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Characterization, Pharmacology and *In-silico* study of 2,4 Ditertiary Butylphenol Isolated from the leaves of *Ficus auriculata* Lour.

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ABSTRACT

Ficus auriculata belongs to the family of dicotyledonous plant. The general phytochemical screening for Ethanol, Hexane, Chloroform and Water extracts are done. Since ethanol shows the presence of maximum compounds, it is subjected to the isolation of phenolic compounds by TLC and Column Chromatography. On repeating this process, a white crystalline solid results, whichon the basis of UV-Vis, FT-IR, GC-MS, ^{1}H NMR, confirms that the isolated compound is 2,4 Ditertiary Butyl Phenol (2,4 DTBP). The isolated compound is further studied for its solventeffect and subjected to anti-microbial and Cytotoxicity studies with AGS cancer cell line and HIEC-6 (Human Normal Intestinal Epithelial cell line). Docking studies is carried out using PatchDock server, with 2,4 DTBP as guest and Occludin, which is a Tight Junction Protein (TJP) as host. The resulted structure is further subjected to Lipinski Rule of five. The present studyconcludes that 2,4 DTBP shows intermediate resistance against *Gram-positive* and *Gram-negative* bacteria like *S. aureus*, *E. coli, Klebsiela pneumonia*, strongly resistant to fungi *Candida sps.* According to cytotoxic and *In silico* studies, the isolated compound has excellent anticancer properties and is thus used in the treatment of gastric cancer. From the Lipinski rule, it is confirmed that the drug can be administered orally.

Keywords: *Ficus auriculata*, Phytochemicals, Isolation, 2,4 Ditertiary ButylPhenol, Pharmacology, *In-silico* studies, Gastric cancer.

INTRODUCTION

Medicinal plants act as a natural resource, providing opportunities for the discovery of new drugs. Plants used in traditional medicine contains a variety of substances that can be used for healing chronic and infectious dxiseases. Thousands of phytochemicals from plants are safer than other effective methods with minimal side effects.[1] Phytochemicals, commonly referred to as secondary metabolites, produced by plants through several chemical methods can be beneficial in the function of human cells.[2] Polyphenols exhibit many protective functions such as hypolipidemic, antiproliferative, anti-inflammatory and antioxidative effects which

reduce theonset of the disease.[3] Extraction is a very important step in analyzing existing nutrients plant.[1] *Ficus auriculata* is a small, perennial evergreen tree which is cultivated in South and Southeast Asia and Brazil for its edible fruits.[4] There are few studies on the benefitsof *F. auriculata* to human health due to its phytochemical compounds.[5] 2,4 DTBP was alsoreported in different groups of plants, like Phaeodacty lumtricornutum Bohlin (diatom), *Marchantia polymorpha* L (liverwort), *Osmunda regalis* L and *Adiantum venustum* D (ferns).[6] It has been reported that 2,4-Di-tert-butylphenol exhibit moderate cytotoxicity against HeLa and MCF-7, high percentage of antioxidant, anti-bacterial activity and induces senescence inhuman gastric adenocarcinoma.[7,18] A global issue in the field of surgery is postoperative wound infections, which are linked to prolonged hospital stays. An infection in the tissues around the incision and the surgical site is referred to as a postoperative wound infection, and it typically develops five to thirty days following surgery. *Streptococcus species*, Proteus species, *Enterobacter species*, *Klebsiella species*, *Coagulase negative* and *Pseudomonas species*, are the most common pathogen.[8]

The effects of several solvents (including ethanol, water, butanol, ether, hexane, carbon tetrachloride, and chloroform) on 2,4 DTBP using UV-Vis spectra were taken into consideration.

The findings revealed that the UV region experienced majority of absorptions, and that the primary electronic transitions are connected to n-σσ* and σ-σ*. The absorption values in different solvents are influenced by dielectric constants of the solvents. The solvent polarizability tends to move the absorption maximum towards lower wavelength.[9] Molecular docking studies predict the preferred orientation of one molecule to another when they are bonded together to form a stable complex.[20] The objective is to determine the correct interaction between two molecules.[10] Lipinski's rule of five, which has been used for nearly 20 years as a broad "rule of thumb"for valuing drug-like qualities, is a widely used way to forecast a drug's performance, mostly for oral medications.[11]

The focus of my work is to identify the Phytochemicals present in various solvents, Isolation of bioactive phenolic component 2,4 Ditertiary Butylphenol, its Characterization using various spectral analysis, and study the effect of various solvents using UV-Vis spectra. The compound is also tested for its anti-microbial activity, cytotoxicity and its drug-likeness is evaluated by in-silico study.

