Indian Journal of Experimental Biology
Vol. 64, June 2026, pp.483-499
DOI: 10.56042/ijeb.v64i06.23973

Exploration of phytochemicals, GC-MS analysis, and assessment of *in vitro* cytotoxic potential of *Justicia adhatoda* (L.) against Dalton's Lymphoma Ascites (DLA) murine tumor model

Mary Devi[1], Akalesh Kumar Verma[2] & Namram Sushindrajit Singh[1]*

[1]Department of Zoology, Murkong Selek College, Jonai, Dhemaji 787060, India

[2]Department of Zoology, Cotton University, Panbazar, Guwahati 781001, India

Received 19 September 2025; revised 17 January 2026

Researchers worldwide are presently delving into natural substances as prospective solutions for cancer treatment to develop drugs that minimize side effects. In this context, the present study investigated the *in vitro* cytotoxic potential of *Justicia adhatoda* (L.), a medicinal plant traditionally employed to address a range of health concerns. The phytochemical analysis using standard tests showed the presence of important metabolites such as carbohydrates, alkaloids, proteins, polyphenols, tannins, saponins, terpenoids, flavonoids, phenols, and triterpenoids. Additionally, GC-MS analysis exhibited the presence of about 20 phytocompounds. Antioxidant potency determined through phosphomolybdate reduction and DPPH (2,2-diphenyl 1-picrylhydrazyl) free radical scavenging assays showed a dose-dependent antioxidant activity compared to the standard ascorbic acid. Moreover, the *in vitro* cytotoxicity assays, such as the trypan blue exclusion method, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium), and LDH (Lactate dehydrogenase) demonstrated an effective cytotoxic response of the extract proportional to dose, indicated with a low IC_{50} value. Furthermore, morphological observation of the extract-treated cells through dual fluorescence staining of ethidium bromide/acridine orange (EtBr/AO) showed apoptotic features. These findings highlight the antitumor efficacy of *J. adhatoda* (L.) extract specifically against the DL cell line. Further *in vivo* studies and analysis of its active constituents are recommended.

Keywords: Anticancer, antioxidant, apoptosis, natural product, secondary metabolites

Medicinal plants have played a vital role as a source of food and human health since ancient times. The therapeutic potential of medicinal plants is attributed to the presence of various phytochemical constituents, including phenols, flavonoids, terpenoids, alkaloids, saponins, steroids, cardiac glycosides, volatile oils, mucilage, and resins[1]. Moreover, the presence of such phytoconstituents is found to be responsible for exhibiting various pharmacological properties, including neuroprotective, antidiabetic, cytotoxic, antioxidant[2], antibacterial, anti-inflammatory, and anticancer effects[3]. This represents the ability of the plant to address various health concerns, such as cancer, diabetes, obesity, and Alzheimer's disease[2]. Hence, currently, researchers are focusing on the development of potent herbal drugs for the treatment of various ailments. Consequently, plant extracts are prepared in different solvents of varying polarity to isolate the active biomolecules that exhibit therapeutic effectiveness[4]. Various *in vitro* studies have been conducted to screen the cytotoxic properties of plant extracts against numerous cancer cell lines[5,6].

Justicia adhatoda (L.), (Acanthaceae) also known as Malabar nut tree and Adulsa in English, Vasaka in Hindi, and Amalaka in Sanskrit[7], with purple or white inflorescence[8], is a well-known medicinal plant in the Unani and Ayurvedic systems of medicine for treating respiratory disorders such as bronchitis, asthma, cold, and cough[9]. The other notable medicinal uses include joint pain, rheumatism, malaria, urinary tract infections, gonorrhea, tumors, mental disorders, diabetes, dysentery, venereal diseases, sprains, eczema[10]. Moreover, the leaves, roots, and flowers are used as part of the herbal formulations to cure cancer and tuberculosis[11,12]. The plant is also reported to exhibit varied pharmacological properties such as anti-inflammatory, analgesic[13], antioxidant activity, abortifacient, cardioprotective, antibacterial, antimutagenic, bronchodilator activity, anti-ulcer, insecticidal, hepatoprotective, and anti-cholinesterase potential[14]. Previous studies have conducted

*Correspondence:
Phone: 7005149283
E-mail: ssdrajt@gmail.com
nssingh@cottonuniversity.ac.in

in vitro anticancer studies on breast cancer cell line, MCF-7, and adenocarcinoma cells, A549[15,16]. However, no current study has reported the anticancer potency of *J. adhatoda* (L.) against Dalton's Lymphoma (DL) cells, a suitable liquid tumor model of murine origin for anticancer studies. Hence, the present study was designed to evaluate the phytoconstituents and preliminary antitumor efficacy of *J. adhatoda* (L.) leaves against DL cells through its antioxidant potency, cytotoxic effects, and apoptosis study. The outcomes could further lead to the investigation of its active principle (s) and *in vivo* antitumor activity.

Materials and Methods

Sampling and identification of plant material

The mature leaves of *J. adhatoda* (L.) were collected from the premises of the Department of Botany, Cotton University, Guwahati, Assam, India, during February 2022. The authentication and identification were conducted at the Botanical Survey of India, Meghalaya, Shillong, where a herbarium of the sample containing the flora was deposited, and an accession no. 101356 was obtained.

Processing of plant material and extract preparation

The collected fresh leaves were properly washed and shade-dried for 10 to 15 days and then ground into a rough powder. The weight of the dried sample was recorded. The powdered sample was dissolved in 70% methanol and macerated for over 72 hours, with filtration every 24 hours. The collected filtrate was evaporated to dryness using a rotary evaporator at 50ºC to yield a semisolid crude extract, which was stored in a sealed container at 4ºC for further use[17]. In addition, the percentage yield of the extracted sample was also determined[18]. The yield percentage was estimated using the following formula:

$$\text{Percentage (\%) Yield} = \frac{Weight\ of\ solvent\ free\ extract}{Weight\ of\ dried\ sample} \times 100$$

Phytochemical screening

The phytochemical profiling of the extract was performed using standard procedures[19,20]. The presence of phytoconstituents such as carbohydrates, proteins, alkaloids, glycosides, phenols, polyphenols, tannins, flavonoids, triterpenoids, terpenoids, gums and mucilage, steroids, and saponins was analyzed.

Determination of phytoconstituents using GC-MS analysis

The presence of various compounds in the plant extract was determined through GC-MS analysis using the PerkinElmer Clarus 680 GC/600C MS system. The extract was diluted in methanol and loaded into the system through auto-injection mode with a split ratio of 10:1. Initially, the oven was heated to about 60°C for 1min with an increasing rate of 70 °C/min up to 200^0C and a holding time of 3min. Later, the temperature was raised to 300°C with a holding time of 5 minutes. The carrier gas used was an inert gas, helium. The GC-MS analysis represented the presence of various compounds in a chromatogram where the percentage of the peak area corresponded with the relative quantity of compounds present. The identification of the compounds was done by using the computer library, NIST (National Institute of Standards and Technology), along with their retention times[21,22].

Antioxidant studies

DPPH (2,2-diphenyl 1-picrylhydrazyl) assay

DPPH is a stable free radical that is used to assess the antioxidant activities of plant extracts. DPPH contains an odd electron in its structure, and when it encounters an antioxidant, it is scavenged by the release of a hydrogen atom or electron. The antioxidant potency of the extract is measured by the ability to scavenge the free radical, indicated with a color change from purple to yellow[23]. To perform the assay, the plant extract and standard, ascorbic acid, were prepared in various concentrations (10, 20, 40, 50, 100, 150, 200, 250) µg/mL, and the method of Devi *et al.*[24] was followed. The scavenging activity was determined using the following formula:

$$\text{\% DPPH radical scavenging activity} = \{(A_0 - A_1/A_0)\} \times 100$$

Where A_0 = absorbance of the control group and A_1 = absorbance of the sample group. The dose representing 50% scavenging activity was determined for the extract and standard.

Reducing power assay

The extract was further evaluated for its antioxidant capacity by its ability to reduce molybdenum (VI) to molybdenum(V). The experiment was conducted as per the methods of Prieto *et al.*[25] with certain modifications. The plant extract (50µl) prepared in varied concentrations (0.3,0.4, 0.5, 1, 2,4) mg/mL was combined with

500 µl of a reagent solution containing 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid. A blank group containing methanol was also run. Ascorbic acid served as the reference standard. The mixture was incubated at 90°C for 90 minutes, then cooled to room temperature to measure absorbance at 695 nm. The increase in absorbance values specifies the reducing power.

Animal care for the propagation of DL cells

Swiss albino mice were used as an animal model to maintain the DL cells in the laboratory and for an *in vivo* study. The mice were kept in polypropylene cages with paddy husk as bedding at temperature conditions of 23±2ºC under a 12h light and dark cycle. All the mice were fed with standard food pellets[26]. Furthermore, the institutional ethics committee granted prior approval for conducting the experiments and caring for animals, with the ethics approval number being 7/IAEC/CU/05/01/2021.

Maintenance of DL cells in Swiss albino mice

The DL cells were propagated in the laboratory through serial intraperitoneal (i.p.) transplantation in healthy Swiss albino mice. For this purpose, about 1 $\times 10^6$ DL cells (0.25ml) in PBS (Phosphate-buffered saline, pH 7.4) were transplanted *via* the i.p. route. The tumor growth was visible after 4-5 days, and the mice survived for about 19-21 days[27].

Culture of DL cells

DL cells were harvested in RPMI-1640 growth medium supplemented with 10% FBS (Fetal bovine serum), 1% antibiotic solution (penicillin 1000 IU and streptomycin 10 mg/mL), Minimum Essential Medium (MEM), and vitamin solution. The culture was incubated at 37ºC and 5% CO_2. After sufficient growth of ~ 80%, cells were then sub-cultured to perform the *in vitro* studies[28].

