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Research Article

Phytochemical analysis, antioxidant, and antitumor activity of *Ligustrum ovalifolium* leaves grown in Jordan: an *in vitro* and *in vivo* study

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Abstract

Ligustrum ovalifolium (family Oleaceae) is a flowering plant with reported anti-inflammatory, antioxidant, hypotensive, and hypoglycemic activities. The present study investigated the phytochemical components and biological activities of *L. ovalifolium* leaves. Results showed that ethyl acetate extract has the highest potential to reduce cell growth in HeLa, T47D, and MDA-MB-231 cell lines with IC_{50} values of 0.047, 0.07, and 0.072 mg/mL, respectively. Based on the LD_{50} value, the tumor-bearing mice were IP injected with 12.5 mg/kg of ethyl acetate extract. Tumor growth was significantly reduced (-43.1%) compared to the control group. According to the LC-MS analysis, Apigenin-7-O-glucoside was the flavonoid with the highest percentage value in *L. ovalifolium* leaves. DPPH assay exhibited antioxidant activity of ethanol and water extracts with the percentage of scavenging around 91% at a concentration of 200 µg/mL. As well, the assessment of liver and kidney functions of the experimented animals showed no toxicity effect compared to the control result. All things considered, the outcomes of this study revealed an antitumor potential of *L. ovalifolium* leaves extracts, hence this activity may arise from the presence of different potent flavonoids and the antioxidant potential. Nevertheless, further investigations are needed to determine the targets and signaling pathways that are affected by *L. ovalifolium* leaves extracts.

Keywords

L. ovalifolium leaves, antitumor activity, LC-MS analysis, MTT assay, Apigenin-7-O-glucoside

Introduction

For centuries, plants have been a rich source for the discovery of potent pharmacologically active compounds, used in the treatment of different diseases (Khan et al. 2019, 2022). The biologically active principles of the plants, the so-called secondary metabolites are known to exert antioxidant, anti-inflammatory, antitumor, cardio-protective, antimicrobial, and several other activities (Leitzmann 2016). Accordingly, plants and herbs are promising sources for developing new remedies, in particular for the treatment of serious or complicated diseases such as cancer

(Mohammed 2019). Cancer is considered one of the main causes of death worldwide (Cabasag et al. 2022; Kocarnik et al. 2022; Morgan et al. 2023). Statistically, more than 19.3 million new cancer incidences and about 10 million cancer deaths have been recorded in 2020 (Sung et al. 2021). Despite the remarkable advances in cancer therapy still, there are many obstacles needed to be solved for comprehensive success in cancer treatment (Khan et al. 2022). On the other hand, different phytochemicals such as flavonoids, phenolic compounds, terpenoids, phytosterols alkaloids, sulfides, and others have shown potential antitumor properties (Avato et al. 2017; Joshi et al. 2017; Talib et al. 2020; Mahmod et al. 2022; Talib et al. 2022b). In this context, numerous extracts from herbs have been investigated for their possible antioxidant and antiproliferative properties to inhibit tumor cell growth using accepted *in vitro* and *in vivo* models. Promising results for many plant species are reported (Shu et al. 2010; Mahmod and Talib 2021; Al Kury et al. 2022). Certain plant secondary metabolites have been reported to prevent cancer cell proliferation, invasion, and metastasis as well as to reduce chemo-resistance by increasing tumor cell sensitivity to the treatment (Mitra and Bhattacharya 2020; Talib et al. 2022a, 2022b).

Ligustrum ovalifolium Hassk., known by the common names "Korean privet", "California privet", "garden privet", or "oval-leaved privet", belongs to the olive family Oleaceae (Wang et al. 2009; Moldovan et al. 2018). It is a semi-evergreen shrub with thick, fleshy leaves and dark purple to black fruits (Hasskarl 1844; Yamada et al. 2014). It is widely distributed in East Asia and is usually cultivated as an ornamental plant in many other countries, including Jordan (Moldovan et al. 2018). Few studies reported the chemical compositions and biological activities of *L. ovalifolium*. Machida et al. isolated acylated triterpenoids from *L. ovalifolium* flowers (Machida et al. 1997). Secoiridoid glucosides with hypotensive activities were identified in *L. ovalifolium* leaves, grown in Egypt (Hosny et al. 2009). Also, other researchers discovered anti-inflammatory, and antioxidant, effects using. *ovalifolium* extracts (Hosny et al. 2009; Kim et al. 2011, 2012). Since this species with certain pharmacological activities very widely cultivated in Jordan, the present study was designed to investigate *L. ovalifolium* leaf extracts phytochemically, and biologically. The findings of the HPLC-MS screening, antioxidant and *in vivo* and *in vitro* evaluation of the antiproliferative efficacy will be detailed in the current study. Additionally, total phenol and total flavonoid concentrations were determined.

