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Elucidating the synergistic capability of phytoconstituents in *Thespesia populnea* (L.) Soland ex Correa towards enhanced cytotoxicity against K562 leukemic cells

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Abstract
Purpose:*The present study mainly focuses on the synergistic interaction of the active constituents in the chloroform leaf extract (CHLE) of the Thespesia populnea plant for its antiproliferative ability.*
Methods:*Chromatographic techniques were carried out to isolate and identify the bioactive compounds from CHLE and additionally checked for their antiproliferative ability by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The isolation techniques employed for CHLE were silica gel column chromatography and preparative HPLC. The identification of the bioactive constituents was analyzed using GC-MS and LC-MS analysis.*
Results:*The preliminary phytochemical screening showed the presence of alkaloids, terpenoids, phenolics, flavonoids, anthraquinones, and lignans using thin layer chromatographytowards specific reagents.The silica gel column chromatographic fraction collected in the combination of petroleum ether: ethyl acetate (80:20) exhibited antiproliferative activity with an IC_{50} value of 69.36±2.15 µg/ml, and the fraction from preparative HPLC demonstrated an IC_{50} concentration of 64.26±3.24 µg/ml. Further, it proved that the combined action of the fractions mixed equally by weight exhibited enhanced antiproliferative ability with an IC_{50} value of 23.31±0.88 µg/ml.*
Conclusion:*The phytoconstituents present in chloroform leaf extract (CHLE) of T.populnea were identified using various chromatographic techniques and also ascertained that phytoconstituents present in the extract work synergistically to give enhanced cytotoxicity after combining the isolated fractions.*
Keywords*-Antiproliferation, MTT assay, T. populnea, Chromatographic techniques*

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I. Introduction

The benefits of plant-derived drugs have gained much attention in this present scenario due to their diverse action and negligible toxicity exhibited in various disease conditions. For more than 5000 years, plants have been used as medicines and therapies. Many of the drugs used in modern medicine are directly or indirectly derived from plants[1]. Natural products obtained from medicinal plants played an imperative role in managing cancer[2]. Cancer recurrence and severe side effects towards currently prevailing chemotherapeutic drugs reduce these drugs' clinical efficiency. So the importance of plant-derived bioactive molecules exhibiting anticancer properties can provide better treatment strategies. Due to the increasing resistance to chemotherapeutic drugs, much research focuses on traditional natural therapeutics, which are less toxic to normal cells[3]. Hartwell, in his review, reported that more than 3000 plant species were used to treat cancer[4]. Plant-derived natural products such as flavonoids, terpenes, alkaloids etc., have received wide attention due to their diverse pharmacological properties, including cytotoxic in cancer prevention[5]. For this, the identification, screening and isolation of these active compounds will possibly serve as a lead molecule for new semi-synthetic compounds with advanced action[6].

Thespesia populnea (L.) Soland ex Correa comes under Malvaceae family, otherwise known as mallow family. It is mainly distributed in the coastal regions of the Indian and Pacific oceans throughout Oceania. The leaves, flowers, bark and fruits are useful in cutaneous infections such as psoriasis, scabies, ringworm, eczema, and guinea worm[7]. The leaves are used for local application for their anti-inflammatory effects in swollen joints[8]. The seed extract and its fractions were evaluated for analgesic and antipyretic activity in mice[9].

More recently, there is evidence that photochemical of specific combinations are more effective than isolated compounds in protecting against cancer[10].With this background, an attempt has been made to isolate and identify the phytoconstituents present in chloroform leaf extract (CHLE) of *T. populnea* using various

chromatographic techniques and establish that the phytoconstituents present in the extract work synergistically to give enhanced cytotoxicity after combining the isolated fractions.

II. Materials And Methods

Extract preparation of *Thespesia populnea (L.) Soland*

The fully matured leaves of *Thespesia populnea* were collected, washed thoroughly, shade dried and powdered coarsely. Initially, the leaf powder of 100g was extracted sequentially using petroleum ether, chloroform, ethyl acetate and methanol (300 ml each) in increasing order of polarity under room temperature with constant shaking for 48h. The extracts were filtered and dried by evaporation. The dried chloroform extract (CHLE) was used in the present study.

Cell line and cell viability assay

In this study, K562 chronic myelogenous leukaemia cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (Gibco 10270, South American Origin), gentamycin 40 µg/ml, Streptomycin 100 µg/ml and penicillin 50 IU/ml (HiMedia, India) were grown in a humidified atmosphere with 5% CO_2 at 37ºC in an incubator (BB15, Thermoelectron corporation). The viability of the cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay[11]which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. In brief, K562 cells were seeded in a 96 well microtitre plate with a density of 15000 cells/ well[12] and incubated overnight. After incubation, a fresh medium containing varying concentrations (0-100) µg/ml was added to respective wells and incubated for 48 hours in a CO_2 incubator. At the end of the incubation period, the medium was aspirated, replaced with a fresh medium containing MTT (0.5mg/ml), and further incubated for 3-4 hours. Thus, the formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm (MULTISKAN EX Thermo Scientific). The percentage of viability was calculated using,

$$\text{Cell viability (\%)} = \frac{Absorbance\ with\ plant\ extract}{Absorbance of control} \times 100$$