MATERIALS AND METHODS

Plant leaves were collected from Nagercoil locality, washed well with double distilled water, shadow dried for 3-4 weeks, powdered and preserved for further work. Plant authentication was done by Dr. M. U Sharief, Scientist 'E' & Head of Office, Botanical Survey of India, Southern Regional Centre, Coimbatore. The voucher specimen no. BSI/SRC/5/23/2021/Tech-263.

Materials required

Ethanol, Chloroform, Hexane, Water, Lead Acetate, Neutral $FeCl_3$, Fehling Solution (A & B), 4% NaOH, 1% $CuSO_4$, Acetic acid, Wagner Reagent, Dil.HCl, Conc.H_2SO_4, Distilled water.

Preparation of extracts

The powdered plant material (5 g) is extracted with solvents (500 mL) like Ethanol, Hexane, Chloroform, Water using Soxhlet apparatus for 24 hours. The solvent is evaporated using rotary evaporator.

Phytochemical Analysis Isolation of Phenolic compound Column Fractions

Silica gel (mesh size 230-400) is used in column chromatography. The column is packed usingwet packing method, washed with Ethyl acetate solvent. The plant extract is loaded into thepacked column. Ethyl acetate: Hexane (30:70) is used as thee luent, the extract is eluted and the fractions were collected in vials. This is furthersubjectedto TLC.[12]

Test	Observation	Inference
About 1 mL of the extract is shaken with1 mL of Lead acetate solution	Formation of white precipitate	Presence of Tannin.
About 1 mL of the extract is shaken vigorously with water and warmed	Formation of foam	Presence of Saponin.
About 1 mL of extract is shaken with distilled water and Neutral $FeCl_3$.	Formation of dark green colour	Presence of Phenolic compounds
Fehling'sTest: 1 mL of extract is mixed with 1 mL Fehling A&1 mL Fehling B solution. This is mixed and boiled.	Formation of brick red precipitate	Presence of Carbohydrate.
BiuretTest: About 1 mL of extract is shakenwith 4% NaOH and 1% $CuSO_4$ solution	Formation of violet or pink colour	Presence of Protein.
About 1 ml of the extract is shaken with $CHCl_3$, Conc. H_2SO_4 and Acetic acid.	Formation of green colour	Presence of Steroids.
About 1 mL of extract is shaken with neutral $FeCl_3$.	Formation of brown colour	Presence of Flavonoids.
To the extract, about 2 mL of glacial acetic acid in 1 drop $FeCl_3$ is added	Formation of brown colour	Presence of Cardial glycoside.
About 1 mL of the extract, is shaken with 1mL of 10% NaOH.	Formation of yellow colour	Presence of Coumarin compounds.
To the extract add dil.HCl. Filterthis and add Wagner Reagent (I2inKI) to the filtrate.	Formation of Reddish brown precipitate	Presence of Alkaloids.

Thin Layer Chromatography

TL Cready-madesheet (Silicagel 60 F25420 cmx20 cm) is cutin to equal sizes and thin mark of 0.5 cm was made from the bottom to load the sample spots. Ethyl acetate: Hexane (6:14) is usedas the mobile phase. The TLC sheet prepared is placed in the beaker containing the mobilephase. It is removed after the sample spot is raised above the level in mobilephase. This is dried, and placed under Iodine chamber and examined under UV for various spots. From the spot R_f valuecan becalculated by the formula.

R_f=Distance travelled by the solute/Distance travelled by the solvent. Gas chromatography-Mass spectrometry (GC-MS)

The JEOL GCMATE II GC-MS with Data system, a high resolution, double focusing instrument, was used to conduct the GC-MS analysis. 6000-pixel maximum resolution maximum calibrated mass: 1500 Daltonson acapillary column (300.25 mL D 0.25 mdf) fusedto an Elite-5MS (5% diphenyl/95% dimethyl poly siloxane). Utilizing the data bases of the National Institute of Standards and Technology (NIST) and Wiley Spectra Libraries, the interpretation of the mass spectrum GCMS was carried out. The name of the molecule was determined using the molecular weight, molecular formula, and the number of hits from the NIST and Wileyspectralibraries.[13]

HPLC

HPLC is recorde dusing SHIMADZU, LC-10AT VP, at ANJAC, Sivakasi.

