Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Antioxidant properties and anticancer activity of *Olea europaea* L. olive and *Ficus carica* fruit extracts against pancreatic cancer cell lines

Nadia Dekdouk[1], Souad Ameddah[1], Chawki Bensouici[2], Meghboun Ibtissem[2], Menad Ahmed[1], And Fátima Martel[3,4]*

[1]Laboratoire de Biologie et Environnement, Faculté des Sciences de la Nature et de la Vie, Université des Frères Mentouri Constantine1, Algérie
[2]Biotechnology Research Center (CRBt), Ali Mendjli, Constantine, Algérie
[3]Unit of Biochemistry, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal
[4]Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Porto, Portugal

**ABSTRACT**

*Olea europaea* (OFE) and *Ficus carica* (FFE) extracts have shown anticancer activity. In the currents report, we aim to evaluate the phytochemical composition, antioxidant properties and anticancer activity of OFE and FFE against PANC-1 and AsPC-1 pancreatic cancer cell lines. OFE possessed higher phenolic, flavonoid content and antioxidant ability than FFE. OFE and FFE induced a cytotoxic effect and their combination resulted in an increased cytotoxicity. OFE showed an antiproliferative effect while FFE had no effect. Combination of the two extracts resulted in an antiproliferative effect similar to that of OFE. OFE showed an anti-migratory effect, while FFE exhibited a promigratory effect. Interestingly, combination of the two extracts resulted in an anti-migratory effect similar to that caused by OFE. In view of the findings of this study, these extracts could be considered potential sources of natural compounds with antioxidant and anti-pancreatic cancer effects. The combination of these extracts should be further investigated in the context of pancreatic cancer.

**ARTICLE HISTORY**

Received: 16 November 2023
Revised: 25 May 2024
Accepted: 12 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Antioxidant capacity
Anti-pancreatic cancer effect
Antiproliferative
Cytotoxic
Fig extract
Olive extract
Phytochemical composition

## 1. Introduction

Medicinal plants have shown great promise in the fight against cancer, with many studies demonstrating their potent anticancer properties. Several naturally occurring plant-based compounds, such as curcumin, resveratrol, and quercetin, have exhibited anticancer effects and are being investigated as potential adjuvant chemotherapy agents (Greenwell and Rahman, 2015; Khan et al., 2019; Mahdavi and Mohammadhosseini 2022). These plant-derived compounds often have lower toxicity towards healthy cells and a tendency to target abnormal or cancerous cells specifically. Many of the drugs currently on the market are structurally similar to natural compounds found in medicinal plants (Khan et al., 2019; Obafemi and Besong, 2023). Herbal compounds possess various beneficial qualities, including antioxidant, anti-inflammatory, antimutagenic, and apoptosis-inducing activities, which aid in the early diagnosis and treatment of cancer. Adequate dietary intake of these herbal products may help prevent and treat cancer by causing cell cycle arrest, inducing apoptosis, regulating carcinogen metabolism and oncogenic expression, inhibiting cell adhesion, proliferation, and migration, and blocking signaling pathways crucial for cancer progression (Obafemi and Besong, 2023). Plants produce a wide variety of secondary metabolites, such as tannins, alkaloids, terpenoids, flavonoids, pigments, and pigment-like molecules, which have been shown to have biological effects on hematopoietic cells, lipids, and cardiovascular systems, as well as anti-inflammatory, anticancer, and contraceptive effects (Mohammadhosseini et al., 2019a; Mohammadhosseini et al., 2019b; Mohammadhosseini et al., 2021).

Pancreatic cancer is one of the most aggressive cancers with poor prognosis and survival despite surgical and adjuvant therapies. Currently, it constitutes the fourth

* Corresponding author: Fátima Martel
Tel: 351 22 0426654; Fax: 351 22 5513624
E-mail address: fmartel@med.up.pt, **doi:**

cause of cancer mortality in occidental countries (Siegel et al., 2016). Treatment with gemcitabine, a pyrimidine agonist, has been the standard of care for metastatic pancreatic cancer for more than a decade. However, survival for most patients treated with this drug has not improved beyond 6 months (Moore et al., 2007; Park et al., 2021). One of the main problems posed with anticancer drugs is that they target not only tumor cells, but also other cell types, leading to toxicity to both abnormal and normal cells (Baldo and Pham, 2014). Another problem related to anticancer therapy is the development of resistance of cancer cells against anticancer treatments (Bar-Zeevet et al., 2017). This is the case of pancreatic cancer cells, which are highly resistant to current chemotherapy and radiotherapy treatments (Vendrely et al., 2017). Therefore, finding new therapeutic options is urgently needed to help manage this deadly cancer.

Nutrition and diet are good candidate agents for protection against various types of cancer (Kazeminia et al., 2022). Epidemiological studies have shown a reduction in the risk of pancreatic cancer through increased consumption of fruits and vegetables (Zheng et al., 2017; Gianfredi et al., 2022). The Mediterranean diet has been recognized as a healthy diet with a preventive effect against chronic diseases associated with oxidative damage, including cardiovascular disease, diabetes, and cancer (Schwingshackl and Hoffmann, 2014; Saha et al., 2017; Mentella et al., 2019; Guasch-Ferré and Willett, 2021). These effects seem to be mediated by many individual components of this diet, including polyphenolic phytochemicals present in fruits such as figs and olive oil (Owen et al., 2000b; Solomon et al., 2006; Lopez-Miranda, 2010).

The olive tree, *Olea europaea*, produces the olive fruit and is one of the vital components of the Mediterranean diet. Olive and its products have many health benefits, including cancer (Markellos et al., 2022; Romani et al., 2019). Preclinical *in vitro* and *in vivo* studies have demonstrated an antitumoral effect of olive fruit extracts (OFE) against hepatic and colorectal cancer (Maalejet al., 2017; Shirazi et al., 2018; Celano et al., 2019). The components of olives and olive oil that have been most consistently associated with a protective effect on carcinogenesis are vitamin E, squalene, and phenolic compounds (Guo et al., 2018). The most abundant phenolic compounds in olive oil are the lignans (+)-1-acetoxypinoresinol and (+)-pinoresinol, followed by the oleuropein-derived simple phenols hydroxytyrosol and tyrosol and the secoiridoids oleuropein, ligstroside aglycone and their respective decarboxylated dialdehyde derivatives (Owen et al., 2004). In contrast, polyphenols such as the flavonoid luteolin, the phenolic acid gallic acid, the phenolic alcohols hydroxytyrosol and tyrosol and the secoiridoids oleuropein and verbascoside are most abundant in olive fruits (Owen et al., 2004; Dekdouket al., 2015). Previous studies have clearly evidenced an antitumor effect of these phenolic compounds against certain types of cancers, namely breast (Han et al., 2009; González et al., 2013; Carrera- Akl et al. 2014; Sepporta et al., 2014), colorectal (Owen et al., 2000a; Carrera-González et al., 2013; Hormozi et al., 2020), gastric (Kountouri et al., 2009), hepatic (Pei et al., 2016), skin cancer (Carrera-González et al., 2013) and leukemia (Fabiani et al., 2002), and also pancreatic cancer (LeGendre et al., 2015; Goldsmith et al., 2018).

*Ficus carica* is a species characteristic of the Mediterranean region where its cultivation and use constitute an ancient tradition. Figs are consumed both fresh and dried (Baby and Justin, 2011). Figs are an excellent source of minerals, vitamins, carbohydrates, and dietary fiber, they are fat- and cholesterol-free and rich in several amino acids (Cruz et al., 2022; Olaoluwa et al., 2022; Ijoma et al., 2023; Popwo Tameye et al., 2023). Figs have traditionally been used for their medicinal benefits as laxative and cardiovascular, respiratory, antispasmodic and anti-inflammatory remedies (Guarrera, 2005). However, figs also exhibit anticancer properties (Morovatiet al., 2022). Namely, preclinical *in vitro* and *in vivo* models revealed anticancer activity of *F. carica* latex or fruit extracts (FFE) in relation to several types of cancers, including breast (Ghandehari et al., 2018), colorectal (Soltana et al., 2019), gastric (Hashemi et al., 2011), hepatic (Purnamasariet al., 2019) and, recently, also in relation to pancreatic cancer (Ou et al., 2022). An important organic compound in figs is 2-benzhydrylsulfinyl-*N*-hydroxyacetamide-Na, which is less abundant in other fruits and plants, and exhibits anticancer activity *in vitro* (Al Salman et al., 2020). Moreover, *Ficus carica* is a rich source of polyphenols. Quercetin and quercetin-related compounds are the main phenolic compounds present in this fruit (Khadhraoui et al., 2019; Arvaniti et al., 2019), and are well known to possess antitumor activity against several types of cancer, including pancreatic cancer (Rauf et al., 2018).

Several *in vitro* and *in vivo* preclinical studies have demonstrated varying degrees of efficacy of natural products against pancreatic cancer (Singh et al., 2015), and numerous dietary and pharmacological agents have been proposed as alternative strategies for the prevention and/or treatment of this disease (Dhar et al., 2018; Selvarajoo et al., 2022).

To the best of our knowledge, no report on the effects of a combination of OFE and FFE on pancreatic cancer cells is available in the literature. Keeping this point in view, the present study was conducted to evaluate the *in vitro* antioxidant activities and the cytotoxic, antiproliferative and antimigratory effects of OFE and FFE, alone and in combination, on two pancreatic cancer cell lines.

## 2. Experimental

### 2.1. Chemicals and reagents

$^{3}$H-Thymidine ([methyl-$^{3}$H]-thymidine, 79 Ci/mmol) was purchased from GE Healthcare GmbH (Freiburg, Germany). Dimethylsulfoxide (DMSO), hydrochloric acid (HCl), triton X-100, trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). Aluminium chloride ($AlCl_3$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteus's phenol reagent, ascorbic acid, sodium carbonate ($Na_2CO_3$), potassium ferricyanide [$K_3Fe(CN)_6$], 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate ($K_2S_2O_8$),

2,6-di-tert-butyl-4-hydroxy toluene (BHA), butylated hydroxyltoluene (BHT), α-tocopherol, iron (III) chloride ($FeCl_3$), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), antibiotic/antimicotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B), Dulbecco's Modified Eagle's Medium-high glucose, fetal bovine serum, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), RPMI 1640 medium, sodium hydroxide (NaOH) and trypsin-ethylenediamine-tetraacetic acid (EDTA) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## 2.2. Extraction and identification of phenolic compounds

### 2.2.1. Plant material

Fruits of the *F. carica* 'Mkaousi' cultivar were harvested in the Constantine region (Algeria) in September 2017. Olives of the *Chemlal* cultivar of *Olea europaea* were harvested in Algeria in December 2017. The olives were handpicked during the harvest season and were immediately frozen in liquid nitrogen to block enzyme activities. Healthy fruits (100 g), without any physical damage or type of infection, were selected, and lyophilized. A reference specimen for each sample is deposited at the Herbarium of the Faculty of Natural and Life Sciences (Université des Frères Mentouri Constantine, Algeria). The plant samples were authenticated by Mendil, M, Director General of Technical Institute of Fruit Arboriculture and Vine (ITAF), Algeria.

### 2.2.2. Extraction procedure

The lyophilized fruits of *O. europaea* (100 g) were defatted with hexane, and then extracted for 48 h with $CHCl_3$, and MeOH, successively, to give 28.33, 2.7, and 5.37 g of the respective residues. The polyphenols from lyophilized fruits of *F. carica* were extracted by maceration using ethyl acetate to give 2.07 g. It has been demonstrated that this solvent is better than others to extract phenolics (Fki et al., 2005). Solvent was renewed 3 times and the obtained extracts were dried using a rotary evaporator.

### 2.2.3. Determination of total phenolic content (TPC)

The TPC of crude OFE and FFE was estimated spectrophotometrically using Folin-Ciocalteu method (Le et al., 2007). In brief, 200 µL of each extract were added to 1 mL of Folin-Ciolcalteu reagent (10% (*w/v*)). After 4 min, 800 µL of sodium carbonate solution (75 g/L) was added. The mixture was allowed to react for 2 h in darkness at room temperature.
After incubation, the absorbance was recorded at 765 nm using UV-Vis spectrophotometer (VIS-7220G). Gallic acid (5-200 µg/mL) was used as reference to establish the calibration curve from which the concentration of polyphenols was calculated, and the results are expressed as TPC (mg EGA/100 g extract).

### 2.2.4. Determination of total flavonoid content (TFC)

The quantification of TFC was performed by the trichloroaluminum method (Turkoglu et al., 2007). Briefly, 1 mL of $AlCl_3$ (2.0 (*w/v*)) solution was added to 1 mL of OFE or FFE. The mixture was vigorously stirred and incubated for 10 min at room temperature, and then the absorbance of each sample was read at 430 nm. Quercetin (1.25-25 µg/mL) was used to establish the calibration curve used to estimate the concentration of flavonoids found in the crude extracts. The results are given as TFC (mg EQ/100 g extract).

## 2.3. Antioxidant activity of OFE and FFE

### 2.3.1. DPPH$^{\bullet}$ (1,1-DIphenyl-2-picrylhydrazil) scavenging assay

The antiradical activity of OFE and FFE was evaluated by the free radical DPPH$^{\bullet}$ assay (Blois,1958). In brief, 25 µL of different dilutions of the extracts or standards BHA (butylated hydroxyl anisole) and BHT (butylhydroxytoluene) were added to 975 µL of DPPH (0.025 mg/mL) prepared in methanol. The obtained mixture was incubated for 30 min in obscurity. Then, the absorbances were measured using a UV-Vis spectrophotometer at 517 nm and the percentage of DPPH$^{\bullet}$ free radical-scavenging activity of each extract was calculated as follows, where I(%) is the percentage of inhibition and $A_C$ and $A_S$ are the absorbances of the control and the test sample, respectively (Eqn. 1):

$$I(\%) = [(A_C - A_S)/A_C] \times 100 \quad \text{(Eqn. 1)}$$

The results were compared to the standard antioxidant BHA and BHT.

### 2.3.2. ABTS$^{\bullet+}$ (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) scavenging assay

The ABTS$^{\bullet+}$ scavenging activity of OFE and FFE was performed spectrophotometrically (Re et al., 1999). 2 mL of the diluted ABTS cation solution was added to 1 mL of sample solution: Extracts or standards (BHT and BHA) at different concentrations (0.0156-1 mg/mL). After 30 min of incubation, the absorbance was measured at 734 nm and the percentage of ABTS scavenging effect of each extract was calculated using Eqn. 1, where I(%) is the percentage of inhibition and $A_C$ and $A_S$ are the absorbances of the control and the test sample, respectively. The results were compared to the standard antioxidant BHA and BHT.

### 2.3.3. Cupric acid reducing antioxidant capacity (CUPRAC)

Fifty µL of a solution of copper(II) chloride (10 mM) were added to 50 µL of the neocuprine solution (7.5 mM) and 60 µL of ammonium acetate buffer solution (1 M, pH = 7.0). Different concentrations of OFE, FFE and standards (BHT and BHA) were added to the initial mixture to make a final volume of 200 µL. The samples were shielded from light and the absorbance was measured at 450 nm after 1 h of incubation. The results were calculated as A 0.5 (µg/mL) (the absorbance whose inhibition is equivalent to 50%), and the reduction capacity of the extracts was compared with those of BHA and BHT as

standards (Apak et al., 2004).

#### 2.3.4. Reducing power assay

The reducing power of OFE and FFE were examined according to the previous method (Oyaizu, 1986). Accordingly, 100 µL of sample solution (extracts or references) prepared at different concentrations were added to 0.5 mL of phosphate buffer (0.2 M pH 6.6) and 0.5 mL of potassium ferricyanide (1.0 (*w/v*)). The mixture was incubated at 50 °C for 20 min. Then, 500 µL of $CCl_3COOH$ (10% (*w/v*)) was added to the mixture, which was centrifuged (10 min/3000 rpm). A volume of 500 µL of the supernatant solution was mixed with 500 µL of distilled water and 125 µL of $FeCl_3$ (1.0% (*w/v*)) freshly prepared. The absorptions were read at 700 nm and the results were calculated as A 0.5 (µg/mL) (the absorbance whose inhibition is equivalent to 50%). The reducing power of the various extracts was compared with those of ascorbic and tannic acids as standards.

### 2.4. Anticancer effect OFE and FFE

#### 2.4.1. Cell culture and treatments

PANC-1 cell line (human pancreatic epithelioid carcinoma cell line) and AsPC-1 cell line (human pancreatic ascites adenocarcinoma cell line) were obtained from the American Type Culture Collection (ATCC) and used between passage numbers 36-38. Cells were maintained in a humidified atmosphere of 5% $CO_2$-95% air and were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 10 mM sodium bicarbonate, 10 (*v/v*%) heat-inactivated fetal bovine serum and 1.0 (*v/v*%) antibiotic/antimycotic (PANC-1 cells) or in RPMI 1640 supplemented with 2 mM of L-glutamine, 10 mM of sodium bicarbonate, 1 mM of sodium pyruvate, 10 (*v/v*%) heat-inactivated fetal bovine serum and 1.0 (*v/v*%) antibiotic/antimycotic (AsPC-1 cells). Culture medium was changed every 2-3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25 (*w/v*%) trypsin-EDTA, 37 °C), split 1:4 (PANC-1) or 1:3 (AsPC-1) and sub-cultured in plastic culture dishes (21 $cm^2$; Ø 60 mm, TPP®, Trasadingen, Switerzland). For determination of migration, viability and cell proliferation, cells were seeded on 24-well plastic cell culture dishes (2 $cm^2$; Ø 16 mm; TPP®, Trasadingen, Switerzland) and were used after 7 (PANC-1 cells) or 11 days (AsPC-1 cells) (90% confluence). The OFE and FFE to be tested were dissolved in DMSO. The final concentration of the solvent in the culture medium was 1.0 (*v/v*%). Controls for the extracts were run in the presence of this solvent.

#### 2.4.2. Determination of cell viability

Cell viability was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were exposed to OFE and/or FFE (or to the solvent) for 24 h. After the treatment period, 30 µL of MTT solution (5 mg/mL) was added to each well and the cells were further incubated for 3 h at 37 °C. Afterwards, the MTT solution was removed and the cells were lysed by the addition of 200 µL of DMSO followed by plate shaking for 10 min at room temperature. Optical density (OD) for the solution in each well was determined at both 540 and 660 nm. The OD at 660 nm corresponds to unspecific light absorption and was subtracted from the OD at 540 nm to give the OD value specific to formazan crystals derived from MTT cleavage, which is proportional to the number of viable cells with active mitochondria.

#### 2.4.3. Determination of cell proliferation

Cell proliferation rate was determined by the $^3$H-thymidine incorporation assay. Briefly, cells were exposed to OFE and/or FFE (or the solvent) for 24 h, being incubated with$^3$H-thymidine 0.025 µCi/mL in the last 5 h of treatment. After removal of excess $^3$H-thymidine by a wash with 300 µL of 10% TCA (*w/v*) for 1 h at 4 °C, drying for 30 min and addition of 1 M NaOH (280 µL/well), the lysate was neutralized with 5 M HCl prior to the addition of scintillation fluid. The radioactivity of the samples was then quantified by liquid scintillometry (LKB Wallac 1209 Rackbeta, Turku, Finland). Cellular DNA synthesis rate was expressed as incorporation of $^3$H-thymidine (µCi/mg protein).

#### 2.4.4. Determination of cell migration rates

Cell migration rates were determined by a scratch injury assay. Briefly, the cell monolayers were scratched with a 10 µL pipette tip and were afterwards treated for 24 h with OFE and/or FFE (or the solvent). Images were obtained at 0 and 24 h after the scratch, and quantification was performed using the ImageJ software (NIH, Bethesda, MD, USA).

#### 2.4.5. Protein determination

The protein content of cell monolayers was determined as described by Bradford (1976), using human serum albumin as standard.

### 2.5. Calculations and statistics

Arithmetic means are given with standard error of the mean (SEM) or standard deviation (SD). The value of n indicates the number of replicates of at least 2 or 3 different replicates.

The extract/drug concentration for 50% inhibition ($IC_{50}$) was estimated by plotting percentage inhibition with respect to control against treatment concentration *in vitro* tests. $A_{0.5}$ values were determined from absorbance curves at the corresponding wavelength.

Statistical significance of the difference between various groups was evaluated by analysis of variance test followed by the Student-Newman-Keuls test. For comparison between two groups, Student's t test was used. Differences were considered significant when $p < 0.05$.

## 3. Results and Discussion

### 3.1. Total polyphenol content (TPC) and flavonoid contents (TFC) of OFE and FFE

The total phenol and flavonoid content of OFE and FFE extracts is shown in Table 1. OFE (174.7 ± 6.0 mg EGA/100 g extract) was proven to have a higher total polyphenol content when compared to FFE (83.2 ± 4.4 mg EGA/100 g extract); similarly, the total flavonoid content of OFE (59.9 ± 2.9 mg EQ/100 g extract) was higher than that of FFE (27.6 ± 1.2 mg EQ/100 g extract). The TPC of OFE reported in the present work (174.7 mg EGA/100 g) agrees with a previous determination of TPC in olive fruits of seven different cultivars of *Olea europaea,* collected in Italy (*Coratina*, *Frantoio*, *Leccino*, *Maiatica*, and *Ogliarola* cultivars) and Algeria (*Sigoise* and *Chemlal* cultivars), which ranged from 147.1 to 290.2 EGA/100 g. However, the TPC was slightly lower than the previously determined in the same olive fruit variety (272.8 EGA/100 g) (Dekdouk et al., 2015). This value is also similar to those observed in the *Chetoui* variety of olive fruits, from Tunisia, which were 313.6, 309.8, 36.1 and 83.3 mg hydroxytyrosol equivalents/100 g during the extraction with hexane, dichloromethane, ethyl acetate and methanol, respectively, although a different standard (hydroxytyrosol equivalents) was used (Brahmi et al., 2015). This variation in TPC may be related to the solvent system used (Brahmi et al., 2015) and the varieties of olive fruits (Dekdouk et al., 2015).

In the case of figs, the TPC content was 83.2 mg EGA/100 g extract, which is lower than that reported by Khadhraoui et al. (2019) for dark dried fig of *Saoudi Douiret* cultivar, but similar to that of green dried fig of *Bayoudhi Douiret* cultivar from Tunisia (TPC of 201.8 and 73.7 mg EGA/100 g, respectively). This value is also lower than those reported for dark colored (*Aberkane*, *Azandjar* and *Bouankik* varieties) and light colored (*Abiarous*, *Azegzaw*, *El-bakour*, *Taamriwth*, *Tahyounte* and *Taghanimt* varieties) dried figs from northern Algeria: the median for dark and light varieties of figs were 619 and 515 mg EGA/100g, respectively (Bachir Bey and Louaileche, 2015). Moreover, they are also lower than the TPC estimated for Iranian (1120-2682 mg EGA/100 g; Pourghayoumi et al., 2017), Indian (331.9 mg EGA/100 g; Vijaya Kumar Reddy et al. (2010) and Turkish (169.4 mg EGA/100 g; dried figs (Capanoglu, 2014). These differences in TPC could be due to many parameters such as the geographical origin, varieties, extraction conditions, and postharvest storage conditions (Bachir Bey et al., 2013; Hoxha et al., 2015).

