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RESEARCH ARTICLE

Phytochemical Screening of *Diospyros paniculata* Bark and *In Vitro* Cytotoxic Study on Human Breast Cancer Cell Line

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Abstract

Medicinal plants are the resource of traditional medicines and modern medicine is also developed indirectly from plants. In traditional medicinal practices such as *Ayurveda* and Unani, *Diospyros paniculata* has been recognised for its medicinal properties. Different parts of the plant, including the leaves, bark, fruits, and seeds, are utilised to prepare various herbal remedies and these species show a richness of triterpenoids, naphthoquinones and naphthaldehydes which majorly contribute to the anticancer activity. *D. paniculata* bark was extracted with methanol and fractionated by low-polar to high-polar solvents. *In-vitro* cytotoxic activity of each solvent fraction was studied on the MCF-7 (Human Adenocarcinoma) cell line by MTT assay. The ethyl acetate fraction shows a significant IC_{50} value of 23.47 µg/ml which might be due to the presence of principal compounds in the fraction. Methanol fraction showed a moderate IC_{50} value. To isolate chemicals, the chloroform fraction underwent column chromatography. Fourier Transform Infrared Spectroscopy (FTIR), ^{1}H NMR (Nuclear Magnetic Resonance Spectroscopy), and ^{13}C NMR were used to characterise the isolated chemicals. Two compounds were isolated from chloroform fraction identified as Betulin and Lupeol.

Keywords: Betulin, Cytotoxicity, *Diospyros paniculata,* Lupeol, MCF-7

1. Introduction

Cancer is a condition marked by unchecked cell division and the capacity to invade or disseminate the tumour to various parts of the body[1]. An estimated 10.0 million cancer-associated deaths and 19.3 million new cases were reported globally in 2020[2]. The survival rate with cancer treatment is not beneficial to surgery, radiation therapy, chemotherapy, and immunotherapy. Chemotherapy produces side effects like vomiting, hair loss, reduction of cell count in the bone marrow and some drugs result in drug resistance[3]. Drug discovery with natural products is evolving as a successful strategy in the development of novel therapeutic drugs. Many diseases like cancer, AIDS, degenerative diseases, and disorders that are getting complicated by synthetic drugs are cured by natural products[4]. Approximately, 80% of the world's population is dependent on herbal medicine in one or the other aspect for primary health care. 60% of the available chemotherapeutic drugs are derived from natural sources (for example: vincristine, vinblastine, phodophyllotoxin, taxol, etc)[5].

Ebenaceae is a family having medium-sized pan tropical plants with a wide variety of species in Asia and the Indo-Pacific region[6]. It includes two subfamilies such as Ebenoideae and Lissocarpoideae and four important genera, *Diospyros, Euclea, Royena* and *Lissocarpa*. This family principally contains two genera such as *Diospyros* and *Euclea* with 500-600 species[7]. As an anti-inflammatory drug, *Diospyros* species are important in traditional medical systems like Chinese, *Ayurvedic* and Tibetan medicine systems.

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The genera comprises about 240 species out of which 59 are found in India. These species are located mostly in the evergreen forests of Assam, Deccan, and Bengal and some in North India. The morphological features of species are trees, some are shrubs, leaves alternate, flowers are green, white or yellow with few to many big fruits with seeds ranging from 1 to 10, soft sapwood in white and hard heartwood in black[8].

Diospyros paniculata belongs to the Ebenaceae family and is used as folk medicine in the *Ayurveda* system. It is a moderate-sized tree about 50 feet in height. The fruits are green and have soft whitish sapwood. It includes secondary metabolites such as terpenoids, alkaloids, flavonoids, steroids, naphthoquinones, and naphthaldehydes[9,10]. The parts of the tree have different uses. The leaves are used in fish poison, fruits to heal burns, a decoction of fruits for gonorrhoea and blood poisoning, and stem bark in rheumatism and ulcers, seeds of the plant have been traditionally used as a remedy for respiratory ailments, such as coughs and asthma, In some regions, the twigs and roots of *D. paniculata* are employed in folk medicine to manage conditions like diabetes and hypertension[11]. The present work focused on the phytochemical profile of methanol extract and its fractionation with different solvents by separating funnel method, *in vitro* cytotoxicity of all the above fractions tested against MCF-7 cell line by MTT assay, isolation of bioactive from the CF by column chromatography and their characterisation by FTIR, ^{1}H NMR, and ^{13}C NMR.

2. Materials and Methods

2.1 Chemicals

Methanol, ethyl acetate, hexane, chloroform, and ethanol were all acquired from Sigma and were all of analytical quality. Fetal Bovine Serum (FBS), antibiotic-antimycotic 100X solution (Thermofisher Scientific), doxorubicin and Dulbecco's Modified Eagle Media (DMEM) with low glucose (Gibco, Invitrogen).

2.2 Plant Material

The plant material *D. paniculata* (bark and heartwood) was collected from the forest region Tillari, district Kolhapur, Maharashtra, India, and authenticated at Shri B. M. K. Ayurvedic Mahavidyalaya, Belagavi, Karnataka (CRF/auth45/2021) (Figure 1).