UV-Vis Spectroscopy

The UV absorbtion spectra is recorded using systronics smart double beam spectrophotometer-2203, in Scott Christian College, Nagercoil.

FT-IR Spectroscopy

The FT-IR is recorde dusing Shimadzu FT-IR Spectrometer, in ANJAC, Sivakasi.

^{1}H-NMR

^{1}H-NMR spectros copy was carried out using Bruker 300MHz FTNMR Spectrometer, in Gandhi Gram Rural University, Dindugal.

Anti-microbial activity of 2,4 DTBPAnti-bacterial activity Sample Preparation Test Organism

The given sample was dissolved in a mixture of aqueous and ethanol solvents at a concentration of 0.1 g/1 mL.

Clinical samples were used to isolate *Staphylococcus aureus, E. coli, Klebsiella pneumoniae, Enterococcus faecalis, Bacillus subtilis*, and *Pseudomonas aeruginosa* to study theirantimicrobial properties.

The lack of zone inhibition was taken to mean that there was no activity. When the zone of inhibition is smaller than 7mm, the activities are classified as resistant moderate 8–10mm, and sensitive greater than 11mm.[14-16]

Anti-fungalactivity Test Organism

Thetest Fungi used *Candida sp* sisisolated from thee nvironment for antifungal analysis. Antifungi assayis performed by disc diffusion method.[17]

MTT Assay Principle

MTT Assay Utilises cellular metabolic activity as a gauge of cell viability, proliferation, and cytotoxicity. This colorimetri cassay relies on the transformation of purple for mazan crystalsin to methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by metabolically active cells. The MTT is converted to formazan by NAD(P) H-dependent oxidoreductase enzymes found in live cells.[21] An ELISA plate reader is used to measure theabsorbance at 570nm after the insoluble formazan crystals have been dissolved using asolubilizingsolution (100% DMSO).

The cytotoxic effect of the isolated compound is tested in both HIEC-6 (Human Normal Intestinal Epithelial cellline) and Gastric Adenocarcinoma (AGS) celllines.

All experiments were carried out intriplicates. The cell viability is determined using the following formula:

$$Percentage\ of\ cellviability = \frac{Average\ absorbance\ of\ treated}{Average\ absorbance\ of\ control} \times 100$$

IC_{50} value

The sample's half maximal inhibitory concentration is known as the IC_{50} value. The average absorbance of the various concentrations of the test sample (6.25-100 g/mL) were plotted in Microsoft Excel, and the equation for slope (y=mx+C) was derived.

Molecular Docking Studies

The 3D structural data of 2,4 DTBP was obtained from PubChem database in SDF format, it istranslated into PDB format using PYMOL software and the 3D structural data of Occludin, a Tight Junction (TJ) protein is obtained from Protein Data Bank using the search interface. Using the Patch Dock server, the 2,4 DTBP ligand and Occludin, the receptor molecule, are uploadedalong with the 3D coordinate data file in order to dock the guest 2,4 DTBP into the cavity of thehost Occludin. Each conformation is given a docking score by the Patch Dock server.

Lipinski Ruleof Five

Lipinski rule of 5 helps is used to distinguish the drug like and non-drug likeness of molecules. Lipinski's rule states that, an orally active drug should not violate more than one of the following criteria:

- Molecular mass <500Dalton
- Lipophilicity (LogP<5)
- Hydrogen Bond Donors <5
- Hydrogen Bond Acceptors<10
- Molar Refractivity~40-130

RESULTS AND DISCUSSION

Table 1 shows the column fractions eluted using various solvents like Methanol: Chloroform, Ethyl acetate: Hexane in variousratios.

Table 1: Column fraction seluted

S.No	Solvent system	Ratio	Volume	Fractions
1	Methanol:Chloroform	4:16	20	6
2	Ethyl acetate:Hexane	5:95	100	5
3	Ethyl acetate:Hexane	6:14	20	7

Table 2 shows the TLC of solvents Methanol: Chloroform and Ethyl acetate: Hexane invariousratios. Out of which Fraction IV issu bjected to further evaluation.

Table 2: Thin Layer Chromatography using solvents of various ratios

S.No	Solvent system	Ratio	Volume(mL)	No of Spots
1	Methanol:Chloroform	4:16	20	Nospot
2	Ethyl acetate:Hexane	1:19	20	Nospot
3	Ethyl acetate: Hexane	2:18	20	4
4	Ethyl acetate:Hexane	4:16	20	4
5	Ethyl acetate:Hexane	6:14	20	3
6	Ethyl acetate:Hexane	8:12	20	Nospot

Table 3 shows the phytochemicals present in various solvents, from the above data, it is concluded that, Ethanol is the most preferred solvent, since it shows the presence of majority of the components.