Isolation, preparation, and viability study of splenocytes

The effect of the extract on normal lymphocytes was also studied to evaluate the specificity of cytotoxic effect towards cancerous and non-cancerous cells. For this purpose, the spleen was excised from normal mice, cut into small pieces, and squeezed between microscopical slides. The separated cells were drained with PBS to prepare a single-cell suspension. Further, to remove the traces of erythrocytes, 0.85% ammonium chloride (NH_4Cl) was mixed with cells and kept for 2-3minutes. Centrifuged to obtain a cell pellet and resuspended in PBS to prepare a single-cell suspension. To observe the effect of the extract, the lymphocytes were harvested in RPMI 1640 growth medium (10% FBS and vitamin solution) for 24h and treated with the extract at varying doses similar to that of the DL cells treatment, and the viability was assessed through various *in vitro* assays[29,30].

***In vitro* cytotoxicity studies**

Trypan blue dye exclusion method

The cell viability of the DL cells and splenocytes (normal cells) on treatment with *J. adhatoda* (L.) extract at concentrations of 25, 50, 100, 500, and 1000 µg/mL was assessed using the trypan blue dye method, following the procedure of previous studies[24]. The cells were incubated with the extract for 24h, containing growth medium RPMI 1640, added with 10% FBS, vitamins, and antibiotic solution. The cell viability on treatment with the standard drug, cisplatin, was considered as a reference. The viable and non-viable cells were counted after incubation with 0.4% trypan blue dye using a cell counter. under a microscope. The viable cells appeared with clear cytoplasm, while the dead or damaged cells showed blue cytoplasm due to the penetration of trypan blue dye through the disintegrated membrane. Further, the photomicrographs of the DL cells in each treated group were captured using a digital camera attached to the microscope at 40x magnification to observe the changes in morphological features compared to the control group. The % of cell viability was calculated using the following formula:

$$\%\ Cell\ viability = 1 - \frac{No.of\ blue\ cells}{Total\ no.of\ cells} \times 100$$

The concentration exhibiting a 50% decrease in cell viability was calculated for the extract treatment and that of the standard drug, cisplatin.

MTT assay

The *in vitro* cytotoxicity of *J. adhatoda* (L.) extract was further assessed through a spectrophotometric method using a yellow dye, MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium). DL and normal cells were cultured in a 96-well plate containing RPMI 1640 medium supplemented with 10% FBS and an antibiotic solution. The cells were then treated with various concentrations (25, 50, 100, 500, and 1000 µg/mL) of the extract and the reference drug, cisplatin. The treated cells were incubated for 24h at 37ºC and 5% CO_2. The cells were then further

added with MTT dye (5mg/mL) for 2h to form formazan crystals, which were then solubilized with Dimethyl sulfoxide (DMSO). The absorbance was recorded at 550nm in a microplate reader[31]. All the readings were conducted in triplicate, and the percentage of cell viability was determined:

$$\% \text{ Cell viability}=\frac{Sample\ O.D.-Control\ O.D.}{Blank\ O.D.-Control\ O.D.}\times 100$$

To estimate the inhibitory effect of the extract in comparison with the reference drug, cisplatin, the IC_{50} dose was determined.

Lactate Dehydrogenase (LDH) assay

To substantiate the cytotoxic effect of *J. adhatoda* (L.) extract further, an LDH assay was performed. The amount of LDH enzyme in cell supernatant shows correspondence with cell cytotoxicity[32]. The assay was performed using the Lactate Dehydrogenase Cell assay kit, HiMedia (CCK036), as per the instructions of the manufacturer. Briefly, the cultured DL and normal cells were treated with various concentrations (25, 50, 100, 500, 1000) µg/mL of the extract and incubated at 37ºC at 5% CO_2 for 24h. A standard group was run, treated with lysis solution, which showed maximum LDH release. The control untreated group received PBS as treatment, a background control group contained ascites fluid of DL cells, and the volume correction group contained a mixture of PBS and lysis solution. Post incubation, the supernatant (50µL) of each group was incubated with 50µL of LDH reagent for 30 minutes. Then 50µL of stop solution was added, and absorbance was recorded at 450nm. The cytotoxicity at each treatment dose was determined using the following formula:

% cytotoxicity=100×A-C/B-C where
A = Absorbance of test group - Absorbance of untreated control group,
B = Absorbance of maximum LDH release group-Absorbance of volume correction control group,
C = Absorbance of untreated control-average absorbance of background control.

The IC_{50} dose exhibiting 50% cytotoxicity was determined on treatment with both the extract and the reference drug cisplatin.

Apoptosis study through fluorescent staining

Apoptosis is a regulated process of cell death where the cell exhibits certain distinct morphological features, such as blebbing of the plasma membrane, cell shrinkage, chromatin condensation, pyknosis, and development of apoptotic bodies[33]. To assess the apoptosis-inducing potential of the extract, the fluorescent dyes ethidium bromide (EtBr) and acridine orange (AO) were used. The treated DL cells were incubated and stained with 10µL of AO and EtBr (100µg/mL) for 3 minutes. Moreover, a negative control group of cells receiving only PBS was also run. The images of the cells were then captured using a fluorescence microscope connected to a digital camera[34].

Determination of apoptotic cell death

To quantify the cell death due to the occurrence of apoptosis, about 1000 cells were analyzed in each of the treated groups[35]. The apoptotic cell death was estimated as per the following calculation:

$$\% \text{ of Apoptotic cells}=\frac{Total\ no.of\ apoptotic\ cells}{Total\ no.of\ cells}\times 100$$

Statistical analysis

All the data are represented as Mean± SEM (Standard Error Mean), and the *in vitro* experiments were conducted in triplicate. Shapiro-Wilk's W-test was employed to see the normality of the data. The data obtained in the experiments were then analyzed through two-way ANOVA (Analysis of variance) followed by a Bonferroni post hoc test. The correlation of dose with treatment was investigated using regression(r^2) analysis. Comparison of means of the IC_{50} values for standard and treated groups was analyzed using an unpaired Student's t-test. Statistical significance level was considered as $P<0.05$. The GraphPad Prism software version 8.0 was used for data analysis.

Results

Percentage yield

The preparation of *J. adhatoda* (L.) extract involved using 70% methanol as a solvent, resulting in a dark green semisolid substance. The process was optimized to yield a significant amount of extract, quantified as 16.32% based on the weight of the dry plant material relative to the weight of the extract produced. The drying phase of the extraction process was carefully controlled at a temperature of around 40°C to ensure that the extract's bioactive components were preserved while achieving the desired concentration and physical characteristics of the final product.

Qualitative phytochemical estimation

Phytochemical profiling of *J. adhatoda* (L.) leaves methanol extract exhibited the presence of important

phytoconstituents in various degrees, as depicted in Table 1. The proteins and alkaloids are found in the highest concentration, followed by carbohydrates, phenols, tannins, polyphenols, and flavonoids, which were found in moderate concentration. On the other hand, triterpenoids, terpenoids, and saponins showed low concentration. Glycosides, steroids, and gums, mucilage were absent in the extract.

Identification of compounds using GC-MS

GC-MS analysis revealed the presence of various phytocompounds in *J. adhatoda* (L.) leaves methanol extract. These compounds are depicted by the total ion chromatogram in Fig. 1, eluted at different retention times. Identification of about 20 compounds was performed using the NIST library represented in Table 2, showing the parameters such as peak percentage area, retention time, molecular weight, and molecular formula. Moreover, the compounds belong to different classes, which are represented in Table 3 along with their structures. The peak percentage area of the 20 compounds corresponding to their retention time is represented in Fig. 2. Analysis of the peak percentage area showed that the compound methyl 8,11,14-heptadecatrienoate was highest (4.08%), followed by 1-(3,3,3-trifluoro-2-hydroxypropyl) piperidine and phytol, showing a peak area of 2.16% and 2.01%, respectively. The compound phytol exhibited the highest retention time of 31.29, and subsequently methyl 8,11,14-heptadecatrienoate (31.16) was eluted. The least retention time required to elute was for the compound 1-(3,3,3-trifluoro-2-hydroxypropyl) piperidine (9.33) (Fig. 2).

Table 1 — Phytochemical profiling of the *J. adhatoda* (L.) leaves methanol extract

Phytochemical tests		Results
Carbohydrates	Fehling's test	++
	Benedict's test	++
	Molisch's test	++
	Millon's test	++
Proteins	Biuret test	+++
Alkaloids	Mayer's test	+++
	Wagner's test	+++
	Dragendroff test	+++
	Borntrager's test	+++
Glycosides	Keller-Killiani test	++
Phenols	Lead acetate test	++
Polyphenols	Lead acetate test	++
Tannins	Ferric chloride test	++
Flavonoids	Alkaline reagent test	++
Triterpenoids	Salkowaski test	+
	Libermann Bruchard's test	+
Terpenoids	Salkowaski test	+
Steroids	Libermann Bruchard's test	–
	Salkowaski test	–
Gums and mucilage	Ruthenium test	–
Saponins	Foam test	+

[+++ indicates high concentration, ++ moderate concentration, + low concentration, and – absence of phytoconstituents]

Antioxidant study

DPPH assay

The antioxidant study depicted a potent free radical scavenging ability of the extract as shown in Fig. 3. The results showed statistically significant scavenging effect compared to that of standard, ascorbic acid (Two-way ANOVA: Dose-F (7, 32) = 421.2, $P<0.0001$; Treatment-F (1, 32) = 15541, $P<0.0001$; Interaction-F (7, 32) = 99.64, $P<0.0001$; (Fig. 3a). The extract exhibited a maximum scavenging effect of 45% at the highest dose of treatment, whereas ascorbic acid reached a maximum scavenging effect of 96% at 150µg/mL with no further increment at higher doses. Furthermore, the IC_{50} value (50% inhibition of DPPH radical) of the extract was found to be 139.2±0.59µg/mL, and that of the standard was 27.33±1.58µg/mL, which were statistically significant (student's t-test: $P<0.0001$; (Fig. 3b). Additionally, the scavenging effects showed significant ($P<0.05$) proportionality with dose as depicted by regression analysis (Fig. 3c).