Materials and methods

Plant collection

The fresh leaves of the *L. ovalifolium* plant were collected from the campus of the Applied Science Private University (ASU) (Amman, Jordan) in March-April 2022. The taxonomic identity of the plant was authenticated by Prof. Fatma Afifi using herbarium samples and descriptive references. Herbarium samples are deposited in the Department of Pharmaceutical Chemistry and Pharmacognosy (ASU) (FMJ-OLE1).

Extracts preparation

Fresh leaves of *L.ovalifolium* were soaked separately in three solvents with different polarities (ethyl acetate, 70% ethanol, and water) and heated until boiling with continuous stirring (1:10 w/v). The extracts were covered and kept overnight at room temperature. After filtration, the solvents were evaporated using a rotary evaporator until dry. The obtained dried crude extracts were kept at -20 °C until use.

Determination of total phenol content

Total phenol content was determined according to Folin-Ciocalteu (F-C) method (Folin and Ciocalteu 1927; Aboalhaija et al. 2022; Mahmod and Talib 2023). To calculate the concentration of total phenol content, a calibration curve standard of gallic acid (400–3.125 µg.mL was used. The result was expressed in terms of mg of gallic acid equivalent per gram of dry extract (mg GAE/g). Methanol was utilized as a blank and each reading was taken in triplicate.

Determination of total flavonoid content

Total flavonoid content was investigated using the Aluminum chloride ($AlCl_3$) method as described earlier (Hossain and Rahman 2011; Mahmod and Talib 2023). The result was expressed in terms of mg of rutin equivalent per gram of dry extract (mg RE/g). Each reading was taken in triplicate.

Phytochemical analysis of ethyl acetate extract using LC-MS analysis

To prepare the sample, ethyl acetate extract was dissolved in 2 mL dimethyl sulfoxide (DMSO) completing the volume up to 50 mL with acetonitrile solvent. At 4000 rpm, the sample was centrifuged for 2 min, followed by moving 1 mL to the autosampler (injection volume was 3 µL). The analysis was performed using Burker Daltonik (Berman, Germany) impact II ESI-Q-TOF system equipped with the Burker Dalotonik Elute UPLC system (Bremen, Germany) as described by Al-Mterin et al.(Al-Mterin et al. 2021).

Antioxidant activity (DPPH assay)

The antioxidant potential of ethyl acetate, ethanol, and aqueous extracts of *L. ovalifolium* leaves was investigated according to the radical scavenging activity of the stable synthetic free radical 2, 2-diphenyl-1-picrylhydrazyl

(DPPH) as demonstrated in the literature (Blois 1958). In brief, a mixture was prepared by adding 3 mL of (0.1 mM) methanol solution of DPPH to 2 mL of plant extracts solution using different concentrations (200–1.65 µg/mL). After 30 min of incubation, the absorbance (Ax) was measured using a spectrophotometer (BEL PHOTONIC, Italy) at 517 nm. The solution of DPPH dissolved in methanol represents the blank sample (A°). IC_{50} value was determined by plotting the percentage of inhibition against extract concentration. The percentage of inhibition was estimated based on the following equation 1:

$$\%I = (A° - Ax) / A° \times 100 \quad (1)$$

In vitro evaluation of antiproliferative activity

Cell lines and cell culture conditions

To investigate the antiproliferative activity of *L. ovalifolium* extracts, six cancer cell lines were used. Human breast cancer cells (T47D and MDA-MB-231), human colon cancer (Caco-2), human prostate cancer (PC3), human cervical cancer (HeLa), and non-cancerous fibroblast cells were provided from the University of Jordan. Mouse mammary sarcoma cells (EMP6/P) were purchased from the European Collection of Cell Cultures (Salisbury, UK). The cells were cultured in a complete medium and incubated in proper conditions, including 37 °C, 5% CO_2, and 95% humidity. The type of culture media varied according to the cell line. In particular, T47D and PC3 cell lines were cultured in a completed RPMI 1640 medium (PAN-biotech, Germany), while MDA-MB-231, Caco-2, HeLa, and fibroblast were cultured in a complete DMEM medium (PAN-biotech, Germany). As well, a completed MEM medium (PAN-biotech, Germany) was used for EMT6/P culturing. All types of media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco, UK), 1% penicillin-streptomycin (Sigma, USA), 1% L-glutamine (Sigma, USA), and 0.1% gentamycin (EuroClone, Italy).