Table 3: Phytochemicals present invarious solvents

Phytochemical constituent	Ethanol	Hexane	Chloroform	Water
Tannin	+	-	-	-
Phenolic Compound	+	-	-	-
Protein	-	-	+	+
Flavonoids	+	-	+	+
Cardiac Glycoside	+	-	+	+
Coumarin	+	+	-	-
Saponin	+	+	-	-
Alkaloids	+	-	+	+
Carbohydrate	+	-	+	+
Steroid	+	+	+	-

Structural elucidation of the Isolated compound GC-MS of Fraction IV

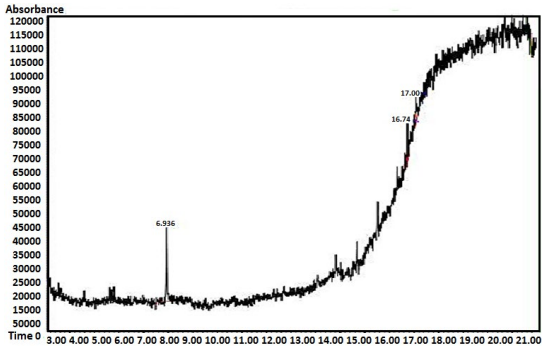


Fig. 1. GCMS Spectra of the isolated compound

The isolated compound' s molecular mass is determined to be 206.32 from the m/zvalue.

HPLC

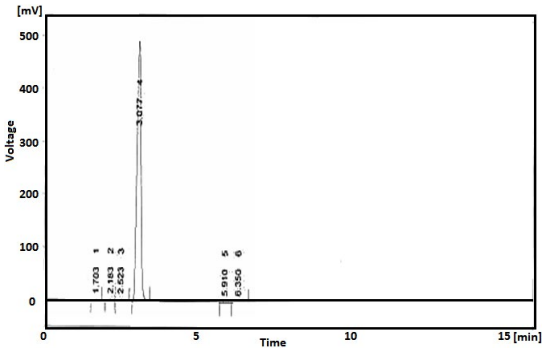


Fig. 2. HPLC spectra of purity of the isolated compound

There tention time of 2,4 DTBP isolated from the ethanolic extract of *Ficus auriculata* leaves was about 3.077.

UV-Visible Spectroscopy

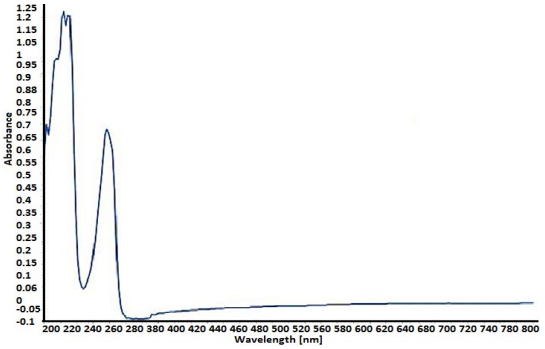


Fig. 3. UV-Visible Spectra of the isolated compound

The UV-Visible spectra of the isolated compound is performed over a wavelength range of 200-800nm. Tertiary butyl group absorbs in the range 224nm and 276.8nm with intensities at 1.273 and 0.75 respectively. This indicates the presence of phenolic functional group. These data provides additional support to the structure.

FT-IR Spectros copy

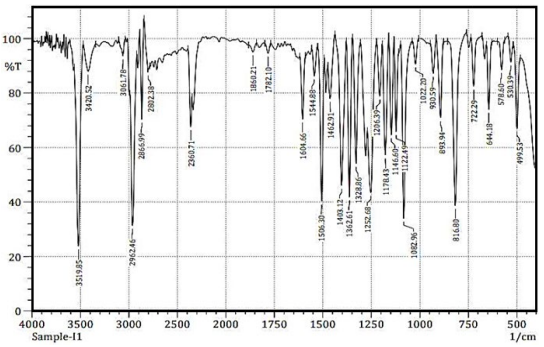


Fig. 4. IR Spectra of the isolated compound

The IR Spectrum of the compound isolated shows various peaks. Out of which, O-Hstretching is represented by 3519.85 cm^{-1}, C-H stretching is represented by 2962.46 cm^{-1}, C-Cstretching of aromatic compounds is represented by 1506.30–1604.66 cm^{-1}, C-H bending in thetertiary butyl group may be responsible for 1362.61 cm^{-1}, and C-O stretching frequency isrepresented by 1252.68 cm^{-1}. Based on all these data, it is clear that the isolated compound contains aromatic ring, tertiary butyl group and phenolic group. Hence the compound may be 2,4 Ditertiary Butyl Phenol.