Flavonoids, a class of polyphenolic secondary metabolites, are one of the most diverse and widespread groups of phytochemicals. These compounds constitute a potential therapeutic alternative for various diseases (Mutha et al., 2021). We verified that the TFC of OFE and FFE were 59.9 ± 2.9 and 27.6 ± 1.2 mg EQ/100 g, respectively. The flavonoid content of olive and Fig fruits are well documented in the literature. The TFC values of OFE obtained is lower than that previously reported for olive extracts of the *Chetoui* variety, from Tunisia, which were 844.6 and 131.2 mg catechin equivalents (EC)/100 g, by dichloromethane and hexane extraction, respectively (Brahmi et al. 2015). Similarly, the TFC of FFE is also slightly lower than those obtained by Bachir Bey and Louaileche (2015), which indicated TFC of dark and light dried fig varieties of 126.6 mg EQ/100 g and 87.2 mg EQ/100 g, respectively. Khadhraoui et al. (2019) reported that the TFC of dried fig extracts from South-Eastern and Middle Eastern Tunisia ranged from 58.0 mg to 112.3 mg EQ/100 g. The variation of TPC could be due to differences in Fig varieties/genotype (Caliskan and Polat, 2012), drying methods (Khadhraoui et al., 2019), solvent used and could also be related to different unit used (catechin or quercetin equivalents).

### 3.2. Antioxidant activity of OFE and FFE

The antioxidant activity of OFE and FFE was determined using different *in vitro* methods: $DPPH^{\bullet}$ free radical scavenging assay, $ABTS^{\bullet+}$ scavenging assay, CUPRAC assay and the Reducing Power assay. The results of all the tests, expressed in terms of $IC_{50}$ (µg/mL) or $A_{0.5}$, are presented in Table 2. The effectiveness of antioxidant properties is inversely correlated with their $A_{0.5}$ and $IC_{50}$ values.

$DPPH^{\bullet}$ or $ABTS^{\bullet+}$ can accept an electron or a hydrogen atom from the antioxidant compound and will thus be converted to a more stable molecule; this reduction is observed as a color switch for $DPPH^{\bullet}$ (from purple to yellow) and as a decolorization for the radical $ABTS^{\bullet+}$ (Re et al., 1999; Magalhães et al., 2006). Table 2 showed that OFE was more effective in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays ($IC_{50}$ value of 29.6 ± 0.8 and 32.4 ± 0.5 µg/mL, respectively) than FFE ($IC_{50}$ > 100 µg/mL in both assays).

In the Reducing power assay, the presence of reductants converts the oxidized form of iron ($Fe^{3+}$) in ferric chloride to ferrous ($Fe^{2+}$), which can be monitored by absorbance measurement at 700 nm (Oyaizu, 1986). The CUPRAC assay is based on the reduction of the neocuproine-copper complex, resulting in the formation of a chromogenic complex of copper-neocuproine (Cu(II)-Nc), which absorbs at 450 nm (Saci et al., 2020). The antioxidant capacity of the extracts evaluated with the CUPRAC and the Reducing power assays is presented as $A_{0.5}$ values, which were determined from absorbance curves at the corresponding wavelength (700 and 450 nm, respectively). This reduction was found to be concentration-dependent (results not shown). OFE exhibited a strong ability to reduce iron ($A_{0.5}$ value: 129.8 ± 3.7 µg/mL) and a weaker ability to reduce copper ($A_{0.5}$ value: 29.8 ± 0.9 µg/mL). FFE showed a lower capacity to reduce both iron and copper ($A_{0.5}$ values were > 200 µg/mL and > 100 µg/mL, respectively) (Table 2).

As expected, BHA and BHT, the standard references for the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and CUPRAC assays, showed a strong scavenger and reducing ability. Ascorbic acid and tannic acids, which were used as standards for the Reducing Power assay, showed also strong reducing activities (Table 2).

Due to the variable responses exerted by a specific antioxidant in various testing systems, it is important to use diverse antioxidant methods to take in account the mechanism of action of each compound (Moukette et al., 2015). The potent antioxidant activity of polyphenols and flavonoids resumes in their ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kasote et al., 2015). The methanolic extract conclusion,

**Table 1**

Total phenolic content (TPC) and total flavonoid content (TFC) of *Olea europaea* (OFE) and *Ficus carica* (FFE) fruit extracts. Total phenolics content is expressed as mg of gallic acid equivalents per 100 mg of extract (mg EGA/100 mg) and total flavonoid content as mg quercetin equivalents per 100 mg of extract (mg EQ/100 g)[a].

| Extract | TPC (mg EGA/ 100 g extract) | TFC (mg EQ/100 g extract) |
|---|---|---|
| OFE | 174.74 ± 6.03 | 59.88 ± 2.91 |
| FFE | 83.19 ± 4.42 | 27.64 ± 1.22 |

[a] Results are expressed as arithmetic means ± SD of three measurements.

**Table 2**

Antioxidant activity of *Olea europaea* (OFE) and *Ficus carica* (FFE) fruit extracts. Results are expressed as $IC_{50}$ (µg/mL) ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays) or $A_{0.5}$ (µg/mL) (CUPRAC and reducing power assays).

| Sample | $DPPH^{\bullet}$ ($IC_{50}$) | $ABTS^{\bullet+}$ ($IC_{50}$) | CUPRAC ($A_{0.5}$) | Reducing Power ($A_{0.5}$) |
|---|---|---|---|---|
| OFE | 29.55 ± 0.77 | 32.40 ± 0.45 | 29.83 ± 0.91 | 129.84 ± 3.72 |
| FFE | >100 | >100 | >100 | >200 |
| BHT | 22.32 ± 1.19 | 1.29 ± 0.30 | 9.62 ± 0.87 | nt |
| BHA | 5.73 ± 0.41 | 1.81 ± 0.10 | 3.64 ± 0.19 | nt |
| Ascorbic acid | nt | nt | nt | 6.77 ± 1.15 |
| Tannic acid | nt | nt | nt | 5.39 ± 0.91 |

The $A_{0.5}$ (Reducing power and CUPRAC assays) and the $IC_{50}$ values ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays) were calculated by linear regression analysis. Results are expressed as arithmetic means ± SD of three measurements. BHA: Butylated hydroxyl anisole, BHT: Butylhydroxytoluene. nt: Not tested.

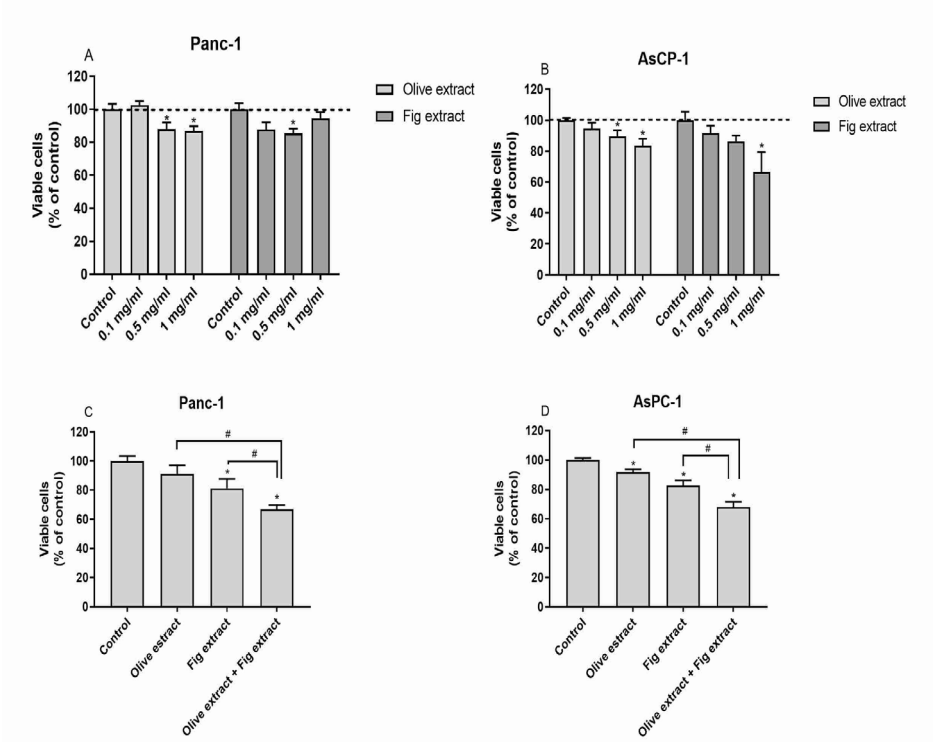



**Fig. 1.** Effects of *Olea europaea* (OFE) and/or *Ficus carica* (FFE) fruit extracts (24 h) on the viability of PANC-1 and AsPC-1 cells. (A-B) Effect of distinct concentrations of OFE and FFE (n=6); (C-D) Effect of the combination of OFE (0.5 mg/mL) and FFE (0.5 mg/mL) (n=8). Results are presented as arithmetic means ± SEM of two measurements. (A-B) * $p < 0.05$ (Student's t test); (C-D) * $p < 0.05$ *vs* control and $^{\#}$ $p < 0.05$ *vs* each other (ANOVA followed by Student-Newman-Keuls test).

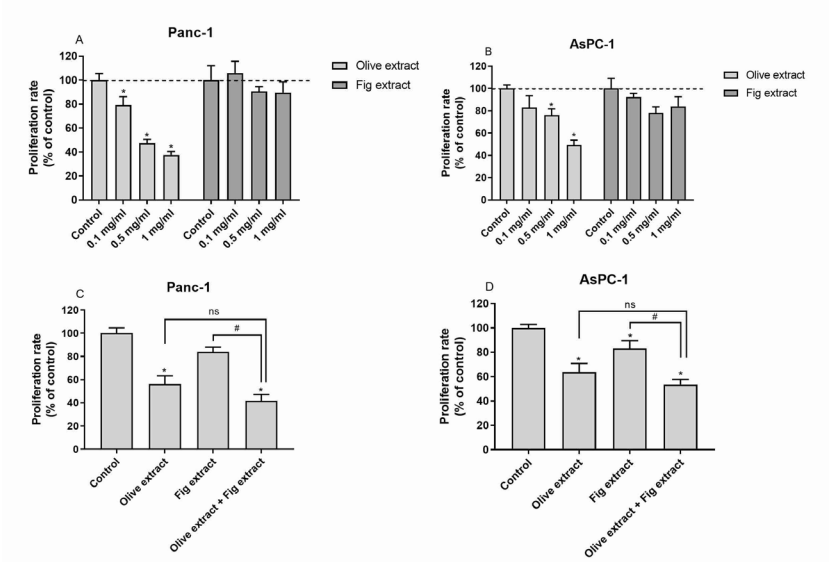



**Fig. 2**. Effects of *Olea europaea* (OFE) and/or *Ficus carica* (FFE) fruit extracts (24 h) on proliferation of PANC-1 and AsPC-1 cells. (A-B) Effect of distinct concentrations of OFE and FFE (n = 4); (C-D) Effect of combination of OFE (0.5 mg/mL) and FFE (0.5 mg/mL) (n = 6). Results are presented as arithmetic means ± SEM of two measurements. (A-B) *$p < 0.05$ (Student's t test); (C-D) * $p < 0.05$ *vs* control and $^{\#}p < 0.05$ *vs* each other (ANOVA followed by Student-Newman-Keuls test).

of olive fruit (OFE) was found to exhibit a more potent activity as a scavenger of free radicals and as a reducing agent than Fig extract (FFE). Olive fruits are known to have a potent antioxidant activity (Brahmi et al., 2015; Dekdouk et al., 2015), and the major contributors to their antioxidant activity appear to be hydroxytyrosol, tyrosol, *p*-hydroxybenzoic acid, and verbascoside (Dekdouk et al., 2015). So, the more potent antioxidant activity of OFE is most probably correlated with its higher phenolic content (Dekdouk et al., 2015; Brahmi et al., 2015).

### 3.3. Anticancer activity

#### 3.3.1. Effects of OFE and/or FFE on cell viability

As shown in Fig. 1, OFE and FFE showed promising cytotoxicity against PANC-1 and AsPC-1 cells (Fig. 1A-B). Combination of the extracts clearly increase the cytotoxic effect against both pancreatic cell lines (Fig. 1C-D).

#### 3.3.2. Effect of OFE and/or FFE on cell proliferation

OFE exhibited a marked concentration-dependent antiproliferative effect in both cell lines. In contrast, FFE caused no significant changes in proliferation rates (Fig. 2A-B). When the two extracts were combined, the inhibitory effect was equal to that caused by OFE, in both cell lines (Fig. 2C-D).

A general consensus exists on the importance of dietary habits, including the quantity and quality of food ingested, and their impact on health. Epidemiological studies have clearly established a protective effect of the Mediterranean diet against several types of cancer (Schwingshackl and Hoffmann, 2014; Saha et al., 2017; Mentella et al., 2019; Guasch-Ferré and Willett, 2021), including colorectal (Bamia et al., 2013), gastric (Buckland et al., 2010) and breast (Buckland et al., 2013) cancers.

A positive relationship between olive oil consumption and a reduction in cancer risk has been reported (Romani et al., 2019; Markellos et al., 2022). In agreement with this, our study revealed that OFE possesses a concentration-dependent cytotoxic and antiproliferative effect in both AsPC-1 and PANC-1 pancreatic cancer cell lines. These findings agree with previous reports showing a cytotoxic and/or antiproliferative effect of OFE against other cancer lines, namely hepatic (HepG2) and colorectal (Caco-2) (Maalej et al., 2017; Celano et al., 2019) and a protective effect in a rat model of hepatocellular carcinoma (Shirazi et al., 2018). Moreover, an antiproliferative effect of extracts from *Corregiola* and *Frantoio varieties* of *Olea europaea* L. leaves were reported in the MIA PaCa-2 pancreatic cancer cell line (Goldsmith et al., 2015). The underlying mechanisms by which olive oil exerts a cancer preventive effect have been attributed to its content in various bioactive natural products, specifically polyphenols. Indeed, polyphenols are known to possess several anticancer mechanisms, namely antioxidant, anti-inflammatory, anti-mutagenic, anti-proliferative, pro-apoptotic and anti-angiogenic

(Gorzynik-Debicka et al., 2018). In support of this the main phenolic compounds present in olive extracts (tyrosol, hydroxytyrosol, oleocanthal, oleuropein, rutin, quercetin and glucoside forms of luteolin) have been shown to have anticancer effects on several types of cancers. For instance, hydroxytyrosol induces apoptosis of the LS180 human colorectal carcinoma cell line (Hormozi et al., 2020). Additionally, oleocanthal has antiproliferative and cell cycle inhibitory effects in hepatocarcinoma cells *in vitro* and suppresses tumor growth in an orthotopic hepatocarcinoma model (Pei et al., 2016). It also inhibits invasiveness and tumor growth in breast cancer models (Akl et al., 2014). As to oleuropein, it decreases the number of breast cancer MCF-7 cells by inhibiting the rate of proliferation and inducing cell apoptosis (Han et al., 2009). Importantly, oleocanthal was reported to induce death of the BxPC-3 pancreatic cancer cell line (LeGendre et al., 2015) and Goldsmith et al. (2018) reported a potential antitumoral effect of oleuropein and hydroxytyrosol through reduction of proliferation, influence of cell cycle and induction of apoptosis in MIA PaCa-2 pancreatic cancer cells. However, these compounds showed no effect on other pancreatic cancer cell lines (BxPC-3 and CFPAC-1) (Goldsmith et al., 2018).

Our current study evidenced that FFE possesses a cytotoxic effect against both AsPC-1 and PANC-1 pancreatic cancer cell lines, although no significant antiproliferative effect was verified. The methanolic extract of *Ficus carica* leaves and fruits was previously shown to possess cytotoxic, pronecrotic and proapoptotic effects in Huh7it cancer liver cells (Purnamasari et al., 2019). Very interestingly, a recent study also showed that a *F. carica* fruit extract reduced viability and effectively suppressed migration, metastasis, invasion, and colony formation of PANC-1 and QGP-1 pancreatic cancer cell lines, by inducing apoptotic cell death and autophagy (Ou et al., 2022). However, in contrast to the present study, antiproliferative effects of fig extracts were observed in other cancer cell types. Namely, *F. carica* leaves extract induced anti-growth effects on triple-negative breast cancer MDA-MB-231 cells by promoting apoptosis and cell-cycle arrest at the S phase (Zhang et al., 2018), an antiproliferative effect of fig tree latex was observed on a stomach cancer cell line (Hashemi et al., 2011), and an antiproliferative and proapoptotic effect of fig leaves, whole fruit and latex extracts was observed on HCT-116 and HT-29 colorectal cancer cell lines (Soltana et al., 2019).

A synergic effect has been widely investigated between polyphenols and therapeutical drugs. For instance, quercetin promoted cisplatin-induced apoptosis *in vitro* in human oral squamous cell carcinoma cell lines by downregulating NF-κB and promoted the apoptotic effect of cisplatin *in vivo* in a mouse xenograph model (Li et al., 2019). Moreover, this same combination had a synergic inhibitory effect on cervical cancer cells via inhibition of proliferation, migration, invasion, and stimulation of apoptosis (Xu et al., 2021). In the current study, we were interested in verifying the effect of combination of the two extracts against AsPC-1 and PANC-1 cell lines. Our findings show a potent additive cytotoxic and antiproliferative effect of combination of OFE and FFE against these two cell lines. This result may be explained by the additive effect of phenolic compounds present in the two extracts, as previously reported when combining quercetin and sulforaphane (Srivastava et al., 2011), isoflavone and curcumin (Wang et al., 2008) and ferulic acid and δ-tocotrienol (Eitsuka et al., 2014).

### 3.3.3. Effect of OFE and/or FFE on cell migration

As shown in Fig. 3, OFE evidenced a concentration-dependent antimigratory effect in PANC-1 cells, but no effect on AsPC-1 migration rates was found. As for FFE, it exhibited a marked and concentration-dependent stimulatory effect on the migration rates of both cell lines (Fig. 3A-B). More interestingly, combination of the two extracts led to a complete abolition of the stimulatory effect of FFE in the two cell lines; instead, an effect very similar to that caused by OFE was observed (Fig. 3C-D).

Directional cell migration plays a key role in pivotal steps that promote tumor metastasis, such as cellular migration and invasion into the surrounding stroma. Given the fact that current chemotherapy drugs for pancreatic cancer only offer an increase in survival of up to six months (Moore et al., 2007; Park et al., 2021), the search for novel agents with an antimigratory effect on pancreatic cancer cells while displaying limited toxicity towards normal cells is thus paramount. The present data clearly demonstrate that:

(1) OFE showed a concentration-dependent antimigratory effect towards PANC-1 cells only.

(2) OFE presented a marked stimulatory effect on the migration of both cell lines.

(3) Very interestingly, combination of the two extracts resulted in a complete abolition of the stimulatory effect of FFE and instead an effect comparable to that caused by OFE was found. Although we have no evidence as to the mechanism involved in this effect of OFE, components in OFE appear to be able to abolish the stimulatory effect of FFE on cell migration.

Several studies have tested the ability of crude extracts rich in phenolic compounds or isolated phenolic compounds in inhibiting migration of pancreatic cancer cells *in vitro* and *in vivo*. Fahrioğlu et al. (2016) reported that ferulic acid exhibits an anticancer effect by inhibiting migration of MIA PaCa-2 pancreatic cancer cells. Similarly, kaempferol effectively inhibited the migratory activity of MIA PaCa-2, PANC-1, and SNU-213 human pancreatic cancer cell lines at relatively low concentrations (Lee and Kim, 2016). From our findings, we believe that the use of OFE, either alone or in combination with pro-migratory agents, can inhibit pancreatic cell migration, which is very interesting.

## 4. Concluding remarks

Collectively, this report demonstrates that OFE, which is very rich in bioactive polyphenolic compounds, possesses anticancer effects against PANC-1 and AsPC-1 pancreatic cancer cell lines. It exhibits cytotoxic, antiproliferative and antimigratory effects. In contrast, FFE exhibits a cytotoxic effect in these two cell lines,

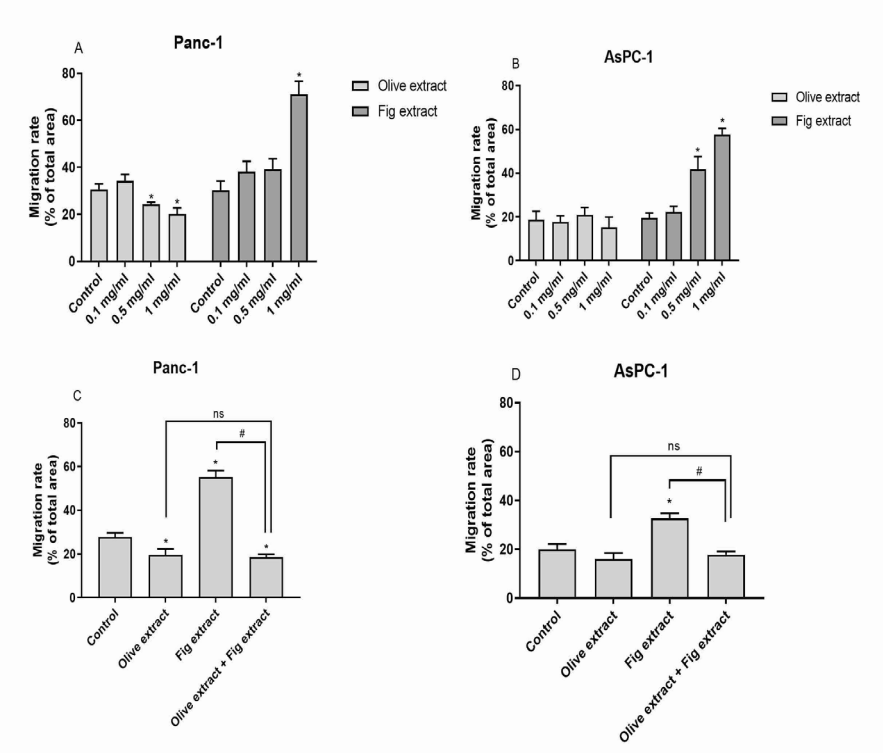



**Fig. 3.** Effects of *Olea europaea* (OFE) and/or *Ficus carica* (FFE) fruit extracts (24 h) on the migration capacity of PANC-1 and AsPC-1 cells. (A-B) Effect of distinct concentrations of OFE and FFE (n = 6); (C-D) Effect of combination of OFE (0.5 mg/mL) and FFE (0.5 mg/mL) (n=8). Results are presented as arithmetic means ± SEM of two measurements. (A-B) *$p < 0.05$ (Student's t test); (C-D) *$p < 0.05$ *vs* control and $^{\#}$ $p < 0.05$ *vs* each other (ANOVA followed by Student-Newman-Keuls test).

but it does not have an antiproliferative effect and even stimulates the migration of these cells. The combination of OFE and FFE led to an increase in the cytotoxic effect in both cell lines and the disappearance of the promigratory effect of FFE. Thus, the combination of these extracts in fact exerts a more interesting anti-cancer effect than either of the extracts alone.