Kingdom: Plantae
Division: Tracheophyta
Order: Ericales
Family: Ebenaceae
Genus: *Diospyros*
Species: *paniculata*

2.3 Extraction

The collected material was cleaned and shade-dried. The dried material was subjected to grinding in a miller to get a coarse powder. The uniform powder was weighed and 1 kg of powder material was carried for extraction. About 1 kg of powdered material was subjected to hot solvent extraction with methanol at 30°C in 3 batches. The solvent recovery was done by distillation. The residue extract was concentrated under reduced pressure using a rotary evaporator at temperatures 30°C, and 90rpm. The methanol extract was stored in an air-tight container until proceeding to the fractionation.

2.4 Fractionation

The four solvents were used to fractionate the methanol extract. Water was used to disperse the methanol extract before it was progressively extracted with n-hexane, chloroform, ethyl acetate, and methanol[12].

2.5 Isolation of Chloroform Fraction

A vertical borosilicate glass column of 60cm in length and 5cm in diameter was used for the isolation. The column was rinsed with acetone and was fully dried before packing. A piece of glass wool was placed inside at the base of the column. The column was packed by the wet packing technique. The column was filled with hexane up to about 3/4th of the volume. A slurry of silica

Figure 1. Different parts of *Diospyros paniculata* plant.

(60-120 mesh) in hexane was prepared and poured into the column containing hexane with gentle tapping using a cork while the columnis ver, dditional points are here include in the results section itself.te-of-the-art infrastructure knob was allowed to drain open to get a uniform bed silica gel approximately up to 2/3 length of the column. The 5g of chloroform fraction was absorbed into silica gel in a China dish and evaporated over a water bath. The chloroform extract-absorbed silica was made into a slurry using a small quantity of n-Hexane and was loaded on the silica bed. A cotton plug was placed on the sample bed. The column was eluted by gradient elution starting with n-hexane followed by chloroform, ethyl acetate, and methanol. In total, around 380 fractions were collected and each fraction was subjected to thin-layer chromatography to confirm the elution of phytoconstituents. Consecutive fractions with similar Thin Layer Chromatography (TLC) spots were pooled together. Chloroform, n-Hexane (50:50) and (70:30) gave the single spots on TLC and were designated as DP-1 and DP-2. These fractions were tested for various phytoconstituents. Both isolated fractions are positive for the Leiberman-Burchard's test indicating the presence of steroids and/or triterpenoids. The fractions were subjected to crystallisation. The isolated phytoconstituents are identified by the melting point, and spectral data of FTIR, ^{13}C, and ^{1}H NMR spectroscopy, and compared with the reference data[13].

2.6 Identification of Isolates

The melting point of a compound is determined by the open capillary method. The FTIR spectra obtained from the KBr technique of sample preparation were used by Shimadzu IR Affinity-1 using a DRS 8000 spectrometer. ^{1}H NMR and ^{13}C NMR spectra was observed with AMX-400, Bruker-400 liquid-state NMR spectrometer, and ECZ 400S, Jeol 400MHz FT-NMR. The basic standard solvent used was Tetramethyl Silane (TMS). $CDCl_3$ was used as a solvent and chemical shifts were recorded as δ (ppm).

2.7 Cell Growth Inhibition Assay for Extracts

The cytotoxic activity of extracts was carried out by MTT assay against MCF-7 (human breast cell line). The live cells were plated on a 96-well flat-bottom microplate and kept at 37°C, 95% humidity, and 5% CO_2 for the next day. These cells were exposed to test samples (solvent fractions) of doxorubicin (standard drugs) at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml, as well as solvent fractions of HF, CF, EF and MF and were incubated for another 48 hours. 20 µL of the MTT staining solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well after the wells had undergone two rounds of PBS washing. The well plate was kept for incubation at 37 °C. The formazan crystals were not damaged during the removal of the supernatant. To measure absorbance at 570 nm, the formed crystals were dissolved in 100 µL of DMSO[14]. Triplicate experiments were carried out and graph pad prism version 5 was used to calculate the concentrations of stem bark extracts (IC_{50}) required to suppress cell growth by 50%.

3. Results

The phytochemical investigation of all solvent fractions indicated the presence of major secondary metabolites which have therapeutic significance. The characteristic features of all the solvent fractions are summarised (Tables 1 and 2). Two identified phytocompounds in the bark extracts of *Diospyros paniculata* that have not yet been isolated from the bark of this species were identified using chromatographic and spectral analyses as well as published data. These compounds include Betulin (DP-1)[15] and Lupeol (DP-2)[16] and were isolated from chloroform extract of the bark (Figure 2). The cytotoxic activity of all four solvent fractions was assessed to elucidate the activity of *D. paniculata* bark.

3.1 Spectral Data of Isolates

The isolated compounds were characterised and identified by melting point, and spectral data of FTIR, UV spectroscopy, ^{13}C and ^{1}H NMR and were compared with the reference data.