Thin layer chromatographic identification of secondary metabolites

The thin layer chromatographic separation of CHLE was performed. The plates were prepared manually using Silica Gel G for thin layer chromatography. The required amount of silica was mixed with distilled water to prepare a suspension. This was then uniformly distributed on the glass plate and kept for air dry. The plates were charged by holding them under 110ºC for 1h. Samples were prepared by dissolving them in chloroform. The sample was loaded 2 cm above its bottom. The plate was loaded with 5µl of the CHLE and then kept in a glass chamber saturated with solvent combination petroleum ether: ethyl acetate (6:4). The mobile phase was allowed to move through the adsorbent phase up to 3/4th of the plate. The plates were then observed under visible, long and short ultraviolet light. Different spraying reagents were used to detect the phytoconstituents in separated bands/regions. Anisaldehyde-sulfuric acid for terpenoids, Dragendorff reagent for alkaloids, Fast blue reagent for phenolics, potassium hydroxide to detect anthraquinones, Conc. H_2SO_4 for detecting cardiac glycosides and lignans, Vanillin-sulphuric acid for essential oil, terpenoids, steroids etc., ethanolic aluminium chloride for detecting flavonoids, ammonia vapours for tetracyclins, iodine reagent to detect compounds having conjugated double bonds and vanillin phosphoric acid used to detect terpenoids, lignans etc.

Column chromatography

The silica gel column chromatography was performed to isolate the phytoconstituents present in them. The silica gel column of mesh size 60-120 was used. The CHLE sample was loaded by mixing with silica gel on the top of the packed column having a height of 25 cm. The solvent system used was petroleum ether: ethyl acetate (100:0, 0:100). Fractions were eluted in the solvent mixture combinations like 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. The eluted fractions were collected, dried and subjected to MTT assay to check the antiproliferative ability. The fraction which showed antiproliferative ability was further analyzed by TLC and GC-MS analysis.

GC-MS analysis

The column fraction which showed the antiproliferative property was subjected to GC-MS analysis. GC-MS analysis of the column chromatographic fraction was carried out in the Aglient gas chromatography 6850 Network GC system. It is connected with a HP-5MS capillary column (30m X 0.25 mm), with a film thickness of 0.25 µm and interfaced with an Agilent 5975C VLMSD with a triple axis mass detector (Agilent Technologies, USA).

Preparative HPLC analysis

For the isolation of active phytoconstituents present in CHLE, preparative HPLC analysis with gradient method was performed. Preparative HPLC system Shimadzu LC 20 AP with column length 250 mm, column id 20 and particle size 10µm. The mobile phase used was methanol: water. The sample concentration was 25mg/ml, and 5ml samples were loaded in a single run. The fractions collected were checked for their antiproliferative property by MTT assay. The active fraction was further analyzed by TLC and LC-MS analysis.

LC-MS analysis

LC-ESI/MS analysis was performed on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 connected with C18 column of 1.8 µm, 2.1 x 50 mm. The gradient elution was carried out with water/0.05% formic acid and methanol at0.8 ml/ min constant flow rate. The MS analysis was examined using ESI in the negative ion detection mode. The conditions for mass spectrometry were: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 40 psig; drying gas temperature 325°C; capillary voltage - 3000 V; fragmentor volt 125V; Oct RF Vpp 750 V. The mass fragmentation was carried out with varying collision energy 4 V/ 100 DA with an offset of 6V.

Statistical analysis

The MTT results were expressed in mean ± standard deviation. Statistical significance was derived from Student's T test, and a p-value less than 0.05 was considered significant and marked as *. In contrast,ap-value less than 0.01 was indicated as **, and a value less than 0.001 was shown as ***. The IC_{50} value was calculated using ed50V10 software.

III. Results And Discussion

Thin layer chromatographic separation of chloroform leaf extract of *T. populnea*

The CHLE showed a very prominent antiproliferative property against different cell lines. The biological activities exhibited by the extract may be due to the presence of various bioactive components present in them. Chloroform extract of *T.populnea* was separated by TLC using petroleum ether: ethyl acetate solvents in the ratio of 6:4, which showed maximum separation illustrated in figure 1. Different compounds in the extract were analyzed using various spraying reagents in Table 1.