This report also demonstrates that OFE and FFE exhibit different antioxidant properties in response to different models, namely DPPH$^{\bullet}$, ABTS$^{\bullet+}$, CUPRAC and Reducing Power models, which may be related to their different polyphenol and flavonoid content. More importantly, the higher phenolic and flavonoid content and antioxidant ability of OFE, in relation to FFE, correlates with a more prominent anticancer effect. Because the biological activity of polyphenols is strongly related to their antioxidant properties (Gorzynik-Debicka et al., 2018), we suggest that the anticancer effect of the extracts results from their antioxidant properties.

This study could lead to new research on pancreatic cancer and the consumption of olive and fig products, and place OFE and FFE/ phenolic compounds as good candidates for further molecular studies aimed at finding a better, and more effective treatment approach for resistant pancreatic cancer. In future work, it would be very interesting to confirm the results of this study in other human pancreatic cancer cell lines, and also in animal pre-clinical models. Moreover, the mechanisms involved in the positive interaction between OFE and FFE should be further explored.

## List of abbreviations

**ABTS**: 2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid; **AsPC-1 Cell Line**: Human Pancreatic Ascites Adenocarcinoma Cell Line; **ATCC**: American Type Culture Collection; **BHA**: 2,6-Di-*tert*-butyl-4-hydroxy toluene; **BHT**: Butylated Hydroxyltoluene; **CUPRAC**: Cupric Acid Reducing Antioxidant Capacity; **DMSO**: Dimethylsulfoxide; **DPPH$^{\bullet}$**: (1,1-Diphenyl-2-picrylhydrazil) Scavenging Assay; **FFE**: *Ficus carica* Extract; HEPES: *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic Acid; **MTT**: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; OFE: *Olea europaea* Extract; **PANC-1 Cell Line**: Human Pancreatic Epithelioid Carcinoma Cell Line; **SD**: Standard Deviation; **SEM**: Standard Error of the Mean; **TFC**: Total Flavonoid Content; **TPC**: Total Phenolic Content.

## Author contribution statement

All authors contributed to the study's conception and

design. Nadia Dekdouk, Souad Ameddah, Chawki Bensouici, Meghboun Ibtissem and Menad Ahmed performed material preparation, experiments, data collection, and analysis. Nadia Dekdouk and Fátima Martel wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Conflicts of interest

The authors declare that there is no conflict of interest.

## Acknowledgments

Nadia Dekdouk thanks very much the Department de Microbiologie et Biochemie-Faculté des Sciences de la Nature et de la Vie-Université Chahid Mostafa Benboulaid-Batna2, Algérie, for the internship granted to the University of Porto, Porto, Portugal.

## References

Akl, M.R., Ayoub, N.M., Mohyeldin, M.M., Busnena, B.A., Foudah, A.I., Liu, Y.Y., Sayed, K.A.E., 2014. Olive phenolics as c-Met inhibitors:(-)-Oleocanthal attenuates cell proliferation, invasiveness, and tumor growth in breast cancer models. PLoS One 9, e97622.

Al-Salman, H.N.K., Ali, E.T., Jabir, M., Sulaiman, G.M., Al-Jadaan, S.A.S., 2020. 2-Benzhydrylsulfnyl-*N*-hydroxyacetamide-Na extracted from fig as a novel cytotoxic and apoptosis inducer in SKOV-3 and AMJ-13 cell lines viaP53 and caspase-8 pathway. Eur. Food Res. Technol. 246, 1591-1608.

Apak, R., Güçlü, K., Özyürek, M., Çelik, S.E., 2008. Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. Microchim. Acta 160, 413-419.

Arvaniti, O.S., Samaras, Y., Gatidou, G., Thomaidis,N.S., Stasinakis, A.S., 2019. Review on fresh and dried figs: Chemical analysis and occurrence of phytochemical compounds, antioxidant capacity and health effects. Food Res. Int. 119, 244-267.

Baby, J., Justin, S.R., 2011. Pharmacognostic and phytochemical properties of *Ficus carica*. Linn. Int. J. Pharm. Tech. Res. 3, 8-12.

Bachir Bey, M., Louaileche, H., 2015. A comparative study of phytochemical profile and *in vitro* antioxidant activities of dark and light dried fig (*Ficus carica* L.) varieties. J. Phytopharmacol. 4, 41-48.

Baldo, B.A., Pham, N.H., 2013. Adverse reactions to targeted and non-targeted chemotherapeutic drugs with emphasis on hypersensitivity responses and the invasive metastatic switch. Cancer Metastasis Rev. 32, 723-761.

Bamia, C., Lagiou, P., Buckland, G., Grioni, S., Agnoli, C., Taylor, A.J., Dahm, C.C., Overvad, K., Olsen, A., Tjønneland, A., Cottet, V., Boutron-Ruault, M.-C., Morois, S., Grote, V., Teucher, B., Boeing, H., Buijsse, B., Trichopoulos, D., Adarakis, G., Tumino, R., Naccarati, A., Panico, S., Palli, D., Bueno-de-Mesquita, H.B., van Duijnhoven, F.J.B., Peeters, P.H.M., Engeset, D., Skeie, G., Lund, E., Sánchez, M.-J., Barricarte, A., Huerta, J.-M., Ramón Quirós, J., Dorronsoro, M., Ljuslinder, I., Palmqvist, R., Drake, I., Key, T.J., Khaw, K.-T., Wareham, N., Romieu, I., Fedirko, V., Jenab, M., Romaguera, D., Norat, T., Trichopoulou, A., 2013. Mediterranean diet and colorectal cancer risk: results from a European cohort. Eur. J. Epidemiol. 28, 317-328.

Bar-Zeev, M., Livney, Y.D., Assaraf, Y.G., 2017. Targeted nanomedicine for cancer therapeutics: Towards precision medicine overcoming drug resistance. Drug Resist. Updat. 31, 15-30.

Basha, R., Connellyd, S.F., Sankpala, U.T., Purnachandra, N.G., Patel, H., Vishwanathaa, J.K., Shelakea, S., Tabor-Simecka, L., Shoji, M, Simecka, J.W., El-Rayes, B., 2016. Small molecule tolfenamic acid and dietary spice curcumin treatment enhances antiproliferative effect in pancreatic cancer cells via suppressing Sp1, disrupting NF-kB translocation to nucleus and cell cycle phase distribution. J. Nutr. Biochem. 31, 77-87.

Blois, M.S., 1958. Antioxidants determination by the use of a stable free radical. Nature. 26, 1199-1200.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brahmi, F., Mechri, B., Dhibi, M., Hammami, M., 2015. Antioxidant capacity and phenolic content of olive fruits from Tunisia, extracted with different solvents. J. Plant Sci. 3, 8-12.

Buckland ,G., Travier, N., Cottet, V., González, C.A., Luján-Barroso, L., Agudo, A., Trichopoulou, A., Lagiou, P., Trichopoulos, D., Peeters, P.H., May, A., Bueno-de-Mesquita, H.B., Bvan Duijnhoven, F.J., Key, T.J., Allen, N., Khaw, K.T., Wareham, N., Romieu, I., McCormack, V., Boutron-Ruault, M., Clavel-Chapelon, F., Panico, S., Agnoli, C., Palli, D., Tumino, R., Vineis, P., Amiano, P., Barricarte, A., Rodríguez, L., Sanchez, M.J., Chirlaque, M.D., Kaaks, R., Teucher, B., Boeing, H., Bergmann, M.M., Overvad, K., Dahm, C.C., Tjønneland, A., Olsen, A., Manjer, J., Wirfält, E., Hallmans, G., Johansson, I., Lund, E., Hjartåker, A., Skeie, G., Vergnaud, A.C., Norat, T., Romaguera, D., Riboli, E., 2013. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. Int. J. Cancer 132, 2918-2927.

Buckland, G, Agudo A., Luján L., Jakszyn P., Bueno-de-Mesquita, H.B., Palli, D., Boeing, H., Carneiro, F., Krogh, V., Sacerdote, C., Tumino, R., Panico, S., Nesi, G., Manjer, J., Regnér, S., Johansson, I., Stenling, R., Sanchez, M.-J., Dorronsoro, M., Barricarte, A., Navarro, C., Quirós, J.R., Allen, N.E., Key, T.J., Bingham, S., Kaaks, R., Overvad, K., Jensen, M., Olsen, A., Tjønneland, A., Peeters, P.H.M., Numans, M.E., Ocké, M.C., Clavel-Chapelon, F., Morois, S., Boutron-Ruault, M.-C., Trichopoulou, A., Lagiou, P., Trichopoulos, D., Lund, E., Couto, E., Boffeta, P., Jenab, M., Riboli, E., Romaguera, D., Mouw, T., González, C.A., 2010. Adherence to a mediterranean diet and risk of gastric adenocarcinoma within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study. Am. J. Clin. Nutr. 91, 381-390.

Caliskan, O., Polat, A.A., 2012. Effects of genotype and harvest year on phytochemical and fruit quality properties of Turkish fig genotypes. Span. J. Agric. Res. 10, 1048-1058.

Capanoglu, E., 2014. Investigating the antioxidant potential of Turkish dried fruits. Int. J. Food Prop. 17, 690-70.
Carrera-González, M.P., Ramírez-Expósito, M.J., Mayas, M.D., Martínez-Martos, J.M., 2013. Protective role of oleuropein and its metabolite hydroxytyrosol on cancer. Trends Food Sci. Technol. 31, 92-99.
Celano, M., Maggisano, V., Lepore, S.M., Russo, D., Bulotta, S., 2019. Secoiridoids of olive and derivatives as potential coadjuvant drugs in cancer: A critical analysis of experimental studies. Pharmacol. Res. 142, 77-86.
Cruz, J.M.A., Corrêa, R.F., Lamarão, C.V., Kinupp, V.F., Sanches, E.A., Campelo, P.H., Bezerra, J.A., 2022. *Ficus* spp. fruits: Bioactive compounds and chemical, biological and pharmacological properties. Food Res. Int. 152, 110928.
Dekdouk, N., Malafronte, N., Russo, D., Faraone, I., De Tommasi, N., Ameddah, S., Severino, L., Milella, L., 2015. Phenolic compounds from *Olea europaea* L. possess antioxidant activity and inhibit carbohydrate metabolizing enzymes *in vitro*. Evid. Based Complementary Altern. Med. 2015, 684925.
Dhar, D., Raina, K., Agarwal, R., 2018. Mechanisms and drug targets for pancreatic cancer chemoprevention. Curr. Med. Chem. 25, 2545-2565.
Eitsuka, T., Tatewaki, N., Nishida, H., Kurata, T., Nakagawa, K., Miyazawa, T., 2014. Synergistic inhibition of cancer cell proliferation with a combination of δ-tocotrienol and ferulic acid. Biochem. Biophys. Res. Commun. 453, 606-11.
Fabiani, R., De Bartolomeo, A., Rosignoli, P., Servili, M., Montedoro, G.F., Morozzi, G., 2002. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur. J. Cancer Prev. 11, 351-358.
Fahrioğlu, U., Dodurga, Y., Elmas, L., Seçme, M., 2016. Ferulic acid decreases cell viability and colony formation while inhibiting migration of MIA PaCa-2 human pancreatic cancer cells *in vitro*. Gene 576, 476-82.
Fki, I., Bouaziz, M., Sahnoun, Z., Sayadi, S., 2005. Hypocholesterolemic effects of phenolic-rich extracts of Chemlali olive cultivar in rats fed a cholesterol-rich diet. Bioorg. Med. Chem. 13, 5362-70.
Ghandehari, F., Fatemi, M., Avicenna, J., 2018. The effect of *Ficus carica* latex on 7,12-dimethylbenz (a) anthracene-induced breast cancer in rats. Avicenna Phytomed. 8, 286-295.
Gianfredi, V., Ferrara, P., Dinu, M., Nardi, M., Nucci, D., 2022. Diets, dietary patterns, single foods and pancreaticcancerrisk:anumbrellareview of meta-analyses. Int. J. Environ. Res. Public Health 19, 14787.
Goldsmith, C.D., Bond, D.R., Jankowski, H., Weidenhofer, J., Stathopoulos, C.E., Roach, P.D., Scarlett, C.J., 2018. The olive biophenols oleuropein and hydroxytyrosol selectively reduce proliferation, influence the cell cycle, and induce apoptosis in pancreatic cancer cells. Int. J. Mol. Sci. 19, 1937.
Goldsmith, C.D., Vuong, Q.V., Sadeqzadeh, E., Stathopoulos, C.E., Roach, P.D., Scarlett, C.J., 2015. Phytochemical properties and anti-proliferative activity of *Olea europaea* L. leaf extracts against pancreatic cancer cells. Molecules 20, 12992-13004.
Gorzynik-Debicka, M., Paulina Przychodzen, P., Cappello, F., Kuban-Jankowska, A., Gammazza, A.M., Knap, N., Wozniak, M., Gorska-Ponikowska, M., 2018. Potential health benefits of olive oil and plant polyphenols. Int. J. Mol. Sci. 19, 686.
Greenwell, M., Rahman, P.K.S.M., 2015. Medicinal plants: Their use in anticancer treatment. Int. J. Pharm. Sci. Res. 6, 4103.
Guarrera, P.M., 2005. Traditional phytotherapy in central Italy (Marche, Abruzzo, and Latium). Fitoterapia 76, 1-25.
Guasch-Ferré, M., Willett, W.C., 2021. The Mediterranean diet and health: a comprehensive overview J. Intern. Med. 290, 549-566.
Guo, Z., Jia, X., Zheng, Z, Lu, X., Zheng, Y., Zheng, B., Xiao, J., 2018. Chemical composition and nutritional function of olive (*Olea europaea* L.): A review. Phytochem. Rev. 17, 1091-1110.
Han, J., Talorete, T.P., Yamada, P., Isoda, H., 2009. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology 59, 45-53.
Hashemi, S.A., Abediankenari, S., Ghasemi, M., Azadbakht, M., Yousefzadeh, Y., Dehpour, A.A., 2011. The effect of fig tree latex (*Ficus carica*) on stomach cancer line. Iran Red Crescent Med. J. 13, 272-5.
Hormozi, M., Marzijerani, A.S., Baharvand, P., 2020. Effects of hydroxytyrosol on expression of apoptotic genes and activity of antioxidant enzymes in LS180 cells. Cancer Manag. Res. 12, 7913-7919.
Ijoma, K.I., Ajiwe, V.I.E., Odinma, S.C., 2023. The organic extracts from the leaves of *Ficus thonningii* Blume, *Jatropha tanjorensis* J.L Ellis and Saroja and *Justicia carnea* Lindley as potential nutraceutical antioxidants and functional foods. Trends Phytochem. Res. 7(1), 76-85.
Kasote, D.M., Katyare, S.S., Hegde, M.V., Bae, H., 2015. Significance of antioxidant potential of plants and its relevance to therapeutic applications. Int. J. Biol. Sci. 11, 982-991.
Kazeminia, M., Mehrabi, A., Mahmoudi, R., 2022. Chemical composition, biological activities, and nutritional application of Asteraceae family herbs: A systematic review. Trends Phytochem. Res. 6(3), 187-213.
Khadhraoui, M., Bagues, M., Artés, F., Ferchichi, A., 2019. Phytochemical content, antioxidant potential, and fatty acid composition of dried Tunisian fig (*Ficus carica* L.) cultivars. J. Appl. Bot. Food Qual. 92, 143-150.
Khan, T., Ali, M., Khan, A., Nisar, P., Jan, S.A., Afridi, S., Shinwari, Z.K., 2019. Anticancer plants: A review of the active phytochemicals, applications in animal models, and regulatory aspects. Biomolecules 10, 47.
Kountouri, A.M., Kaliora, A.C., Koumbi, L., Andrikopoulos, N.K., 2009. *In-vitro* gastric cancer prevention by a polyphenol-rich extract from olives through induction of apoptosis. Eur. J. Cancer Prev. 18, 33-39.
Le, K., Chiu, F., Ng, K., 2007. Identification and quantification of antioxidants in *Fructus lycii*. Food Chem. 105, 353-563.
Lee, J., Kim, J.H., 2016. Kaempferol inhibits pancreatic cancer cell growth and migration through the blockade of EGFR-related pathway *in vitro*. PLoS ONE 11, e0155264.

LeGendre, O., Breslin, P.A.S., Foster, D.A., 2015. (-)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization. Mol. Cell. Oncol. 2, e1006077.

Li, X., Guo, S., Xiong, X.K., Peng, B.Y., Huang, J.M., Chen, M.F., Wang, F.Y., Wang, J.N., 2019. Combination of quercetin and cisplatin enhances apoptosis in OSCC cells by downregulating xIAP through the NF-κB pathway. J. Cancer 10(19), 4509-4521.

Lopez-Miranda, J., Perez-Jimenez, F., Ros, E, De Caterina, R., Badimón, L., Covas, M.I., Escrich, E., Ordovás, J.M., Soriguer, F., Abiá, R., Alarcón de la Lastra, C., Battino, M., Corella, D., Chamorro-Quirós, J., Delgado-Lista, J., Giugliano, D., Esposito, K., Estruch, R., Fernandez-Real, J.M., Gaforio, J.J., La Vecchia, C., Lairon, D., López-Segura, F., Mata, P., Menéndez, J.A., Muriana, F.J., Osada, J., Panagiotakos, D.B., Paniagua, J.A., Pérez-Martinez, P., Perona, J., Peinado, M.A., Pineda-Priego, M., Poulsen, H.E., Quiles, J.L., Ramírez-Tortosa, M.C., Ruano, J., Serra-Majem, L., Solá, R., Solanas, M., Solfrizzi, V., de la Torre-Fornell, R., Trichopoulou, A., Uceda, M., Villalba-Montoro, J.M., Villar-Ortiz, J.R., Visioli, F., Yiannakouris, N., 2010. Olive oil and health: Summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. Nutr. Metab. Cardiovasc. Dis. 20, 284-294.

Maalej, A., Bouallagui, Z., Hadrich, F., Isoda, H., Sayadi, S., 2017. Assessment of *Olea europaea* L. fruit extracts: Phytochemical characterization and anticancer pathway investigation. Biomed. Pharmacother. 90, 179-186.

Magalhães, L.M., Santos, M., Segundo, M.A., Reis, S., Lima, J.L.F.C., 2006. Automatic method for determination of total antioxidant capacity using 2,2-diphenyl-1-picrylhyrazyl assay. Anal. Chim. Acta. 558, 310-318.

Mahdavi, B., Mohammadhosseini, M., 2022. Antioxidant, antimicrobial and anti-prostate cancer activity of the extracts from different parts of *Etlingera velutina* (Ridl.) R. M. Sm (Zingiberaceae). Trends Phytochem. Res. 6(4), 353-362.

Markellos, C., Ourailidou, M.E., Gavriatopoulou, M., Halvatsiotis, P., Sergentanis, T.N., Psaltopoulou, T., 2022. Olive oil intake and cancer risk: a systematic review and meta-analysis. PLoS One 17, e0261649.

Mentella, M.C., Scaldaferri, F., Ricci, C., Gasbarrini, A., Miggiano, G.A.D., 2019. Cancer and Mediterranean diet. Nutrients. 11, 2059.

Mohammadhosseini, M., Frezza, C., Venditti, A., Akbarzadeh, A., 2019a. Ethnobotany and phytochemistry of the genus *Eremostachys* Bunge. Curr. Org. Chem. 23, 1828-1842.

Mohammadhosseini, M., Frezza, C., Venditti, A., Sarker, S., 2021. A systematic review on phytochemistry, ethnobotany and biological activities of the genus *Bunium* L. Chem. Biodivers. 18(11), e2100317.

Mohammadhosseini, M., Venditti, A., Akbarzadeh, A., 2019b. The genus *Perovskia* Kar.: Ethnobotany, chemotaxonomy and phytochemistry: A review. Toxin Rev. 40(4), 484-505.

Moore, M.J., Goldstein, D., Hamm, J., Figer, A., Hecht, J.R., Gallinger, S., Au, H.J., Murawa, P., Walde, D., Wolff, R.A., Campos, D., Lim, R., Ding, K., Clark, G., Voskoglou-Nomikos, T., Ptasynski, M., Parulekar, W.; National Cancer Institute of Canada Clinical Trials Group., 2007. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J. Clin. Oncol. 25, 1960-1966.

Morovati, M.R., Ghanbari-Movahed, M., Barton, E.M., Farzaei, M.H., Bishayee, A., 2022. A systematic review on potential anticancer activities of *Ficus carica* L. with focus on cellular and molecular mechanisms. Phytomedicine 105, 154333.

Moukette, B.M., Pieme, C.A., Njimou, J.R., Biapa, C.P.N., Marco, B., Ngogang, J.Y., 2015. *In vitro* antioxidant properties, free radicals scavenging activities of extracts and polyphenol composition of a non-timber forest product used as spice: *Monodora myristica*. Evidence-Based Complement. Alternat. Med. 48, 1-17.

Mutha, R.E., Tatiya, A.U., Surana, S.J., 2021. Flavonoids as natural phenolic compounds and their role in therapeutics. Futur. J. Pharm. Sci. 7, 25.

Nguyen, L.T., Lee, Y.-H., Sharma, A.R., Park, J.-B., Jagga, S., Sharma, G., et al., 2017. Quercetin induces apoptosis and cell cycle arrest in triple-negative breast cancer cells through modulation of Foxo3a activity. Korean J. Physiol. Pharmacol. 21, 205-213.

Obafemi, F.A., Besong, E.E., 2023. A review of plants with anticancer properties. J. Clin. Med. Res. 5, 138-161.

Olaoluwa, O., Taiwo, O., Nahar, L., Sarker, S.D., 2022. Ethnopharmacology, phytochemistry and biological activities of selected African species of the genus *Ficus*. Trends Phytochem. Res. 6, 46-69.

Ou, A., Zhao, X., Lu, Z., 2022. Autophagy is involved in *Ficus carica* fruit extract-induced anti-tumor effects on pancreatic cancer. Biomed. Pharmacother. 150, 112966.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Spiegelhalder, B., Bartsch, H., 2000a. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur. J. Cancer. 36, 1235-1247.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Würtele, G., Spiegelhalder, B., Bartsch, H., 2000b. Olive oil consumption and health: The possible role of antioxidants. Lancet Oncol. 1, 107-112.

Owen, R.W., Haubner, R., Wurtele, G., Hull, E., Spiegelhalder, B., Bartsch, H., 2004. Olives and olive oil in cancer prevention. Eur. J. Cancer Prev. 13, 319-326.

Oyaizu, M., 1986. Studies on products of browning reactions: antioxidative activities of browning reaction prepared from glucosamine. Jap. J. Nutr. 44, 307-315.

Park, W., Chawla, A., O'Reilly, E.M., 2021. Pancreatic cancer: a review. JAMA 326, 851-862.

Pei, T., Meng, Q., Han, J., Sun, H., Li, L., Song, R., Sun, B., et al., 2016. (-)-Oleocanthal inhibits growth and metastasis by blocking activation of STAT3 in human hepatocellular carcinoma. Oncotarget. 7, 43475-43491.