Betulin (DP-1): It is obtained as a white crystalline powder; (0.22g), M.P:251-253°C FTIR (KBr, cm^{-1}): 3510 (O-H), 2942 (C-H) (Figure 3). The ^{1}H NMR exhibited signals (δ $CDCl_3$, 400 MHz) at 0.91, 1.65 (1H, d, H-1), 1.55, 1.60(1H, d, H-2), 3.2 (1H, dd, 3-OH),) 0.69 (1H, o, H-5), 1.53 (1H, s, H-6), 1.39 (1H, s, H-7), 1.25 (1H, s, H-9), 1.20, 1.42(1H, d, H-11), 1.04, 1.62 (IH, o, H-12), 1.64 (1H, s, H-13), 1.05, 1.70 (1H, d, H-15), 1.2, 1.94 (1H, d, H-16), 1.35 (1H, s, H-18), 2.38 (1H, m, H-19), 1.42, 1.97 (1H, d, H-21), 1.06,

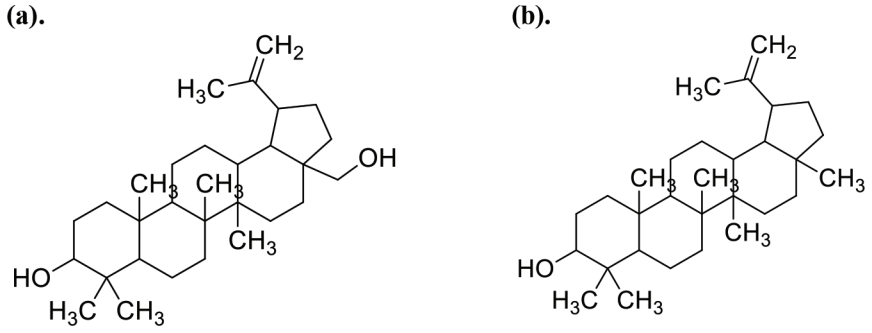

Figure 2. Structure of the isolated compounds: **(a).** Betulin, **(b).** Lupeol.

Table 1. Characteristics of solvent fractions

	HF	CF	EF	MF
Nature	Semisolid	Semisolid	Semisolid	Semisolid
Colour	Yellow	Dark brown	Dark brown	Dark brown
Odour	Distinctive	Distinctive	Distinctive	Distinctive
Taste	Unpleasant	Unpleasant	Unpleasant	Unpleasant
Solubility	Soluble in n-hexane, methanol, ethyl acetate, and chloroform.	Soluble in chloroform, methanol, and benzene.	Soluble in ethyl acetate chloroform, methanol and benzene.	Soluble in methanol, and ethanol.
Weight	1.1g	5g	3.1g	5.0g

HF: n-Hexane Fraction, CF: Chloroform Fraction, EF: Ethyl acetate Fraction, MF: Methanol Fraction.

Table 2. Phytochemical investigation for fractions of methanol extract

Sl.No.	Chemical test	HF	CF	EF	MF
1	Alkaloids	+	+	+	+
2	Steroids and Triterpenoids	+	+	+	+
3	Test for Flavonoids	–	–	+	+
4	Tannins	–	–	+	+
5	Saponins	–	–	–	+
6	Carbohydrates	–	–	–	+

1.86 (1H, d, H-22), 0.94 (3H, s, 23-Me), 0.76 (3H, s, 24-Me), 0.82 (3H, s, 25-Me), 1.02 (3H, s, 26-Me), 0.96 (3H, s, 27-Me), 0.78 (2H, s, 28-CH_2), 3.80 (1H, s, 28-OH), 4.57, 4.69 (2H, s, 29-CH_2), 1.68 (3H, s, 30-Me) (Figure 4). The ^{13}C NMR exhibited signals (δ $CDCl_3$, 100 MHz,) at 38.89 (C-1), 27.58 (C-2), 79.19 C-3 (OH), 39.06 (C-4), 55.47 (C-5), 18.5 (C-6), 34.41 (C-7), 41.06 (C-8), 50.6 (C-9), 37.49 (C-10), 21.02 (C-11), 25.38 (C-12), 37.5 (C-13), 42.90 (C-14), 27.3 (C-15), 29.30 (C-16), 47.98 (C-17), 47.98 (C-18), 48.94 (C-19), 150.7 (C-20), 29.35 (C-21), 34.17 (C-22), 28.18 (C-23 Me), 15.57 (C-24 Me), 16.17 (C-25 Me), 16.31 (C-26 Me), 14.96 (C-27 Me), 60.75 (C-28, CH_2), 109.9 (C-29, CH_2), 19.3 (C-30 Me). The ^{13}C NMR spectra of Betulin. Molecular formula: $C_{30}H_{50}O_2$, molecular weight: 442.72g/mol (Figure 5).

Lupeol (DP-2): It is obtained as a white crystalline powder; (0.14g), M.P:198-200°C FTIR (KBr, cm^{-1}): 3496 (O-H), 2944(C-H), 1624.13 (C=C) represented in (Figure 6). The ^1H NMR exhibited signals (δ $CDCl_3$, 400 MHz) at 0.91, 1.65 (1H, d, H-1), 1.55, 1.60(1H, d, H-2), 3.2 (1H, dd, 3-OH),) 0.69 (1H, o, H-5), 1.53 (1H, s, H-6), 1.39 (1H, s, H-7), 1.26 (1H, s, H-9), 1.20, 1.42(1H, d, H-11), 1.04, 1.62 (IH, o, H-12), 1.64 (1H, s, H-13), 1.05, 1.70 (1H, d, H-15), 1.2, 1.94 (1H, d, H-16), 1.57 (1H, s, H-18), 2.39 (1H, m, H-19), 1.42, 1.98 (1H, d, H-21), 1.06, 1.87 (1H, d, H-22), 0.96 (3H, s, 23-Me), 0.76 (3H, s, 24-Me), 0.82 (3H, s, 25-Me), 1.02 (3H, s, 26-Me), 0.98 (3H, s, 27-Me), 3.34 (2H, s, 28-Me), 4.59, 4.69 (2H, s, 29-CH_2), 1.68 (3H, s, 30-Me) (Figure 7). The ^{13}C NMR exhibited signals (δ $CDCl_3$, 100 MHz,) at 38.90 (C-1), 27.64 (C-2), 79.22 C-3 (OH), 39.06 (C-4), 55.48 (C-5), 18.52 (C-6), 34.47 (C-7), 41.02 (C-8), 50.62 (C-9), 37.2 (C-10), 21.12 (C-11), 25.32 (C-12), 38.24 (C-13), 43.03 (C-14), 27.61(C-15), 35.78 (C-16),