Reducing power assay

The reducing power assay is based on the ability of the antioxidant to reduce phosphomolybdate ion to

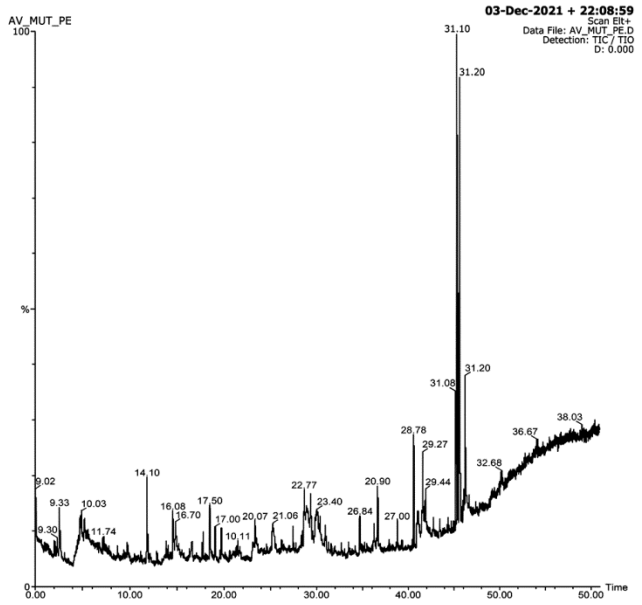
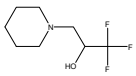
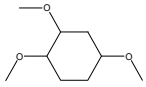
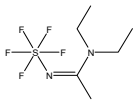
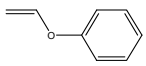
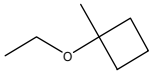
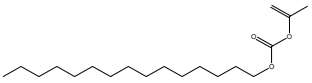
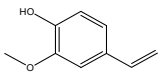


Fig. 1 — Total ion chromatogram of *J. adhatoda* leaves methanol extract showing peaks of different compounds eluted at different retention times.

Table 2 — List of compounds identified in the GC-MS analysis of *J. adhatoda* (L.) leaves methanol extract

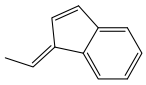
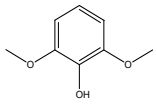
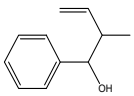
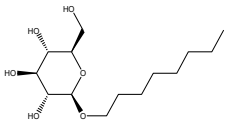
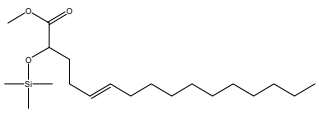
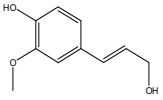
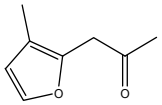
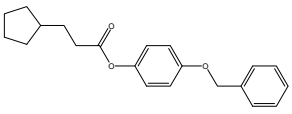
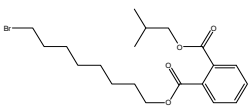
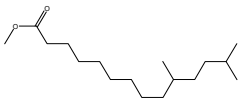
Sl. No.	Compounds name	Retention time	Molecular formula	Molecular weight	Peak % area
1	1-(3,3,3-trifluoro-2-hydroxypropyl) piperidine	9.33	$C_8H1_4F_3NO$	197.2	2.16
2	Cyclohexane, 1,2,4-trimethoxy-, stereoisomer I	10.53	$C_9H_{18}O_3$	174.24	1.57
3	1-[(diethylamino)ethylidenimino] sulfur pentafluoride	14.15	$C_6H_{13}F_5N_2S$	240.24	1.16
4	Benzene, (ethenyloxy)	15.56	C_8H_8O	120.15	0.98
5	1-methyl-1-ethoxy cyclobutane	15.70	$C_7H_{14}O$	114.20	1.14
6	Carbonic acid, pentadecyl prop-1-en-2-yl ester	17.20	$C_{19}H_{36}O_3$	312.49	0.35
7	2-Methoxy-4-vinyl phenol	17.60	$C_9H_{10}O_2$	150.18	1.05
8	1H-indene, 1-ethylidene	17.88	$C_{11}H_{10}$	142.20	0.42
9	Phenol, 2,6-dimethoxy-	18.23	$C_8H_{10}O_3$	154.16	0.55
10	2-methyl-1-phenylbut-3-en-1-ol	20.08	$C_{11}H_{14}O$	162.23	0.92
11	Octyl-beta-d-glucopyranoside	22.77	$C_{14}H_{28}O_6$	292.37	1.36
12	2-trimethylsiloxy-6-hexadecenoic acid, methyl ester	23.15	$C_{20}H_{40}O_3Si$	356.6	1.50
13	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	25.84	$C_{10}H_{12}O_3$	180.20	0.67
14	3-methyl-2-(2-oxopropyl) furan	26.61	$C_8H_{10}O_2$	150.22	0.35
15	3-cyclopentyl propionic acid, 4-methoxyphenyl ester	26.80	$C_{15}H_{20}O_3$	248.32	0.91
16	Phthalic acid, 8-bromoctyl isobutyl ester	27.88	$C_{20}H_{29}BrO_4$	413.35	0.39
17	Tetra decanoic acid, 10,13-dimethyl-, methyl ester	28.78	$C_{17}H_{34}O_2$	270.5	1.14
18	Dodecanoic acid	29.27	$C_{12}H_{24}O_2$	200.32	1.06
19	Methyl 8,11,14-heptadecatrienoate	31.16	$C_{18}H_{30}O_2$	278.4	4.08
20	Phytol	31.29	$C_{20}H_{40}O$	296.53	2.01

Table 3 — Representation of the structure and different classes of the identified compounds in *Justicia adhatoda* (L.) extract

SI No.	Compounds name	Type of compound	Structure
1	1-(3,3,3-trifluoro-2-hydroxypropyl) piperidine	alkaloid	
2	Cyclohexane, 1,2,4-trimethoxy-, stereoisomer I	hydrocarbon	
3	1-[(diethylamino)ethylidenimino] sulfur pentafluoride	lipid	
4	Benzene, (ethenyloxy)	alcohol	
5	1-methyl-1-ethoxy cyclobutane	cycloalkane	
6	Carbonic acid, pentadecyl prop-1-en-2-yl ester	ester	
7	2-Methoxy-4-vinyl phenol	phenol	

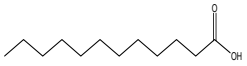
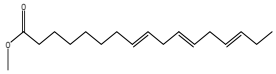
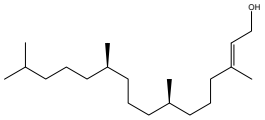
(*Contd.*)

Table 3 — Representation of the structure and different classes of the identified compounds in *Justicia adhatoda* (L.) extract (*Contd.*)

SI No.	Compounds name	Type of compound	Structure
8	1H-indene, 1-ethylidene	hydrocarbon	
9	Phenol, 2,6-dimethoxy-	alcohol	
10	2-methyl-1-phenylbut-3-en-1-ol	alcohol	
11	Octyl-beta-d-glucopyranoside	lipid	
12	2-trimethyl siloxy-6-hexadecenoic acid, methyl ester	fatty acid	
13	4-((1E)-3-hydroxy-1-propenyl)-2-methoxy phenol	alcohol	
14	3-methyl-2-(2-oxopropyl) furan	heteroaromatic	
15	3-cyclopentyl propionic acid, 4-methoxyphenyl ester	Carboxylic acid	
16	Phthalic acid, 8-bromoctyl isobutyl ester	phthalate esters	
17	Tetra decanoic acid, 10,13-dimethyl-, methyl ester	fatty acid	

(*Contd.*)

Table 3 — Representation of the structure and different classes of the identified compounds in *Justicia adhatoda* (L.) extract (*Contd.*)

SI No.	Compounds name	Type of compound	Structure
18	Dodecanoic acid	fatty acid	
19	Methyl 8,11,14-heptadecatrienoate	carotenoid	
20	Phytol	terpenoid	

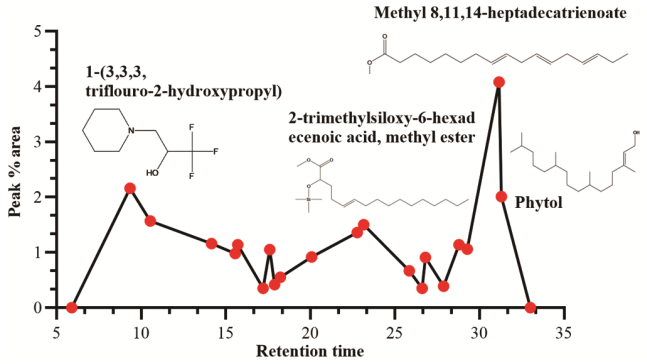


Fig. 2 — Graph representing the peak percentage area of 20 identified compounds corresponding to their retention time.

MoV complex. Fig. 4 depicts the reducing ability of the extract in comparison to the standard, ascorbic acid. The outcome of the reduction assay depicted potent reducing power of the *J. adhatoda* (L.) extract found statistically significant compared to that of standard, ascorbic acid (Two-way ANOVA: Dose: F (5, 24) = 474.5, P<0.0001; Treatment: F (1, 24) = 29389, P<0.0001; Interaction: F (5, 24) = 115.3, P<0.0001; (Fig. 4a). The extract exhibited a continual increase in reducing activity indicated with an increase in O.D values. Furthermore, linear regression analysis indicated a correspondence of dose with an increase in reducing activity (Fig. 4b).

In vitro cytotoxicity studies

Trypan blue method

The results of the trypan blue assay showed effective cytotoxic activity of *J. adhatoda* (L.) extract against DL cells, which was statistically significant compared to the cell viability of cisplatin-treated cells (Two-way ANOVA: Dose-F (4, 30) = 130.6, P<0.0001; Treatment-F (2, 30) = 3369, P<0.0001; Interaction-F (8, 30) = 30.93, P<0.0001; Fig. 5a). Analysis of cell viability in normal and DL cells revealed significant (P<0.0001) differences at higher doses of 500µg/ml and 1000µg/ml. However, no significant differences were observed at lower doses of 25, 50, and 100µg/mL. Additionally, the normal cells did not reach 50% viability even at the highest dose, indicating the extract's specificity towards DL cells. The extract showed 50% cell viability at a dose of 216.4±1.95µg/mL, which was statistically significant (t-test; P<0.0001) compared to cisplatin-treated cells, exhibiting an IC_{50} value of 91.81±2.07µg/mL (Fig. 5b). Moreover, the decrease in cell viability was proportional to the increasing dose for treatments with *J. adhatoda* (L.) (r^2=0.8873, P<0.0001), cisplatin (r^2=0.6782, P=0.0002), and normal cells (r^2=0.6202, P=0.0004) (Fig. 5C). Morphological analysis of DL cells treated with the extract showed changes compared to the control group of DL cells. The control cells maintained an intact membrane (Fig. 6a), whereas cells treated with cisplatin and extract exhibited features such as cell shrinkage, membrane disintegration, and blebbing (Fig. 6b- g).