Antiproliferative assay

The cytotoxicity of *L. ovalifolium* leaves extracts was detected using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described in the literature (Mahmod and Talib 2021). In the present study, the cells were treated with *L. ovalifolium* extracts (5–0.039 mg/mL) and processed as mentioned by Mahmod and Talib (2021). The percentage of cell survival was calculated compared to the negative control (untreated cells) (Equation 2).

$$\text{Percentage of cell viability (\%)} = (\text{OD of treated cell}/\text{OD of control cell}) \times 100 \quad (2)$$

The half-maximum inhibitory concentration (IC_{50}) of the treated cells was determined using SPSS (Statistical Package for the Social Science, Illinois version 24).

In vivo evaluation of the antitumor activity

Animals

The experiments with the animals were approved by the Research and Ethical Committee of Applied Science Private University (Approval Number: 2015-PHA-05). Thirty Balb/C female mice (4–6 weeks old, weight 21–25 g) were pathogen-free housed including convenient temperature (25 °C) and humidity of less than 60% in single cages, and had access to a standard pellet diet and water *ad libitum* before starting the experiments. The required conditions have been applied to keep the animals with ongoing air ventilation.

Acute toxicity of L. ovalifolium ethyl acetate extract

To determine the proper starting dose in the LD_{50} estimation assay, a limit test was performed. A small group of female mice (n=2) was treated (IP injection) with *L. ovalifolium* ethyl acetate extract. After 24 hrs, mortality incidence was observed. In case the mice have tolerated the dose, a gradual increase in the concentration (the dose multiplied by 1.5) is applied; otherwise, the dose will be reduced by 0.7. The maximum non-lethal and minimum lethal doses demonstrated the lower and upper limits, which were used to achieve the LD_{50} estimation assay (Akhila et al. 2007).

LD_{50} estimation assay was carried out by treating (IP injection) three groups of mice (n=4) with three doses of ethyl acetate extract (100, 300, and 500 mg/kg). All the investigated doses were within the upper and lower range that was recognized in the limit test. The vital conditions of the treated mice were observed for 24 hrs. The arithmetical method of Karber was considered to estimate the LD_{50} of *L. ovalifolium* leaves (Akhila et al. 2007).

Antitumor activity in an animal model

EMT6/P cells were collected and prepared to be inoculated in Balb/C mice (n=18) with a density of 1×10^6 cells/0.1 mL via subcutaneous injection. After 10 days, the size of the growing tumors was measured using a digital caliper (Vogel, Germany). Tumor-bearing mice were divided into a control group (n=9) (with no treatment) and a treatment group (n=9) (treated with ethyl acetate extract). In particular, ethyl acetate extract was prepared for IP injection in a concentration of 12.5 mg/kg which is 10% of the estimated LD_{50} value. The treatment stage was carried on for 10 days and during that tumor measurement was reported and serum samples were collected for further analysis. At the end of the experiment, the mice were sacrificed using cervical dislocation method and all the tumors were extracted and kept in 10% formalin. The volumes of the tumors were calculated according to the following equation (3) (Agrawal et al. 2004):

$$\text{Volumes of the tumors} = A \times B^2 \times 0.5 \quad (3)$$

Whereas (A) = the length of the longest aspect of the tumor, (B) = the length of the perpendicular to A

Assessment of kidney and liver functions in the experimental animals

Creatinine was measured to evaluate nephrotoxicity. Alanine transaminase (ALT) and aspartate transaminase (AST) were evaluated to assess liver function. Quantification of these biomarkers was achieved by following the protocol of specific kits including ALAT (GPT) FS* (Cat. No. 1 2701 99 10 972, Holzheim, Germany), ASAT (GOT) FS* (Cat. No. 1 2601 99 10 021, Holzheim, Germany), and Creatinine FS* (Cat. No. 1 1711 99 10 021, Holzheim, Germany) using DiaSys Respons 920 analyzer (Holzheim, Germany).

Statistical analysis

Data was demonstrated using the mean ± SEM (Standard Error of Mean). The statistical significance between groups was detected utilizing SPSS student's *t*-test. Variation between groups was approved significantly when the *p*-value is less than 0.05 ($p < 0.05$). IC_{50} values were determined by applying non-linear regression in SPSS (Statistical Package for the Social Science, Chicago, Illinois version 24).