43.21 (C-17), 47.19 (C-18), 48.49 (C-19), 151.22 (C-20), 29.91 (C-21), 40.20 (C-22), 28.19 (C-23 Me), 15.58 (C-24 Me), 16.17 (C-25 Me), 16.33 (C-26 Me), 14.7 (C-27 Me), 18.21 (C-28, Me), 109.5 (C-29, CH_2), 19.5 (C-30 Me). The ^{13}C NMR spectrum of Lupeol. Molecular formula: $C_{30}H_{50}O$, molecular weight: 426.72g/mol (Figure 8).

3.2 Anticancer Activities

Cytotoxic activity of chloroform fraction, ethyl acetate fraction, hexane fraction, and methanol fraction were performed against the MCF-7 cell line. The IC_{50} values were determined to be 628±15.55, 23.47±0.22, 235.90±10.17 and 37.53±0.71 respectively and standard doxorubicin was found to be 3.16±0.10. Among these ethyl acetate fraction showed less IC_{50} value of 23.47 µg/ml on MCF-7 cell line. Figure 9 shows the significant reduction of MCF-7 cells on treatment with all extracts of *D. paniculata*. The mean IC_{50} values of all the extracts against MCF-7 cancer cells are summarised in Table 3.

4. Discussion

Cancer will be the main cause of mortality in the twenty-first century. Naturally obtained products serve as a major source of potent drugs for the treatment of cancer. *D. paniculata* has a rich history of use in traditional medicine for treating a wide range of ailments. Its various plant parts have been valued for their purported therapeutic effects, and ongoing scientific investigations seek to validate and understand the bioactive compounds responsible for these properties. Incorporating the knowledge of traditional medicine into modern research may offer new insights into the potential of this plant as a source of novel and effective natural remedies. The phytochemical investigation of *D. paniculata* bark extract showed the presence of most of the secondary metabolites mainly triterpenoids which show numerous biological activity. The isolation of phytoconstituents from chloroform extract by column chromatography results in the elution of Betulin in n: hexane: chloroform 50:50 and Lupeol in n: hexane: chloroform 30:70.