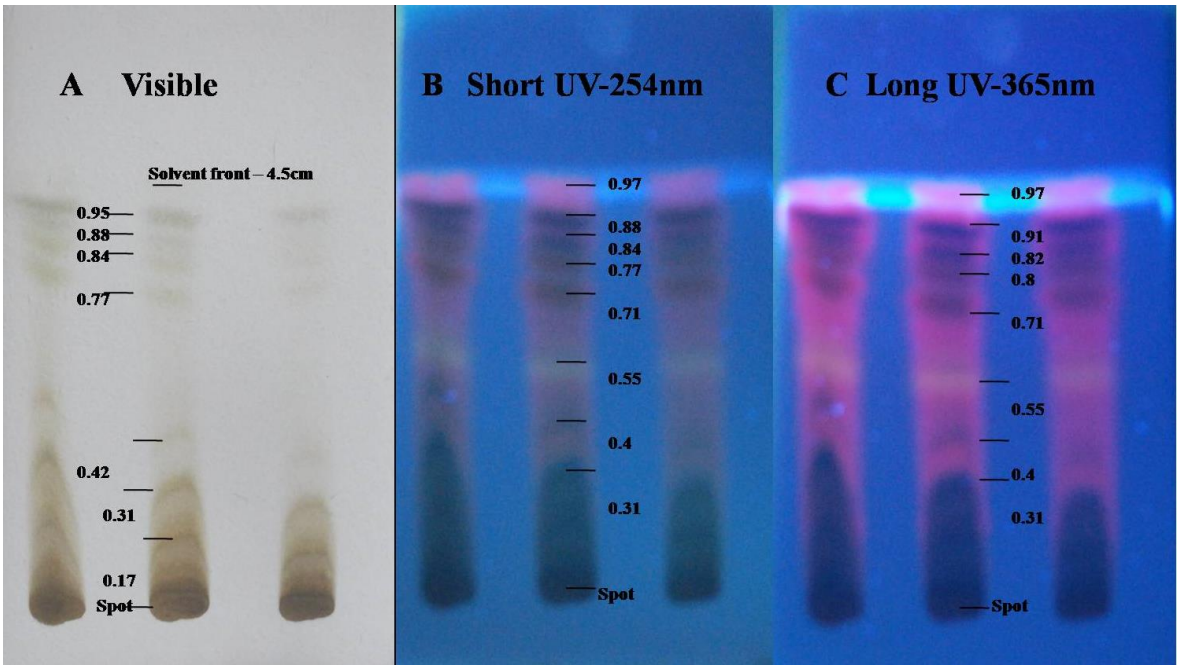


Figure 1: Thin layer chromatogram of chloroform leaf extracts of *T. populnea* observed under A-visible light, B-short UV 254nm and C-long UV 365nm after separating using petroleum ether: ethyl acetate (6:4)

Table 1: Preliminary phytochemical screening of CHLE using various spraying reagents

S No	Spraying reagent/test	Phytoconstituents	Detection in CHFE
1	Anisaldehyde Sulphuric acid	Terpenois, phenolic compounds	Present
2	Dragendoff's	Alkaloids	Present
3	Fast blue salt	Phenolic compounds	Present
4	Iodine	Compounds having conjugated bond	Present
5	Potassium hydroxide	Anthraquinones	Present
6	Con. H_2SO_4	Cardiac glycosides, lignans	Present
7	Vanillin Phosphoric acid	Terpenoids	Present
8	Vanillin Sulphuric acid	Essential oils (terpenoids)	Present
9	Ethanolic Aluminium chloride	Flavonoids	Present
10	Ammonia vapors	Tetracyclins	Absent
11	Ninhydrin	Aminoacids	Absent
12	Boric acid and Citric acid	Quinolines	Absent
13	Urea Hydrochloric acid	Sugars	Absent
14	Iodine Chloroform	Ipecacuanha alkaloids	Absent

The preliminary screening of the chloroform extract of *T.populnea* showed the presence of alkaloids, terpenoids, phenolics, flavonoids, anthraquinones, and lignans in the specific spraying reagents. Generally, medicinal plants contain various chemical components, and their biological activities are not generally attributed to a single moiety [13].

3.2 *Silica gel column chromatography:* For the isolation of the bioactive constituent present inCHLE that promotes the antiproliferative property, silica gel column chromatography was performed. The solvent combination used to separate compounds was petroleum ether: ethyl acetate. The fractions were eluted accordingly using petroleum ether: ethyl acetate solvent mixture (100:0-0:100). The fractions were collected and checked for their cytotoxicity in K562 cells by MTT assay. The fraction contained in the combination 80:20 exhibited antiproliferative activity with an IC_{50} value of 69.36±2.15 µg/ml. The TLC chromatogram of the active fraction showed in figure 2, and the viability percentage graph is illustrated in figure 3. The TLC profile of the active fraction showed the presence of a variety of compounds. Further GC-MS analysis of this fraction was performed.

