2-hydroxy-	C ₅ H ₆ O ₂	98
3.	4.905	2-furancarboxaldehyde, 5-methyl-	C ₆ H ₆ O ₂	110
4.	5.07	Phenol	C ₆ H ₆ O	94
5.	5.295	2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	C ₆ H ₈ O ₄	144
6.	6.085	Benzeneacetaldehyde	C ₈ H ₈ O	120
7.	6.17	4-methyl-2-prop[(1'-enyl)-1,3-dioxolan	C ₇ H ₁₂ O ₂	128
8.	6.425	Propane, 1,1,3-triethoxy-	C ₉ H ₂₀ O ₃	176
9.	6.6	2,5-furandicarboxaldehyde	C ₆ H ₄ O ₃	124
10.	6.705	Furyl hydroxymethyl ketone	C ₆ H ₆ O ₃	126
11.	7.585	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C ₆ H ₈ O ₄	144
12.	7.705	Benzoic acid	C ₇ H ₆ O ₂	122
13.	8.22	2,3-dimethylcyclohexanol	C ₈ H ₁₆ O	128
14.	8.52	1-octanamine, N-methyl-N-nitro-	C ₉ H ₂₀ N ₂ O ₂	188
15.	8.585	7-chloro-1,3-dihydro-5-phenyl-2H-1,4-dibenzodiazepin-2-one	C ₁₈ H ₁₉ ClN ₂ OSi	342
16.	8.69	5-hydroxymethylfurfural	C ₆ H ₆ O ₃	126
17.	9.78	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1R-(1.alpha.,2.beta.,5.alpha.)]	C ₁₀ H ₂₀ O	156
18.	11.555	Xanthosine	C ₁₀ H ₁₂ N ₄ O ₆	284
19.	12.49	Phenol, 2,4-bis(1,1-dimethylethyl)	C ₁₄ H ₂₂ O	206
20.	14.48	3-furoic acid, TBDMS derivative	C ₁₁ H ₁₈ O ₃ Si	226
21.	15.38	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228
22.	16.23	10-undecen-2-ol, (.+.-)-	C ₁₁ H ₂₂ O	170
23.	16.3	1-undecenal	C ₁₁ H ₂₄ O	172
24.	16.685	16-heptadecenal	C ₁₇ H ₃₂ O	252
25.	17.375	5H,10H-dipyrrolo[1,2-A:1',2'-D]pyrazine-5,10-dione, octahydro-, (5As-cis)-	C ₁₆ H ₁₄ N ₂ O ₂	194
26.	17.465	l-(+)-ascorbic acid 2,6-dihexadecanoate	C ₃₈ H ₆₈ O ₈	652
27.	19.385	Undecanoic acid	C ₁₁ H ₂₂ O ₂	186

Table 3. Major phytochemicals of *Turnera subulata* from the ethanol extract by Gas Chromatography-Mass Spectrometry with (%) abundance

S. no	Retention time (mins.)	Major Component name	Molecular weight	Molecular formula	Abundance %
1	5.07	Phenol	94	C ₆ H ₆ O	6.28
2	6.425	1,1,3-triethoxy-propane	176	C ₉ H ₂₀ O ₃	11.79
3	7.585	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	C ₆ H ₈ O ₄	2.75
4	8.69	5-Hydroxymethylfurfural	126	C ₆ H ₆ O ₃	63.67
5	11.555	Xanthosine	284	C ₁₀ H ₁₂ N ₄ O ₆	1.96
6	15.38	Tetradecanoic acid	228	C ₁₄ H ₂₈ O ₂	7.86
7	17.465	L-(+)-ascorbic acid 2,6-dihexadecanoate	652	C ₃₈ H ₆₈ O ₈	4.32

Table 4. Antimicrobial activity of *Turnera subulata* EtOH extract

Test organisms	Zone of Inhibition (in mm)	
	Sample	Standard
<i>Escherichia coli</i> (NCIM 2065)	14	38
<i>Candida albicans</i> (NCIM 3102)	12	32
<i>Staphylococcus aureus</i> (NCIM 2079)	18	35
<i>Aspergillus niger</i> (NCIM 105)	15	35

**Fig. 1. Antimicrobial (A) and MTT assay (B) of *Turnera subulata* extract**

We have observed that compounds such as benzoic acid, furaneol (PubChem: CID 19309), benzeneacetaldehyde (PubChem: CID 99748) and undecanoic acid (PubChem: CID 243) in *T. subulata* as minor peaks and all weighed about ~0.1% (Fig. 5).

Peculiar compound 1,1,3-triethoxy-propane (PubChem CID: 223797) that weighed ~12% , doesn't have any activity but similar large propane compound as 2,2'-((propane-1,3-diyldis(azanylylidene))bis(ethan-1-yl-1-ylidene)) bis(4-chloronaphthalen-1-ol) (II) a Schiff base

exhibit excellent antibacterial property against *E. coli* and *Salmonella enterica* serovar *Typhi* [10]. Furthermore investigation should be experimented on 1,1,3-triethoxy-propane that was obtained from *T. subulata* extract.