MTT assay

The effect of *J. adhatoda* (L.) extract on DL cell viability was further assessed through the MTT assay. The outcome of the assay showed a potent decrease in cell viability on treatment with extract compared to that of cisplatin-treated cells which were statistically significant (Two-way ANOVA: Dose-F (4, 30) = 241.7, P<0.0001; Treatment- F (2, 30) = 6123, P<0.0001; Interaction-F (8, 30) = 23.60, P<0.0001; Fig. 7a). The normal cells on treatment with extract at the same concentration range exhibited least cell

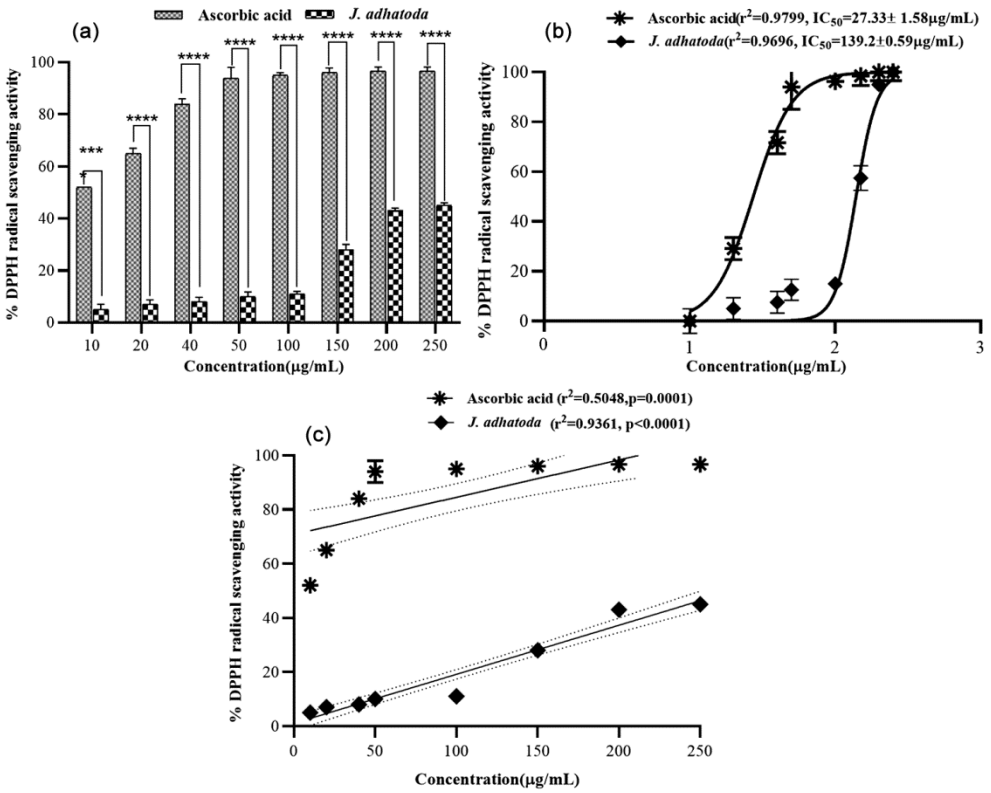


Fig. 3 — (a) Two-way ANOVA analysis of DPPH free radical scavenging activity of *J. adhatoda* extract compared to ascorbic acid, (b) Graph showing the log-transformed and normalized IC_{50} value of extract and standard, (C) Linear regression analysis exhibiting the increase in scavenging activity of both the treatments along with concentration. Results are expressed as Mean±SEM, n=3, Bonferroni post hoc test. The **** asterisk indicates P<0.0001.

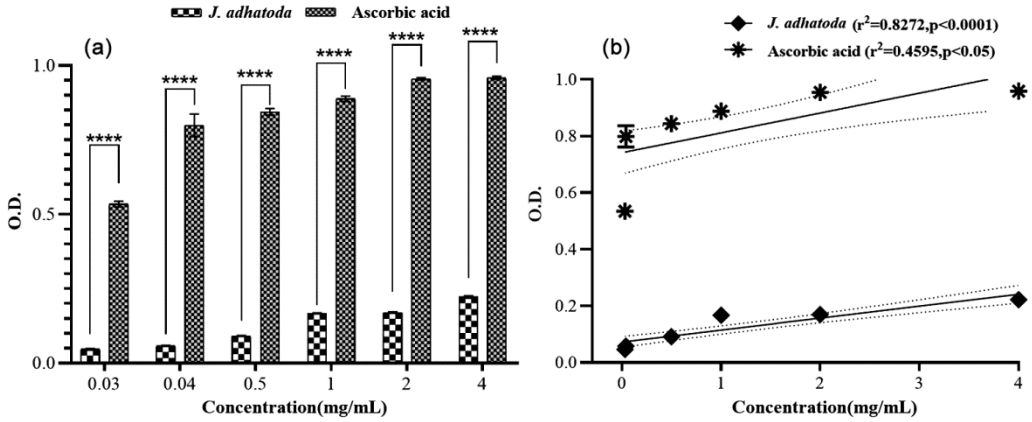


Fig. 4 — (a) Two-way ANOVA assessment of reducing power of *J. adhatoda* extract to ascorbic acid, (b) Linear regression representation of an increase in reducing power of extract corresponding to dose. Results are expressed as Mean±SEM, n=3, Bonferroni post hoc test. The asterisk indicate**** P<0.0001.

death even at the highest treatment dose compared to the viability of the DL cells. However, at the least treatment dose of 25µg/mL, no significant differences in the percentage of cell viability were observed between normal and DL cells. Moreover, the dose at which 50% inhibition of cell viability was observed on treatment with extract (167.6±0.50µg/mL) was found to be statistically significant (t-test, P<0.0001) compared to that of the IC_{50} dose of cisplatin treatment (79.14±1.30µg/mL) (Fig. 7b). Additionally, the increment in concentration of the treatments and decrease in cell viability was found to show

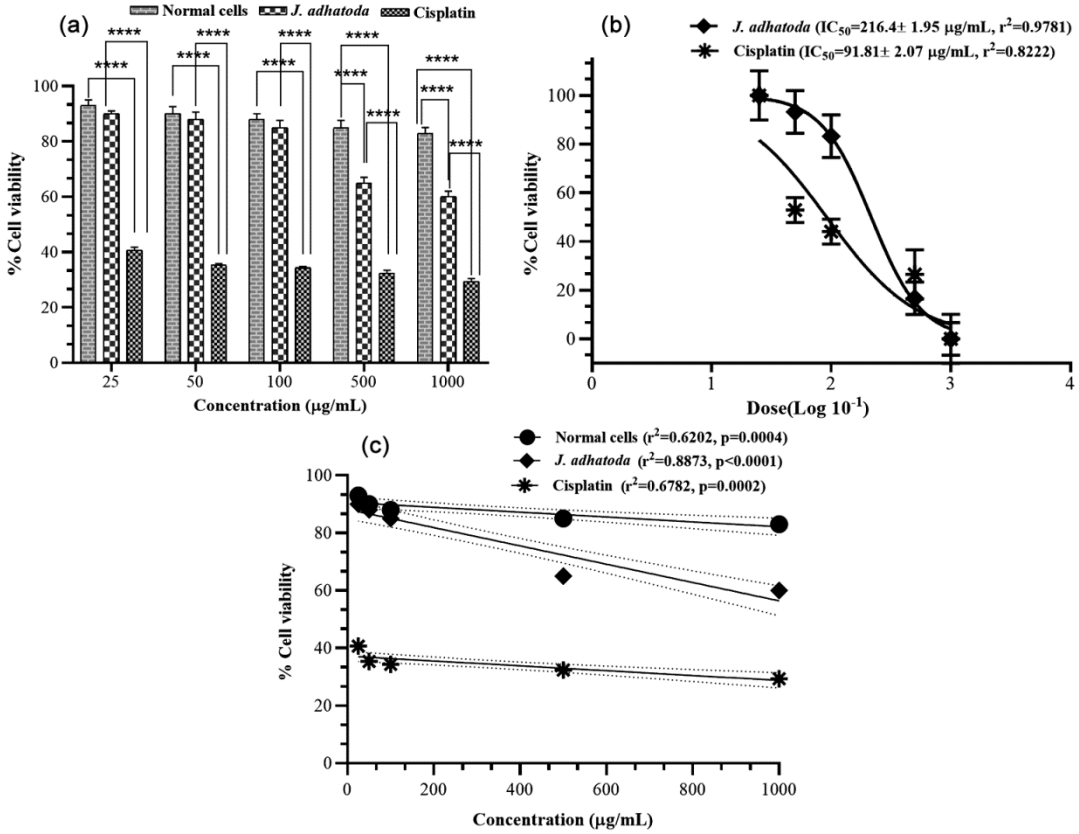


Fig. 5 — (a) Two-way ANOVA analysis representing changes in viability of DL and normal cells determined through the trypan blue dye method on treatment with extract compared to cisplatin-treated DL cells, (b) Log transformed and normalized IC_{50} value of *J. adhatoda* extract and cisplatin, (c) Linear regression analysis exhibiting proportionality of decrease in cell viability with dose. Results are expressed as Mean± SEM, n=3, Bonferroni post hoc test. Asterisk indicate ****P<0.0001.

proportionality (*J. adhatoda* (L.): r^2=0.8298, P<0.0001; cisplatin: r^2=0.6307, P=0.0004; normal cells: r^2=0.8284, P<0.0001; Fig. 7C).