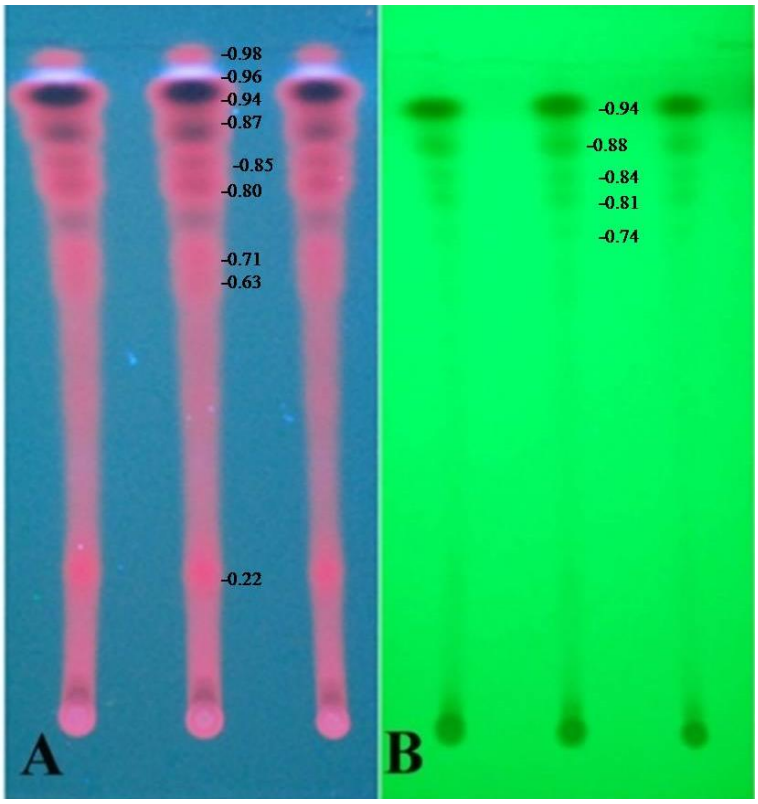


Figure 2: TLC chromatogram of column chromatographic fraction showing antiproliferative property A-under 365nm and B-254nm.

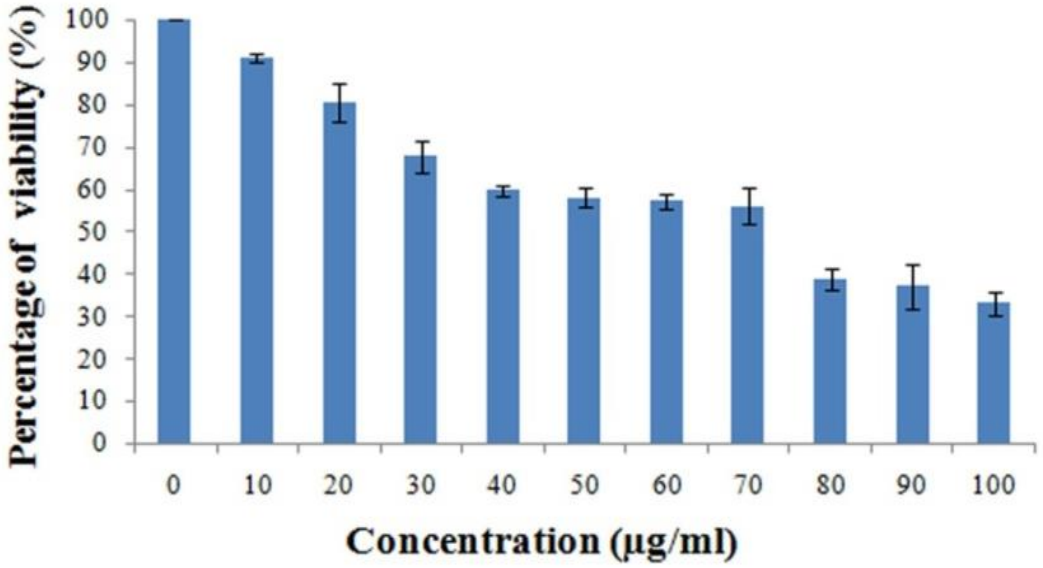


Figure 3: Graph representing the percentage of viability of column chromatographic fraction against K562 cell line and exhibited an IC_{50} value of 69.36±2.15µg/ml.

3.3 *GC-MS analysis*: To separate and identify volatile compounds, GC MS analysis was performed. The gas chromatograms of column chromatographic fraction of chloroform leaf extract of T. populnea showed antiproliferative activity and confirmed the presence of various compounds with their retention time (RT), as shown in figure 4. The compounds were identified through mass spectrometry attached with gas chromatography. The identified compounds' retention time and peak area with more than 95% similarity were shown in table 2. The identification of the compounds is mainly based on their retention time and by comparison with the reported mass spectral fragmentation pattern in the available literature and also based on MS library

(NIST database NIST08 and 0.8L) by the National Institute of standards and technology US. The compounds identified in the GC-MS spectrum showed the presence of oleic acid[14] and hexadeconoic acid[15] are reported to have the antiproliferative ability. So the antiproliferative ability of the active column chromatographic fraction may be due to the presence of this compound.