The 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (PubChem CID:119838) a phytochemical that first identified with *T. subulata* and weighed ~3% of the ethanol extract. It has been noted as a novel anticancer agent in ethyl acetate leaf extract (ELE), methanol leaf extract (MLE) of *Callistemon lanceolatus*. It has excellent anticancer activity against Hep G2 cells [11].

Another major source compound for antitumor activity acquiring is 5-hydroxymethylfurfural (5-HMF) (PubChem CID: 237332) identified along with other compounds in *T. subulata* has weighed ~64% of the EtOH extract. It was confirmed that the outcomes indicated that 5-

HMF showed higher antiproliferative activity on human melanoma A375 cells than other cell lines. 5-HMF could keep the peroxidation from the source to secure the erythrocytes. The morphological change of erythrocytes was additionally confirmed by utilizing atomic force microscopy. The inhibitory impact on 5-HMF on human cancer cell proliferation was examined by MTT assay, flow cytometric examination, and the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI) costaining assay [12]. It has anti oxidative, anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, and anti-hyperuricemic effects when it is further converted into 5-sulfoxymethylfurfural [13]. Xanthosine (PubChem CID:64959) was observed in the extract that uniquely revealed but comparing with other components it has less abundance of ~2%. Fortunately, xanthine derivatives caffeine, theophylline and xanthine/chalcone hybrids produce promising anticancer activity [14-16].