Results and discussion

Despite all technological advancements in cancer prevention and treatment, cancer is still one of the main causes of death around the world (Khan et al. 2022). Conventional treatment is associated with many side effects that may cause other critical disorders (Raina et al. 2014; Khan et al. 2022). Therefore, the search for new drugs for treatment with low cost and fewer adverse effects is the main goal in cancer research development (Raina et al. 2014; Mitra and Bhattacharya 2020). Natural plant compounds such as flavonoids, terpenoids, phenols, alkaloids, and others are rich sources of potential anticancer agents. Several chemotherapeutic agents, currently used in the treatment are derived from plants and their semisynthetic derivatives (Shaikh et al. 2016; Mitra and Bhattacharya 2020). According to the US National Cancer Institute (NCI), more than 35 thousand plant species had been evaluated. Vincristine, vinblastine, taxol, indicine-N-oxide, etoposide analogs, and many others are the results of these investigations (Shaikh et al. 2016). To benefit from nature, more research and experimentation are required using edible, medicinal, and ornamental plants (Raina et al. 2014). Although *Ligustrum* is an ornamental plant it has medicinal uses in traditional Chinese medicine. It is known to stimulate the immune system, improve osteoporosis, and enhance poor vision (Starr et al. 2003; Pang et al. 2015). *L. ovalifolium*, another representative of this genus, exerts various medicinal properties including anti-inflammatory, antioxidant, hypoglycemic, and hypotensive activities (Harsha et al. 2013). In the current study, LC-MS analysis, antioxidant, and antiproliferative assays were conducted to investigate the possible medicinal value of *L. ovalifolium* leaves extracts. The results revealed promising antioxidant and antiproliferative activity. Regarding extraction results, the percentage yield of the extracts was varied according to the solvent used in the extraction. Water extract exhibited the highest percentage followed by ethanol extract. (Table 1). Ethyl acetate extract showed the lowest percentage of yield.

Table 1. Extraction yield results of the three solvent extracts.

Source	Extraction solvent	% of dried extracts yield
Ligustrum ovalifolium leaves	Ethyl acetate	3.73%
	Ethanol	12.14%
	Water	17.24%

Based on MTT results, ethyl acetate inhibited tumor cell growth at a low concentration of around 40 to 70 µg/mL particularly in cervical and breast cancer cell lines (Table 2).

As well, ethanol and aqueous extracts were able to reduce the percentage of survival in both PC3 and MDA-MB-231 cell lines (Fig. 1). On contrary, all the extracts showed less toxicity ($IC_{50} > 5$ mg/mL) toward the fibroblast, the normal model cell line (Table 2). Using normal fibroblast cells the safety of these extracts was established.

Based on the limit test result, the non-lethal dose of ethyl acetate extract IP injection was 100 mg/kg. By following the arithmetical method of Karber, the estimated LD_{50} of ethyl acetate extract was 125 mg/kg (Table 3).

Table 2. IC_{50} values of *L. ovalifolium* extracts against different cancer cell lines.

	T47D IC_{50} (mg/mL)±SEM	MDA-MB-231 IC_{50} (mg/mL)±SEM	PC3 IC_{50} (mg/mL)±SEM	Caco-2 IC_{50} (mg/mL)±SEM	HeLa IC_{50} (mg/mL)±SEM	EMT6/P IC_{50} (mg/mL)±SEM	Fibroblast IC_{50} (mg/mL)±SEM
Ethyl acetate extract	0.07±0.08	0.072±0.03	0.17±0.02	0.16±0.06	0.047±0.08	0.21±0.05	>5
Ethanol extract	0.87±0.12	0.74±0.14	0.57±0.03	1.2±0.12	0.87±0.16	1.8±0.03	>5
Aqueous extract	>5	1.88±0.1	0.70±0.09	1.53±0.12	2.22±0.09	1.8±0.16	>5

Table 3. Acute toxicity assay of *L. ovalifolium* ethyl acetate extract.