Lactate Dehydrogenase assay

The findings of LDH assay showed an increase in percentage of cytotoxicity with dose on treatment with *J. adhatoda* (L.) extract as shown in Fig. 8. Further, the increase in cytotoxicity was found statistically significant as compared to that of cisplatin-treated cells (Two-way ANOVA: Dose-F (4, 30) = 65.26, P<0.0001; Treatment-F (2, 30) = 2339, P<0.0001; Interaction-F (8, 30) = 11.75, P<0.0001; Fig. 8A). Additionally, the normal cells showed the least cytotoxic response with about 10% cell death even at the maximum treatment dose. Furthermore, the half maximal inhibitory concentration at which the extract exhibited 50% cytotoxicity (IC_{50}) was found at a dose of 217.2± 2.0µg/mL and that of cisplatin was found at a dose of 89.12± 1.1µg/mL (student's t-test: P<0.001, Fig. 8b). Moreover, the increase in cytotoxicity was found to show positive correlation with dose (cisplatin: r^2=0.8450, P<0.0001; *J. adhatoda* (L.): r^2=0.7461, P<0.0001; normal cell: r^2=0.4396, P=0.0071) (Fig. 8C).

Apoptosis study through fluorescent staining

Evaluation of the occurrence of apoptosis on extract treated DL cells using fluorescent dyes AO/EtBr revealed characteristics changes in cell morphology of the treated groups compared to that of the control group of cells (Fig. 9). The control cells showed green fluorescence, with round and intact nuclei indicating the presence of viable cells (Fig. 9a). On the other hand, the positive control group of cells treated with the reference drug, cisplatin (1000µg/mL) appeared in red/orange fluorescence. Moreover, the cells were found to have specific structural features, which included blebbing of the membrane, reduction of cell size, and condensed chromatin (Fig. 9b). Similarly, the cells treated with *J. adhatoda* (L.) *extract* showed red/orange

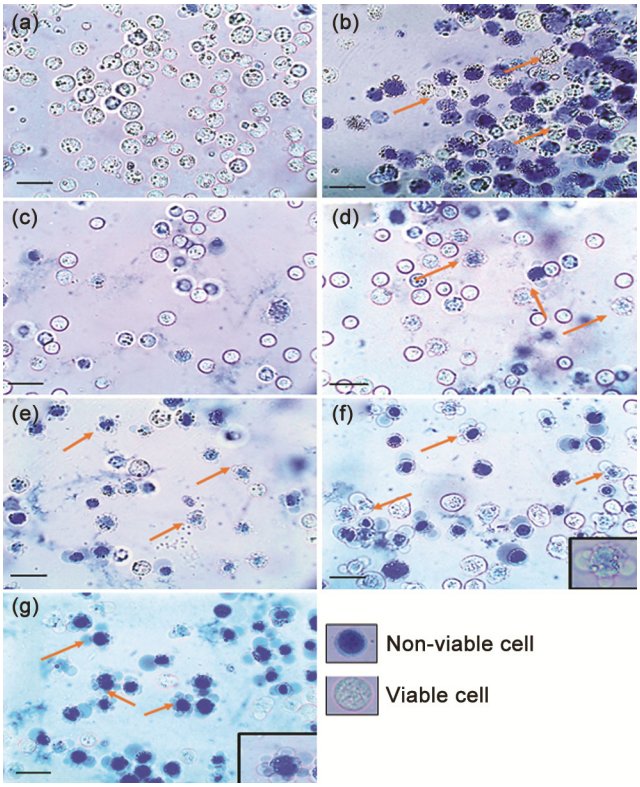


Fig. 6 — Microphotographs representing the morphological features of DL cells observed on treatment with *J. adhatoda* extract at different concentrations, (a) Negative control (b) Cisplatin treated (c) 25µg/mL (d) 50µg/mL (e) 100µg/mL (f) 500µg/mL, (g) 1000µg/mL. The orange arrows represent the apoptotic features such as membrane blebbing, membrane disintegration, and cell shrinkage. The images are represented at 40x magnification. Each bar in the image represents 10µm.

fluorescence, indicating apoptotic cell death, and the occurrence of typical apoptotic features was also observed (Fig. 9c-g).

Apoptotic index

The analysis of percentage of apoptotic cell death on treatment with *J. adhatoda* (L.) extract at different concentrations showed significant changes in cell death compared to cisplatin-treated DL cells (Two-way ANOVA: Dose-F (4, 20) = 136.3, P<0.0001; Treatment-F (1, 20) = 77.76, P<0.0001; Interaction- F (4, 20) = 0.8100, P=0.5334, Fig. 10A). Cell death of 42% was observed at the maximum dose of treatment compared to that of cisplatin treated cells which showed an apoptotic cell death of 35% at the same dose. The number of apoptotic cell deaths corresponded with an increment in dose (*J. adhatoda* (L.): r^2= 0.8879, P<0.0001; cisplatin: r^2=0.9103, P<0.0001, Fig. 10B).

Discussion

According to GLOBOCAN (Global Cancer Observatory), it is estimated that the cancer cases in India would increase to about 2.08 million, which accounts for a 57.5 percent increase in 2040 from 2020. Moreover, as per the reports of the National Cancer Registry Programme 2020, the number of cancer cases in the year 2022 was about 14,61427 cases, which was estimated to increase by 12.8 percent in 2025 compared to 2020[36]. Thus, cancer burden seems to rise continually, creating concern for its effective treatment. Although advancements have been made in the traditional treatment methods of cancer, which include surgery, chemotherapy, radiation therapy, and hormonal therapy. However, they are found with limitations such as toxic effects, resistance to drugs, side effects, damage to normal tissues, and lower efficacy are observed[37]. Hence, currently, researchers worldwide are focusing on the development of anticancer drugs from natural products, which are found to be more effective and safer due to their unique molecular characteristics. Compounds such as phenols, flavonoids, terpenoids, and alkaloids have played a crucial role in the development of novel anticancer drugs[38]. Various anticancer drugs sourced from natural products include vincristine, paclitaxel, taxol, etoposide, camptothecin, etc.[39]. This has led the researchers to screen the traditionally used medicinal plants for their anticancer activity.

The present study reports the presence of phytochemical constituents, antioxidant potential, and dose-dependent *in vitro* cytotoxicity of a widely used traditional medicinal plant, *J. adhatoda* (L.), against DL cells. The methanolic extract exhibited a considerable amount of yield value, about 16.32%, which could be corroborated by the findings where the methanol extract showed the highest yields compared to extraction in solvents ethanol, n-hexane, and chloroform[40,41]. Furthermore, the leaves proved to be a promising source of important secondary metabolites, which included proteins, carbohydrates, phenols, flavonoids, alkaloids, polyphenols, terpenoids, tannins, saponins, and triterpenoids (Table 1). The presence of such phytochemicals is in line with the results of the studies conducted earlier[42,43]. These secondary metabolites are reported to have various pharmacological properties. Alkaloids, an important class of phytoconstituents, are found to act as antioxidants, anti-carcinogenic, anti-

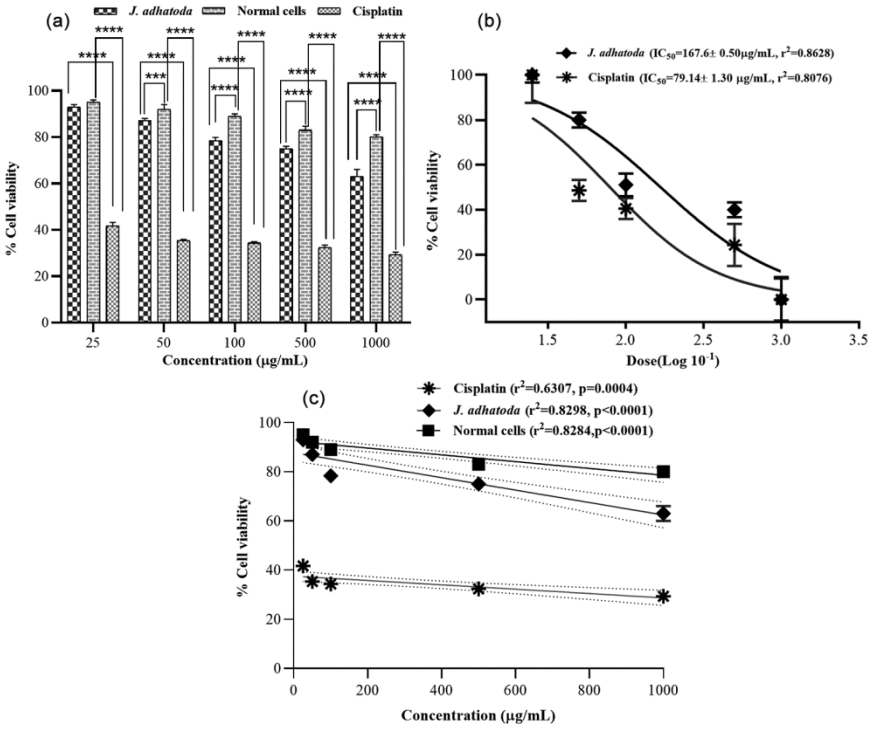


Fig. 7 — (a) Two-way ANOVA representing the viability of cells on treatment with extract and reference drug, cisplatin, determined through MTT assay, (b) IC_{50} values of *J. adhatoda* extract and reference drug, cisplatin, through log transformation and normalization, (c) Linear regression analysis of decrease in cell viability with increment in dose. Results are expressed as Mean± SEM, n=3, Bonferroni post hoc test. Asterisk indicate ****P<0.0001, ***P<0.001.