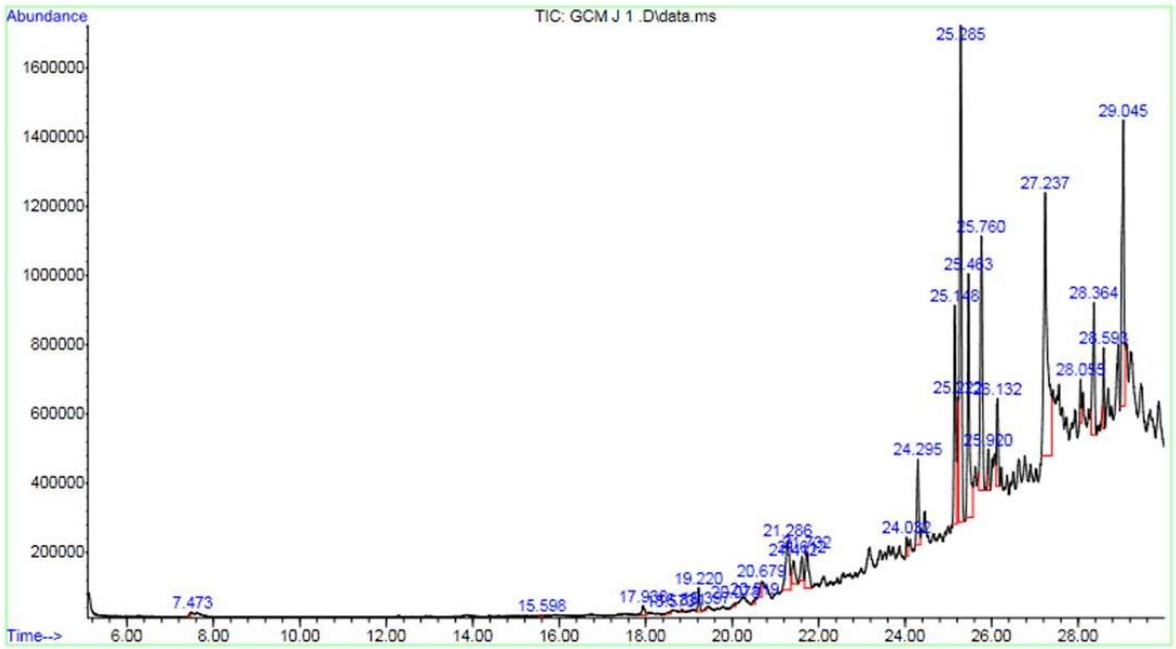
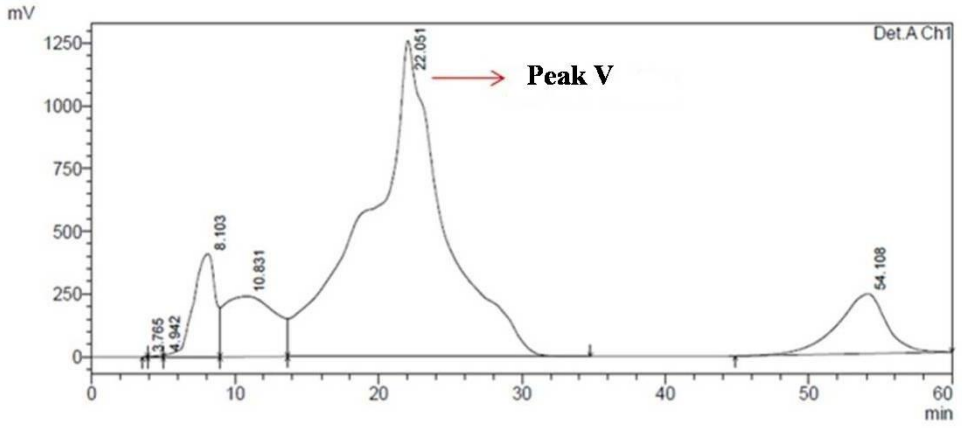


Figure 4: GC-MS chromatogram of column chromatographic fraction showing CHLE.

Table 2: Compounds identified in the chloroform leaf extract of *T. populnea* by GC-MS analysis.

S No	Retention time (RT)	Peak area (%)	Compound name	Molecular weight (amu)
1	17.944	0.0767	Humulene	204.36
2	19.2258	0.259	2,4-Di-tert-butylphenol	206.32
3	20.7878	0.3481	2-Methyl-Z,Z-3,13-octadecadienol	280.45
4	21.2857	1.6681	9-Octadecenal-(Z)-	266.469
5	24.038	0.2311	Linoelaidic acid	256.43
6	25.4684	4.2036	n-Hexadecanoic acid	268.438
7	26.5041	1.0383	Heptadecanolide	268.434
8	27.2365	9.4297	Cis-Vaccenic acid	282.468
9	27.637	1.4622	Oleic acid	282.47
10	27.7286	1.6899	Trans-13-Octadecenoic acid	282.468
11	27.8774	0.3988	Cis-13-Octadecenoic acid	282.46
12	28.9302	2.8233	Squalene	410.718
13	29.222	3.9273	Cyclopropaneoctanal, 2-octyl-	280.49

3.4 Preparative HPLC: To isolate the active constituents attributing to the cytotoxicity in chloroform leaf extract of *T. populnea*, preparative HPLC with gradient method was performed. The fractions were collected and checked for their antiproliferative ability. The peak, which showed maximum intensity at retention time 22.051 detected at 274 nm, exhibited cytotoxicity, and this region was marked as peak V. The preparative HPLC chromatogram was shown in figure 5.



Detector A Ch1 274nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.765	5543	405	0.001	0.019
2	4.942	189671	6328	0.031	0.293
3	8.103	42937884	410436	6.945	19.028
4	10.831	59504921	241180	9.624	11.181
5	22.051	458633929	1260242	74.179	58.425
6	54.108	57009645	238419	9.221	11.053
Total		618281594	2157010	100.000	100.000

Figure 5: Preparative HPLC chromatogram of CHLE using the gradient method. The peak V showed maximum intensity at retention time 22.051 detected at 274 nm, flow rate 8 ml/min.