Groups (n=4)	Dose (mg/kg)	No. of mortality	Dose difference (a)	Mean mortality (b)	Probit (a × b)
1	100	0	0	0	0
2	300	2	200	1	200
3	500	3	200	2.5	500

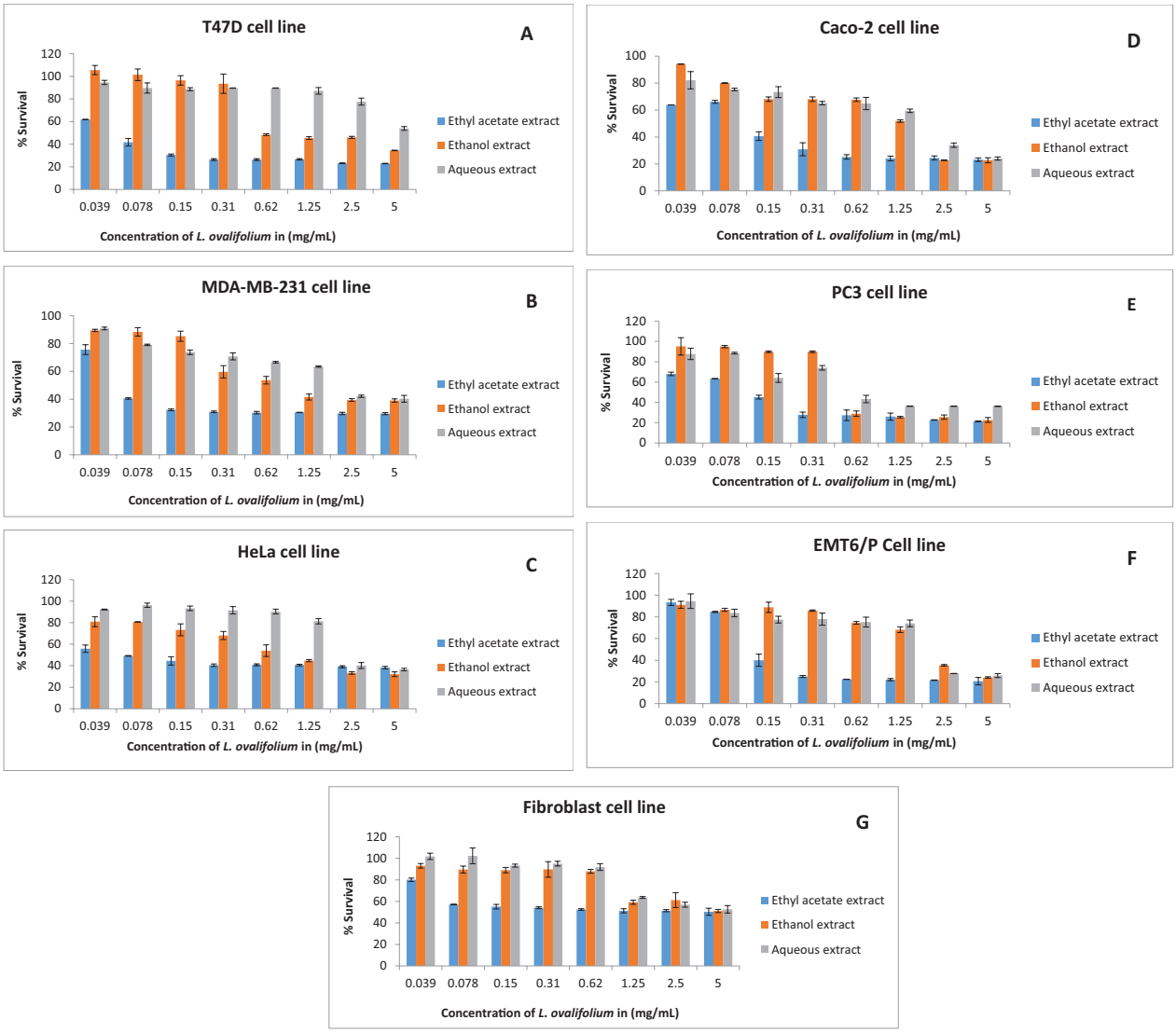

Figure 1. The antiproliferative activity of *L. ovalifolium* extracts against (**A**) T47D cell line (**B**) MDA-MB-231 cell line (**C**) HeLa cell line (**D**) Caco-2 cell line (**E**) PC3 cell line (**F**) EMT6/P cell line (**G**) Fibroblast cell line. Results are expressed as means of three independent experiments (bars) ± SEM (lines).

In the animal model experiment, Ethyl acetate extract was selected for the *in vivo* assay since it exhibited the best antiproliferative efficacy in the *in vitro* assessment among the three extracts. After treating tumor-bearing mice (n=9) with *L. ovalifolium* ethyl acetate extract (12.5mg/kg), results revealed a -43.1% reduction in tumor size compared to the negative control (48.1%) (Table 4) (Figs 2, 3).