^{1}H Nuclear Magnetic Resonance (^{1}HNMR) Spectroscopy

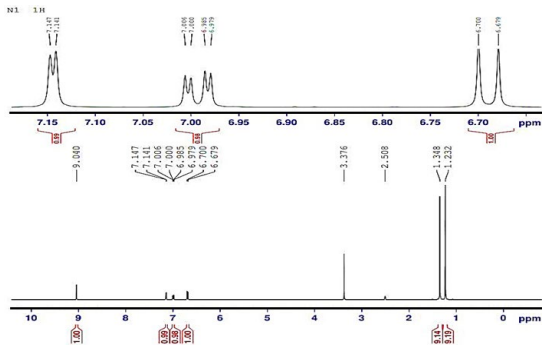


Fig. 5. ^{1}H-NMR Spectra of the isolated compound

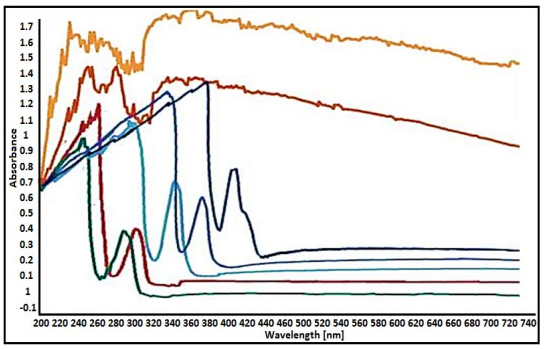
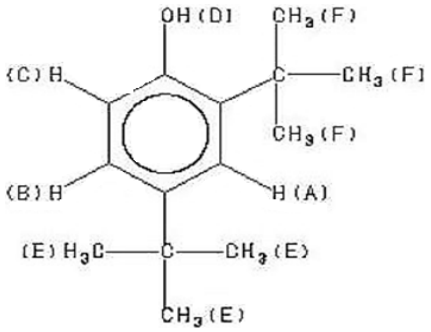


Fig.6. Effect of Solventon isolated compound

Figure 5 shows the ^{1}H NMR spectra of the compound isolated. The signals revealed the presence of phenolic proton (D) at 3.376, Methylene proton (R-CH_2-R) (F) at 1.232, Methine proton (R3C-H) (E) at 1.348 and aromaticprotons (A) (B)&(C) around 7.147, 7.141 and 7.006 respectively.

The existence of each of these signals supports the isolated compound's structure.

The Phytochemical screening, Column Fractions, TLC Studies, GC-MS Spectra, UV-Vis, FT-IRand ^{1}HNMR confirms that the isolated compound is 2,4DTBP and its structureis.



Effect of Solvent

The solvent effects on the electronic absorption is used to identify the electronic transitions in amolecule. The light absorbed by the compound shifts from low energy, long wavelength to high energy, short wavelength as the solvents become more polar.

Table 4: Effect of Solventon isolated compound

Solvent	Wavelength	Absorbance
Water	221.6	1.237
Ethanol	224	1.273
Ether	231.2	1.351
Butanol	224	1.282
Chloroform	243.2	1.512
Carbon tetrachloride	243.2	1.515
Hexane	219.2	1.122

Figure 6 & Table 4 shows the Effect of Solvent on isolated compound. Saturated compounds with one hetero atom(ethanol, butanol, ether, CCl_4, $CHCl_3$, water) undergoes n $\rightarrow \sigma^*$ transition, where as in Hexane only sigma bond is available and hence $\sigma \rightarrow \sigma^*$ transition occurs, which requires higher energy and lower wavelength.