Popwo Tameye, S.C., Ndom, J.C., Nguemfo, E.L., Wansi, J.D., Vardamides, J.C., Azebaze, A.G.B., 2020. Ficusanol, a new cinnamic acid derivative and other constituents from the roots of *Ficus exasperata* Vahl. (Moraceae) with antioxidant and cytotoxic activities. Trends Phytochem. Res. 4, 3-8.

Pourghayoumi, M., Bakhshi, D., Rahemi, M., Noroozisharaf, A., Jafari, M., Salehi, M., Chamane, R., Hernandez, F., 2017. Phytochemical attributes of some dried fig (*Ficus carica* L.) fruit cultivars grown in Iran. Agric. Conspec. Sci. 3, 161-166.

Purnamasari, R., Winarni, D., Permanasari, A.A., Agustina, E., Hayaza, S., Win, D., 2019. Anticancer activity of methanol extract of *Ficus carica* leaves and fruits against proliferation, apoptosis, and necrosis in Huh7it cells. Cancer Inform. 18, 1-7.

Rauf, A., Imran, M., Khan, I.A., Ur-Rehman, M., Gilani, S.A., Mehmood, Z., Mubarak, M.S., 2018. Anticancer potential of quercetin: A comprehensive review. Phytother. Res. 32, 2109-2130.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26, 1231-1237.

Romani, A., Ieri, F., Urciuoli, S., Noce, A., Marrone, G., Nediani, C., Bernini, R., 2019. Health effects of phenolic compounds found in extra-virgin olive oil, by-products, and leaf of *Olea europaea* L. Nutrients 11, 1776.

Saci, F., Bachir Bey, M., Louaileche, H., Gali, L., Bensouici, C., 2020. Changes in anticholinesterase, antioxidant and related bioactive compounds of carob pulp (*Ceratonia siliqua L.*) during ripening stage. J. Food Measur. Character. 14, 937-945.

Saha, S.K., Lee, S.B., Won, J., Choi, H.Y., Kim, K., Yang, G.-M., Dayem, A.A., Cho, S.-G., 2017. Correlation between oxidative stress, nutrition, and cancer initiation. Int. J. Mol. Sci.18, 1544.

Schwingshackl, L., Hoffmann, G., 2014. Adherence to Mediterranean diet and risk of cancer: A systematic review and meta-analysis of observational studies. Int. J. Cancer. 135, 1884-1897.

Selvarajoo, N., Stanslas, J., Islam, M.K., Sagineedu, S.R., Lian, H.K., Lim, J.C.W., 2022. Pharmacological modulation of apoptosis and autophagy in pancreatic cancer treatment. Mini Rev. Med. Chem. 22, 2581-2595.

Sepporta, M.V., Fuccelli, R., Rosignoli, P., Ricci, G., Servili, M., Morozzi G., Fabiani, R., 2014. Oleuropein inhibits tumour growth and metastases dissemination in ovariectomised nude mice with MCF-7 human breast tumour xenografts. J. Funct. Foods. 8, 269-273.

Shirazi, F.H., Piri, M., Keshavarz, S., Gholamib, S., Hosseinic, S.H., Noubaranib, M., Andalibb, S., Kamalinejad, M., Adiban, H., Eskandari, M.R., 2018. Olive fruit (*Olea europaea* L.): Chemopreventive effect in the rat model of hepatocellular carcinoma. PharmaNutrition 6, 207-214.

Siegel, R.L., Miller, K.D., Jemal, A., 2016. Cancer statistics. CA Cancer J. Clin. 66, 7-30.

Singh, D., Upadhyay, G., Srivastava, R.K., Shankar, S., 2015. Recent advances in pancreatic cancer: Biology, treatment, and prevention. Biochim. Biophys. Acta. 1856, 13-27.

Solomon, A.S., Golubowicz, Z., Yablowicz, S., Grossman, M., Bergman, H.E., Gottlieb, A., Altman, Z., Kerem, M.A., 2006. Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). J. Agric. Food Chem. 20, 7717-7723.

Soltana, H., Pinon, A., Limami, Y., Zaid, Y., Khalki, L., Zaid, N., Salah, D., Sabitaliyevich, U.Y., Simon, A., Liagre, Bertrand., Hammami, M., 2019. Antitumoral activity of *Ficus carica* L. on colorectal cancer cell lines. Cell. Mol. Biol. 65, 6-11.

Srivastava, R.K., Tang, S.N., Zhu, W., Meeker, D., Shankar, S., 2011. Sulforaphane synergizes with quercetin to inhibit self-renewal capacity of pancreatic cancer stem cells. Front. Biosci. 3, 515-28.

Turkoglu, A., Duru, M.E., Mercan, N., Kivrak, I., Gezer, K., 2007. Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. Food Chem. 101, 267-273.

Vendrely, V., Peuchant, E., Buscail, E., Moranvillier, I., Rousseau, B., Bedel, A., Brillac, A., de Verneuil, H., Moreau-Gaudry, F., Dabernat, S., 2017. Resveratrol and capsaicin used together as food complements reduce tumor growth and rescue full efficiency of low dose gemcitabine in a pancreatic cancer model. Cancer Lett. 390, 91-102.

Vijaya Kumar Reddy, C., Sreeramulu, D., Raghunath, M., 2010. Antioxidant activity of fresh and dry fruits commonly consumed in India. Food Res. Int. 43, 285-288.

Wang, Z., Desmoulin, S., Banerjee, S., Kong, D., Li, Y., Deraniyagala, R.L., Abbruzzese, J., Sarkar, F.H., 2008. Synergistic effects of multiple natural products in pancreatic cancer cells. Life Sci. 83, 293-300.

Xu, W., Xie, S., Chen, X., Pan, S., Qian, H., Zhu, X., 2021.Effects of quercetin on the efficacy of various chemotherapeutic drugs in cervical cancer cells. Drug Des. Devel. Ther. 15, 577-588.

Zhang, Y., Wan, Y., Huo, B., Li, B., Jin, Y., Hu, X., 2018. Extracts and components of *Ficus carica* leaves suppress survival, cell cycle, and migration of triple-negative breast cancer MDA-MB-231 cells. OncoTargets Ther.11, 4377-4386.

Zheng, J., Guinter, M.A., Merchant, A.T., Wirth, M.D., Zhang, J., Stolzenberg-Solomon, R.Z., Steck, S.E., 2017. Dietary patterns and risk of pancreatic cancer: A systematic review. Nutr. Rev. 75, 883-908.

Trends Phytochem. Res. 8(2) 2024 131-140

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Exploring the biological potential of *Cruciata taurica* (Pall. ex Willd.) Ehrend.: Evaluating antibacterial, antioxidant, antidiabetic, and cytotoxic properties

Shiva Khalil-Moghaddam[1]*, Roya Moghimi[2], Thomas Efferth[3], And Sajedeh Mousavian[4]

[1]Department of Biology, Yadegar-e-Imam Khomeini (RAH) Shahre Rey Branch, Islamic Azad University, Tehran, Iran
[2]Department of Organic Chemistry, Faculty of Chemistry, University of Mazandaran, Babolsar, Iran
[3]Department of Pharmaceutical Biology, Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg University Mainz, Germany
[4]Departmant of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

**ABSTRACT**

This study focused on the biological properties of *Cruciata taurica*, a plant found in the Irano-Turanian region, which has not been extensively studied in terms of its phytochemical and biological activities. The methanolic extract of the plant was obtained and its cytotoxic, antibacterial, antidiabetic, and antioxidant properties were investigated. The antibacterial activity of the fractions was evaluated using broth microdilution method, while the antioxidant properties were assessed through the DPPH assay and the cytotoxic properties were determined using the RRA assay. This study revealed that the residual aqueous fraction exhibited the highest extraction yield and the most potent antioxidant activity. The EtOAc fraction showed strong antibacterial activity against certain bacteria, while the *n*-hexane fraction exhibited greater antidiabetic effects. Additionally, the EtOAc fraction was found to be the most cytotoxic. Overall, the study suggested that *C. taurica* has potential therapeutic applications due to its various biological properties.

**ARTICLE HISTORY**

Received: 06 February 2024
Revised: 29 May 2024
Accepted: 17 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Bioactivity
Cancer
*Cruciata taurica* (Pall. ex Willd.) Ehrend.
Cytotoxic
Ethnopharmacology
Multidrug resistance
Plant extract

## 1. Introduction

The Rubiaceae family, also known as the Madder family, Bedstraw family, or coffee family, is named after the Madder genus *Rubia*. Currently, the Rubiaceae family comprises approximately 637 genera and over 13,000 species, making it one of the six largest angiosperm families in terms of both genus and species diversity (Wooster et al., 2008; Avoseh et al., 2020; Nyobe et al., 2020; Wonkam et al., 2022). It shares this distinction with the Asteraceae, Orchidaceae, Fabaceae, Poaceae, and Euphorbiaceae families (Bremer and Eriksson, 2009). Rubiaceae species are predominantly found in warmer and tropical regions across the globe. This family exhibits a wide range of growth forms, with shrubs being the most common, although its members can also be trees, lianas, or herbs. While the majority of species are woody, less than 20% of the genera are herbaceous (Karou et al., 2011). The *Cruciata* genus includes only nine species (Tava et al., 2020). *Cruciata* genus has central hermaphrodite and lateral male flowers (Huysmans et al., 2003). The species within the *Cruciata* genus have diverse applications in traditional medicine. For instance, *C. laevipes* has been traditionally recognized for its wound-healing properties, has historically been used for both external and internal purposes (De Rosa et al., 2003). The internal uses of this herbal species include treatment of stomach and bowel obstructions, appetite stimulation, rheumatism, and dropsy (Tava et al., 2020). *C. taurica* is distributed in Crimea, East Greece, Southwest Asia, and Iran (De Rosa et al., 2003). This plant is a small shrub with yellow flowers. It is a perennial, semi-woody, herbaceous plant with primary roots and sometimes adventitious roots. The stems are typically upright from the base, with erect to ascending branches that are quadrangular, and often have sparse or no trichomes. The leaves are ovate to almost circular, approximately 30 mm long and 15

* Corresponding author: Shiva Khalil-Moghaddam
Tel: +989127752895; Fax: +989127752895
E-mail address: shiva.moghaddam@yahoo.com, **doi:**

mm wide, and tend towards yellow or purplish colors. After flowering, the leaves are retrorsed. The secondary leaves are ovate to almost round, about 15 mm long and 12 mm wide, and may have trichomes that are roughened. The flower has a golden yellow to yellowish cup that is ovate to oblong and sharp-beaked. The fruit has a nut-like, usually solitary, globose structure without trichomes or sometimes with appressed trichomes (Ehrendorfer et al, 2005). The midpoint of diversification and the source of this species is the Irano-Turanian area (Ehrendorfer and Schönbeck-Temesy, 1982; Salim and Necattin, 2018). Phytochemical studies have shown that *C. taurica* contains coumarins, *e.g.*, umbelliferon and scopoletin (Borisov, 1974), flavonoids, *e.g.*, hyperoside, quercetin, and iridoid glycosides (Ergun et al., 1984; De Rosa et al., 2003). *C. taurica* has been used in traditional medicine as a mixed juice of crushed material with fresh milk (Salim and Necattin, 2018).

Herbal products have different biological activities including anti-inflammatory (Karakas et al., 2017; Lopez-Corona et al., 2022; Yeshi et al., 2022), antibacterial (Singh, 2022; Eruygur et al., 2023), anticancer (Ng et al., 2022), antioxidant (Ghagane et al., 2017; Alam et al., 2022a; Sbieh et al., 2022), cardioprotective (Tomou et al., 2023), neuroprotective (Zieneldien et al., 2022), antidiabetic (Alam et al., 2022b), antiparasitic (Ranasinghe et al., 2023), and antiviral properties (Saifulazmi et al., 2022). Diabetes mellitus is characterized by impaired and prolonged insulin activity in target tissues, as well as disruptions that affect the metabolism of carbohydrates, fats, and proteins (Choudhury et al., 2018). One of the most important remedies for diabetes is controlling postprandial hyperglycemia (Méril-Mamert et al., 2022). Low and stable blood glucose levels can be achieved by delaying glucose absorption through inhibiting enzymes such as α-amylase and α-glucosidase, which break down carbohydrates in the digestive system (Khadayat et al., 2020). There are numerous commercial products available worldwide that are designed to manage diabetes and are derived from medicinal plants (Ansari et al., 2022). Natural products with antioxidant properties neutralize free radicals and safeguard cells against damage and degradation (Zhang et al., 2023). These herbal products exhibit notable bioactivities and can help inhibit the progression of diabetes mellitus, cancer, and heart disease. Cancer is a major global cause of mortality (Tohma et al., 2019; Behdarvand et al., 2020; Goli et al., 2021). Recently, there has been increased focus on natural bioactive compounds from medicinal plants that exhibit anticancer activity (Abd Wahab et al., 2020; Khan et al., 2022; Yuan et al., 2022). To the best of our knowledge, there are only few studies on the biological activity of *C. taurica* (De Rosa et al., 2002, 2003). Furthermore, there is a lack of research on the liquid-liquid extraction of *C. taurica*. This study represents the first investigation of the cytotoxic effects of *C. taurica* extracts on leukemia cells. In this study, we report on the methanolic extract of the aerial parts of the plant, obtained through maceration and liquid-liquid extraction. The plant was fractionated into different fractions (MeOH, EtOAc, *n*-hexane, and residual aqueous fractions), and their biological activities were investigated, including antioxidant, antidiabetic, antibacterial, and cytotoxic activities.

## 2. Experimental

### 2.1. Plant collection

The aerial parts of the *C. taurica* plant were collected from Iran, specifically from the Kurdistan Province, Marivan City, and Miraji Mountain, in May 2021 (Fig. 1). The plant was collected and identified by Dr. Nastaran Jalilian. A voucher number (RANK-8420) was deposited at the herbarium of the Agricultural and Natural Resources Research and Education Center in Kermanshah, Iran. The specimen was obtained from an area characterized by inceptisol soil composition, which consists of minimally developed soils interspersed with rock fragments. The region's climate is Mediterranean and cold. The plant collection was conducted under the authority of the official national plant protection organization.

### 2.2. Chemicals and reagents

The following compounds were used: DPPH (Merck Millipore), α-amylase (Merck-Millipore), Starch (Merck Millipore), acarbose (Sigma-Aldrich, Taufkirchen, Germany), 3,5-dinitrosalicylic acid (Sigma-Aldrich), resazurin reagent (Sigma-Aldrich), RPMI 1640 culture medium (Gibco BRL, Eggenstein, Germany), fetal bovine serum (FBS; Gibco), penicillin and streptomycin sulfate (Biochrom, Germany). All solvents were purchased from Merck Millipore. All chemicals used in this study were of 99% purity or higher. An Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) was used for fluorescence measurement. Also, UV absorbance was conducted using a spectrophotometer (Jasco V-750, Japan).

### 2.3. Plant extract

The maceration method was used for the preparation of the plant extracts. The cleaned aerial parts of the plant were dried at room temperature and then powdered. Subsequently, 200 g of *C. taurica* powder was extracted with methanol (MeOH) at room temperature for 48 h under shaking conditions (150 rpm), at plant material-to-solvent ratio of 1:10 (*w/v*%). The plant material was removed from the solvents by filtration through Whatman paper, and the filtrate was concentrated using a rotary evaporator. The MeOH extract was suspended in distilled water, and further extraction was performed using EtOAc and *n*-hexane solvents (1:1 *v/v*%) by the liquid-liquid extraction method (Mazzola et al., 2008).

### 2.4. Yield of extraction

The extraction yield was represented as the dry matter content of the extracts and was calculated using the following equation (Eqn. 1). For this purpose, MeOH, aqueous, EtOAc, and *n*-hexane fractions were dried using a freeze dryer at -60 °C. The residual aqueous solution was obtained from the remaining liquid-liquid extraction process.

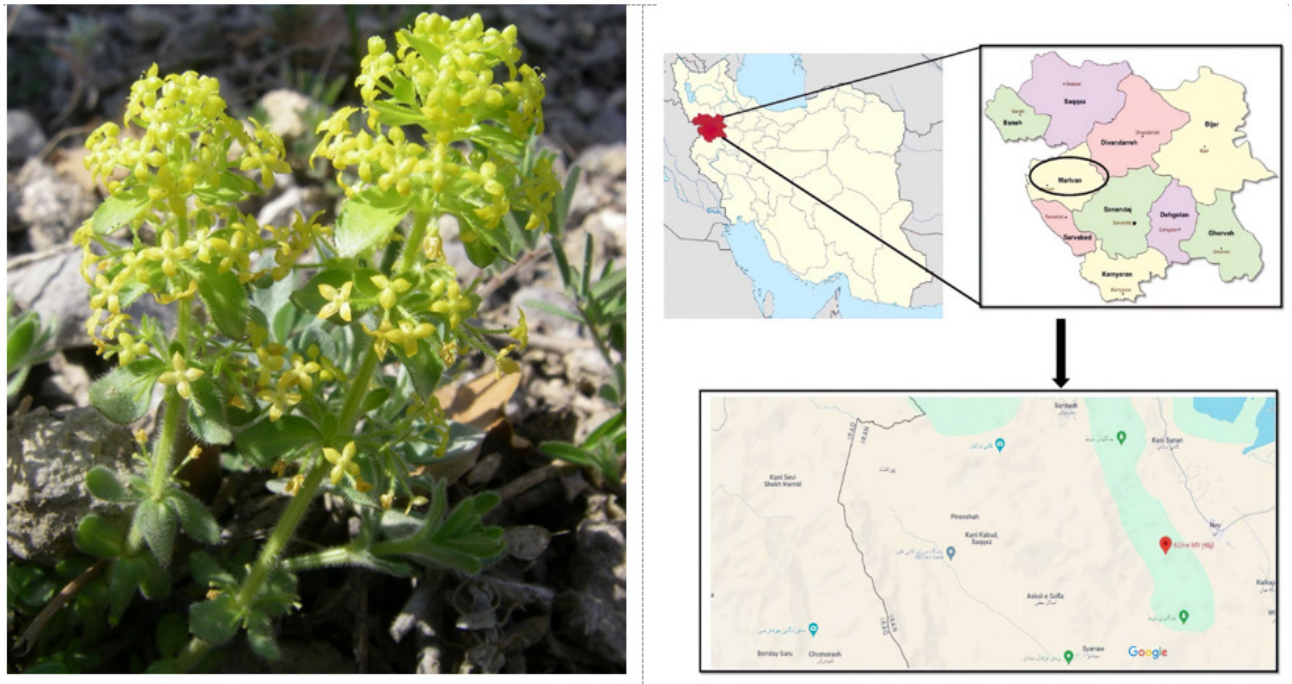



**Fig. 1. A**: The photograph of *C. taurica* plant and **B:** Map showing the location of the sampling (Kurdistan Province, Marivan City, Miraji Mountain, Iran).

Extraction yield = $(W_1/W_2) \times 100$ (Eqn. 1)

Where $W_1$ is the dry weight of the MeOH extract or fractions, and $W_2$ is the weight of the dried aerial parts of *C. taurica*.

### 2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH free radical scavenging assay, as recently described with slight modifications (Khalil-Moghaddam et al., 2021) was used to determine the antioxidant activity of various plant fractions. In summary, diluted DMSO solutions of the samples were prepared at concentrations ranging from 0.009 to 10 mg/mL. A solution of DPPH (80 µg/mL) was then prepared in MeOH, and 150 µL of this solution was added to the different concentrations of the plant fractions. Sample blanks were also prepared by substituting MeOH for the DPPH solution. The plates were then incubated at 25 °C for 30 minutes, followed by measurement of the absorbance of each well at 517 nm using a microplate reader. As a positive control, butylated hydroxytoluene (BHT) was used.
The percent inhibition was calculated as follows (Eqn. 2):

Inhibition (%) = $(A_{Blank}\text{-}A_{Sample}/A_{Blank}) \times 100$ (Eqn. 2)

The terms $A_{Blank}$ and $A_{Sample}$ respectively account for the corresponding absorbance of the control reaction (DPPH solution), and the plant extraction combined with DPPH, both at 517 nm. Furthermore, the solution concentration that led to 50% inhibition ($IC_{50}$) was determined by plotting the inhibition percentage against each plant extraction concentration.

### 2.6. Antibacterial activity

#### 2.6.1. Microbial strain

Four bacterial standard strains were evaluated in this study, *i.e., Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11788, and *Escherichia coli* ATCC 25922.

#### 2.6.2. Determination of the minimum inhibitory concentration (MIC)

MICs were assigned using a broth microdilution assay according to CLSI method (CLSI, 2006) in a 96-well microtiter plate. Different fractions of the *C. taurica* extract were dissolved in DMSO to produce an initial concentration of 36 mg/mL. Subsequently, aliquots of the samples were consecutively diluted in a 96-well plate containing Mueller-Hinton Broth (MHB) medium to yield a concentration range of 0.017-36 mg/mL. A standardized bacterial suspension (100 µL, $10^6$ CFU/mL) was inoculated in each well and incubated overnight at 37 °C. The lowest concentration that showed no visible growth was considered the minimum inhibitory concentration. The antibacterial activity of DMSO was also analyzed, and no antibacterial activity was observed at the examined concentrations.

### 2.7. α-Amylase inhibitory activity

The α-amylase inhibition assay was conducted using the 3,5-dinitrosalicylic acid (DNSA) method with certain

modifications (Wickramaratne et al., 2016). Acarbose was employed as the positive control. To prepare the DNSA reagent, DNSA (1.0 g) and sodium potassium tartaric acid (30 g) were dissolved in 20 mL of NaOH (2.0 N). The sample was then diluted to 100 mL using distilled water. The plant extract was dissolved in a minimal amount of dimethyl sulfoxide (DMSO: 50 mg/mL). In the initial step, two concentrations (3 and 300 µg/mL) of various fractions were evaluated to determine the most effective fraction with significant antidiabetic activity. Subsequently, concentration ranges of 0.3, 3, 30, and 300 µg/mL were analyzed for the best fraction (*n*-hexane), while concentrations of 0.3, 0.75, 1.5, 3.75, and 7.5 were assessed for acarbose. Additionally, the $IC_{50}$ values for both the *n*-hexane fraction and acarbose were calculated during this stage. To initiate the assay, 200 µL of α-amylase solution (1.0 mg/mL) was added to 200 µL of plant extract and incubated for 30 min at 37 °C. Subsequently, 200 µL of an aqueous starch solution (1.0% *w/v* in buffer solution) was added to each sample and incubated at 37 °C for 3 min. The absorbance of each tube was measured at 540 nm using a UV-Visible spectrophotometer.

### 2.8. Cytotoxic effect

#### 2.8.1. Cell lines and culture

Human CCRF-CEM acute lymphoblastic leukemia cells and their multidrug resistant subline CEM/ADR5000 Axel Sauerbrey (Department of Pediatrics, University of Jena, Jena, Germany) were used for cytotoxicity assays. The generation of CEM/ADR5000 cells was described by selection with doxorubicin and the characterization of their multidrug resistance phenotype (Efferth et al., 2008). The cell lines were maintained in RPMI 1640 culture medium supplemented with fetal bovine serum (FBS, 10.0%), (penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL). The resistance phenotype of CEM/ADR5000 was maintained by treating the cells every other week with 5000 ng/mL doxorubicin. The cells were incubated at 37 °C in a $CO_2$ incubator (5.0% $CO_2$).