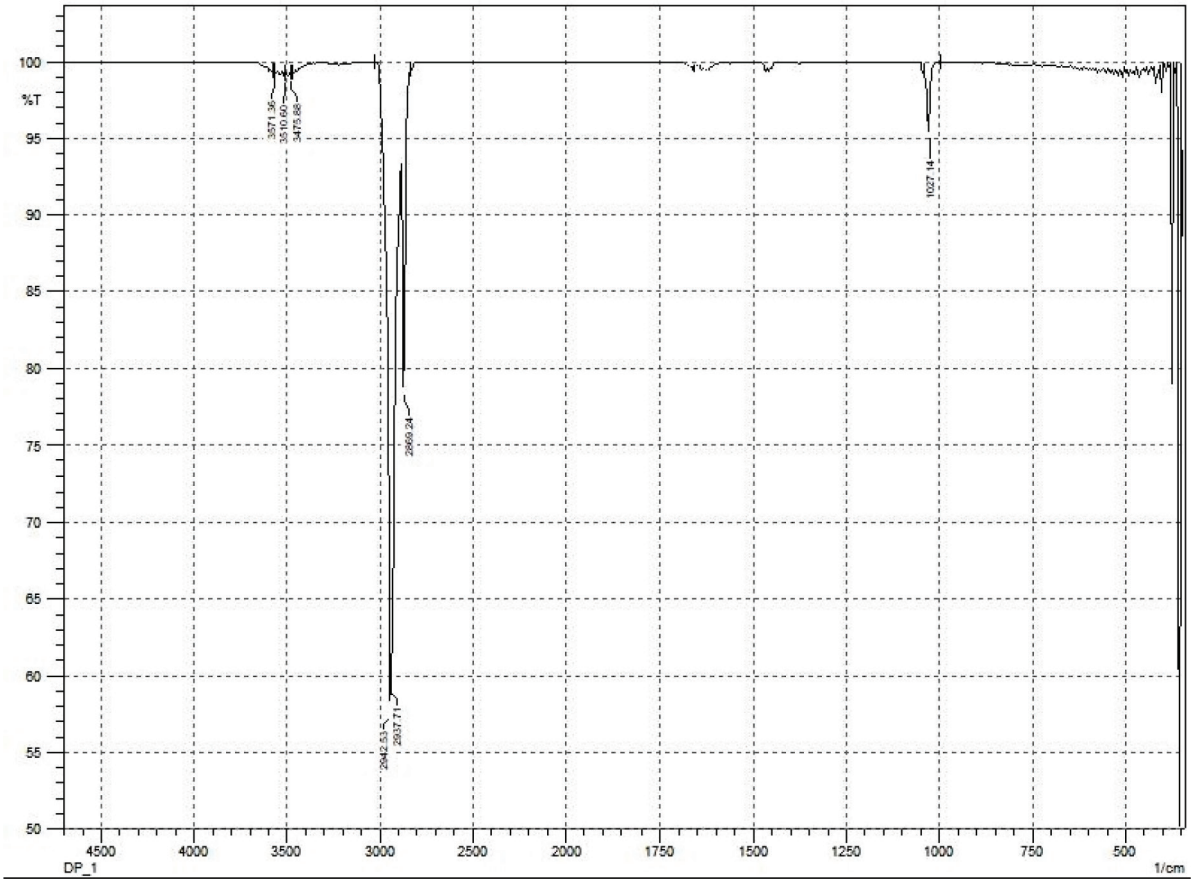

Figure 3. FTIR spectra of Betulin DP-1.

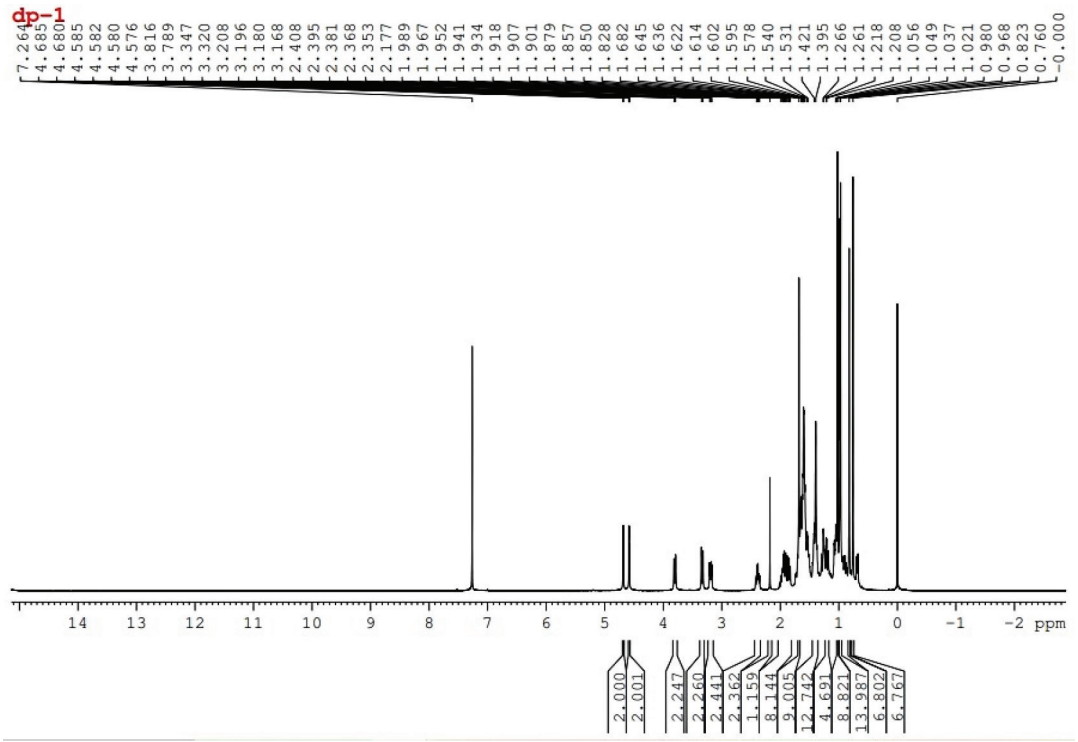

Figure 4. Proton NMR of Betulin DP-1.