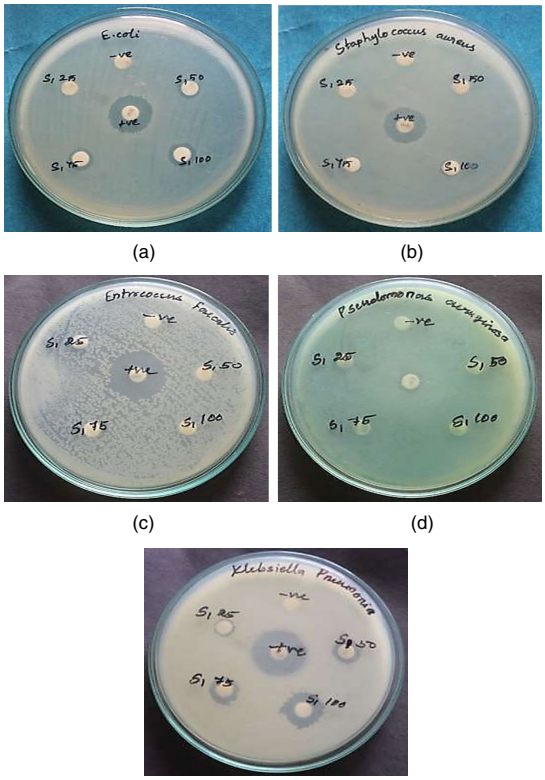
Anti-microbial property of 2,4 DTBP

Table 5: Anti-Bacterial activity of 2,4 DTBP

Name of Bacteria Strains	Samples Zone of inhibition (mm in diameter)					
	S1 25µg	S1 50µg	S1 75µg	S1 100µg	Positive control	Negative control
E. coli (G-)	9	10	10	11	16	-
Staphylococcus aureus(G+)	-	-	8	9	16	-
Enterococcus faecalis(G+)	-	-	-	-	17	-
Pseudomonasae ruginosa(G-)	-	-	-	-	10	-
Klebsiella pneumonia(G-)	8	11	12	14	20	-

The anti-bacterial activity of 2,4 DTBP was analyzed with various bacteria like: *E.coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumonia. Enterococcus faecalis, Pseudomonas aeruginosa has no inhibition. E.coli, Staphylococcusaureus, Klebsiella pneumonia* shows inhibition. Out of these three, *Klebsiella pneumonia* shows higher

inhibition than *E. coli, Staphylococcus aureus.* Hence it is concluded that, 2,4 DTBP stronglyinhibits the activityof bacteria *Klebsiella pneumonia*.



(a) (b)

(c) (d)

(e)

Fig. 7. Anti-bacterial activity of the isolated compound on *(a) E.coli (b) Staphylococcus aureus (c) Enterococcus faecalis (d) Pseudomonas aeruginosa (e) Klebsiella pneumonia*

Table 6: Anti-Fungal activity of 2,4 DTBP

Fungai name	Samples Zone of inhibition(mm in diameter)					
	S1 25µg	S1 50µg	S1 75µg	S1 100µg	Positive control	Negative control
Candida sps	6	10	11	12	14	-

Figure 8 depicts the anti fungal activity of the compound isolated, 2,4 DTBP with *Candida albicans.* This fungi shows dense zone of inhibition. The compound is very active against the fungi.

Fig. 8. Anti-fungal activity of the compound isolated on *Candida albicans*

Cytotoxic Effect MTT Assay

Table 7: % cell viability values of 2,4 DTBP against HIEC-6 cells after the treatment period of 24 hours

Culture condition	%cell viability	IC_{50} conc. (µg/mL)
Untreated	100	
Std control (Dox-5µM)	56.20	
S1-10µg/mL	99.41	
S1-25 µg/mL	94.89	NA
S1-50 µg/mL	89.08	
S1-75 µg/mL	78.87	
S1-100 µg/mL	69.50	