#### 2.8.2. Resazurin reduction assay (RRA)

The investigation into the cytotoxicity of the plant fractions was conducted by employing the RRA assay, with certain modifications (Youmbi et al., 2023). Following appropriate growth for experimental purposes, the cell lines underwent a series of procedures, including washing, trypsinization, and resuspension in a fresh medium. At the initial stage, the cells were cultured in 96-well plates, with or without varying concentrations of the plant fractions (10 and 100 µg/mL), for a duration of 72 hours. Subsequently, the most potent fraction exhibiting the most effective antiproliferative activity was chosen for $IC_{50}$ determination. To this end, ten concentrations of the optimal fraction (ranging from 0.01 to 1000 µg/mL) were prepared and tested against two different cell lines and the $IC_{50}$ representing the concentration at which 50% of the cells were killed was subsequently determined. The fluorescence emitted during the experiment was measured using an Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany). The excitation and emission wavelengths were 544 nm and 590 nm, respectively.

### 2.9. Statistical analysis

All experiments were performed in triplicate with each of the six parallel measurements. The results are presented as means ± standard deviation (SD). Statistical analysis was performed employing Graph Pad Prism software version 9.2.0. The comparison between the three groups was accomplished by the use of one-way analysis of variance (ANOVA).

## 3. Results and Discussion

### 3.1. Extraction yield and antioxidant activity

The results of the extraction yield and DPPH assays for the MeOH, EtOAc, *n*-hexane, and residual aqueous fractions are presented in Table 1. As can be seen in this table, the lowest and highest extraction yield was obtained for the EtOAc (0.2%) and the residual aqueous (2.2%) fractions, respectively. The DPPH assay showed that the residual aqueous fraction had the lowest $IC_{50}$ (10.0 ± 2.6 µg/mL). A comparison of the antioxidant activity of different plant fractions with BHT revealed that *C. taurica* extracts had potent antioxidant activity, and the $IC_{50}$ of different fractions was comparable to that of the synthetic standard antioxidant compound (BHT). As far as we know, there are no comprehensive studies on the antioxidant activity of the *Cruciata* genus in the literature. In one study, some compounds isolated from *Cruciata articulata* did not exhibit antioxidant or antibacterial activity (Liu et al., 2021). The antioxidant properties of the Rubiaceae family have been discussed in a recently published review, which highlights that the family contains active biological compounds with potent antioxidant activity, capable of preventing oxidative stress (González-Castelazo et al., 2023). The antioxidant activity of *C. cruciata* may be attributed to the presence of flavonoids such as hyperoside, quercetin, and rutin (De Rosa et al., 2003). Flavonoids with antioxidant properties can effectively modulate essential stages of cell growth and differentiation, thereby influencing the regulation of overall plant development and formation of individual organs (Agati et al., 2012).

### 3.2. Antibacterial activity

The antibacterial activities of the plant fractions were investigated against two Gram-negative bacteria (*E. coli* and *P. aerosinosa*) and two Gram-positive bacteria (*B. cereus* and *S. aureus*). Among the plant fractions screened, the EtOAc fraction showed the most significant inhibitory effect against *E. coli* and *B. cereus*, with an MIC value of 0.1 mg/mL (Table 2). Moreover, the *n*-hexane fraction had more excellent antibacterial activity against *P. aeruginosa* than the other fractions (MIC: 2.2 mg/mL). On the other hand, the residual aqueous fraction showed the weakest antibacterial activity among all fractions against *E. coli* (MIC: 18 mg/mL). Several studies have reported MIC values of less

**Table 1**
Yield of plant extraction and antioxidant activity of different *C. taurica* fractions.

| Samples | Yield of extraction (%) | DPPH assay $IC_{50}$ (µg/mL) |
|---|---|---|
| MeOH | 1.8 | 150.0 ± 12.0 |
| EtOAc | 0.2 | 20.0 ± 2.0 |
| *n*-Hexane | 1.2 | 1010.0 ± 81.0 |
| Water | 2.2 | 10.0 ± 2.6 |
| BHT | - | 10.0 ± 2.0 |

**Table 2**
MIC values of *C. taurica* fractions (mg/mL).

| Samples | *S. aureus* ATCC 25923 | *P. aeruginosa* ATCC27853 | *B. cereus* ATCC 11778 | *E. coli* ATCC 25922 |
|---|---|---|---|---|
| MeOH | 4.5 | 4.5 | 0.6 | 1.1 |
| EtOAc | 1.1 | 4.5 | 0.1 | 0.1 |
| *n*-Hexane | 1.1 | 2.2 | 0.3 | 0.3 |
| Water | 4.5 | 4.5 | 4.5 | 18 |
| Chloramphenicol | 2.0[a] | 64 | 1 | 8 |

[a] MIC value of Chloramphenicol was represented as µg/mL.

than 0.5 mg/mL, indicating the potent and significant antibacterial activity of plant extracts (Sartoratto et al., 2004; Mogana et al., 2020). As a result, the *n*-hexane and EtOAc fractions exhibited strong antibacterial activity against *B. cereus* and *E. coli*.
*C. cruciata* is a rich source of aromatic monoterpenoid glycosides. These glycoside compounds can be extracted using methanol and exhibit significant antimicrobial activity (De Rosa et al., 2003). Therefore, the antibacterial activity observed in different fractions of *C. cruciata*'s methanolic extract can be attributed to the presence of these compounds. A previous research has examined the antibacterial activity of many plants belonging to the Rubiaceae family (Chassagne et al., 2021). The aqueous and ethanol extracts of *Mtracarpus villosus* leaves, for instance, were shown to have antibacterial activity in a research. In accordance with this study, the results indicated that the MICs against *S. aureus*, *B. subtilis*, *S. faecalis*, and *E. coli* ranged from 0.1 to 8.0 mg/mL (Irobi and Daramola, 1994). In another study, the ethanolic extracts from the leaves of *M. scabrum* were tested for their potential antibacterial efficacy against different pathogens including *E. coli* and *S. aureus*. In this connection, the greatest results were obtained with the hydroalcoholic extract with an MIC of 50 mg/mL (Ouadja et al., 2018). A previous study reported the antibacterial and antifungal activities of selected plants (75 crude extracts) from the Rubiaceae family. Specifically, 85% of *n*-hexane extracts from these plants demonstrated significant antibacterial activity. Our results are consistent with the previous study, which found that the *n*-hexane extract of plants belonging to the Rubiaceae family exhibited significant antibacterial activity (Niño et al., 2012).

### 3.3. α-Amylase inhibitory assay

The α-amylase inhibitory activity of the different plant fractions was plotted as a function of the extract concentration (Fig. 2A), and the $IC_{50}$ values were subsequently calculated (Fig. 2B). As seen, the results for the two concentrations of each fraction were significantly different ($p < 0.05$). In addition, significant differences were found between the $IC_{50}$ values of the sample and the standard ($p < 0.001$). Evaluation of the inhibitory activity of the plant fractions against α-amylase at two concentrations (3 and 300 µg/mL) revealed that the *n*-hexane fraction was the most potent. The $IC_{50}$ of the *n*-hexane fraction was determined to be 183.1 ± 8.6 µg/mL. In comparison, the standard positive control, acarbose, exhibited an $IC_{50}$ of 3.8 ± 0.1 µg/mL. Our findings also showed that all plant fractions had relatively weak antidiabetic activity compared to the standard compound, acarbose. Several studies have previously evaluated the α-amylase inhibitory activities of plant extracts (Borhan et al., 2013; Namjoyan et al., 2015; Wickramaratne et al., 2016; Sai et al., 2019; Abdollahzadeh et al., 2021; Abd Elkader et al., 2022; Kumar et al., 2022). The α-amylase inhibitory activity of different extracts of loquat (*Eriobotrya japonica*) leaves

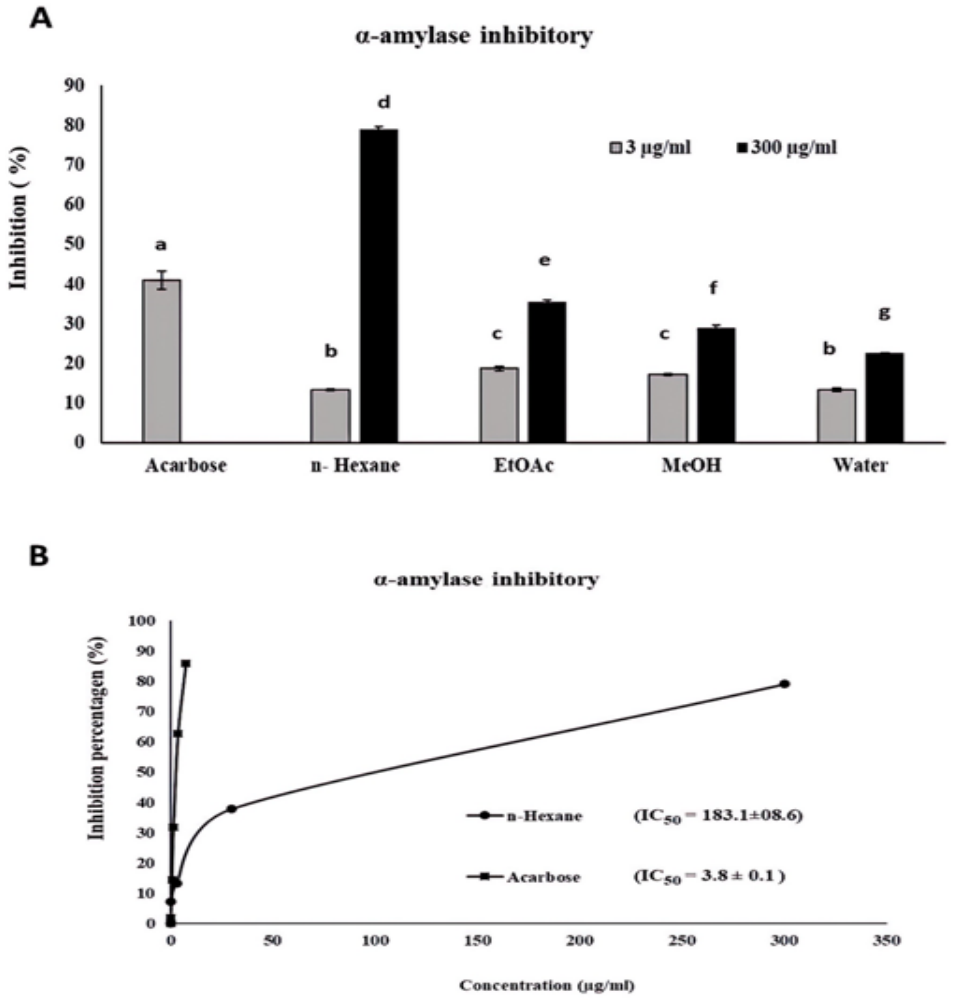



**Fig. 2.** Enzyme inhibition (%) (**A**) For different fractions of *C. taurica* at two concentrations (3 and 300 µg/mL), (**B**) As a function of optimum fraction (*n*-hexane) extract concentration for determination of $IC_{50}$ *(*Note: Different letters in the same pattern represent significant difference ($p < 0.05$).

has been the subject of a recently published report (Mogole et al., 2020). In another study, the α-amylase inhibitory activity of nine traditional plant extracts from Southeastern Nigeria was investigated. The results indicated that eight of the plant extracts exhibited moderate α-amylase inhibitory activity (Oyedemi et al., 2017). The α-amylase enzyme facilitates the process of breaking down starch through a double substitution mechanism, which involves the formation and subsequent breakdown of a covalent β-glycosyl enzyme intermediate within its active site. Inhibiting the α-amylase with plant-derived compounds appears to be a promising approach for treating diabetes. Coumarins and iridoid glycoside are a potent source for antidiabetic effect of *C. cruciate* extracts (Chen et al., 2016; Li et al., 2017). Also, flavonoids had high inhibitory capacity against α-amylase. The proposed inhibitory capacity of flavonoids action mechanism correlates inhibition potency of these compounds with the number of hydroxyl groups in the B ring of the flavonoid (Shah et al., 2018). Typically, flavonoids exhibit desirable structural characteristics as enzyme inhibitors, which encompass adaptable backbones, a hydrophobic composition, and multiple accessible hydrogen bond (H-bond) donors and acceptors (Rasouli et al., 2017). Our findings are consistent with previously published studies, which reported moderate to weak antidiabetic effects of plant extracts. We hypothesized that the results might be improved if the α-amylase inhibitory activity of pure active compounds from these plants was investigated.

### 3.4. Cytotoxic activity

The plant extracts were analyzed at concentrations of 10 and 100 µg/mL while using doxorubicin as a positive control drug on human CEM-CCRF lymphoblastic leukemia cells (drug-sensitive) (Fig. 3A) and their multidrug-resistant sub-line CEM/ADR5000 (multidrug-resistant) (Fig. 3B). Among the plant fractions tested, the EtOAc fraction demonstrated cytotoxic activity on cell proliferation of both cell lines at a concentration

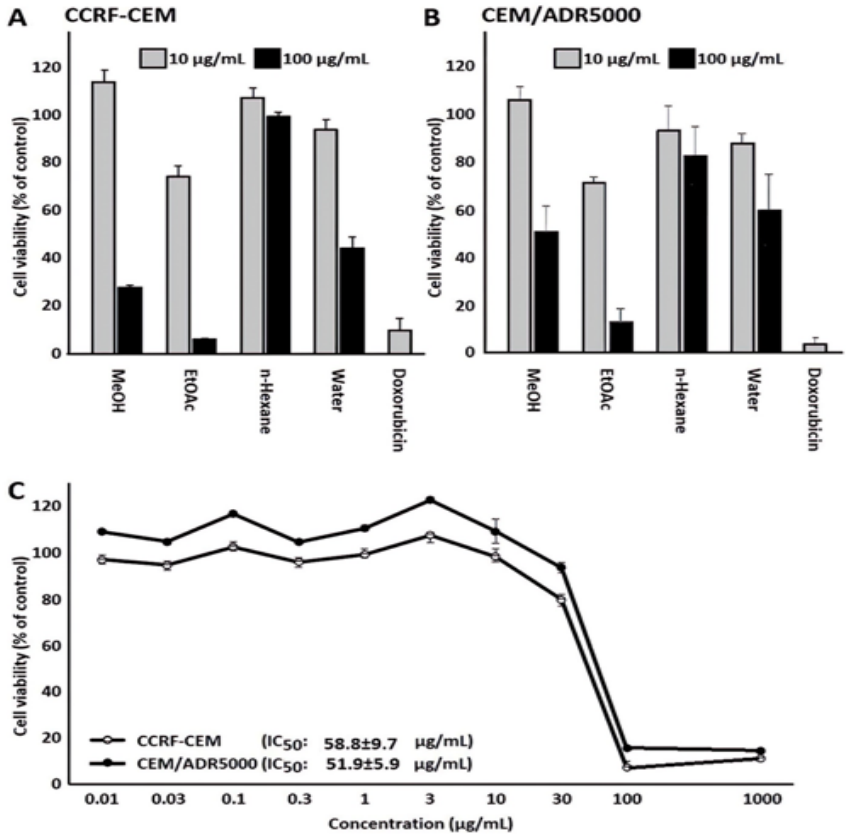



**Fig. 3.** Growth inhibition of *C. taurica* plant fractions. Effect of fixed concentrations (10 and 100 µg/mL) towards: **A**: Drug-sensitive CCRF-CEM, **B:** Multidrug-resistant CEM/ADR5000 and **C:** Effect of a dose range from 0.01 to 1000 µg/mL EtOAc fraction to both CEM-CCRF and CEM/ADR5000 cells.

of 100 µg/mL. In contrast, the other fractions showed no apparent cytotoxic effects at the concentrations used. Consequently, the EtOAc fraction was selected as the optimal fraction, exhibiting the best cytotoxic activity. Further investigation of the EtOAc fraction at various concentrations (0.01-1000 µg/mL) led to the determination of the $IC_{50}$ value (Fig. 3C). The $IC_{50}$ values obtained were 58.8 ± 9.7 and 51.9 ± 5.9 µg/mL for CCRF-CEM and CEM/ADR5000 cells, respectively. Although the $IC_{50}$ values indicate relatively weak activity of the extract, there was no cross-resistance of CEM/ADR5000 cells to the *C. taurica* extract. The presence of flavonoids is a crucial factor in the cytotoxic properties of the *C. taurica* extract (Sak, 2014). This is noteworthy since CEM/ADR5000 cells exhibited high resistance to doxorubicin and many other clinically established anticancer drugs (Efferth et al., 2008). Multidrug resistance is a frequent cause of failure of chemotherapy, which leads to the death of many cancer patients (Volm and Efferth, 2015). Therefore, the cytotoxic potency of plant extracts and natural products that can inhibit multidrug-resistant cells with similar efficacy to drug-sensitive tumor cells may have the potential to overcome drug resistance.

Numerous studies have focused on the anticancer activities of plant extracts and isolated phytochemicals (Molnár et al., 2010; Kumar and Jaitak, 2019; Luo et al., 2019). In one of the recently published reports, multidrug-resistant. However, many inhibited sensitive and multidrug-resistant cells with similar efficacies (Atanasov et al., 2021). This may open a new horizon to treat multidrug-resistant tumors with plant extracts and natural products.

## 4. Concluding remarks

In this study, the methanol (MeOH) extract of *C. taurica* was obtained. Subsequently, several fractions were made from the initial extract, including residual aqueous, *n*-hexane, and EtOAc fractions. To gain a better understanding of the plant's biological activity, some complimentary tests were conducted to assess its antibacterial, antioxidant, anticancer, and antidiabetic properties. The results confirmed that the residual aqueous fraction had remarkable antioxidant activity. The EtOAc fraction was potent against *E. coli* and *B. cereus*, with a minimum inhibitory concentration (MIC) of 0.1 mg/mL. The *n*-hexane and EtOAc fractions exhibited the best antidiabetic and anticancer effects, respectively. Different plant extracts can be a valuable natural resource for isolating active ingredients with potent biological activity. The investigations presented here demonstrate that *C. taurica* could be a rich source of natural bioactive compounds with antibacterial, anticancer, antidiabetic, and antioxidant properties.

The key limitation of this work was the assessment of the biological activity of the crude extracts. Therefore, further investigation is recommended to confirm the potential of the extracts as phytotherapeutic agents. This includes: i) Isolating and characterizing the main active compounds from the extracts. ii) Exploring the biological activities and mechanisms of action of the isolated compounds. By addressing these aspects, the full potential of *C. taurica* as a source of natural bioactive compounds can be more thoroughly explored and validated.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Author contribution

Shiva Khalil-Moghaddam: Investigation and methodology, Project administration, Supervision; Roya moghimi: Conceptualization, writing-review & editing; Thomas Efferthc: Investigation and methodology; Sajedeh Mousavian: Investigation and methodology.

## Acknowledgments

Not applicable.

## List of Abbreviations

**BHT:** Butylated hydroxytoluene; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **DNSA:** 3, 5-Dinitrosalicylic Acid; **FBS:** Fetal Bovine Serum; **$IC_{50}$:** Inhibitory Concentration (50%); **MHB:** Mueller-Hinton Broth; **MIC:** Minimum Inhibitory Concentration; **RRA:** Resazurin Reduction Assay; **UV:** Ultraviolet.

## References

Abd Elkader, A.M., Labib, S., Taha, T.F., Althobaiti, F., Aldhahrani, A., Salem, H.M., Saad, A., Ibrahim, F.M., 2022. Phytogenic compounds from avocado (*Persea americana* L.) extracts; antioxidant activity, amylase inhibitory activity, therapeutic potential of type 2 diabetes. Saudi J. Biol. Sci. 29, 1428-1433.

Abd Wahab, N.Z., Azizul, A., Ibrahim, N., 2020. Phytochemistry, cytotoxicity and antiviral activity of *Catharanthus roseus*. Iran. J. Microbiol. 12, 460.

Abdollahzadeh, E., Nematollahi, A., Hosseini, H., 2021. Composition of antimicrobial edible films and methods for assessing their antimicrobial activity: A review. Trends Food Sci. Technol. 110, 291-303.

Agati, G., Azzarello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci. 196, 67-76.

Alam, M.W., Najeeb, J., Naeem, S., Usman, S.M., Nahvi, I., Alismail, F., Abuzir, A., Farhan, M., Nawaz, A., 2022a. Electrochemical methodologies for investigating the antioxidant potential of plant and fruit extracts: A review. Antioxidants 11, 1205.

Alam, S., Sarker, M.M.R., Sultana, T.N., Chowdhury, M.N.R., Rashid, M.A., Chaity, N.I., Zhao, C., Xiao, J., Hafez, E.E., Khan, S.A., 2022b. Antidiabetic phytochemicals from medicinal plants: Prospective candidates for new drug discovery and development. Front. Endocrinol. (Lausanne) 13.

Ansari, P., Akther, S., Hannan, J.M.A., Seidel, V., Nujat, N.J., Abdel-Wahab, Y.H.A., 2022. Pharmacologically active phytomolecules isolated from traditional antidiabetic plants and their therapeutic role for the management of diabetes mellitus. Molecules 27, 4278.

Atanasov, A.G., Zotchev, S.B., Dirsch, V.M., Orhan, I.E., Banach, M., Rollinger, J.M., Barreca, D., Weckwerth, W., Bauer, R., Bayer, E.A., Majeed, M., Bishayee, A., Bochkov, V., Bonn, G.K., Braidy, N., Bucar, F., Cifuentes, A., D'Onofrio, G., Bodkin, M., Diederich, M., Dinkova-Kostova, A.T., Efferth, T., El Bairi, K., Arkells, N., Fan, T.P., Fiebich, B.L., Freissmuth, M., Georgiev, M.I., Gibbons, S., Godfrey, K.M., Gruber, C.W., Heer, J., Huber, L.A., Ibanez, E., Kijjoa, A., Kiss, A.K., Lu, A., Macias, F.A., Miller, M.J.S., Mocan, A., Müller, R., Nicoletti, F., Perry, G., Pittalà, V., Rastrelli, L., Ristow, M., Russo, G.L., Silva, A.S., Schuster, D., Sheridan, H., Skalicka-Woźniak, K., Skaltsounis, L., Sobarzo-Sánchez, E., Bredt, D.S., Stuppner, H., Sureda, A., Tzvetkov, N.T., Vacca, R.A., Aggarwal, B.B., Battino, M., Giampieri, F., Wink, M., Wolfender, J.L., Xiao, J., Yeung, A.W.K., Lizard, G., Popp, M.A., Heinrich, M., Berindan-Neagoe, I., Stadler, M., Daglia, M., Verpoorte, R., Supuran, C.T., 2021. Natural products in drug discovery: Advances and opportunities. Nat. Rev. Drug Discov. 20, 200-216.