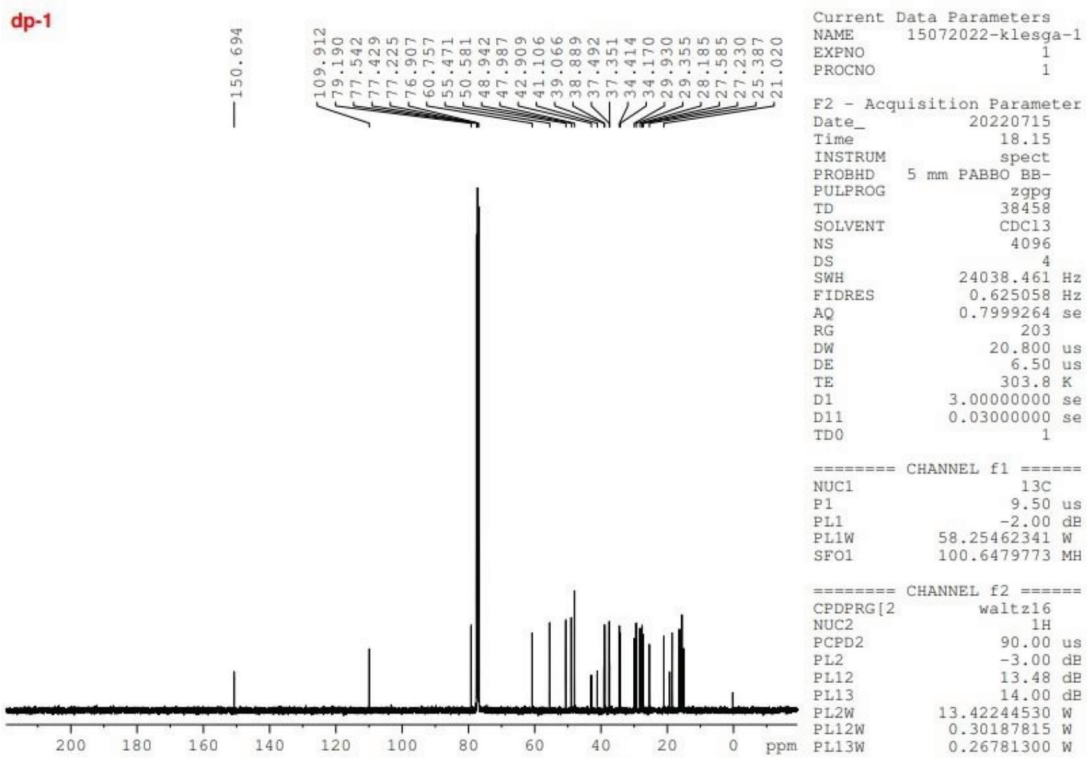

Figure 5. carbon 13 NMR of Betulin DP-1.

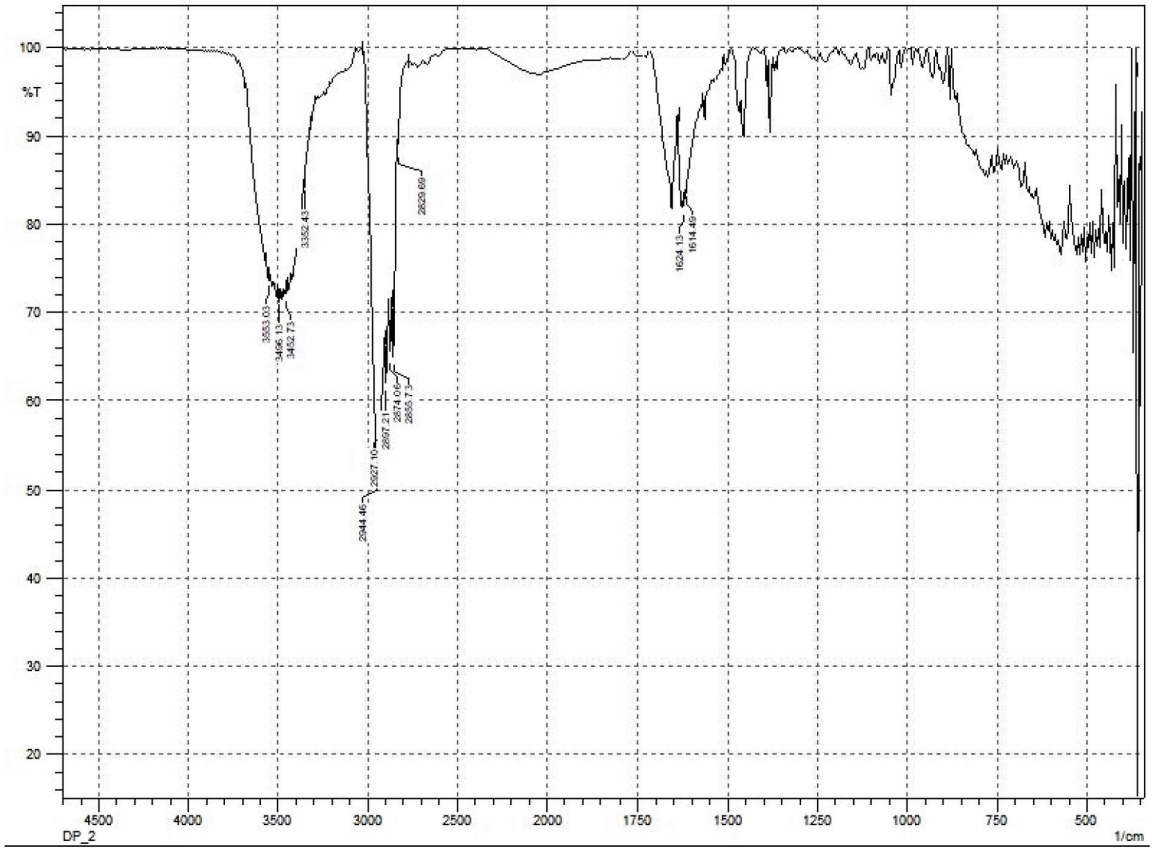

Figure 6. FTIR spectra of Lupeol DP-2.