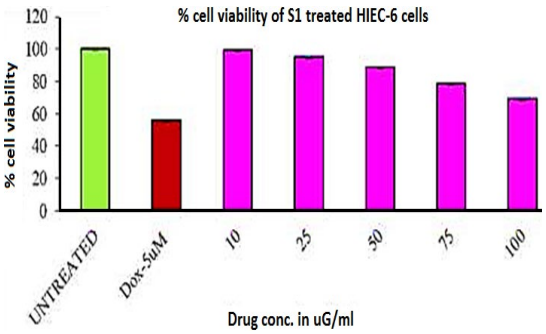
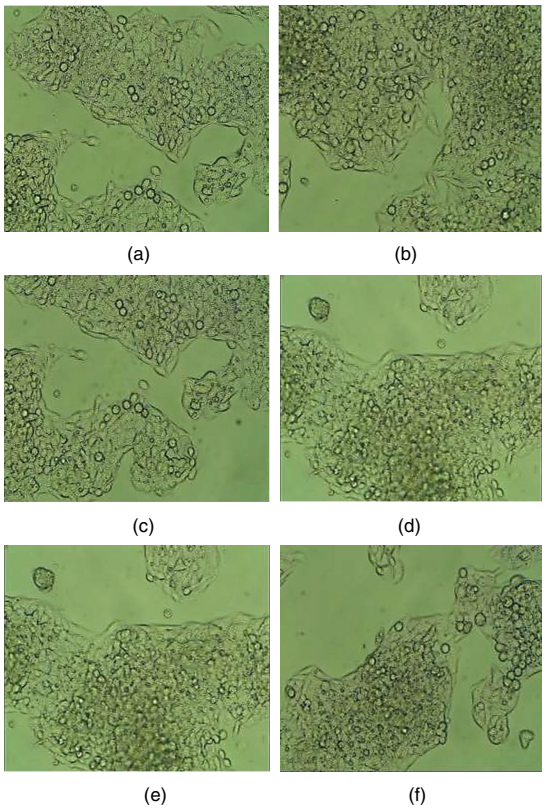


Fig. 9. %cell viability values 2,4 DTBP against HIEC-6 cells after the incubation period of 24 hours



(a) (b)

(c) (d)

(e) (f)

Fig. 10. Effect of Concentration of isolated compound on HIEC-6 cells (a) untreated (b) 10 *µg*/mL (c) 25 *µg*/mL (d) 50 *µg*/mL (e) 75 *µg*/mL (f) 100 *µg*/mL

Figure 9 is the graphical representation of the viability of HIEC-6 cells with the isolated compound, and Fig.10 shows the effect of concentration of the isolated compound on HIEC-6 cells.

The observed results clearly confirmed the Non-toxic efficacy of the isolated compoundon Normal human intestinal epithelial cells with % cell viability value of 69%, at the highest concentration of 100 μg/mL after the incubation period of 24 hours.

Table 8: Cell Viability of AGS cells with 2,4DTBP at various concentrations

Samples	Triplicate 1	Triplicate 2	Triplicate 3	Average	Percentage of Viability	IC_{50}
Control	0.679	0.668	0.653	0.66667	-	83.18
6.25	0.633	0.625	0.617	0.625	93.75	
12.5	0.584	0.571	0.565	0.57333	86	
25	0.495	0.486	0.477	0.486	72.9	
50	0.395	0.416	0.407	0.406	60.9	
100	0.287	0.305	0.313	0.30167	45.25	

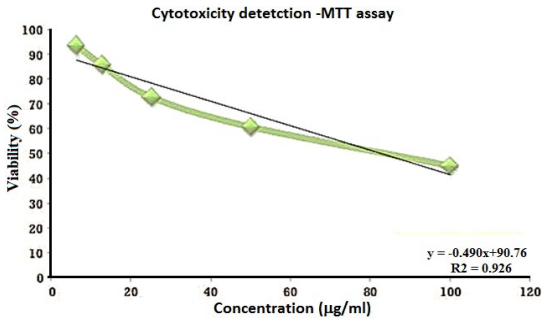


Fig. 11. Cell Viability of AGS cellline with isolated compound

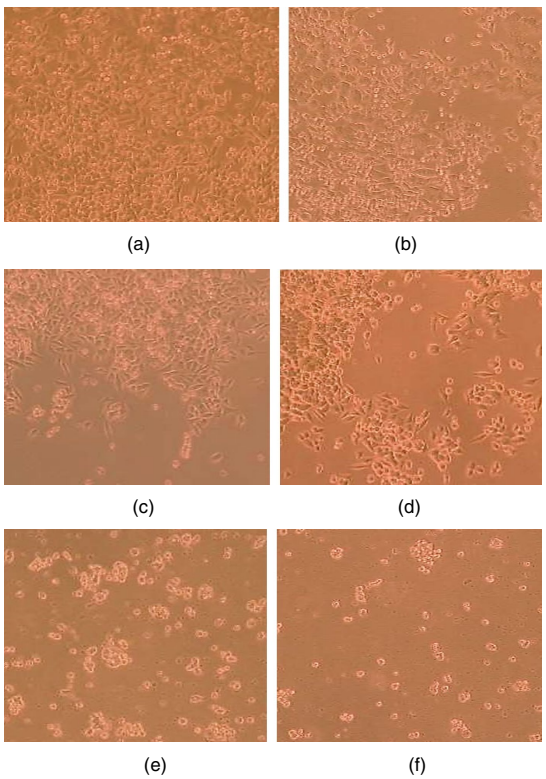


Fig. 12. Effect of Concentration of isolated compound on AGS cellline (a) untreated (b) 6.25 *μ*g/mL (c) 12.5 *μ*g/mL (d) 25 *μ*g/mL (e) 50*μ*g/mL (f) 100 *μ*g/mL