Avoseh, N.O., Lawal, O.A., Ogunwande, I.A., Ascrizzi, R., Flamini, G., Amoo, E., 2020. *In vivo* anti-inflammatory and anti-nociceptive activities, and chemical constituents of essential oil from the leaf of *Gardenia jasminoides* J. Ellis (Rubiaceae). Trends Phytochem. Res. 4, 203-212.

Behdarvand, N., Bikhof Torbati, M., Shaabanzadeh, M., 2020. Tamoxifen-loaded PLA/DPPE-PEG lipid-polymeric nanocapsules for inhibiting the growth of estrogen-positive human breast cancer cells through cell cycle arrest. J. Nanoparticle Res. 22, 262.

Borhan, M.Z., Norhidayah, A., Ahmad, R., Rusop, M., Abdullah, S., 2013. Production of herbal nanopowders: Effect of milling time. Adv. Mater. Res. 686, 3-7.

Borisov, M.I., 1974. Coumarins of the genera *Asperula* and *Galium*. Chem. Nat. Compd. 10, 78.

Bremer, B., Eriksson, T., 2009. Time tree of Rubiaceae: Phylogeny and dating the family, subfamilies, and tribes. Int. J. Plant Sci. 170, 766-793.

Chassagne, F., Samarakoon, T., Porras, G., Lyles, J.T., Dettweiler, M., Marquez, L., Salam, A.M., Shabih, S., Farrokhi, D.R., Quave, C.L., 2021. A systematic review of plants with antibacterial activities: A taxonomic and phylogenetic perspective. Front. Pharmacol. 11, 2069.

Chen, Y., Wu, Y., Gan, X., Liu, K., Lv, X., Shen, H., Dai, G., Xu, H., 2016. Iridoid glycoside from *Cornus officinalis* ameliorated diabetes mellitus-induced testicular damage in male rats: Involvement of suppression of the AGEs/RAGE/p38 MAPK signaling pathway. J. Ethnopharmacol. 194, 850-860.

Choudhury, H., Pandey, M., Hua, C.K., Mun, C.S., Jing, J.K., Kong, L., Ern, L.Y., Ashraf, N.A., Kit, S.W., Yee, T.S., Pichika, M.R., Gorain, B., Kesharwani, P., 2018. An update on natural compounds in the remedy of diabetes mellitus: A systematic review. J. Tradit. Complement.

Med. 8(3), 361-376.
CLSI, W., 2006. Clinical and Laboratory Standards Institute Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approv. Stand. M7-A7, CLSI, Seventh Ed, PA, USA.
De Rosa, S., Mitova, M., Handjieva, N., Çalış, İ., 2002. Coumarin glucosides from *Cruciata taurica*. Phytochemistry 59, 447-450.
De Rosa, S., Mitova, M., Handjieva, N., Ersoz, T., Calis, I., 2003. Aromatic monoterpenoid glycosides from *Cruciata taurica*. Nat. Prod. Res. 17, 109-113.
Efferth, T., Konkimalla, V.B., Wang, Y.-F., Sauerbrey, A., Meinhardt, S., Zintl, F., Mattern, J., Volm, M., 2008. Prediction of broad spectrum resistance of tumors towards anticancer drugs. Clin. Cancer Res. 14, 2405-2412.
Ehrendorfer, F., Schönbeck-Temesy, E., 1982. Flora of Turkey and the East Aegean Islands.
Ergun, F., Küsmenoglu, Ş., Şener, B., 1984. High-performance liquid chromatographic determination of iridoids in *Cruciata Taurica*. J. Liq. Chromatogr. 7, 1685-1689.
Ehrendorfer, F., Schonbeck-Temesy, E., Puff, C., Rechinger, W., 2005. Rubiaceae. In: Rechinger, K H (ed.) Flora Iranica, No. 176-Graz, p. 443.
Eruygur, N., Buyukyildirim, T., Tetik Rama, S., Ayaz, F., Tekin, M., Tuzcu, M., Akcakavak, G., Abdullah Yilmaz, M., 2023. Phytochemical profiling and biological activity of *Achillea sintenisii* Hub.-Mor. Chem. Biodivers. 20, e202201258.
Ghagane, S.C., Puranik, S.I., Kumbar, V.M., Nerli, R.B., Jalalpure, S.S., Hiremath, M.B., Neelagund, S., Aladakatti, R., 2017. *In vitro* antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. Integr. Med. Res. 6, 79-87.
Goli, P.P., Torbati, M.B., Parivar, K., Khiavi, A.A., Yousefi, M., 2021. Preparation and evaluation of gemcitabin and cisplatin-entrapped Folate-PEGylated liposomes as targeting co-drug delivery system in cancer therapy. J. Drug Deliv. Sci. Technol. 65, 102756.
González-Castelazo, F., Soria-Jasso, L.E., Torre-Villalvazo, I., Cariño-Cortés, R., Muñoz-Pérez, V.M., Ortiz, M.I., Fernández-Martínez, E., 2023. Plants of the Rubiaceae family with effect on metabolic syndrome: Constituents, pharmacology, and molecular targets. Plants 12, 3583.
Huysmans, S., Dessein, S., Smets, E., Robbrecht, E., 2003. Pollen morphology of NW European representatives confirms monophyly of Rubieae (Rubiaceae). Rev. Palaeobot. Palynol. 127, 219-240.
Irobi, O.N., Daramola, S.O., 1994. Bactericidal properties of crude extracts of *Mitracarpus villosus*. J. Ethnopharmacol. 42, 39-43.
Karakas, F.P., Turker, A.U., Karakas, A., Mshvildadze, V., Pichette, A., Legault, J., 2017. *In vitro* cytotoxic, antibacterial, anti-inflammatory and antioxidant activities and phenolic content in wild-grown flowers of common daisy—A medicinal plant. J. Herb. Med. 8, 31-39.
Karou, S.D., Tchacondo, T., Ilboudo, D.P., Simpore, J., 2011. Sub-Saharan Rubiaceae: A review of their traditional uses, phytochemistry and biological activities. Pak. J. Biol. Sci. 14, 149-169.
Khadayat, K., Marasini, B.P., Gautam, H., Ghaju, S., Parajuli, N., 2020. Evaluation of the alpha-amylase inhibitory activity of Nepalese medicinal plants used in the treatment of diabetes mellitus. Clin. Phytosci. 6, 1-8.
Khalil-Moghaddam, S., Aliahmadi, A., Jalilian, N., Tabad, M.A., 2021. The study of antioxidant and cellular toxicity effects of methanol, ethyl acetate, aqueous and *n*-hexane extracts of *symphytum kurdicum* plant. J. Chem. Heal. Risks 11, 105-111.
Khan, M.I., Bouyahya, A., Hachlafi, N.E.L., Menyiy, N. El, Akram, M., Sultana, S., Zengin, G., Ponomareva, L., Shariati, M.A., Ojo, O.A., 2022. Anticancer properties of medicinal plants and their bioactive compounds against breast cancer: A review on recent investigations. Environ. Sci. Pollut. Res. 29, 24411-24444.
Kumar, A., Aswal, S., Chauhan, A., Semwal, R.B., Singh, R., Andola, H.C., Joshi, S.K., Semwal, D.K., 2022. Antidiabetic effect of aqueous-ethanol extract from the aerial parts of *Artemisia roxburghiana*. Nat. Prod. Res. 36, 1300-1305.
Kumar, A., Jaitak, V., 2019. Natural products as multidrug resistance modulators in cancer. Eur. J. Med. Chem. 176, 268-291.
Li, H., Yao, Y., Li, L., 2017. Coumarins as potential antidiabetic agents. J. Pharm. Pharmacol. 69, 1253-1264.
Liu, X., Wang, Y., Alizade, V., Khutsishvili, M., Atha, D., Borris, R.P., Clark, B.R., 2021. Cruciasides C-G, monoterpenoid glycosides from *Cruciata articulata*. Phytochemistry 189, 112821.
Lopez-Corona, A.V., Valencia-Espinosa, I., González-Sánchez, F.A., Sánchez-López, A.L., Garcia-Amezquita, L.E., Garcia-Varela, R., 2022. Antioxidant, anti-inflammatory and cytotoxic activity of phenolic compound family extracted from raspberries (*Rubus idaeus*): A general review. Antioxidants 11, 1192.
Luo, H., Vong, C.T., Chen, H., Gao, Y., Lyu, P., Qiu, L., Zhao, M., Liu, Q., Cheng, Z., Zou, J., 2019. Naturally occurring anti-cancer compounds: Shining from Chinese herbal medicine. Chin. Med. 14, 48.
Mazzola, P.G., Lopes, A.M., Hasmann, F.A., Jozala, A.F., Penna, T.C. V, Magalhaes, P.O., Rangel-Yagui, C.O., Pessoa Jr, A., 2008. Liquid-liquid extraction of biomolecules: An overview and update of the main techniques. J. Chem. Technol. Biotechnol. Int. Res. Process. Environ. Clean Technol. 83, 143-157.
Méril-Mamert, V., Ponce-Mora, A., Sylvestre, M., Lawrence, G., Bejarano, E., Cebrián-Torrejón, G., 2022. Antidiabetic potential of plants from the Caribbean basin. Plants 11, 1360.
Mogana, R., Adhikari, A., Tzar, M.N., Ramliza, R., Wiart, C., 2020. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates. BMC Complement. Med. Ther. 20, 55.
Mogole, L., Omwoyo, W., Mtunzi, F., 2020. Phytochemical screening, anti-oxidant activity and α-amylase inhibition study using different extracts of loquat (*Eriobotrya japonica*) leaves. Heliyon 6(8), e04736.
Molnár, J., Engi, H., Hohmann, J., Molnár, P., Deli, J., Wesolowska, O., Michalak, K., Wang, Q., 2010. Reversal of multidrug resistance by natural substances from plants. Curr. Top. Med. Chem. 10, 1757-1768.
Namjoyan, F., Dastjerdi, Z.M., Azemi, M.E., 2015. Alpha amylase inhibition activity of some plants extract of

*Teucrium* species. Eur. J. Biol. Sci. 7, 26-31.
Ng, C.X., Affendi, M.M., Chong, P.P., Lee, S.H., 2022. The potential of plant-derived extracts and compounds to augment anticancer effects of chemotherapeutic drugs. Nutr. Cancer 74, 3058-3076.
Niño, J., Mosquera, O.M., Correa, Y.M., 2012. Antibacterial and antifungal activities of crude plant extracts from Colombian biodiversity. Rev. Biol. Trop. 60, 1535-1542.
Nyobe, J.C.N., Bikélé, D.M., Fodouop, M.B., Mpondo, E.M., Ndom, J.C., 2020. A new pyrrolidinyl-piperazine alkaloid derivative from *Oxyanthus speciosus* DC.(Rubiaceae). Trends Phytochem. Res. 4, 109-116.
Ouadja, B., Anani, K., Djeri, B., Ameyapoh, Y.O., Karou, D.S., 2018. Evaluation of the phytochemical composition, antimicrobial and anti-radical activities of *Mitracarpus scaber* (Rubiaceae). J. Med. plants Res. 12, 493-499.
Oyedemi, S.O., Oyedemi, B.O., Ijeh, I.I., Ohanyerem, P.E., Coopoosamy, R.M., Aiyegoro, O.A., 2017. Alpha-amylase inhibition and antioxidative capacity of some antidiabetic plants used by the traditional healers in Southeastern Nigeria. Sci. World J. 2017, 3592491.
Ranasinghe, S., Armson, A., Lymbery, A.J., Zahedi, A., Ash, A., 2023. Medicinal plants as a source of antiparasitics: An overview of experimental studies. Pathog. Glob. Health 1-19.
Rasouli, H., Hosseini-Ghazvini, S.M.-B., Adibi, H., Khodarahmi, R., 2017. Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: A virtual screening perspective for the treatment of obesity and diabetes. Food Funct. 8, 1942-1954.
Sai, K., Thapa, R., Devkota, H.P., Joshi, K.R., 2019. Phytochemical screening, free radical scavenging and α-amylase inhibitory activities of selected medicinal plants from Western Nepal. Medicines 6, 70.
Saifulazmi, N.F., Rohani, E.R., Harun, S., Bunawan, H., Hamezah, H.S., Nor Muhammad, N.A., Azizan, K.A., Ahmed, Q.U., Fakurazi, S., Mediani, A., 2022. A review with updated perspectives on the antiviral potentials of traditional medicinal plants and their prospects in antiviral therapy. Life 12, 1287.
Sak, K., 2014. Cytotoxicity of dietary flavonoids on different human cancer types. Pharmacogn. Rev. 8, 122.
Salim, M., Necattin, T., 2018. A survey on wild plants with ethnobotanical use in the Bahçe and Hasanbeyli districts of Osmaniye, Turkey. GSC Biol. Pharm. Sci. 05, 28-35.
Sartoratto, A., Machado, A.L.M., Delarmelina, C., Figueira, G.M., Duarte, M.C.T., Rehder, V.L.G., 2004. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Brazilian J. Microbiol. 35, 275-280.
Sbieh, R., Al-Lahham, S., Jaradat, N., 2022. Antioxidant, antimicrobial and cytotoxic properties of four different extracts derived from the aerial parts of *Chiliadenus iphinoides*. Eur. J. Integr. Med. 54, 102149.
Shah, S.B., Sartaj, L., Ali, F., Shah, S.I.A., Khan, M.T., 2018. Plant extracts are the potential inhibitors of α-amylase: A review. MOJ Bioequiv. Availab. 5, 270-273.
Singh, A.K., 2022. A review on plant extract-based route for synthesis of cobalt nanoparticles: Photocatalytic, electrochemical sensing and antibacterial applications. Curr. Res. Green Sustain. Chem. 100270.
Tava, A., Biazzi, E., Ronga, D., Avato, P., 2020. Identification of the volatile components of *Galium verum* L. and *Cruciata leavipes* Opiz from the Western Italian Alps. Molecules 25.
Tohma, H., Altay, A., Köksal, E., Gören, A.C., Gülçin, İ., 2019. Measurement of anticancer, antidiabetic and anticholinergic properties of sumac (*Rhus coriaria*): Analysis of its phenolic compounds by LC-MS/MS. J. Food Meas. Charact. 13, 1607-1619.
Tomou, E.-M., Papakyriakopoulou, P., Skaltsa, H., Valsami, G., Kadoglou, N.P.E., 2023. Bio-actives from natural products with potential cardioprotective properties: Isolation, identification, and pharmacological actions of apigenin, quercetin, and silibinin. Molecules 28, 2387.
Volm, M., Efferth, T., 2015. Role of P-glycoprotein for Resistance of Tumors to Anticancer Drugs: From bench to Bedside. Resistance to Targeted ABC Transporters in Cancer. pp. 1-26.
Wickramaratne, M.N., Punchihewa, J.C., Wickramaratne, D.B.M., 2016. *In-vitro* alpha amylase inhibitory activity of the leaf extracts of *Adenanthera pavonina*. BMC Complement. Altern. Med. 16, 1-5.
Wooster, T.J., Golding, M., Sanguansri, P., 2008. Impact of oil type on nanoemulsion formation and Ostwald ripening stability. Langmuir 24, 12758-12765.
Wonkam, A.K.N., Njanpa, C.A.N., Ateba, J.E.T., Fongang, Y.S.F., Awantu, A.F., Bankeu, J.J.K., Chouna, J.R., Boyom, F.F., Sewald, N., Lenta, B.N., 2022. Constituents of the ripe fruits of *Nauclea latifolia* Sm. (Rubiaceae) and their antileishmanial activities. Trends Phytochem. Res. 6(4), 292-300.
Yeshi, K., Turpin, G., Jamtsho, T., Wangchuk, P., 2022. Indigenous uses, phytochemical analysis, and anti-inflammatory properties of Australian tropical medicinal plants. Molecules 27, 3849.
Youmbi, L.M., Makong, Y.S.D., Mbaveng, A.T., Tankeo, S.B., Fotso, G.W., Ndjakou, B.L., Wansi, J.D., Beng, V.P., Sewald, N., Ngadjui, B.T., 2023. Cytotoxicity of the methanol extracts and compounds of *Brucea antidysenterica* (Simaroubaceae) towards multifactorial drug-resistant human cancer cell lines. BMC Complement. Med. Ther. 23, 48.
Yuan, M., Zhang, G., Bai, W., Han, X., Li, C., Bian, S., 2022. The role of bioactive compounds in natural products extracted from plants in cancer treatment and their mechanisms related to anticancer effects. Oxid. Med. Cell. Longev. 2022.
Zhang, J., Chu, W.-C., Li, L.-Z., 2023. Isolation and structure elucidation of antioxidant compounds from stem and root barks of *Daphne giraldii*. J. Asian Nat. Prod. Res. 1-10.
Zieneldien, T., Kim, J., Cao, C., 2022. The multifaceted role of neuroprotective plants in Alzheimer's Disease treatment. Geriatrics 7, 24.

Trends Phytochem. Res. 8(2) 2024 122-130

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Biological activities of protocorms and stems extracts of *Dendrobium transparens*

Pusp Raj Joshi[1,2], Bir Bahadur Thapa[1], Krishna Chand[2], Lasta Maharjan[2], Mukti Ram Paudel[1], Basant Pant[2,3], And Bijaya Pant[1]✉*

[1]Central Department of Botany, Tribhuvan University, Kirtipur 44613, Kathmandu, Nepal
[2]Annapurna Research Center, Tanka Prasad Ghumti Sadak 44600, Kathmandu, Nepal
[3]Annapurna Neurological Institute & Allied Sciences, Tanka Prasad Ghumti Sadak 44600, Kathmandu, Nepal

**ABSTRACT**

This study assessed the phenolic and flavonoid contents, and antioxidant, antibacterial, as well as cytotoxic properties of the protocorm extract of *Dendrobium transparens* and compared it to its wild equivalents. Methanol was used to extract compounds from the stems (DTSE) and protocorms (DTPE). DTSE contained 61.889 mg flavonoid and 82.00 mg phenolic content per gram extract equivalent to quercetin and gallic acid, respectively. At a concentration of 191.23 µg/mL, DTSE exhibited a 50% DPPH radical scavenging efficiency. Compared to the 3T3 cell line (2108.87 µg/mL), the DTPE's cytotoxic ability against the HeLa (229.30 µg/mL) and U251 (213.90 µg/mL) cell lines was found to be significantly stronger. However, the U251 cell line was strongly cytotoxic to DTSE (75.84 µg/mL). At a dose of 2000 mg/kg, neither DTSE nor DTPE caused any discernible harm in mice. They could inhibit the growth of *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Based upon the experimental results, the wild stems and protocorms were found to be alternatives suitable for creating pharmacologically bioactive substances.

**ARTICLE HISTORY**

Received: 15 September 2023
Revised: 28 May 2024
Accepted: 16 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Antibacterial activity
Antioxidant activity
*Dendrobium transparens*
MTT
Protocorms
Total flavonoid content (TFC)
Total phenolic content (TPC)

## 1. Introduction

Medicinal plants have long been recognized for their remarkable ability to produce a diverse array of biologically active compounds that exhibit a wide range of therapeutic effects (El Jabboury et al., 2023; Kota et al., 2023). These natural products, often the result of millions of years of evolutionary adaptation, have the potential to treat various ailments, from inflammation and pain to cancer and infectious diseases (Hapuarachchi et al., 2022). By harnessing the power of medicinal plants, researchers and healthcare professionals can develop novel drugs and treatments that are both effective and safe, offering hope to those suffering from debilitating conditions (Mohammadhosseini et al., 2019; Mohammadhosseini et al., 2021). As we continue to explore the vast and largely untapped potential of these botanical wonders, we move closer to unlocking the secrets of nature's pharmacy and improving the quality of life for people around the world.

*Dendrobium transparens* (common name: Translucent Dendrobium) is a medium to large-sized rare orchid growing on trees, with slender, basally swollen, erect to pendulous stems. Translucent Dendrobium is found in the Himalayas, from Kumaon, Nepal to Bhutan, NE India, Laos, and Myanmar at altitudes of 800-2000 m (eFloras, 2020). It is a horticulturally important orchid. Besides horticulture, it has been widely important in traditional medicine to treat fractured, dislocated bones and geriatric diseases (Gutierrez, 2010). The wild resource of this orchid is depleting day by day due to habitat loss, and collection from hobbyists to large-scale illegal trade. The rate of vegetative multiplication of this orchid is extremely slow producing 2-4 shoots per year, however, its seed germination process is very complex as it needs a specific mycorrhiza due to the lack of

✉ Corresponding author: Bijaya Pant
Tel: +977-1-4331322; Fax: +977-1-4331322
E-mail address: bijaya.pant@cdb.tu.edu.np, **doi:**

endosperm, and about 2.0-5.0% are germinated (Otero et al., 2002; Pant et. al., 2017). Plant tissue culture has overstated the endeavours in plant conservation and would be the most appropriate substitute to reduce the burden on wild orchids (Pfab and Scholes, 2004; Pant, 2013, 2014), thus assisting in their sustainable utilization. Furthermore, it is useful for delivering plant-derived bioactive components (Bourgaud et al., 2001; Vanisree et al., 2004; Hussain et al., 2012; Ochoa-Villarreal et al., 2016; Espinosa-Leal et al., 2018). Orchid protocorms are unique, highly organized tissues that emerge from *in vitro* seed cultures and undergo differentiation into mature plants. These tissues are characterized by the accumulation of a high concentration of secondary metabolites (Paudel et al., 2020; Pant et al., 2021). The major purpose of orchid protocorm development is to form plantlets and the isolation of bioactive secondary metabolites (Yeung, 2017).

This research was assumed on Translucent Dendrobium to (i) Perceive the effect of different phytohormones on seed germination, protocorms formation, shoot and root initiation, (ii) Determine the phenolic and flavonoid contents of wild plants and tissue culture-raised protocorms, and (iii) Evaluate the antioxidant, antibacterial and cytotoxicity of wild plant and *in vitro* developed protocorms.

## 2. Experimental

### 2.1. Plant materials

The mature capsule and stems of Translucent Dendrobium were collected from the Bhaktapur district of central Nepal (27.6451° N, 85.4427° E) in June and July 2018. The voucher specimen was deposited at the Tribhuvan University Central Herbarium (TUCH) (voucher number P08), Kathmandu, Nepal. The seeds were used as explants in the production of protocorms and plantlets.

### 2.2. Sterilization of capsule

The capsule was sterilized with 1-2 drops of Tween-20 and rinsed for at least half an hour under running tap water. The capsule was submerged in a sodium hypochlorite solution (1.0%) for 15 minutes and in ethanol (70%) for 10 minutes. Then, it was washed with sterile water.

### 2.3. Culture medium and culture conditions

Full and half strengths of Murashige and Skoog (MS) basal medium were used for protocorm development from seeds. BAP (6-benzylaminopurine) and NAA (1-naphthaleneacetic acid) were used as organic additives. The pH of the medium was adjusted to 5.8 before being sterilized in an autoclave at 121 °C and 1 kPa for 20 minutes. The cultures were kept in a room at 25 ± 2 °C under a 16/8-hour (light/dark) photoperiod of 30 µmol/$m^2$/s intensity.