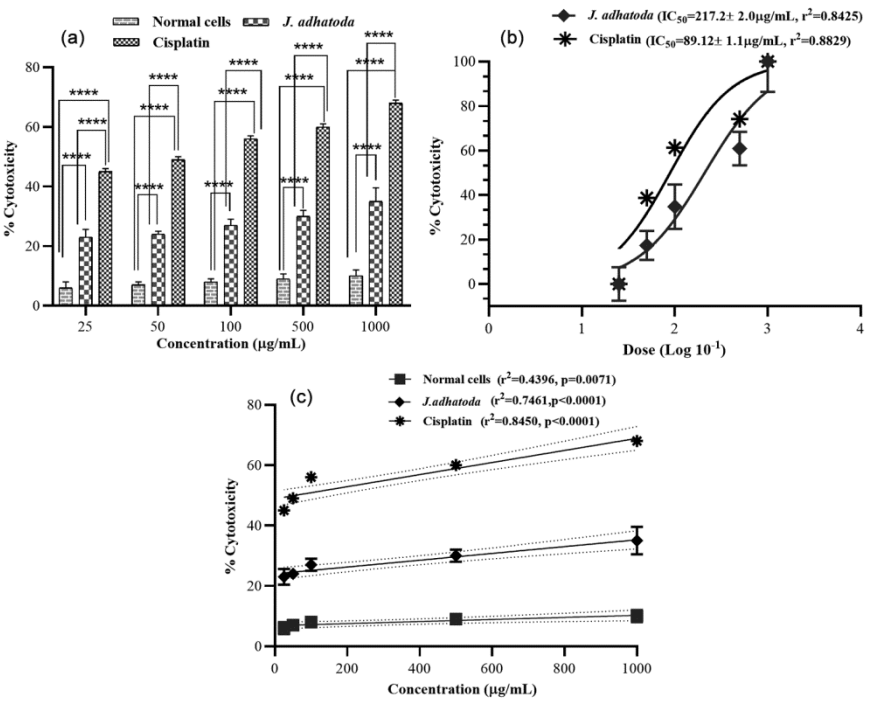


Fig. 8 — (a) Two-way ANOVA representing the percentage of cell cytotoxicity on treatment with *J. adhatoda* extract and reference drug, cisplatin, determined through LDH assay, (b) Representation of IC_{50} values of *J. adhatoda,* and cisplatin determined through log transformation and normalization, (c) Linear regression analysis demonstrating an increase in cytotoxicity of DL cells along with concentration. Results are expressed as Mean ± SEM, n=3. Bonferroni post hoc test. Asterisk indicate**** P<0.0001.

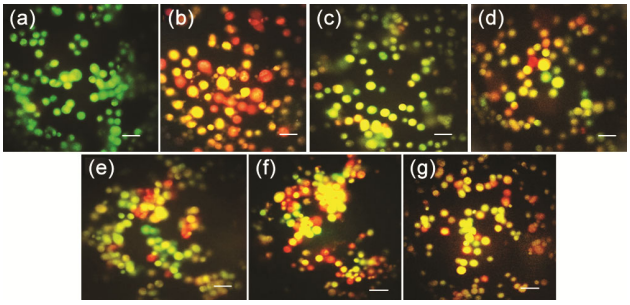


Fig. 9 — Apoptosis study of DL cells through acridine orange (AO) and ethidium bromide (EtBr) fluorescent staining, (a) untreated DL cells, (b) cisplatin-treated cells, DL cells treated with different concentrations of *J. adhatoda* extract, (c) 100µg/ml (d) 150µg/mL (e) 200µg/mL (f) 500µg/mL (g) 1000µg/mL. The live cells appear green, and the apoptotic cells are observed in red/orange fluorescence. Each bar in the image represents 20µm at 40x magnification.

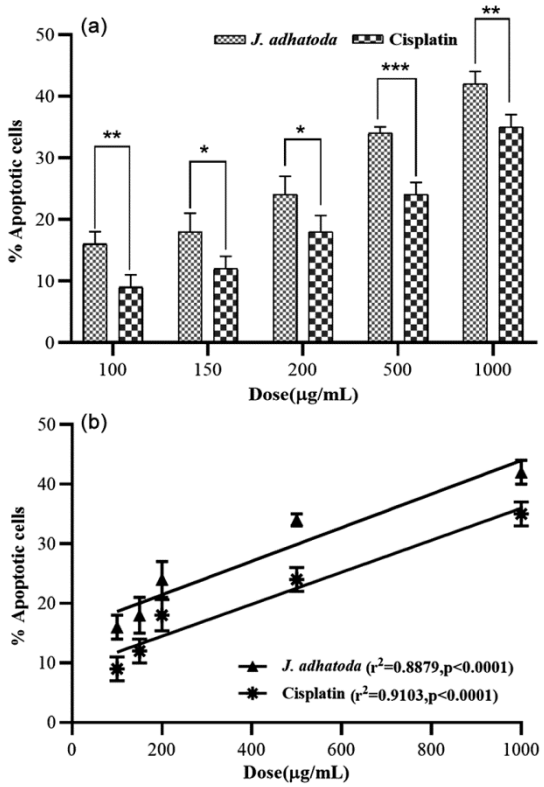


Fig. 10 — (a) Two-way ANOVA analysis representing percentage of apoptotic cell death on treatment with *J. adhatoda* extract and the reference drug, cisplatin, (b) Linear regression study showing correspondence of apoptotic cell death with dose. Bonferroni post hoc test. Asterisks indicate * $P<0.05$, ** $P<0.01$, *** $P<0.001$

microbial, and anti-mutagenic compounds[44]. Flavonoids are natural antioxidants that humans consume through food sources. Various epidemiologic studies have reported that antioxidants in foods are effective against Parkinson's disease, reduce the incidence of dementia, reduce blood cholesterol and pressure, and also provide protection against cardiovascular disease. Besides, flavonoids are also reported to exhibit anti-cancer, hepatoprotective, anti-viral, and anti-inflammatory activities[45]. Natural polyphenols also exhibit a range of pharmacological activities such as antioxidant, cardioprotective, anti-inflammatory, anti-microbial, anti-cancer, and protective for skin and kidney diseases[46]. Terpenoids, being the largest and most diverse secondary metabolites, are reported to have anti-inflammatory, antibacterial, anti-tumor, anti-malarial, anti-viral, and cardioprotective properties. It also lowers blood sugar levels[47]. Thus, the qualitative phytochemical estimation revealed that *J. adhatoda* (L.) leaves methanol extract is a storehouse of active phytoconstituents, which are of varied pharmacological importance. These outcomes scientifically validate the traditional use of *J. adhatoda* (L.) for treating various disorders.

Furthermore, the analysis of phytoconstituents through gas chromatography also reported the presence of varied phytocompounds (Fig. 1). A total of 20 compounds were identified (Fig. 2) eluted at different retention times. The identified compounds occupied different percentage areas at specific retention times and belonged to different classes, which included terpenoids, carotenoids, alkaloids, fatty acids, phenols, hydrocarbons, cycloalkane, and esters (Table 2,3), validating the results of our phytochemical analysis. The identified compounds were found to exhibit varied pharmacological properties, as per the previous findings, such as the compound methyl 8,11,14-heptadecatrienoate, which is reported to have anti-inflammatory activity[48]. An alkaloid, 1-(3,3,3-trifluoro-2-hydroxypropyl) piperidine, has anti-microbial properties. Phytol, a terpenoid, is reported to have antitumor, anti-inflammatory, antibacterial, antiviral, and antimalarial activities[49]. Earlier studies on GC-MS analysis of the acetone extract of *J. adhatoda* (L.) also reported the presence of phytol[50]. Moreover, the compound cyclohexane, 1,2,4-trimethoxy-, stereoisomer I, is reported to have antimicrobial and antioxidant properties[51]. Another compound, 1-methyl-1-ethoxycyclobutane, acts as an acidifier, inhibitor of arachidonic acid, and also increases the activity of aromatic amino acid decarboxylase. It was also found to inhibit the production of uric acid and act as a urine acidifier[52]. Octyl-beta-d-glucopyranoside, a lipid, was reported to have anticancer properties. The *in vitro*

studies showed its cytotoxic response against HeLa cell lines and were also found to exhibit hemolytic, thrombolytic, and anti-bacterial activities[53]. The compounds, 2-trimethylsiloxy-6-hexadecenoic acid methyl ester and tetradecanoic acid, 10,13-dimethyl-, methyl ester, are fatty acids reported with antioxidant activity[54,55] and another fatty acid, dodecanoic acid, was also reported with anti-bacterial and antioxidant activities[56].

Antioxidants are substances that neutralize free radicals and prevent damage caused by them. The antioxidants may play a protective role against the DNA damage caused by oxidative stress and prevent cancer progression. Moreover, natural antioxidants obtained from medicinal plants are safe and less toxic compared to synthetic antioxidants[57]. Thus, the antioxidant potency of *J. adhatoda* (L.) was evaluated using the DPPH and reducing power assays. The antioxidant potential of *J. adhatoda* (L.), ascertained through DPPH free radical scavenging assay, showed positive results, indicated by an increase in scavenging effect with an increment in dose. Furthermore, a low IC_{50} value in comparison to the standard was observed (Fig. 3). Similarly, the findings of the reducing power assay showed an increase in reducing ability proportional to dose (Fig. 4). The outcome of the present antioxidant study coincides with the conclusions of the earlier studies, where a positive correlation was found between antioxidant potency and dose[13]. However, a variation in IC_{50} value was observed in the DPPH study, which could be related to the different geographical locations of sample collection, affecting the phytochemical composition. Further, the observation of such antioxidant potential could be attributed to the presence of phytochemicals such as flavonoids, phenols, and polyphenols determined in the phytochemical tests and GC-MS analysis. These phytoconstituents are reported to act as natural antioxidants in several studies[41]. Moreover, various studies have found that plants exhibiting antioxidant properties are rich in total phenol and flavonoid contents[1,3].

The investigation of *in vitro* antitumor potential showed noteworthy results as determined using the trypan blue dye method, MTT, and LDH assay. The outcome of the cytotoxic assays was compared to that of a reference drug, cisplatin, which is an effective chemotherapeutic drug for the treatment of cancers[58]. The results of the trypan blue method and MTT assay showed a significant decrease in the viability of DL cells as compared to cisplatin-treated cells (Fig. 5,6). The cytotoxic effect was also evidenced through observation of morphological features such as cell shrinkage, membrane blebbing, and disintegration in treated cells compared to untreated cells (Fig. 7). Further, an increase in percentage of cell cytotoxicity on treatment with extract was also observed in the LDH assay (Fig. 8). The observed *in vitro* cytotoxicity showed proportionality with increment in dose. However, the normal cells (splenocytes) exhibited contrary results compared to those of the DL cells, indicating the specificity of *J. adhatoda* (L.) towards cancerous cells. This could be corroborated by the similar *in vitro* study conducted on other cancer cell lines, MCF-7 (breast cancer cell line), and A549 (human lung adenocarcinoma cell line), in a dose-dependent manner, without affecting the non-cancerous cell line HEK 293[15]. Likewise, the gold nanoparticles synthesized from *J. adhatoda* (L.) leaf extract showed promising anticancer potential against the A549 cancer cell line[59]. Additionally, another study also revealed potent cytotoxicity of *J. adhatoda* (L.) methanolic extract against MCF7, compared to that of the cytotoxicity shown by the ethanolic extract[60]. These findings support the outcome of the present *in vitro* cytotoxic study of *J. adhatoda* (L.) against DL cells, indicating its potential as an effective anticancerous drug.