The antiproliferative ability of V peak at retention time 22.051 was checked against K562 cell lines with different concentrations ranging from 0-100 µg/ml for 48h and exhibited cytotoxicity in a concentration-dependent manner in figure 6. The IC_{50} concentration was found to be 64.26±3.24 µg/ml.

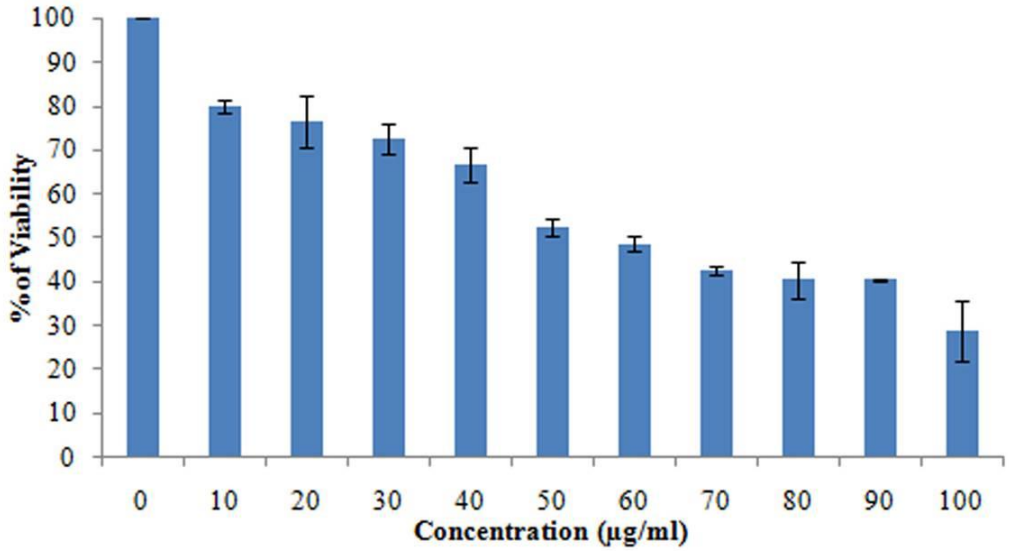


Figure 6: Graph representing percentage viability of peakV from preparative HPLC against K562 cell line showed an IC_{50} of 64.26±3.24 µg/ml.

The peak V of preparative HPLC was further separated using TLC. After separation, showed 4 separate areas on the TLC plate, namely V(1), V(2), V(3) and V(4), using a solvent combination of petroleum ether:ethyl acetate 6:2 shown in figure 7 A. Each separated region was checked for its antiproliferative potential; it was observed that only the peakV (4) region exhibited a significant antiproliferative ability shown in figure 7 B.

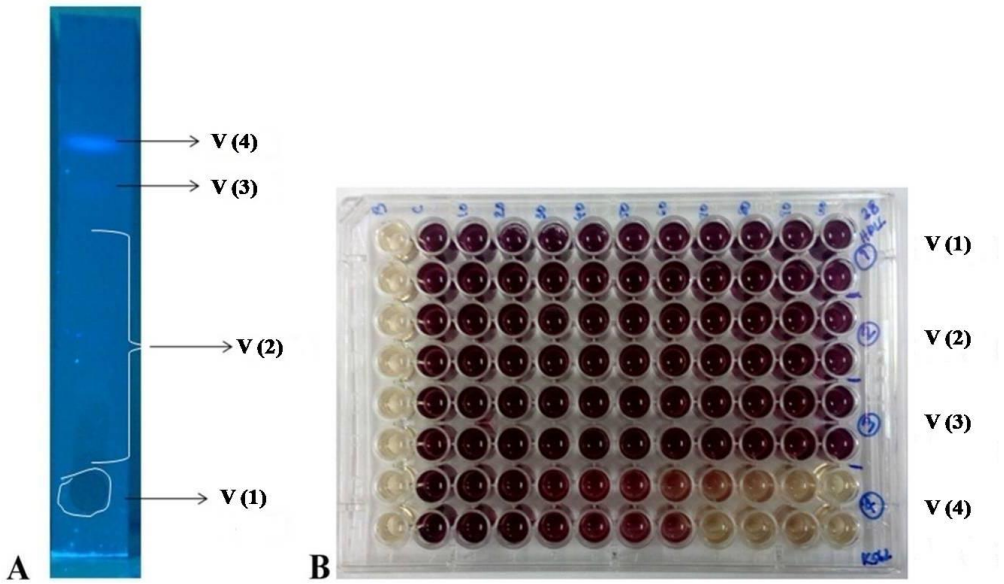
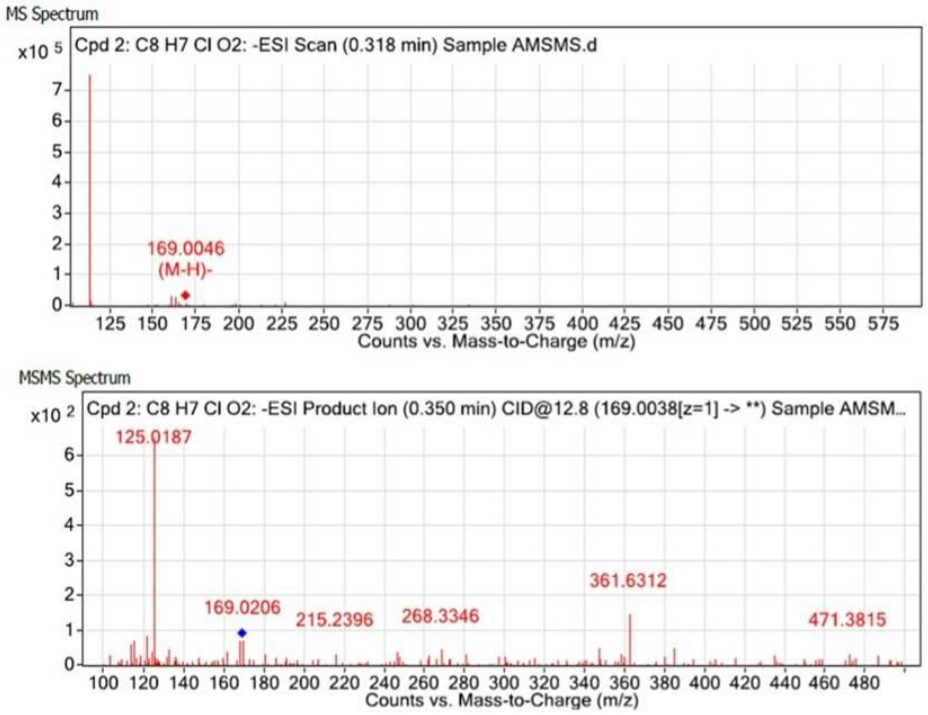