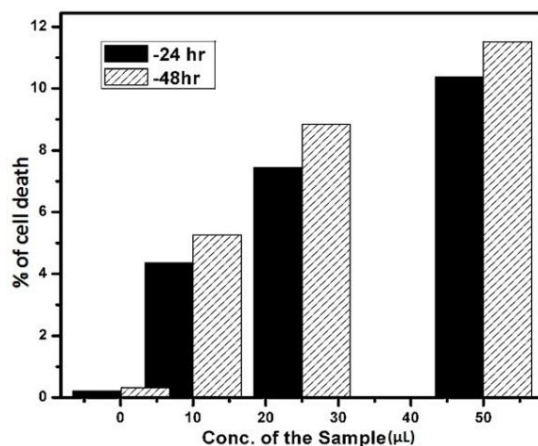


Fig. 2. Column graph for percentage of cell death

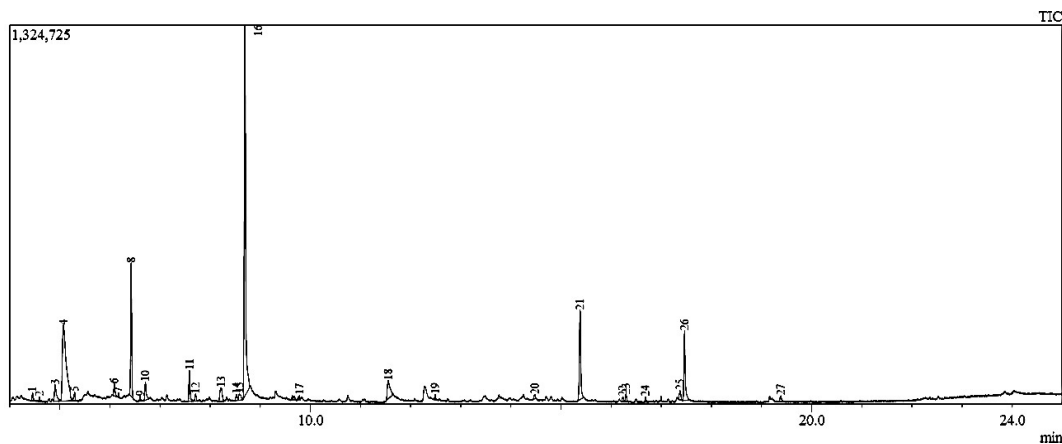


Fig. 3. Chromatogram of EtOH extract of *Turnera subulata*

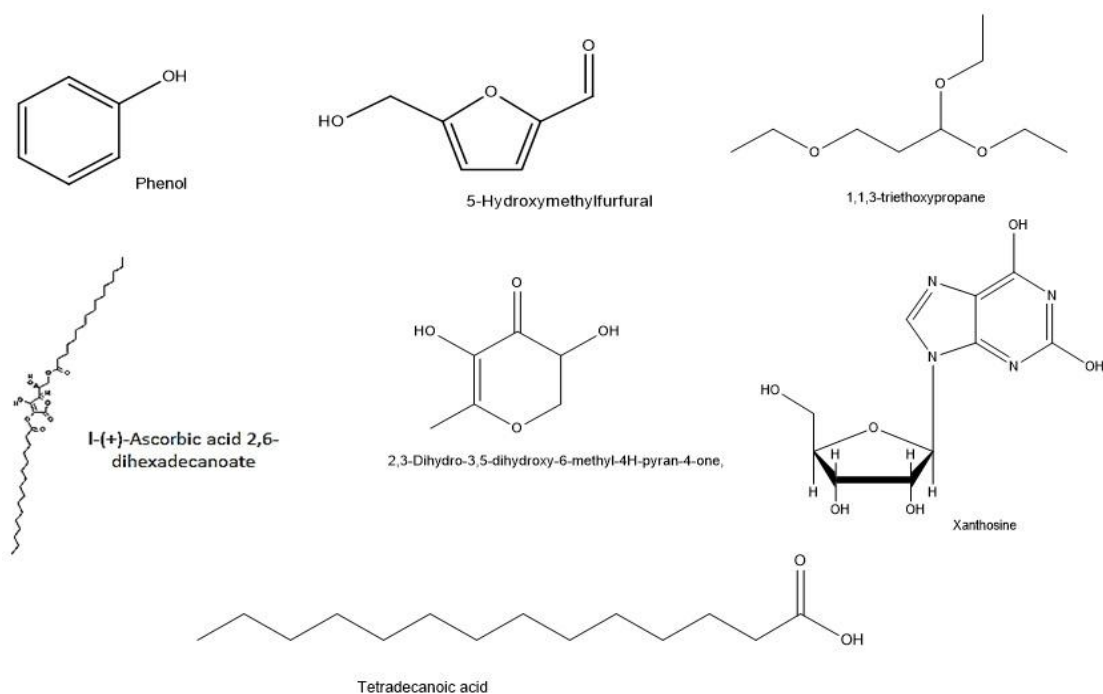


Fig. 4. Chemical structures of the major components in *Turnera subulata* EtOH extract

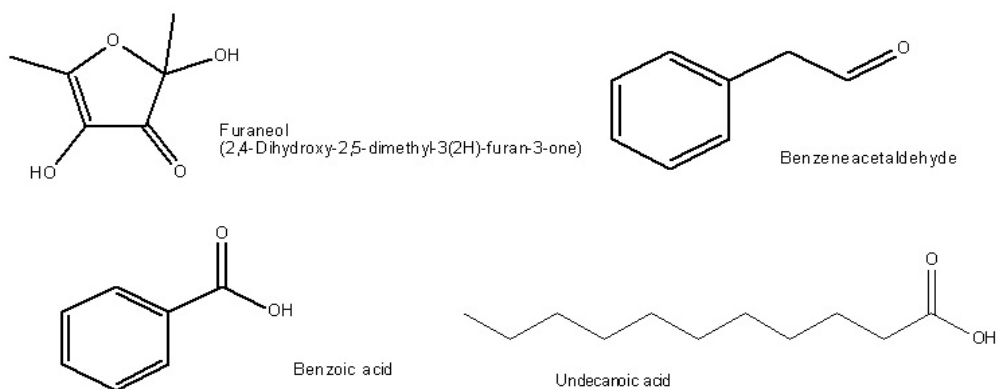


Fig. 5. Chemical structures of other compounds with abundance ~0.1%

Furthermore, two acidic compounds were identified tetradecanoic acid (PubChem CID: 11005) also called as myristic acid and I-(+)-ascorbic acid-2,6-dihexadecanoate (PubChem CID :54722209) these two weighed ~8% and ~4 % respectively. Myristic acid identified and indicated that the separate of *Xestospongia testudinaria* can be used as a natural anticancer toward HeLa cell [17,18].

I-(+)-ascorbic acid 2,6-dihexadecanoate, as far as, with respect to literature survey it has only antioxidant property [19] further research and

progress in future will provide insight information on anticancer property possibilities.

4. CONCLUSION

The present study demonstrated for the first time for antitumor activity in *Turnera subulata* ethanol extract against Hep G2 human liver cancer cell line. The cell death was measured by well-established assay i.e MTT assay and the aqueous part was inactive. The antibacterial activity against *Staphylococcus aureus* that exhibit has higher inhibition zone than

Escherichia coli. In the case of antifungal property, *Aspergillus niger* has higher inhibition zone comparing to *Candida albicans* by results obtained from disc diffusion method. Through GC-MS, phytochemicals in ethanolic extract from *T. subulata* were identified and determined the abundance of salient active components in the extract. The important phytochemical that was exceptionally having antitumor properties are 5-hydroxymethylfurfural, 1,1,3-triethoxy-propane (an antioxidant part), tetradecanoic acid (Myristic acid), phenol, l-(+)-ascorbic acid 2,6-dihexadecanoate, and 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (Fig. 4). Additionally, Furaneol (2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one), benzeneacetaldehyde, benzoic acid and undecanoic acid (PubChem CID: 8180 an antifungal agent) were also identified that considered to be supporting molecules in the system. As far as from the results the major compounds represent and exhibits as an important role in antitumor properties in *T. subulata*. Therefore these isolated major compounds with exceptional abundance value can be used as an antitumor agents and it will be a possible alternative to cancer treatment. For future studies like *in vivo* study for evaluating toxicity, efficacy and mechanism of action and enhancement of the bioactivity of the compounds are required.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

The authors thank the Heber Analytical Instrumentation Facility (HAIF), Periyar College of Pharmaceutical Sciences and Biological & Bioinformatics Research Centre (BBRC) for providing instrumentation facility and biological studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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