In the present study, the antiproliferative efficacy of the ethylacetate extract of *L. ovalifolium* leaves is for the first time demonstrated while for some other species of the genus *Ligustrum*, cytotoxic effects against different cancer cells were reported. A recent study revealed the antiproliferative activity of different phytochemicals isolated from *L. japonicum* fruits (Kim et al. 2022). These compounds have suppressed cell growth and invasion by inhibiting MMP-2 and MMP-9 in HT 1080 fibrosarcoma (Kim et al. 2022). Also, ethanol extract of *L. lucidum* leaves decreased cell migration and invasion of hepatocellular carcinoma (Tian et al. 2019). Aqueous extract of *L. robustum* prevented tumor cell proliferation both, *in vitro* and *in vivo* by promoting apoptosis (Zuo et al. 2019). Moreover, methanol extract prepared from *L. vulgare* leaves and fruits showed cytotoxic effects against human leukemia cells (Zaric et al. 2021). The cytotoxic effect observed in the present study might be due to the high concentration of flavonoids,

Table 4. Antitumor effect of *L. ovalifolium* ethyl acetate extract in the animal model.

Treatment groups (n=9)	Initial tumor size (mm^3) ± SEM	Final tumor size (mm^3) ± SEM	% Change in tumor size	% of mice with no detectable tumor	Number of death	Average tumor weight (mg)
Control group	332.2±15	492.2±11	48.1	11.1	1	512.8
Ligustrum ovalifolium group	367.3±22	208.7±16	-43.1	55.1	0	228.2

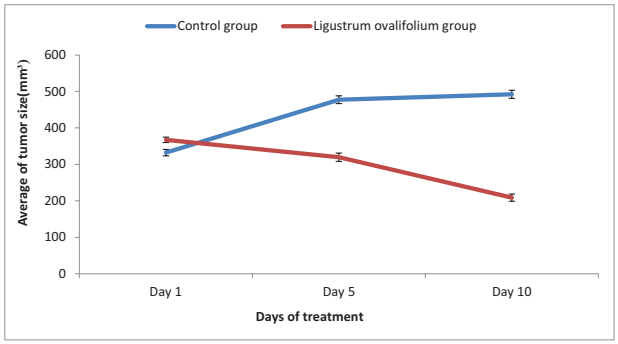

Figure 2. A plot verifying the changes in average tumor size (mm^3) vs time (days) of treatment with *L. ovalifolium* ethyl acetate extract in Balb/C mice inoculated with the EMT6/P cell line.

such as apigenin-7-O-glucosides (34%), luteolin (19%), baicalein (19%), and luteolin-7-O-glucosides (15%) detected in the ethylacetate extract using LC-MS (Table 5).

Additionally, ethyl acetate extract revealed the highest phenol content (154.7 mg GAE/g), and flavonoids (100.3 mg RE/g) as well as a high value of IC_{50} (33 µg/mL) according to DPPH assay (Table 6). Ethanol and aqueous extracts showed scavenging activity at a concentration of 200 µg/mL (Fig. 4).

The natural flavonoid apigenin-7-O-glucoside with multiple biological activities has high stability and good solubility (Kowalski et al. 2005; Smiljkovic et al. 2017). Its cytotoxic effect was reported against colon cancer (Smiljkovic et al. 2017), and prostate and cervical cancers (Srivastava and Gupta 2009; Liu et al. 2020; Minda et al. 2020). Liu et al. have suggested that apigenin-7-O-glucoside mediated cell apoptosis by modulating PTEN/PI3K/AKT pathway and prevented cell migration in cervical cancer cells (Liu et al. 2020). A study has shown that apigenin-7-O-glucoside was more active than apigenin in inhibiting the cell growth of HeLa human cervical cancer cells (Minda et al. 2020). Baicalein is another flavonoid detected in *L. ovalifolium* leaves. This flavonoid has also various pharmacological activities such as antioxidant, anti-inflammatory, antiviral, antibacterial, and anticancer activities (Wang et al. 2014; Gao et al. 2016; Nik Salleh et al. 2020). Bacailein has been tested on many cancer cell lines and showed high potency as an antiproliferative agent (Gao et al. 2016). Zhang et al. suggested that baicalein inhibited cell growth in osteosarcoma through the upregulation of IncRNA-NEF, which suppressed the Wnt/β-catenin signaling pathway that led to prevent tumor growth *in vitro* and *in vivo* (Zhang et al. 2022). Moreover, baicalein exhibited an anti-metastatic effect against breast cancer cells via blocking

Table 5. LC-MS analysis of *L. ovalifolium* ethyl acetate extract.