To ascertain the apoptosis-inducing property of the extract, the DL cells were studied using fluorescent dyes AO/EtBr. The outcome of the study showed the occurrence of apoptosis in DL cells treated with extract, indicated with red/orange fluorescence, contrary to the untreated group of cells exhibiting green fluorescence (Fig. 9). Further, observation of apoptotic features such as extensive membrane blebbing, membrane disintegration, reduction of cell size was also seen in the treated cells compared to cisplatin treated cells (Fig. 9B-G). Similar morphological alterations and apoptotic features were observed in A549 cancer cells treated with *J. adhatoda* (L.) extract[59]. Additionally, quantitative estimation of cell death occurring due to apoptosis showed an increase in cell death percentage proportional to dose, found statistically significant ($P<0.05$) when equated to cisplatin-treated cells at similar concentrations (Fig. 10). Similar observations on the occurrence of apoptosis and percentage of cell death in a dose-dependent manner by *J. adhatoda* (L.)

leaves methanol extract against the cancer cell line, MCF-7 was reported by Kumar *et al.*[15,61]. One of the vital factors in the development and progression of cancer is the suppression of apoptosis[35]. Hence, researchers are focusing on cancer therapies that target apoptosis of cancer cells, as it is considered one of the most effective non-surgical methods with specificity for only cancer cells. Thus, the observation of various morphological changes in the DL cells treated with *J. adhatoda* (L.) extract compared to the untreated cells substantiates its apoptosis-inducing potential. The findings of the apoptosis study also validate the in *vitro* cytotoxic effect of *J. adhatoda* (L.) extract against DL cells. Overall, the findings of the present study suggest the antitumor effectiveness of *J. adhatoda* (L.) against DL cells.

Conclusion

The findings of the present study exhibited the occurrence of important secondary metabolites such as phenols, polyphenols, alkaloids, flavonoids, terpenoids, triterpenoids, tannins, and saponins, which corroborate the immense therapeutic potential of *J. adhatoda* (L.) as a traditional medicinal plant. Additionally, GC-MS analysis showed the occurrence of about 20 phytocompounds, which are reported to have diverse biological properties such as antiviral, anticancer, antimicrobial, antimalarial, anti-inflammatory, and antioxidant activity. Moreover, the extract showed potent antioxidant ability as compared to the standard, ascorbic acid, indicating its ability to act as an antioxidant and protect the cells from damage due to the generation of free radicals. Additionally, the *in vitro* cytotoxic assays displayed an effective cytotoxic response against DL cells as compared to the reference drug, cisplatin. The extract, however, showed the least cytotoxic effect towards the normal cells (splenocytes), demonstrating its specificity towards cancerous cells. Such observations would support its effectiveness for development as an anticancer drug. The cytotoxic effect observed was further substantiated by the findings of an apoptosis study, where the extract-treated DL cells exhibited apoptotic features compared to the control, untreated DL cells, which appeared round with intact plasma membranes. The occurrence of apoptosis in DL cells emphasizes the efficacy of the extract as a potent anticancer agent. The outcome of the present study provides the first report of anticancer potential of *J. adhatoda* (L.) methanol extract against lymphomas, which would serve as a guide to conduct further *in vivo* studies to treat lymphomas and explore the underlying molecular mechanisms involved.

Ethical statement

Furthermore, the institutional ethics committee granted prior approval for conducting the experiments and caring for animals, with the ethics approval number being 7/IAEC/CU/05/01/2021.

Acknowledgements

The authors acknowledge Guwahati Biotech Park for providing the GC-MS facility and Prof. Surya Bali Prasad, Department of Zoology, NEHU, Shillong, for providing the Dalton's Lymphoma cell line. We express our sincere gratitude to the DBT for their generous funding support through the DBT Builder project (Grant No: BT/INF/22/SP45376/2022). We also acknowledge the UGC, New Delhi (India), for financial assistance to Ms. Mary Devi (Savitribai Jyoti Rao Phule Single Girl Child research fellowship: 82-7/2022(SA-III).

Conflict of interest

The authors declare no conflict of interest.

References

1 Al Qaisi Y, Alfarrayeh I, Alsarayreh A, Khleifat K & Abu-Nwas N. Assessment of antioxidant potential, cytotoxicity and anticancer activity of methanolic extracts from selected wild medicinal plants. *Phytomed Plus*, 4 (2024) 100534.

2 Ahmed S, Zengin G, Fernández-Ochoa A, Cádiz-Gurrea ML, Jiménez FL, Elkiran O, Cakilcioglu U, Akgul BH, Pereira CG & Custódio L. Exploration of UHPLC-ESI-QTOF-MS Profiles and the Neuroprotective, Antidiabetic, Antioxidant and Cytotoxic Effects of Extracts from *Achillea maritima* (L.) Ehrend. & Y.P.Guo (Asteraceae) Collected in Türkiye. *Plant Foods Hum Nutr*, 80 (2025) 66.

3 Dar RA, Shahnawaz M, Ahanger MA, & Majid I. Exploring the diverse bioactive compounds from Medicinal plants: A Review. *J Phytopharmacol*, 12 (2023) 189.

4 Uba AI, Zengin G, Montesano D, Cakilcioglu U, Selvi S, Ulusan MD, Caprioli G, Sagratini G, Angeloni S, Jugreet S, Hasan MM & Mahoomodally MF. Antioxidant and Enzyme Inhibitory Properties, and HPLC–MS/MS Profiles of Different Extracts of *Arabis carduchorum Boiss*.: An Endemic Plant to Turkey. *Appl Sci*, 12 (2022) 6561.

5 Kurt-Celep İ, Zengin G, Uba AI, Caprioli G, Mustafa AM, Angeloni S, Cakilcioglu U, Guler O, Kaplan A, Sharmeen J & Mahomoodally MF. Unraveling the chemical profile, antioxidant, enzyme inhibitory, cytotoxic potential of different extracts from *Astragalus caraganae*. *Arch Pharm*, 356 (2023) 2300263.

6 Zengin G, Fernández-Ochoa A, Cádiz-Gurrea ML, Jiménez FL, Uba AI, Gunes AK, Soomro SI, Balos M, Cakilcioglu U, Rodrigues MJ, Pereira CG & Custódio L. Cytotoxic, antioxidant, and enzyme inhibitory activities of *Centaurea stapfiana* extracts and their HPLC-ESI-QTOF-MS profiles:

Insights into an unexplored *Centaurea species*. *Fitoterapia*, 179 (2024) 106207.
7 Isha, Kumar P & Singh AN. An overview of *Justicia Adhatoda*: A Medicinal Plant but Native Invader in India. *Conservation*, 5 (2025) 2.
8 Khaing WW, Yhu YM & Yee NN. Morphological and Anatomical studies on *Justicia adhatoda* L. and *Justicia ventricosa* Wall. *3rd Myanmar-Korea Conf Res J,* 3 (2020) 1088.
9 Dhankhar S, Kaur R, Ruhil S, Balhara M, Dhankhar S & Chhillar AK. A review on *Justicia adhatoda*: A potentialsource of Natural Medicine. *Afr J Plant Sci*, 5 (2011) 620.
10 Jayaweera U, Shivashekaregowda NKH, Herapathdeniya SKMK & Paranagama PA. Ethnopharmacological uses, phytochemistry, pharmacological activities and toxicity of *Justicia adhatoda* L.: a review. *Discover Plants*, 1 (2024) 41.
11 Pandey G. Anticancer herbal drugs of India with special reference to Ayurveda, *Indian medical science series*, (Sri Satguru Publications, Delhi), 2002, 18.
12 Barry VC, Conalty ML & Rylance HJR. Antitubercular effects of an extract of *Adhatoda vasica*. *Nature*, 176 (1955) 119.
13 Basit A, Shutian T, Khan A, Khan SM, Shahzad R, Khan A, Khan S & Khan M. Anti-inflammatory and analgesic potential of leaf extract of *Justicia adhatoda L.* (Acanthaceae) in Carrageenan and Formalin-induced models by targeting oxidative stress. *Biomed Pharmacother*, 153 (2022) 113322.
14 Sobia, Nawaz H, Khan S & Nadeem F. Use of Malabar nut (*Justicia adhatoda* L.) from traditional medicine to pharmacopeia- A review study. *Int J Chem Biochem Sci*, 13 (2018) 46.
15 Kumar S, Singh R, Dutta D, Chandel S, Bhattacharya A, Ravichandran V & Sukla S. *In vitro,* anticancer activity of methanolic extract of *Justicia adhatoda* leaves with special emphasis on human breast cancer cell Line. *Molecules*, 27 (2022) 8222.
16 Duraipandiyan V, Al-Dhabi NA, Balachandran C, Ignacimuthu S, Sankar C & Balakrishna K. Antimicrobial, antioxidant, and cytotoxic properties of vasicine acetate synthesized from vasicine isolated from *Adhatoda vasica* L. *BioMed Res Int*, 2015 (2015) 727304.
17 Handa SS. An Overview of Extraction Techniques of Medicinal and Aromatic plants in *Extraction Technologies for Medicinal and Aromatic Plants*, (2008)21.
18 Abbas A, Naqvi SAR, Rasool MH, Noureen A, Mubarak MS & Tareen RB. Phytochemical analysis, antioxidant and antimicrobial screening of *Seriphidium oliverianum* plant extracts. *Dose-Response*, 19 (2021) 15593258211004739.
19 Trease EC & Evans WC. Phytochemistry, *Pharmacognosy* (Saunders Elsevier, Philadelphia), 2009, 133.
20 Balamurugan V, Fatima MAS & Velurajan S. A guide to Phytochemical analysis. *Int J Adv Res Innov Ideas Educ*, 5 (2019) 236.
21 Perumal PC, Sowmya S, Pratibha P, Vidya B, Anusooriya P, Starlin T, Vasanth R, Jeya SSD & Gopalakrishnan VK. Identification of novel PPARγ agonist from GC-MS analysis of ethanolic extract of *Cayratiatrifolia* (L.): A computational molecular simulation studies. *J Appl Pharm Sci*, 4 (2014) 6.
22 Nonglang FP, Khale A, Wankhar W & Bhan S. Pharmacognostic evaluation of Eranthemum indicum extracts for its *in-vitro* antioxidant activity, acute toxicology, and investigation of potent bioactive phytocompounds using HPTLC and GCMS. *Beni-Suef Univ J Basic Appl Sci*, 11 (2022) 129.
23 Owolabi OO, James DB, Sani I, Andongma BT, Fasanya OO & Kure B. Phytochemical analysis, antioxidant and anti-inflammatory potential of *Feretia apodanthera* root bark extracts. *BMC Complement Altern Med*, 18 (2018) 12.
24 Devi M, Verma, AK, Singh NS, Das J, Dutta K, Gogoi M & Dutta D. Phytochemical investigation and *in vitro* cytotoxicity of *Phlogacanthus thyrsiformis* (Roxb. Ex Hardw.) Mabb. against Dalton's Lymphoma Ascites (DLA) cells. *Adv Tradit Med*, 24 (2023) 593.
25 Prieto P, Pineda M & Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal Biochem*, 269 (1999) 337.
26 Chakrabarti S, Chakrabarti A & Pal AK. Chromosome Analysis of Dalton's Lymphoma Adapted to the Swiss Mouse: Clonal Evaluation and C-Heterochromatin Distribution. *Cancer Genet Cytogenet*, 11 (1984) 417.
27 Verma PK, Singh RK, Kumar S, Shukla A, Kumar S, Gond MK, Bharty MK & Acharya A. Cobalt (III) complex exerts anti-cancer effects on T cell lymphoma through induction of cell cycle arrest and promotion of apoptosis. *Daru*, 30 (2022) 127.
28 Segeritz CP & Vallier L. Cell Culture: Growing Cells as Model Systems *in vitro. Basic Science Methods for clinical Researchers,* 7 (2017) 151.
29 Lefort CT & Kim M. Human T lymphocyte isolation, culture and analysis of migration *in vitro*. *J Vis Exp*, 40 (2017) 1.
30 Prasad SB & Turnia I. Antitumor activity with no toxicity of propolis from Meghalaya, India in ascites Dalton's lymphoma-bearing mice. *Indian J Nat Prod Resour*, 11 (2020) 267.
31 Kumar P, Nagarajan A & Uchil PD. Analysis of cell viability by the MTT assay. *Cold Spring Harb Protoc*, 6 (2018) 469.
32 Kumar P, Nagarajan A & Uchil PD. Analysis of cell viability by the Lactate dehydrogenase assay. *Cold Spring Harb Protoc*, 6 (2018) 465.
33 Elmore S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol*, 35 (2007) 495.
34 Prasad R & Koch B. Antitumor activity of ethanolic extract of *Dendrobium formosum* in T-cell lymphoma: An *in vitro* and *in vivo* study. *BioMed Res Int*, 2014 (2014) 753451.
35 Verma AK & Singh S. Phytochemical analysis and *in vitro* cytostatic potential of ethnopharmacological important medicinal plants. *Toxicol Rep*, 7 (2020) 443.
36 Sathishkumar K, Chaturvedi M, Das P, Stephen S & Mathur P. Cancer incidence estimates for 2022 & projection for 2025: Result from National Cancer Registry Programme, India. *Indian J Med Res*, 156 (2022) 598.
37 Zafar A, Khatoon S, Khan MJ, Abu J & Naeem A. Advancements and limitations in traditional anti-cancer therapies: a comprehensive review of surgery, chemotherapy, radiation therapy, and hormonal therapy. *Discov Oncol*, 16 (2025) 607.
38 Chunarkar-Patil P, Kaleem M, Mishra R, Ray S, Ahmad A, Verma D, Bhayye S, Dubey R, Singh HN & Kumar S. Anticancer Drug Discovery Based on Natural Products: From Computational Approaches to Clinical Studies. *Biomedicines*, 12 (2024) 201.

39 Huang M, Lu JJ & Ding J. Natural Products in Cancer Therapy: Past, Present and Future. *Nat Prod Bioprospect*. 11 (2021) 5.

40 Krishnamoorthy S. Influence of drying temperature on quality parameters of *Adhatoda vasica* leaves. *Int J Life Sci Biotechnol Pharma Res*, 3 (2014) 280.

41 Srinivasan K & Kumaravel S. A comparative Study: The Impact of solvent extraction on phytochemical profiling of *Adhatoda vasica*. *Int J Pharm Res Health Sci*, 3 (2015) 874.

42 Jose AB, Deepali, Malsawmtluangi, Priscilla N, Rashmi & Francis D. Antimicrobial, antioxidant and anti-inflammatory activities of *Justicia adhatoda L.* leaf extract. *Med Plants*, 15 (2023) 595.

43 Nasir M, Ramash R, Fatima H, Ashraf S, Munir I, Asghar S, Adnan M, Masood A & Chaudhari SK. Phytochemical Characterization and Assessment of crude Extracts from *Justicia adhatoda* for phytotoxic and cytotoxic activity. *Scientifica*, 2024 (2024) 1374346.

44 Adamski Z, Blythe LL, Milella L & Bufo SA. Biological activities of alkaloids: from toxicology to Pharmacology. *Toxins (Basel)*, 12 (2020) 210.

45 Kumar S & Pandey AK. Chemistry and biological activities of flavonoids: An overview. *Sci World J*, 2013 (2013) 162750.

46 Li AN, Li S, Zhang YJ, Xu XR, Chen YM & Li HB. Resources and biological activities of natural polyphenols. *Nutrients*, 6 (2014) 6020.

47 Yang W, Chen X, Li Y, Guo S, Wang Z & Yu X. Advances in Pharmacological Activities of Terpenoids. *Nat Prod Commun*, 15 (2020) 1.

48 Ali A, Khan N, Qadir A, Warsi MH, Ali A & Tahir A. Identification of the phytoconstituents in methanolic extract of *Adhatoda vasica* L. leaves by GC-MS analysis and its antioxidant activity. *J AOAC Int*, 105 (2022) 267.

49 Tamilselvi S, Dharani T, Padmini S, Nivetha S, Sangeetha M, Das A & Balakrishnaraja R. GC-MS Analysis of *Albizia amara* and *Phyla nodiflora* ethanolic leaf extracts. *Int J Recent Technol Eng*, 7 (2018) 466.

50 Malathi R, Kaviyarasan D & Chandrasekar S. Study on preliminary phytochemicals and GC-MS Analysis of *Justicia adhatoda* leaves extract. *J Drug Deliv Ther,* 9 (2019) 547.

51 Al-Seadi HL, Sabti MZ & Taain DA. GC-MS Analysis of Papaya Leaf Extract (*Carica papaya* L.). *IOP Conf. Ser.: Earth Environ Sci*, 910 (2021) 012011.

52 Perumal GM, Prabhu K, Rao MRK, Janaki CS, Kalaivannan J & Kavimani M. The GC-MS analysis of ethyl acetate extract of one Herbal plant, '*Canthiumparviflorum*'. *Nat Vol Essent Oils*, 8 (2021) 4041.

53 Dinesh KG, Karthik M & Rajakumar R. GC-MS analysis of bioactive compounds from ethanolic leaves extract of *Eichhornia crassipes* (Mart) Solms. and their pharmacological activities. *Pharma Innovation*, 7 (2018) 459.

54 Shaaban MT, Ghaly MF & Fahmi SM. Antibacterial activities of hexadecanoic acid methyl ester and green-synthesized silver nanoparticles against multidrug-resistant bacteria. *J Basic Microbiol*, 61 (2021) 557.

55 Renugadevi K, ValliNachiyar C & Zaveri M. Bioactivity of dodecanoic acid extracted from Geitlerinema sp. TRV57. *Indian J Pharm Educ Res*, 55 (2021) 224.

56 Tareq AM, Hossain MM, Uddin M, Islam F, Khan Z, Karim MM, Lyzu C, Ağagündüz D, Reza ASMA, Emran TB & Capasso R. Chemical profiles and pharmacological attributes of *Apiscerana indica* beehives using combined experimental and computer-aided studies. *Heliyon,* 9 (2023) 15016.

57 Mo YN, Cheng F, Yang Z, Shang XF, Liang JP, Shang RF, Hao BC, Wang XH, Zhang HJ, Wali A, Lu CF & Liu Y. Antioxidant Activity and the Potential Mechanism of the Fruit from *Ailanthus altissima* Swingle. *Front Vet Sci*, 8 (2021) 784898.

58 Dasari S & Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol*, 740 (2014) 364.

59 Latha D, Prabhu P, Arulvasu C, Manikandan R, Sampurnam S & Narayanan V. Enhanced cytotoxic effect on human lung carcinoma cell line (A549) by gold nanoparticles synthesized from *Justicia adhatoda* leaf extract. *Asian Pac J Trop Biomed*, 8 (2018) 540.

60 Batool R, Salahuddin H, Mahmood T & Ismail M. Study of anticancer and antibacterial activities of *Foeniculum vulgare*, *Justicia adhatoda* and *Urtica dioica* as natural curatives. *Cell Mol Biol*, 63 (2017) 109.

61 Ramaya B, Khusro A, Indra V, Agastian P, Almutairi MH & Almutairi BO. Green synthesis of silver nanoparticles using *Justicia adhatoda* leaves extract and its anticancer effect on human lung carcinoma via induced apoptosis mechanism. *Res Chem*, 7 (2024) 101472.