### 2.4. Induction of protocorms and plant regeneration

For protocorm formation, mature seeds were placed on a hormone-free MS solid medium and supplemented with BAP (0.5-2.0 mg/L) and NAA (0.5-1.0 mg/L). Protocorms were cultivated on hormone-free MS medium as control and on MS medium comprising NAA, BAP or kinetin with concentrations ranging from 0.5 to 2.0 mg/L for shoot initiation. For subsequent growth, healthy shoots were sub-cultured on the basal MS medium. MS medium with NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) at 1-1.5 mg/L as well as coconut water (100 mL/L) were used for root initiation.

### 2.5. Biomass determination and extraction

*In-vitro* growth protocorms were removed by mesh filtering and the biomass was quantified after surface drying. The biomass of protocorms was dried up at room temperature. The *in vitro* developed protocorms and wild plant stems were soaked in methanol for 48 hours and centrifuged at 4000x for 15 minutes. The supernatant was collected and dried in a rotary evaporator. Both protocorms extract (DTPE) and stem extract (DTSE) of Translucent Dendrobium were stored at 4 °C until further use.

### 2.6. Determination of total phenolic content (TPC)

Total phenolic content of DTPE and DTSE was determined using the Folin-Ciocalteu (FC) reagent colorimetric method. In brief, 25 µL of extract (1.0 mg/mL) was mixed with 25 µL of FC reagent (10.0%) followed by 75 µL of distilled water in a 96-well plate. A similar procedure was applied for a gallic acid standard (25-200 mg/mL). The blank was prepared by replacing the plant sample with absolute methanol. After a steady reaction, 100 µL of $Na_2CO_3$ (1.0 M) was added to each sample. The plate was covered and incubated in the dark for 90 minutes. The absorbance of each solution was then read at 765 nm using a microplate reader (Azure Biosystems Microplate Reader). The experiment was done in triplicate. Total phenolic content was stated in terms of milligram of gallic acid equivalent per gram of plant extract (mg GAE/g).

### 2.7. Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) of DTPE and DTSE was determined using the aluminium chloride ($AlCl_3$) colorimetric method. In summary, 25 µL of extract (1 mg/mL) was mixed with 75 µL of $AlCl_3$ (10.0%) and then 50 µL of potassium acetate (1.0 M) in a 96-well plate. A similar procedure was applied for the quercetin as the TFC standard (25-200 mg/mL). The blank was prepared by replacing the plant sample with absolute methanol. The plate was then covered and incubated at room temperature for 30 minutes. Finally, the absorbance was recorded at 415 nm using a microplate reader (Azure Biosystems Microplate Reader). The experiment was done in triplicate and the TFC was stated in terms of a milligram of quercetin equivalent per gram of plant extract (mg QE/g).

### 2.8. Determination of antioxidant activity

The antioxidant activity of DTPE and DTSE was determined by the DPPH free radicals scavenging method (Babili et al., 2022). Briefly, 50 µL of extract at a concentration ranging from 25 to 200 µg/mL was mixed up with 150 µL of DPPH (0.2 mM) in a 96-well plate. The blank was prepared by replacing the same volume of plant sample with absolute methanol. After 30 minutes of incubation in the dark at room temperature, the absorbance of the plate was read at 517 nm using a microplate reader (Azure Biosystems Microplate reader). The antioxidant capacity of the extract ($IC_{50}$) was calculated using a polynomial regression equation derived from the percentage of DPPH scavenging activity at various concentrations of the extract.

### 2.9. Determination of cytotoxic activity

The cytotoxic activity of DTPE and DTSE was determined by employing the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay in HeLa, U251 and 3T3 cell lines. Briefly, 1 x$10^4$ cells were transferred to a 96-well plate with 100 µL of DMEM (Dulbecco's Modified Eagle Medium) including 10.0% fetal bovine serum (FBS), penicillin/streptomycin (1.0%) and L-glutamine (1.0%). The plate was incubated in a 5.0% $CO_2$ incubator at 37 °C to reach a confluence of 80-90%. The extract at 50, 100, 200, and 400 µg/mL concentrations was put into the well and incubated for 48 hours. After incubation, the supernatant was substituted with 150 µL of medium and 50 µL of MTT. Purple formazan crystals appeared after 4 hours of incubation and were dissolved by DMSO (0.1%). The absorbance of the plate was read at 595 nm using a microplate reader (Azure Biosystems Microplate Reader) with a commercially available cisplatin serving as a positive control. The cytotoxic capacity of extract ($IC_{50}$) was calculated using a polynomial regression equation obtained from percentage cell growth inhibition at different concentrations of extract.

### 2.10. Acute toxicity assay

Twenty albino mice, approximately 8-10 weeks old, were selected, with 10 males and 10 females, weighing 28 ± 4 g. The mice were kept under control conditions for five days according to OECD (2008) test guidelines. Before dosing, the mice were fasted overnight for approximately 16 hours. They were then orally administered a dose of 20 mL/kg of DTPE and DTSE extracts at a concentration of 2000 mg/kg body weight. The control group consisted of five mice that were fed an equivalent volume of sterile water. After dosing, the mice were fed their regular diet. Furthermore, both groups were closely monitored for any signs of abnormality for 14 days.

### 2.11. Determination of antibacterial activity

The pure culture of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* in nutrient broth (HiMedia, India) was incubated in a shaker at 37 °C and 120 rpm overnight. Each bacterial strain was inoculated on the Mueller-Hinton agar plate. The antibacterial activity of the extracts was determined by using the well diffusion method. Five wells of 7 mm diameter were made on a nutrient agar plate. Three wells were filled with 100 µL of each DTSE and DTPE (2.5, 5, 10 and 15 mg/mL), one with DMSO (negative control) and one with antibiotics (positive control). After incubation overnight at 37 °C, the zone of inhibition was measured in millimetres (mm).

### 2.12. Statistical analysis

The data were presented as mean ± SD of three replicates. One-way analysis of variance was applied to compare the values using SPSS version 20.

## 3. Results and Discussion

### 3.1. Results

The seeds of Translucent Dendrobium (Fig. 1) were cultured on full-strength MS medium and full-strength MS medium supplemented with BAP (0.5 to 2.0 mg/L) and NAA (1 mg/L). The seeds were germinated and developed into protocorms on full-strength MS medium as early as 4 weeks of culture (Table 1). The full-strength MS medium supplemented with 2.0 mg/L BAP and 1 mg/L NAA also induced protocorm formation after 6 weeks of seed culture, and these protocorms developed into shoots and roots within 8 and 11 weeks of culture, respectively (Table 1, Fig. 1). The protocorms and wild stem extracts (DTPE and DTSE) were evaluated for their total phenolic and flavonoid contents and antioxidant activity. Accordingly, DTSE demonstrated comparable high total phenolic content (82.00 mg GAE/g) and total flavonoid content (61.889 mg QE/g) as that of DTPE (Table 2). In view of the obtained findings of this study, DTSE was capable of scavenging 64.91% of DPPH radical, while DTPE scavenged only 53.79-56.10% at 100-200 µg/mL concentration. However, ascorbic acid (as a positive control) scavenged 62.15-74.58% at the same concentration (Fig. 2). The DTSE performed the highest DPPH radical scavenging activity and thus showed strong antioxidant capacity by scavenging 50% DPPH radicals at 191.23 µg/mL as compared to DTPE (Table 2).

The cytotoxic activity of DTSE and DTPE was evaluated against two cancer cell lines, namely HeLa and U251 and a normal cell line, namely 3T3. DTPE exhibited a significantly high percentage of cell growth inhibition in HeLa (56.63%) and U251 (60.29%) cell lines compared to the 3T3 cell line (7.6%) at a concentration of 400 µg/mL (Fig. 3 and Fig. 4). The cytotoxic capacity ($IC_{50}$) of DTPE against HeLa (229.30 µg/mL) and U251 (213.90 µg/mL) cell lines was found to be significantly stronger than that against the 3T3 cell line (2108.87 µg/mL) (Table 3). Similarly, DTSE showed significantly strong cytotoxic capacity towards both cancer cell lines, whereas it was least cytotoxic towards normal cell lines (data retrieved from our previous publication, Joshi et al. 2020). However, commercially available cisplatin exhibited

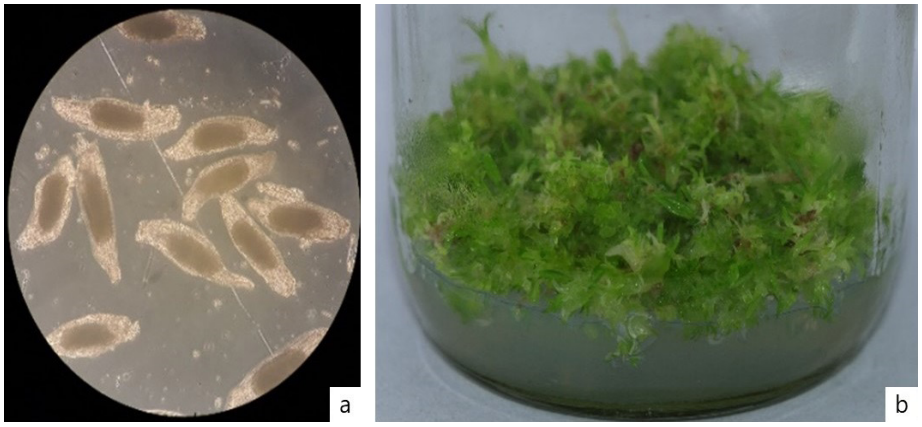



**Fig. 1.** Seed germination and protocorms development: Ocular view of seeds under inverted microscope at x200 magnification before culture (a), Protocorms developed on full strength MS medium with 2 mg/L BAP and 1 mg/L NAA (b).

**Table 1**

Effect of different strengths of MS medium with or without BAP and NAA for *in vitro* culture of seeds of Translucent Dendrobium.

| S.N. | Medium | PGRs | | Initiation of events observed in a week | | | |
|---|---|---|---|---|---|---|---|
| | | BAP | NAA | Germination | Protocorms | Shoot | Root |
| 1 | HMS | 0 | 0 | 5±0.3 | 7±0.6 | 10±0.5 | 12±0.7 |
| 2 | FMS | 0 | 0 | 4±0.4* | 6±0.5* | 9±0.7 | ∞±0 |
| 3 | FMS | 0.5 | 1 | 7±0.5 | 9±0.3 | ∞±0 | ∞±0 |
| 4 | FMS | 1 | 1 | 6±1.6 | 7±0.5 | ∞±0 | ∞±0 |
| 5 | FMS | 1.5 | 1 | 6±1.1 | 7±0.7 | 10±0.2 | 14±0.7 |
| 6 | FMS | 2 | 1 | 5±0.4 | 6±1.5* | 8±0.3* | 11±0.2* |

*The values with an asterisk (*) are significantly different at $p < 0.05$.

**Table 2**

Total phenolic, total flavonoid, and antioxidant activity of Translucent Dendrobium extracts.

| S.N. | Extract | Total Phenolic Content (mg GAE/g) | Total Flavonoid Content (mg QE/g) | Antioxidant capacity ($IC_{50}$) (µg/mL) |
|---|---|---|---|---|
| 1 | Wild stem (DTSE) | 82 | 61.89 | 191.23 |
| 2 | Protocorms (DTPE) | 72.33 | 56.89 | 295.83 |
| 3 | Ascorbic acid | - | - | 33.72 |

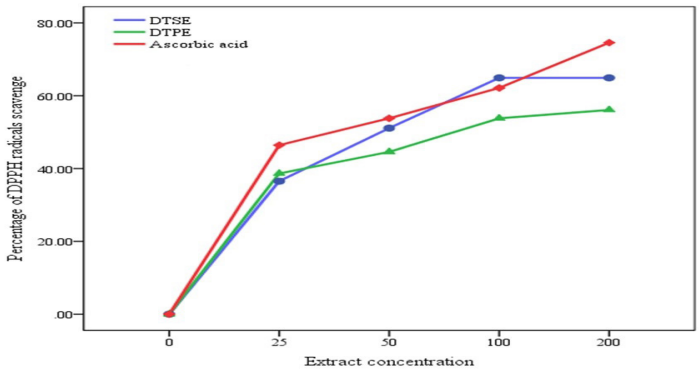



**Fig. 2.** Percentage of DPPH radical scavenging by Translucent Dendrobium extracts and Ascorbic acid.

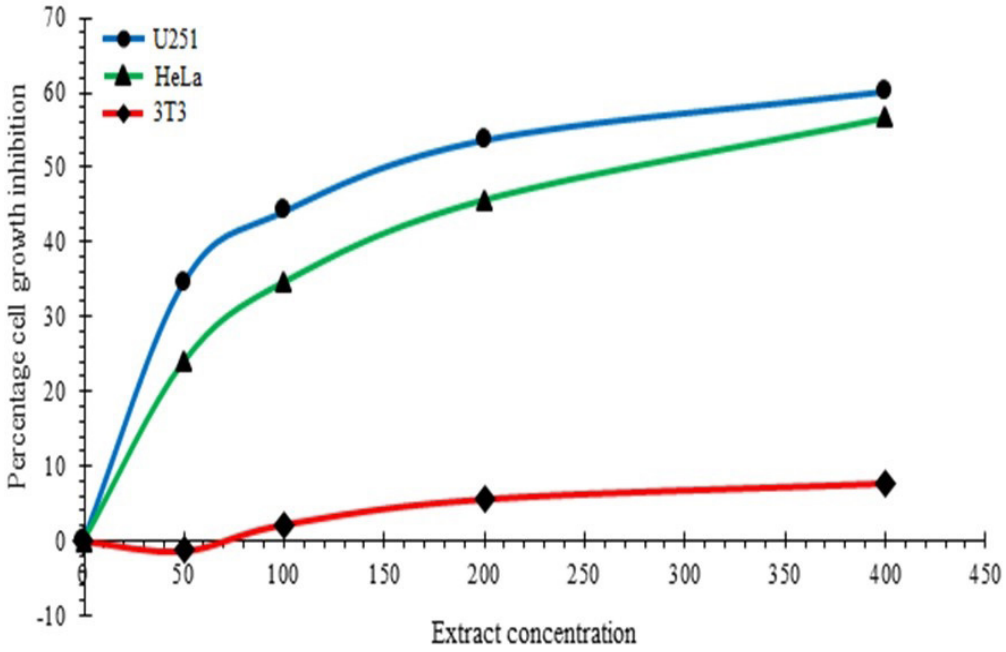



**Fig. 3.** Percentage of cell growth inhibition by Translucent Dendrobium protocorm extract (DTPE).

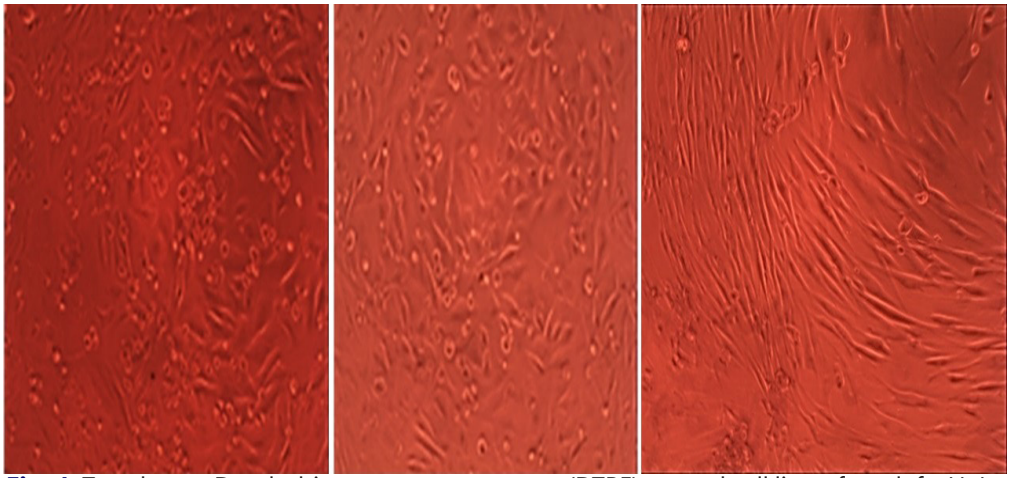


**Fig. 4.** Translucent Dendrobium protocorm extract (DTPE) treated cell lines; from left- HeLa, U251 and 3T3.

significantly strong anticancer activity towards both cancer cell lines compared to the extracts tested (Table 3). The acute toxicity of DTSE and DTPE was assessed in albino mice. It was observed that all tested animals survived until the 14-day observation period, and there was no significant change compared to the control group of mice. The lethal dose (LD) value ranged above 2000 mg/kg body weight for both extracts (Table 4).

Antibacterial activity of the DTSE and DTPE was evaluated against the five ATCC-type bacteria, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* (Fig. 5). Regarding the experimental findings, both of the extracts were capable of inhibiting the growth of all the bacterial strains used, but only the higher concentration of extracts inhibited the growth

**Table 3**
Cytotoxic capacity ($IC_{50}$) of Translucent Dendrobium extracts and cisplatin towards HeLa, U251 and 3T3 cell lines.

| S.N. | Extract/Control | $IC_{50}$ for HeLa cells (µg/ mL) | $IC_{50}$ for U251 cells (µg/ mL) | $IC_{50}$ for 3T3 cells (µg/ mL) |
|---|---|---|---|---|
| 1 | Wild stem (DTSE) | 382.14* | 75.84* | 2329.17 |
| 2 | Protocorms (DTPE) | 229.30* | 213.90* | 2108.87 |
| 3 | Cisplatin drug | 25.00** | 25.00** | - |

**Table 4**
Acute toxicity test in albino mice.

| S.N. | Extract | $LD_{50}$ value | Remarks |
|---|---|---|---|
| 1 | Wild stem (DTSE) | >2000 mg/kg BW | Acute oral toxicity test according to OECD TG 425 Up and down procedure, 2008 |
| 2 | Protocorms (DTPE) | >2000 mg/kg BW | |

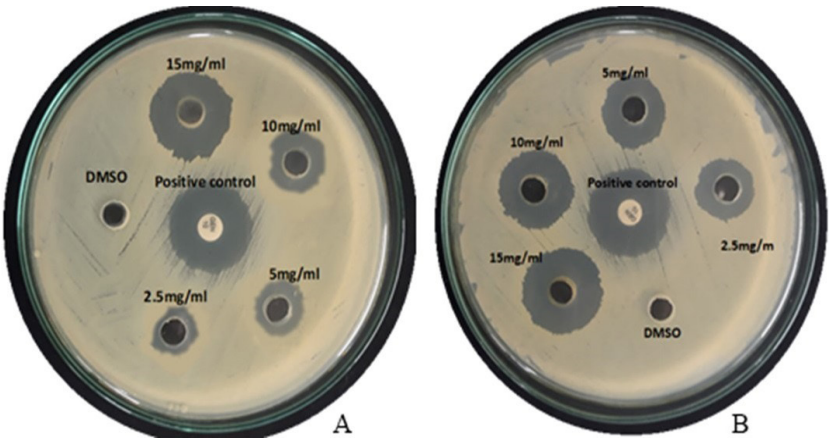



**Fig. 5.** Inhibition of growth of *Acinetobacter baumannii* by DTSE (A) and DTPE (B).

of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Table 5). However, commercial antibiotics (gentamycin) have significantly inhibited the growth of bacterial strains.

### 3.2. Discussion

Orchids are gaining popularity as a means of preventing and treating a variety of ailments. They are considered safe and free of major side effects, encouraging consumers to use natural treatments (Pariyani et al., 2015). Orchids are also equally important in the field of horticulture. However, their large-scale consumption from natural habitats has led to a decrease in their population. To address this, orchids are being conserved through plant tissue culture techniques, which can synthesize bioactive secondary metabolites in their tissues. Protocorms, highly organized tissues produced through seed culture, are particularly noteworthy as they accumulate these valuable bioactive secondary metabolites. Protocorm cultures have been accepted for the mass propagation of valuable orchid species (Park et al., 2000, 2020; Paudel et al., 2020) and the production of bioactive compounds including polysaccharides (Zha et al., 2007). In this study, a full-strength MS medium was found to produce protocorms for further biological assays. Production of the metabolite from *in vitro* cultures is dependent on the composition of media, pH, the density of inoculum used, and environmental conditions like temperature, light density, and aeration because of which conditions need to be optimized for efficient metabolite production (Pant 2014; Ochoa-Villarreal et al., 2016; Isah et al., 2018). Macronutrients and micronutrients, vitamins, sugars, amino acids

and plant hormones in a culture medium might have influenced the metabolite formation in protocorms (Cardoso et al., 2019), due to which protocorms have subsequently shown better antioxidant, antibacterial and cytotoxic activity including a significant number of polyphenols comparable to its wild counterpart.

Previous studies by Park et al. (2000), Zha et al. (2007), and Cui et al. (2015) support our claim that protocorms can be a proper source for isolating bioactive components with decent biological activity. In this study, we produced biomass from *in vitro*-developed protocorms for the synthesis of bioactive secondary metabolites and evaluated their antioxidant, antibacterial, and cytotoxic activities.

Our results show that the wild sample exhibited slightly superior antioxidant, antibacterial, and cytotoxic activity compared to the protocorm extract, which had higher flavonoid and phenolic content. Antioxidant-rich substances found in plants can scavenge free radicals and reactive oxygen species produced in the human body, potentially preventing cancer. These antioxidant-rich substances can halt the cell cycle and induce apoptosis, as reported by Pham-Huy et al. (2008) and Roleira et al. (2015). In a previous study, *in-vitro*-developed protocorms from *Dendrobium densiflorum* were also found to have significant cytotoxicity and antioxidant activity compared to their wild counterpart (Pant et al., 2021). A similar result was also reported in *Coelogyne stricta* with cytotoxic activity against HeLa cells (Thapa et al., 2020). The existence of bioactive compounds varies depending on which section of the plant is inoculated, and variations in the nutrient components of organic supplements have a significant impact on the accumulation of such compounds (Zha et al., 2007; Cui et al., 2015). Similar mechanisms of action of antioxidant-rich substances found in wild counterparts and protocorms were seen in *Dendrobium longicornu*, *D. amoenum, D. crepidatum, D. moniliforme, D. chryseum* and *D. densiflorum* on the HeLa and U251 cell lines (Sayin et al., 2014; Gali-Muhtasib et al., 2015; Paudel et al., 2017, 2018, 2019; Pant et al., 2021). Many folk medicinal plants have been reported to exhibit toxic effects (Roleira et al., 2015; Saleem et al., 2016). Initial toxicological evaluation is crucial for confirming the safety of herbal medications. However, acute oral toxicity studies are required to determine the appropriate dosage to manage the clinical signs and symptoms of the drugs (Saleem et al., 2016). Mice were used in the present study to collect data on lethal doses as they are more suitable for anticipating toxic effects (Walum et al., 1995). The acute toxicity test revealed that the extracts of Translucent Dendrobium (DTSE and DTPE) are non-toxic and reliable, with no evidence of toxicity observed up to a dose of 2000 mg/kg body weight.