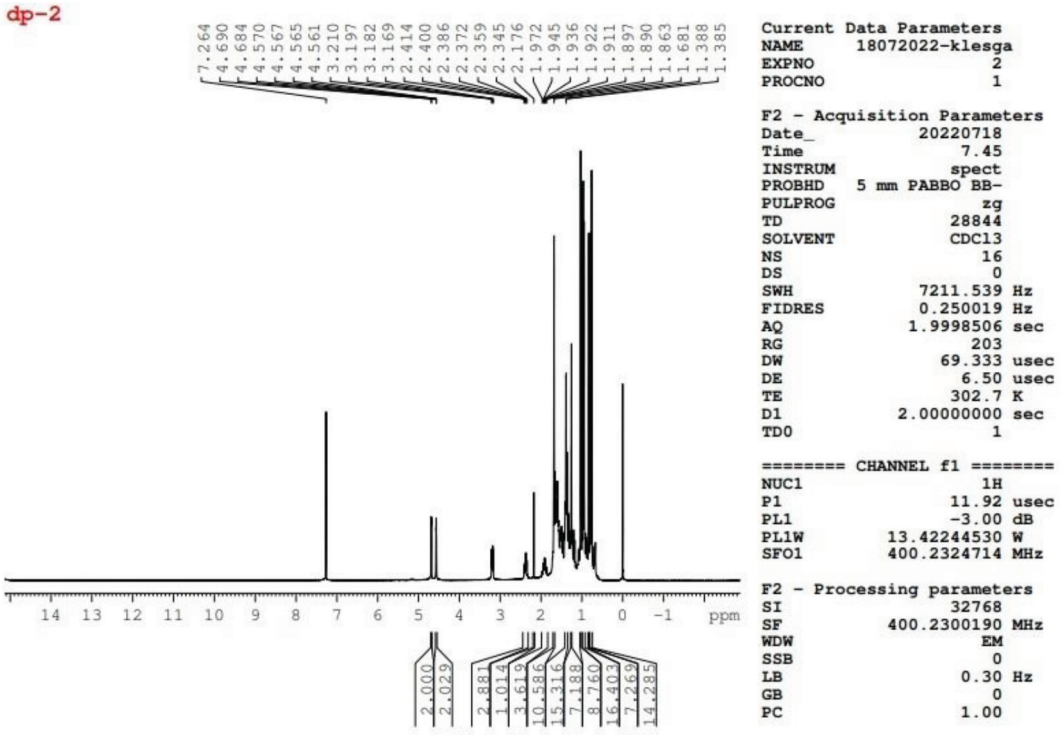

Figure 7. Proton NMR of Lupeol DP-2.

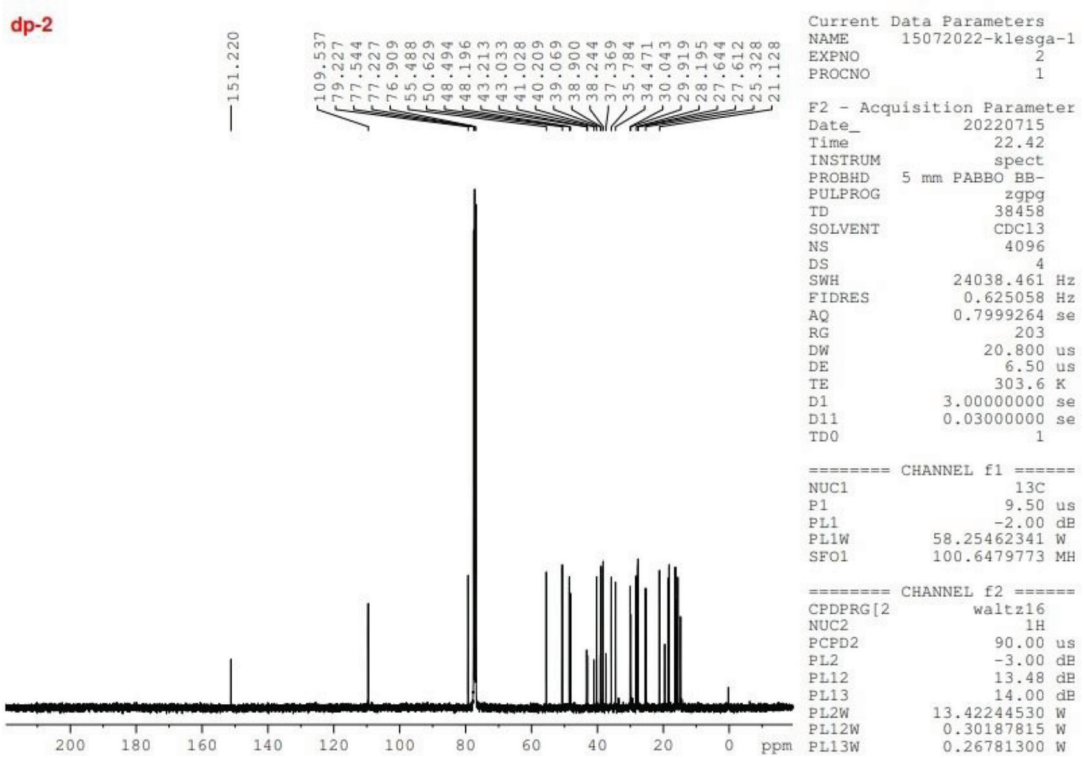

Figure 8. Carbon 13 NMR of Lupeol DP-2.