Figure 7: A- TLC profile of peak V and B- MTT picture of TLC isolated and separated regions.

3.5 LC-MS analysis of TLC isolated V peak: The TLC separated region of V(4), which exhibited the antiproliferative ability, was further analyzed for LC-MS to identify the molecular mass of the bioactive compound. The LC-MS spectrum is depicted in figure 8. The MS spectrum of peak V(4) showed similarity towards the MS spectrum of gallic acid or its derivative[16]. To evaluate the cytotoxic effect of gallic acid, an MTT assay was performed against K562 cells and compared with the antiproliferative effect of peak V(4) using concentrations ranging from (0-100µg/ml) for 48h. The result was encouraging, showing a dose-dependent response against K562 by peakV(4) and gallic acid figure 9. The IC_{50} concentration was calculated and was found to be 38.47±1.033 µg/ml for peak V(4) and for gallic acid found to be 32.8±1.2 µg/ml.



MS/MS Spectrum Peak List

m/z	*Calc m/z*	Diff (ppm)	Abund	Formula
112.9771	112.98	25.21	58	C5 H2 Cl O
114.9574	114.9592	16.11	71	C4 Cl O2
121.0281	121.0295	11.32	85	C7 H5 O2
124.9511			211	
125.0187	125.0164	-18.92	649	C7 H6 Cl
167.9682			70	
169.0206			70	
346.5611			50	
361.6312			149	
384.3168			51	

Figure 8: LC-MS spectrum of peak V(4)

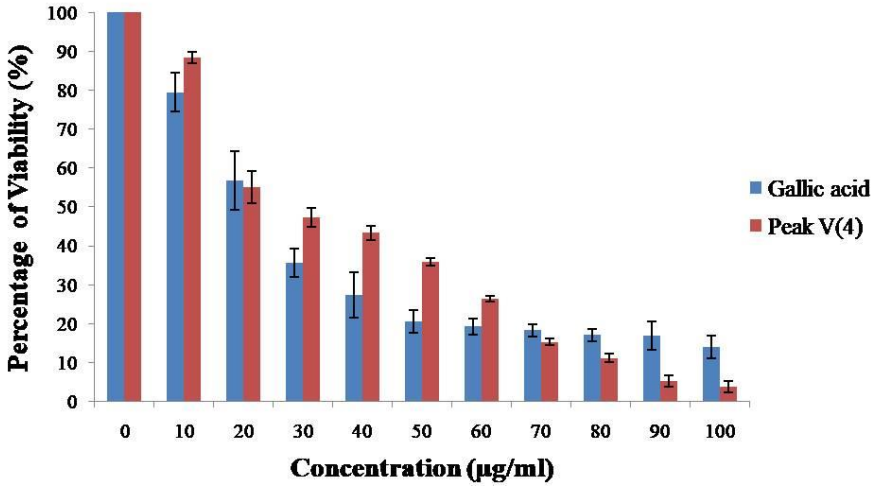


Figure 9: Graph representing the antiproliferative ability of gallic acid and peakV(4) against the K562 cell line.