NO	Compounds	RT (Retention time)	Formula	Relative % (ethyl acetate extract)
1	2,4-Dihydroxyacetophenone	5.41	$C_8H_8O_3$	0.130681
2	Luteolin 7-O-glucoside (Cynaroside)	5.88	$C_{21}H_{20}O_{11}$	15.84325
3	Isorhoifolin	6.54	$C_{27}H_{30}O_{14}$	7.621954
4	Apigenin-7-O-glucoside (Apigetrin)	6.77	$C_{21}H_{20}O_{10}$	34.6314
5	7-Glu Chrysoeriol (NMR)	7.11	$C_{22}H_{22}O_{11}$	2.079451
6	Luteolin	8.57	$C_{15}H_{10}O_6$	19.05762
7	Tiliroside	8.8	$C_{30}H_{26}O_{13}$	0.139487
8	Oct-1-en-3-yl Ara (1-6)Glu (NMR)	8.9	$C_{19}H_{34}O_{10}$	0.344667
9	naringenin	9.53	$C_{15}H_{12}O_5$	0.104087
10	Baicalein	9.86	$C_{15}H_{10}O_5$	19.7901
11	Madecassic acid	15.56	$C_{30}H_{48}O_6$	0.090174
12	Hederagenin	21.62	$C_{30}H_{48}O_4$	0.084362
13	Ursolic acid	27.19	C30H48O3	0.082776

Table 6. Total phenol, flavonoid content, and antioxidant activity of *L. ovalifolium*.

L. ovalifolium leaves extracts	TFC* (mg RE/g)	TPC* (mg GAE/g)	DPPH assay IC_{50} (µg/mL)
Ethyl acetate extract	100.3±0.4	154.7±0.4	33±9.2
Ethanol extract	41.5±0.1	115.2±1.1	46.7±11
Aqueous extract	22.6±0.3	87.9±0.1	52.2±8.6
Ascorbic acid			1.74±0.2

* TFC = total flavonoid content, TPC = total phenol content.

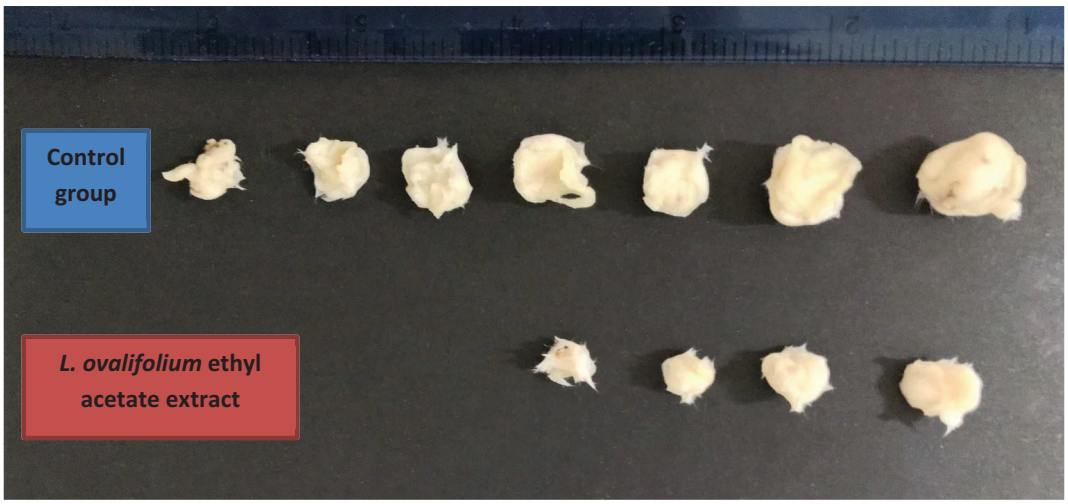

Figure 3. *L. ovalifolium* ethyl acetate extract effect on tumor size and cure percentage. Compared to the control group, treating tumor-bearing mice with *L. ovalifolium* has reduced tumor size and improved the cure percentage. (n=9 per group).