Figure 11 is the graphical representation of the viability of AGS Cell line with the isolatedcompound, and Fig. 12 shows the effect of concentration of the isolated compound on AGS cellline. As the concentration increases, the viability decreases. At 100 μg/mL concentration, the viability of the cancer cell is minimum. (ie):45.25%.

With varied quantities of the sample supplied to SKMEL cancer cells, a dose-dependent reduction in cell viability was seen. 83.18 μg/mL is the IC_{50} value obtained for the sample.

Molecular Docking Study

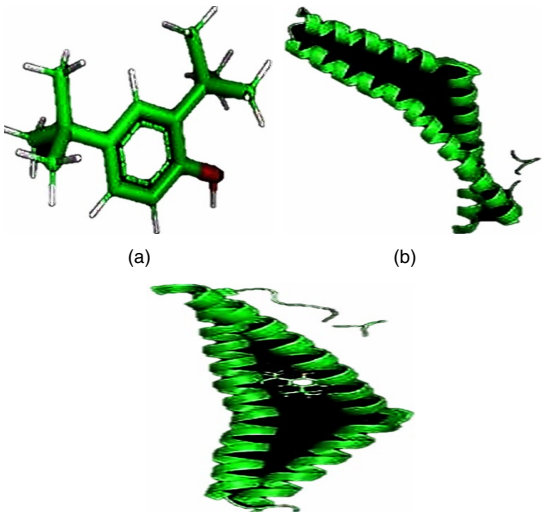


Fig. 13. 3-D Structure of (a) 2,4 DTBP (b) Occludin (c) 2,4DTBP Docked with Occludin

Figure 13 (a),(b) shows the 3-D structures of 2,4 DTBP, Occludin respectively. These structures are obtained in PDB format and viewe dusing PyMol software. Out of all the models, the one with score 2846 for the area 358.70 is chosen as a the most favourable model, which is represented in Fig. 11(c).

Table 9: Set of PatchDock results showing the docking structures of Occludin with 2,4 DTBP

S. No	Score	Area	Atomic Contact Energy(ACE)	Transformation
1	2846	358.70	-57.23	-3.021.14 -1.87 6.06 15.72 0.80
2	2828	352.50	-101.00	0.081.24 -2.43 2.85 14.97 8.88
3	2790	331.70	-84.85	-0.49-1.03 0.30 2.75 14.84 9.02
4	2726	366.90	14.29	1.290.07 3.07 6.43 18.21 -6.28
5	2696	314.20	-73.90	-2.03-0.33 2.07 3.83 13.90 7.91
6	2684	368.00	7.26	-1.450.19 0.04 7.3618.54 -6.72
7	2680	304.10	-24.97	-1.67-0.35 1.68 1.29 12.73 17.69
8	2666	306.10	-1.60	-0.05-1.20 1.94 9.92 20.16 -14.58
9	2646	345.80	-18.61	-2.090.07 2.79 8.0016.19 -2.19
10	2632	372.20	-61.13	0.64-1.12 1.99 6.4816.07 -0.33

Lipinski Rule of Five

Table 10: Lipinski Rule for 2,4 DTBP with Occludin

Mass	206
Hydrogen Bond Donor	1
Hydrogen Bond Acceptor	1
Log P	3.987
Molar Refractivity	65.506

From the above results, it is clear that the compound 2,4 DTBP doesn't violate anycriteria, henceit can be orally administered.

CONCLUSION

Phytochemicals present in various extracts of *Ficus auriculata* leaf has been analyzed. From the Chromatographic, GC-MS and Spectroscopic studies, and comparing the results fromvarious literatures, it is found that the isolated compound is 2,4 Ditertiary Butyl phenol, which is abiologically active compound, and from the *In vitro* & cytotoxicity studies, it is found to possess anti-microbial & anti-cancer activity. The 3-D structure obtained from Molecular docking studies, is subjected to Lipinski rule of 5, and from the data obtained it can be concluded that 2,4 DTBP can beadministered orally.

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