3.6 Combined effect of column chromatographic fraction and preparative HPLC fraction against K562 cell lines: The bioassay-guided isolation and characterization of anticarcinogenic compounds from chloroform leaf extract was performed using chromatographic techniques. One fraction in the column chromatography exhibited antiproliferative ability against K562 cells and represented an IC_{50} value of 69.36±2.15µg/ml. Similarly, the prepared HPLC isolated fraction V peak demonstrated an IC_{50} value of 64.26±3.24 µg/ml. The fractions (20mg/ml) were mixed equally by weight, and the antiproliferative ability was checked against K562 cells with different concentrations ranging from 0-50 µg/ml for 48h. The result was so encouraging that the combined effect decreased the IC_{50} value to 23.31±0.88 µg/ml. The graph representing the antiproliferative ability is shown in figure 10.

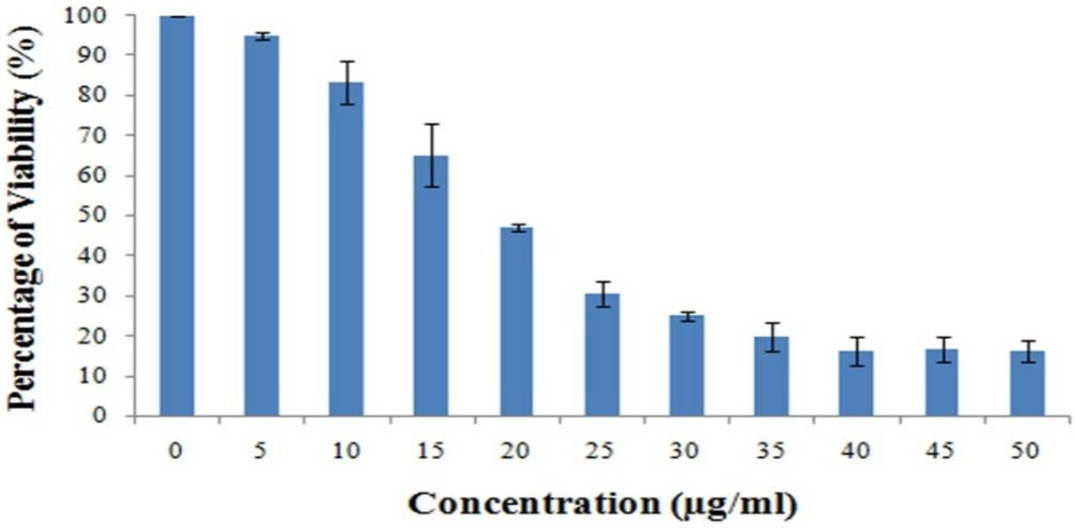


Figure 10: Graph representing the combined effect of column chromatographic fraction and preparative HPLC V peak exhibited IC_{50} value of 23.31±0.88 µg/ml.

In this study, the cytotoxic ability of chloroform leaf extract of *T. populnea* on chronic myelogenous leukemic cells is demonstrated for the first time. Their semipurified fractions from silica gel column chromatography and preparative HPLC fraction inhibit the cell viability in a concentration-dependent manner. After combining both fractions by equal proportion by weight being various potency to their parent extract. According to US National Cancer Institute (NCI), the decisive factor of cytotoxicity for crude or semipurified fractions is the value IC_{50} less than 30µg/ml in the preliminary assays[17]. The results showed that the combined effect of the fractions increased the cell death rate at minimal concentrations comparable to that of the parent extract and satisfied the condition of a potential anticancer drug moiety as per the NCI guidelines[18].

It has been reported that when mixtures of two or more compounds demonstrate a more potent therapeutic effect than that of individual compounds at equal concentrations, the effect is described as a synergistic one[19]. Synergy can occur when one compound increases the bioavailability[20], inhibits the detoxification[21]or compromises the export of another compound[22]. A similar study in cranberry extract exhibited enhanced antiproliferative activity than its phytochemicals, suggesting synergistic or additive antiproliferative interactions[23].Here the combined actions of the individual phytoconstituents contribute toward a better and enhanced cytotoxic activity. Therefore, in addition to the characterization of chemo-preventive effects of unique compounds, the evaluation of synergistically acting phytochemicals is of particular interest. Suganuma *et al.* reported in their study that with synergistic effects between green tea polyphenols, whole tea is more efficient for cancer prevention than supplementation with epigallocatechin gallate (EGCG) alone[24]. Synergistic interactions are documented for constituents within a total extract of a single herb and between different herbs. Thus the present investigation supports the concept that the whole or partial purified extracts of herbal preparations offer advantages over a single isolated ingredient[25]. Therefore it is proved that the antiproliferative ability increases with the combined action of fractions rather than its isolated forms. Thus our study strongly supports the synergistic effect of the phytoconstituents on its antiproliferative ability.

IV. Conclusion

Studying the combined effect of bioactive compounds may be a promising approach to optimizing the pharmacological efficiency of cancer prevention and therapy with minimal dose usage. The present study identified a variety of phytochemicals present in the leaf extracts of *T. populnea.* It proved that the

phytoconstituents present in the section work synergistically to give better antiproliferative ability. Thus the medicinal plant *T. populnea* is a promising medical aid in therapeuticals and herbal formulations.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflicts of interest.

FUNDING

None

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