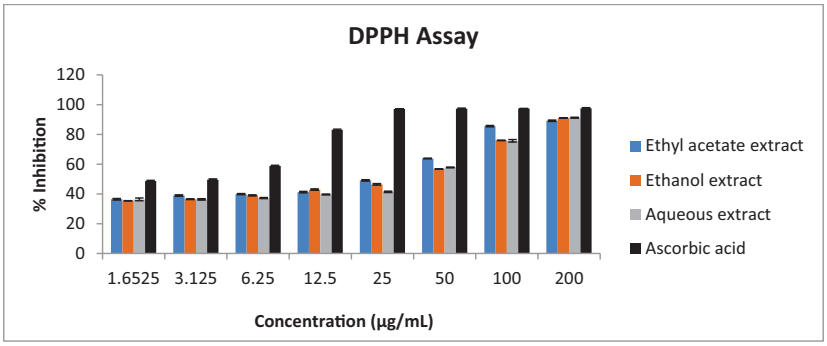

Figure 4. DPPH radical scavenging activity of *L. ovalifolium* extracts. Ascorbic acid is the positive control in this assay. Results are expressed as means of three independent experiments (bars) ± SEM (lines).

STAT3 activity, MMP-2/9 expression, STAB1and Wnt/β-catenin pathway (Wang et al. 2010; Ma et al. 2016; Susmitha et al. 2020). Han et al. demonstrated the anticancer activity of baicalein by inhibiting AKT/NF-kB and STAT3 signaling pathways in cholangiocarcinoma cells (Han et al. 2021). On the other hand, luteolin, a natural flavone, has been reported to have antiproliferative activity against breast cancer (Lee et al. 2012), gastric cancer (Pu et al. 2018), cervical cancer (Chen et al. 2023), lung cancer, and other cancer cell lines (Imran et al. 2019; Ganai et al. 2021). Lee et al. (2012) experimented antitumor effect of luteolin on the MDA-MB-231 cell line, which showed suppression of AKT, PLK1, cyclin B^1, cyclin A, CDC2, CDK2, and Bcl-xL as well as induction of inducing p21 and Bax expression (Lee et al. 2012). Furthermore, luteolin-7-O-glucoside, a glycosyloxyflavone derived from luteolin, exhibited a potent cytotoxic effect on breast cancer cell lines (MCF-7 and MDA-MB-231 cells) (Goodarzi et al. 2020). It inhibited migration and invasion of oral cancer cells by downregulation of MMP-2 expression (Velmurugan et al. 2020) as well as induced apoptosis in human nasopharyngeal carcinoma (Ho et al. 2021).

There is a correlation between the antioxidant effect of plant extracts and their cytotoxic activity. Reactive oxygen species (ROS) can stimulate DNA damage which may lead to converting normal cells into cancerous ones by genetic mutation (Ziech et al. 2010). In fact, the unbalanced redox equilibrium mediates cancer development and progression (Jambunathan et al. 2014). Based on our results, *L. ovalifolium* extracts exhibited antioxidant activity compared to ascorbic acid. The positive correlation among total phenolic content, total flavonoids, and antioxidant activity of *L. ovalifolium* leaves was in agreement with what has been reported by Kim et al. (Kim et al. 2011).

To evaluate the effect of *L. ovalifolium* leaves treatment on the main functional biomarkers of the liver and kidney in mice; serum samples for both, treated and untreated groups of animals were analyzed. There are slight differences between the ethyl acetate group and the control group, which indicate the safety of the *L. ovalifolium* leaves extract with no toxic effects on kidney and liver functions (Table 7).

Table 7. *L. ovalifolium* effect on liver and kidney functions.

	Creatinine (mg/mL)±SEM	ALT (U/L)±SEM	AST (U/L)±SEM
L. ovalifolium ethyl acetate extract group	0.35±0.17	34.75±9	156±7
Control group	0.31±0.12	68.8±1.4	200±6.2
Healthy mice	0.17±0.05	61.3±9.8	150±11

Conclusion

The results of this study suggested that *L. ovalifolium* leaves extracts could be considered a promising medicinal plant with anticancer potential. Ethyl acetate extract of *L. ovalifolium* leaves exhibited high potency in preventing cell growth of various cancer types as well as reduced tumor size and enhanced quick recovery of mice bearing breast cancer. Besides, the phytochemical analysis revealed the richness of the active ethyl acetate extract in flavonoids, which are recognized by their antioxidant and antiproliferative activities. Nevertheless, further investigations are needed to determine the targets and signaling pathways that are affected by *L. ovalifolium* extracts.

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Supplementary material 1

Ligustrum ovalifolium leaves ethyl acetate extract LCMS results

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Data type: docx

Explanation note: Spectra of identified compunds in Ligustrum ovalifolium extraxts using LCMS

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