Table 3. IC_{50} values of all extracts (µg/ml)

Sample	Mean	SD
Chloroform Fraction	628.23	15.55
Ethyl acetate Fraction	23.47	0.22
N-hexane Fraction	235.90	10.17
Methanol Fraction	37.53	0.71
Doxorubicin	3.16	0.10

In the characterisation of isolated compounds, the FTIR spectra of Betulin (DP-1) show the characteristic peaks of OH (3510cm^{-1}) and C-H (2942 cm^{-1}), ^{1}H NMR data gives the two OH proton peaks at 3.2, 3.8 δ ppm and six tertiary methyl groups at), 0.94, 0.76, 0.82, 1.02, 0.96, 1.68δ ppm, ^{13}C NMR data shows the peak of olefin carbons at 150.7, 109.9 δ ppm, carbons with OH groups at 79.19, 60.75δ ppm and six tertiary methyl groups at 28.18, 15.57, 16.17, 16.31, 14.96, 19.3 δ ppm.

The lupeol (DP-2) characterised by FTIR gives a characteristic peak of OH (3496 cm^{-1}), C-H (2944 cm^{-1}), C=C (1624.13), ^{1}H NMR exhibited signals of one OH proton at 3.2δ ppm and seven tertiary methyl protons at 0.96, 0.76, 0.82, 1.02, 0.98, 1.68, 0.78 δ ppm. ^{13}C NMR exhibited signals of O-H peak at 79.22 δ ppm, and olefin peak at 151.22, 109.5 δ ppm, and seven tertiary methyl carbons appeared at 28.19, 15.58, 16.17, 16.33, 14.7, 18.21, 19.5 δ ppm.

The triterpenoids are a class of compounds containing 30 carbons and six isoprene units[16]. *In-vitro* and *in-vivo* literature studies show that terpenes are major secondary metabolites present in plants and show significant antiproliferative activity. The present study successfully isolated two pentacyclic lupine-type triterpenoids Betulin and Lupeol have potential pharmacological activities. Betulin has promising properties like anti-HIV, anti-inflammatory, and most importantly anticancer[15,16]. Lupeol shows curative effects on inflammation, arthritis, mutagenesis, and maleria[17].

The *in vitro* cytotoxic activity of *D. paniculata* plant extract was evaluated using the MTT assay. *D. paniculata*, commonly known as the Indian Persimmon in some regions, is a medicinal plant with various therapeutic properties. Extracts from this plant have shown potential in traditional medicine for treating various ailments. However, the *in vitro* activity of all four extracts on the adenocarcinoma human breast cell line shows

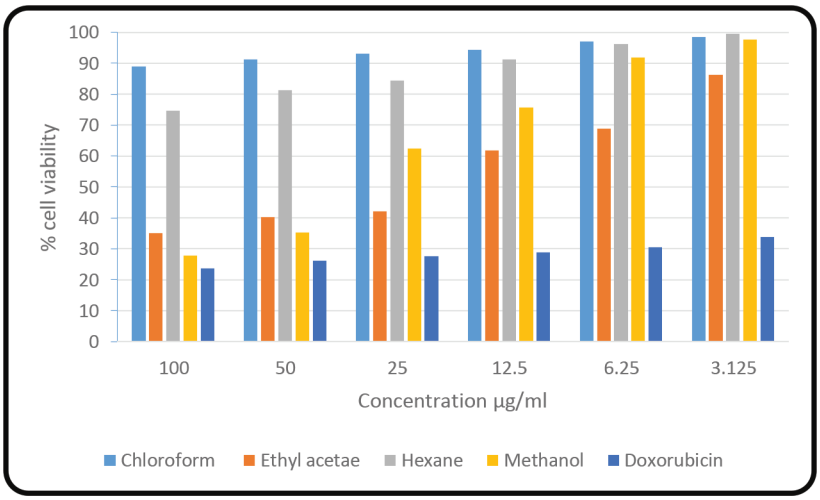

Figure 9. Cytotoxic activity evaluation against breast cancer cell line MCF-7.

dose-dependent inhibition cells. In that ethyl acetate fraction showed potent activity on the MCF-7 cell line. Further studies, including *in vivo* experiments and identification of the active compounds responsible for the observed effects, are warranted to fully understand its therapeutic potential and pave the way for the development of novel and effective cancer treatments based on this plant extract.

The results showed that the plant extracts exhibited significant dose-dependent cytotoxic effects on the cancer cells. Higher concentrations of the extract led to greater inhibition of cell proliferation compared to lower concentrations and the control group. This indicates that the extract has the potential to induce cell death in cancer cells, making it a promising candidate for further investigation as a potential anticancer agent.

Cytotoxic activity HF, CF, EF, and MF were performed against the MCF-7 cell line. Figure 9 shows the significant reduction of breast cancer cells on treatment with all extracts of *D. paniculata*. Ethyl acetate fraction showed less IC_{50} value of 23.47 µg/ml on the MCF-7 cell line Also methanol fraction showed a moderate IC_{50} value of 37.53 µg/ml. The mean IC_{50} values of all the extracts against MCF-7 cancer cells are summarized in Table 3. However, *in vitro* activity of all four extracts on the adenocarcinoma human breast cell line shows dose-dependent inhibition cells. In that ethyl acetate fraction showed potent activity on the MCF-7 cell line. In conclusion, the *in vitro* cytotoxic activity of *D. paniculata* plant extract was assessed using the MTT assay, demonstrating its potential as a cytotoxic agent against cancer cells. Further studies, including *in-vivo* experiments and identification of the active compounds responsible for the observed effects, are warranted to fully understand its therapeutic potential and pave the way for the development of novel and effective cancer treatments based on this plant extract.

5. Acknowledgements

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