## 4. Concluding remarks

On a full-strength MS medium containing 2.0 mg/L BAP and 1.0 mg/L NAA, *D. transparens protocorms* were effectively replicated *in vitro*. *D. transparens* wild stems extract displayed comparable high total phenolic content and total flavonoid content, therefore, scavenged at least 50% DPPH radicals at its 191.23 µg/mL concentration. However, *D. transparens* wild stems and protocorms extracts exhibited cytotoxic effects against HeLa and U251 cell lines, but not normal cell lines (3T3). These extracts showed no overt harmful effects in mice. These extracts also have antibacterial activity against certain ATCC-type bacteria. Therefore, *D. transparens* wild stems and *in-vitro*-raised protocorms may be ideal for synthesizing pharmacologically bioactive substances. Protocorms alone in pharmacology would be used sustainably to lessen the strain on this species' natural population.

## Abbreviations

**BAP:** 6-Benzylaminopurine; **DMEM: Dulbecco's Modified Eagle Medium; DTPE:** *Dendrobium transparens* Protocorms Extract; **DTSE:** *Dendrobium transparens* Stem Extract; **FBS: Fetal Bovine Serum; FC:** Folin-Ciocalteu; IAA: Indole-3-Acetic Acid; **IBA:** Indole-3-Butyric Acid; **LD:** Lethal Dose; **MS:** Murashige and Skoog; **MTT:** (3-[4,5-Dimethylthiazole-2-Yl]-2,5-Diphenyl-Tetrazolium Bromide)**; NAA:** 1-Naphthaleneacetic Acid; **TPC:** Total Phenolic Content; **TUCH:** Tribhuvan University Central Herbarium.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgement

We would like to acknowledge the University Grants Commission (UGC), Nepal for providing a Ph.D. research grant (Grant No.: PhD-76-77-S&T-20) to Pusp Raj Joshi. We are also thankful to Kagoshima University, Department of Neurosurgery, Japan for providing U251 cell lines, and Shikhar Biotech Pvt. Ltd., Nepal for providing HeLa and 3T3 cell lines.

## References

Babili, F.E., Nigon, C., Lacaze, L., Millé, J., Masiala, A., Simm, J., Lamade, V.M., El Haj, A.A., 2022. A new colorimetric DPPH radical scavenging activity method: Comparison with spectrophotometric assay in some medicinal plants used in Moroccan Pharmacopoeia. Pharma. Fronts. 4, e89-e102.

Bourgaud, F., Gravot, A., Milesi, S., Gontier, E., 2001. Production of plant secondary metabolites: A historical perspective. Plant Sci. 161(5), 839-851.

Cardoso, J.C., Oliveira, M.E., Cardoso, F.D.C., 2019. Advances and challenges on the *in vitro* production of secondary metabolites from medicinal plants. Horticult. Brasil. 37, 124-132.

Cui, H.Y., Murthy, H.N., Moh, S.H., Cui, Y.Y., Paek, K.Y., 2015. Establishment of protocorm suspension cultures of *Dendrobium candidum* for the production of bioactive compounds. Horticult. Environ. Biotechnol. 56(1), 114-122.

eFloras, 2020. *Dendrobium transparens* Wall. Ex Lindl. http://www.efloras.org/florataxon.aspx?flora_

id=110&taxon_id=242421917. Accessed: 12 Dec 2021
El Jabboury, Z., Bentaib, R., Stevanovic, Z.D., Ousaaid, D., Benjelloun, M., El Ghadraoui, L., 2023. *Ammi visnaga* (L.) Lam.: An overview of phytochemistry and biological functionalities. Trends Phytochem. Res. 7(3), 141-155.
Espinosa-Leal, C.A., Puente-Garza, C.A., García-Lara, S., 2018. *In vitro* plant tissue culture: Means for production of biological active compounds. Planta 248(1), 1-18.
Gali-Muhtasib, H., Hmadi, R., Kareh, M., Tohme, R., Darwiche, N., 2015. Cell death mechanisms of plant-derived anticancer drugs: Beyond apoptosis. Apoptosis 20(12), 1531-1562.
Gutierrez, R.M.P., 2010. Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. J. Med. Plants Res. 4(8), 592-638.
Hapuarachchi, S.D., Suresh, T.S., Hadunnetthi, S., Senarath, W.T.P.S.K., Ranasinghe, C., 2022. Anti-inflammatory potential of aqueous extract and ethyl acetate fractions of *Munronia pinnata* (Wall) Theob. and the isolated compound, senecrassidiol. Trends Phytochem. Res. 6(3), 247-258.
Hussain, M.S., Fareed, S., Saba-Ansari, M., Rahman, A., Ahmad, I.Z., Saeed, M., 2012. Current approaches toward production of secondary plant metabolites. J. Pharm. Bioall. Sci. 4(1), 10.
Isah, T., Umar, S., Mujib, A., Sharma, M.P., Rajasekharan, P.E., Zafar, N., Frukh, A., 2018. Secondary metabolism of pharmaceuticals in the plant *in vitro* cultures: Strategies, approaches, and limitations to achieving higher yield. Plant Cell Tiss. Organ Cult. 132(2), 239-265.
Joshi, P.R., Paudel, M.R., Chand, M.B., Pradhan, S., Pant, K.K., Joshi, G.P., Bohara, M., Wagner, S.H., Pant, B. Pant, B., 2020. Cytotoxic effect of selected wild orchids on two different human cancer cell lines. Heliyon 6(5), e03991.
Kota, S., Dumpala, P., Sajja, R., Anantha, R., 2023. Phytoconstituents of *Chromolaena odorata* (L.) leaf extract for the synthesis of copper oxide/copper nanoparticles and evaluation of their biological potential in wound healing. Trends Phytochem. Res. 7(3), 186-206.
Mohammadhosseini, M., Frezza, C., Venditti, A., Akbarzadeh, A., 2019. Ethnobotany and phytochemistry of the genus *Eremostachys* Bunge. Curr. Org. Chem. 23, 1828-1842.
Mohammadhosseini, M., Frezza, C., Venditti, A., Sarker, S., 2021. A systematic review on phytochemistry, ethnobotany and biological activities of the genus *Bunium* L. Chem. Biodivers. 18(11), e2100317.
Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M.O., Jin, Y.W., Lee, E.K. Loake, G.J., 2016. Plant cell culture strategies for the production of natural products. BMB Rep. 49(3), 149.
OECD, O., 2008. Guideline 425: Acute Oral Toxicity—Up-and-Down Procedure. OECD Guidelines for the Testing of Chemicals, 2. OECD 425.
Otero, J.T., Ackerman, J.D. Bayman, P., 2002. Diversity and host specificity of endophytic Rhizoctonia-like fungi from tropical orchids. American J. Bot. 89(11), 1852-1858.
Pant, B., 2013. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. African J. Plant Sci. 7(10), 448-467.
Pant, B., 2014. Application of Plant Cell and Tissue Culture for the Production of Phytochemicals in Medicinal plants. In "Infectious Diseases and Nanomedicine II ." Cham: Springer, pp. 25-39.
Pant, B., Chand, K., Paudel, M.R., Joshi, P.R., Thapa, B.B., Park, S.Y., Shakya, S., Thakuri, L.S., Rajbahak, S., Sah, A.K. Baniya, M.K., 2022. Micropropagation, antioxidant and anticancer activity of pineapple orchid: *Dendrobium densiflorum* Lindl. J. Plant Biochem. Biotechnol. 31, 399-409.
Pant, B., Joshi, P.R., Maharjan, S., Thakuri, L.S., Pradhan, S., Shah, S., Wagner, S.H. Pant, B., 2021. Comparative cytotoxic activity of wild harvested stems and *in vitro*-raised protocorms of *Dendrobium chryseum* Rolfe in human cervical carcinoma and glioblastoma cell lines. Adv. Pharmacol. Pharma. Sci. 2021, 8839728.
Pant, B., Shah, S., Shrestha, R., Pandey, S. Joshi, P.R., 2017. An Overview on Orchid Endophytes. In "Mycorrhiza-nutrient Uptake, Biocontrol, Ecorestoration." Cham: Springer, pp. 503-524.
Pariyani, R., Safinar Ismail, I., Azam, A.A., Abas, F., Shaari, K. Sulaiman, M.R., 2015. Phytochemical screening and acute oral toxicity study of Java tea leaf extracts. BioMed Res. Int. 2015(1), 742420.
Park, S.Y., Ho, T.T. Paek, K.Y., 2020. Medicinal orchids: Production of Bioactive Compounds and Biomass. In "Orchid Biology: Recent Trends & Challenges." Singapore: Springer, pp. 439-450.
Park, S.Y., Murthy, H.N. Paek, K.Y., 2000. *In-vitro* seed germination of *Calanthe sieboldii*, an endangered orchid species. J. Plant Bio. 43, 158-161.
Paudel, M.R., Chand, M.B., Pant, B. Pant, B., 2017. Cytotoxic activity of antioxidant-riched *Dendrobium longicornu*. Pharmacog. J. 9(4), 499-503.
Paudel, M.R., Chand, M.B., Pant, B. Pant, B., 2018. Antioxidant and cytotoxic activities of *Dendrobium moniliforme* extracts and the detection of related compounds by GC-MS. BMC Comp. Alt. Med. 18, 134.
Paudel, M.R., Chand, M.B., Pant, B. Pant, B., 2019. Assessment of antioxidant and cytotoxic activities of extracts of Dendrobium crepidatum. Biomolecules 9(9), 478.
Paudel, M.R., Joshi, P.R., Chand, K., Sah, A.K., Acharya, S., Pant, B. Pant, B., 2020. Antioxidant, anticancer and antimicrobial effects of *in vitro* developed protocorms of *Dendrobium longicornu*. Biotechnol. Rep. 28, e00527.
Pfab, M.F. Scholes, M.A., 2004. Is the collection of *Aloe peglerae* from the wild sustainable? An evaluation using stochastic population modelling. Biol. Conser. 118(5), 695-701.
Pham-Huy, L.A., He, H. Pham-Huy, C., 2008. Free radicals, antioxidants in disease and health. Int. J. Biomed. Sci. 4(2), 89.
Roleira, F.M., Tavares-da-Silva, E.J., Varela, C.L., Costa, S.C., Silva, T., Garrido, J. Borges, F., 2015. Plant derived and dietary phenolic antioxidants: Anticancer properties. Food Chem. 183, 235-258.
Saleem, U., Ahmad, B., Ahmad, M., Erum, A., Hussain, K., Irfan Bukhari, N., 2016. Is folklore use of *Euphorbia helioscopia* devoid of toxic effects? Drug Chem. Toxicol. 39(2), 233-237.
Sayin, V.I., Ibrahim, M.X., Larsson, E., Nilsson, J.A., Lindahl, P. Bergo, M.O., 2014. Antioxidants accelerate lung cancer progression in mice. Sci. Transl. Med. 6(221),

221ra15.
Thapa, B.B., Thakuri, L.S., Joshi, P.R., Chand, K., Rajbahak, S., Sah, A.K., Shrestha, R., Paudel, M.R., Park, S.Y., Pant, B., 2020. *Ex-situ* conservation and cytotoxic activity assessment of native medicinal orchid: *Coelogyne stricta*. J. Plant Biotechnol. 47(4), 330-336.
Vanisree, M., Lee, C.Y., Lo, S.F., Nalawade, S.M., Lin, C.Y. Tsay, H.S., 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot. Bull. Acad. Sin. 45(1), 1-22.
Walum, E., Nilsson, M., Clemedson, C. Ekwall, B., 1995. The MEIC program and its implications for the prediction of acute human systemic toxicity. Alt. Meth. Toxicol. 11, 275-282.
Yeung, E.C., 2017. A perspective on orchid seed and protocorm development. Bot. Stud. 58(1), 33.
Zha, X.Q., Luo, J.P., Jiang, S.T. Wang, J.H., 2007. Enhancement of polysaccharide production in suspension cultures of protocorm-like bodies from *Dendrobium huoshanense* by optimization of medium compositions and feeding of sucrose. Process Biochem. 42(3), 344-351.

Trends Phytochem. Res. 8(2) 2024 141-148

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Green synthesis of carbon quantum dots and its composite with Ag/chitosan using citrus fruit extracts

Behnam Mahdavi[1], Ehsan Koushki[2]✉*, Behnaz Noori Dolouie[1] and Majid Mohammadhosseini[3]

[1]Department of Chemistry, Faculty of Sciences, Hakim Sabzevari University, Sabzevar, 96179-76487, Iran
[2]Department of Physics, Faculty of Sciences, Hakim Sabzevari University, Sabzevar, 96179-76487, Iran
[3]Department of Chemistry and Biochemistry, College of Basic Sciences, Shahrood Branch, Islamic Azad University, Shahrood, Iran

**ABSTRACT**

Quantum dots are small semiconductors less than 50 nanometers in size, exhibiting unique optical and electronic properties due to quantum mechanics. Carbon quantum dots (CQDs) are particularly popular due to their size-dependent fluorescence, non-toxicity, biocompatibility, and ease of access. CQDs have significant potential applications in various chemical fields. In this study, CQDs were successfully synthesized using a green, one-step hydrothermal method involving citrus (tangerine). The synthesized CQDs were characterized using UV-VIS, XRD, IR, and TEM methods. The CQDs were then reacted with silver oxide to form CQD/Ag composite, and chitosan polymer was used to synthesize carbon quantum dots/Ag/Chitosan (CQD/Ag/Chit) bio-composite. The product was characterized using ultraviolet and infrared spectroscopy to confirm the bonds. This composite has potential applications in biological fields, as chitosan and silver have strong antibacterial properties, and their combination with active CQDs can enhance these properties.

**ARTICLE HISTORY**

Received: 08 February 2024
Revised: 30 May 2024
Accepted: 20 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Bioimaging
Carbon quantum dots (CQDs)
Carbon quantum dots/Ag/Chitosan (CQD/Ag/Chit)
Characterization
Citrus fruit extracts
Fluorescence
Photoluminescence

## 1. Introduction

Carbon, as the fundamental element in organic chemistry, has a profound impact on human daily life. Furthermore, carbon's versatility in combining with other elements makes it one of the most important and valuable elements in the pharmaceutical and food industries (Demming, 2010; Egbedina et al., 2022). Carbon quantum dots (CQDs) are the smallest members of the carbon family and have emerged as a powerful alternative to numerous fluorophores due to their exceptional electrical, optical, and physical properties (Huang et al., 2019b; Tajik et al., 2020; Kumar et al., 2022; Soumya et al., 2023; Zhao et al., 2023). In addition, CQDs are a desirable alternative to conventional materials in a wide array of applications, spanning from biological to industrial fields. This is due to their advantageous properties compared to other materials, such as extremely low cytotoxicity and excellent biocompatibility (Li et al., 2020; Tungare et al., 2020; Nammahachak et al., 2022; Mat Zaid et al., 2023). The luminescence of CQDs exhibits promising optical properties that function effectively even in solid-state conditions (Magesh et al., 2022; Shan et al., 2023; Sharma et al., 2023; Shan et al., 2024).

Among their notable features, the luminescence and, in particular, the photoluminescence (PL) emission of CQDs have garnered significant attention in recent years. CQDs are capable of emitting photons across a wide spectrum, from the ultraviolet to the near-infrared region (Zhang et al., 2019). Several plausible mechanisms have been proposed to explain the factors influencing the PL emission of CQDs, including surface passivation, aggregation-induced emission (AIE), proximity effects of other CQD particles, and CQD size (Tang et al., 2019; Lai et al., 2020; Ghasedi et al., 2022). Additionally, impurities can significantly alter the optical behavior of

✉Corresponding author: Ehsan Koushki
Tel: +98-51-44013319 ; Fax: +98-51-44012669
E-mail address: ehsan.koushki@hsu.ac.ir, **doi:**

CQDs by introducing new energy states within the band gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), thereby affecting electronic transitions (Yang et al., 2018; Kou et al., 2020). An excited electron can jump to an energy state within the middle of the band gap and then return to its initial state. In the study of Tang et al. (2019), white-emitting CQDs were prepared using a direct one-pot synthesis approach, which offers the advantage of an *in situ* cellulose matrix in the synthesis medium.

On the other hand, the use of green resources to produce nanoparticles and quantum dots has been a focus of extensive research in recent years (Haji and Barzinjy, 2023; Khajavi et al., 2023; Kakhki et al., 2024). The key advantages of these green synthesis methods include simplicity, low cost, and biocompatibility. Recently, our group has synthesized CQDs using the leaves of Aloe Vera (Montazeri et al., 2024). The durability of cellulose fibers in the Aloe Vera paste allowed us to utilize them as *in-situ* matrix networks. In a recently published paper, Hong et al. (2019a) synthesized CQDs through a one-step hydrothermal process using straw plant and bamboo plant residues at 180 °C. Subsequently, they characterized the synthesized sample using spectroscopic methods, demonstrating its high optical properties and quantum performance. Finally, they presented their product for advanced biological imaging applications. In another study, CQDs were synthesized from candle soot and dispersed in a chitosan matrix, yielding a homogeneous CQD-Chit nanocomposite. After extensive experiments and subsequent spectrometric analysis, it was determined that the proposed sensor can be used to measure insulin with high selectivity, accuracy, and sensitivity. Additionally, this sensor offers a simple, cost-effective construction and superior stability compared to other sensors (Abazar and Noorbakhsh, 2020).

In the present study, CQDs were prepared under hydrothermal conditions (in an aqueous environment at 160 °C) using an easily accessible citrus species as a precursor. This method is simple, environmentally friendly, and potentially scalable for mass production. The obtained CQDs contain a significant amount of oxygen-containing functional groups, as confirmed by infrared spectroscopy. A series of analyses, including transmission electron microscopy (TEM), ultraviolet-visible (UV-Vis) spectroscopy, X-ray diffraction (XRD), and Fourier-transform infrared (FT-IR) spectroscopy were conducted to provide sufficient evidence supporting the claims regarding the white emission of CQDs. Additionally, the final product of this study is a CQD-Ag-polymer bio-composite which has potential applications in biological fields, as compounds such as chitosan and silver have strong antibacterial properties, and their combination with active CQDs can enhance these properties.

## 2. Experimental

### 2.1. Synthesis of CQDs

We synthesized CQDs using a hydrothermal/solvothermal method using citrus (tangerine) juice as the carbon source. For this propose, 25 mL of the filtered fruit juice and 25 mL of deionized water were poured into a teflon autoclave, which was protected by a stainless steel. After shaking, the autoclave was tightly lidded reaction container and placed in an oven at 160 °C for 4 hours (Bhunia et al., 2013). After the completeness of the reaction, the autoclave was cooled to ambient temperature. Next, the obtained product was placed in a centrifuge for 10 minutes at a speed of 12000 rpm. Then, the obtained solution was filtered with a 0.22 mm smooth syringe head filter and was finally kept as CQDs. The sediment was also dried and stored in a suitable place for analysis.

### 2.2. Synthesis of CQDs/Ag

To synthesize CQDs/Ag, 10 mL of $AgNO_3$ (1.0 M) was mixed with 10 mL of NaCl (1.0 M). After shaking, the mixture was centrifuged at 8000 rpm for 5 min. Next, 5 mL of NaOH (1.0 M) was added to the residue and shaked vigorously. The centrifuging process was then continued for over 5 min. The residue, as silver oxide, was kept to be used in the next step. In the next step, 0.06 g of silver oxide was added to 5 mL of deionized water and centrifuge was placed in an ultrasonic bath for 30 min at 60 °C. Then, 1 mL of the silver oxide solution and 1 mL of CODs were poured into an autoclave containing 10 mL of deionized water. After tightly lidding, the autoclave was put in an oven at 160 °C for 2 h. The obtained yield was kept cold in a dark place for the synthesis of CQDs/Ag/Chitosan.

### 2.3. Synthesis of CQDs/Ag/Chitosan composite

For the successful synthesis of CQDs/Ag/Chitosan composite, 0.2 g of chitosan was first dissolved in 50 mL of acetic acid (1.0%). Then, 10 mL of CQDs/Ag was added dropwise to chitosan solution at 40 and the mixture was stirred moderately for 24 h. Finally, the obtained powder (CQDs/Ag/Chitosan composite) was kept in a cold place for the relevant analyses (Esmaeilzadeh et al., 2020).

### 2.4. Characterization methods

To prove the formation of CQDs, a UV chamber with a lamp in the wavelength range of 200 to 300 nm was used. For this purpose, a solution of CQDs and NaOH with the ratio of 1 to 2 was poured in a test tube and exposed to UV radiation. In this relation, UV-VIS spectra were used to determine the amount and range of absorption of CQDs. About 1 mL of the product diluted 12 times with water to completely become transparent which was then transferred to the special quartz cell of the device and the absorption spectrum was recorded over the wavelength range of 200 to 800 nm. Absorption spectra were also recorded for the synthesized products of other steps in the same way using a NanoDrop 2000 UV-Vis spectrophotometer. A 150 kV Philips CM30 transmittance electron microscopy (TEM) was used to take TEM images. To obtain Fourier Transform Infrared (FT-IR) spectra of the sample, a PerkinElmer

spectrometer (ASTM E 1252-98) was employed.

## 3. Results and Discussion

### 3.1. Impact of addition of different volumes of CQD/Ag composite to chitosan

The experiment was carried out by adding 1, 4, 7 and 10 mL of CQD/Ag composite to chitosan, and it was found that the higher the volume of CQD/Ag composite, the darker the color of the obtained product that is due to the presence of more CQDs representing optical properties of the sample. As shown in Fig. 1, the product with 4 mL CQD/Ag has lower optical absorption than the product with 10 mL. The greenish photoluminescence of CQDs has been shown in Fig. 1.b and Fig. 1.d.

### 3.2. UV-Vis. absorption spectra of the prepared CQDs, CQDs/Ag composite and Ag NPs

UV-visible spectroscopy is regarded as a simple approach for characterizing carbon quantum dots (CQDs), implying some valuable insights into their optical properties and structural features. The UV-visible spectra of CQDs typically exhibit absorption peaks in the UV region, often accompanied by emission peaks in the
visible range, which can be used to determine the size, shape, and composition of the particles (De and Karak, 2013; Zulfajri et al., 2019). The position and intensity of these peaks can provide information about the functional groups present on the surface of the CQDs, such as carbon, nitrogen, and oxygen, which are critical for their optical and biological applications (De and Karak, 2013; Zulfajri et al., 2019). Furthermore, the UV-visible spectra can be used as proper criteria to evaluate the photoluminescence mechanism of CQDs, which is of paramount importance for understanding their fluorescence properties and potential uses in sensing, imaging, and photocatalysis (Guo et al., 2017; Zhu et al., 2017; Rawat et al., 2023; Danawala et al., 2024). In the literature, UV-visible spectroscopy is referred as a powerful tool for probing the optical and structural characteristics of CQDs, enabling researchers to optimize their synthesis and applications in various fields.
The absorption spectrum of CQDs has shown two peaks between 200 nm and 300 nm, which are caused by π to π* electron transitions related to $sp^2$ orbitals and n to π* electron transitions related to C=O bonds. Fig. 2 (A-E) depicted UV-Visible spectra of all the products in this study.

### 3.3. IR spectra of the synthesized CQD and CQD/Ag/Chit

IR spectroscopy was used to determine the functional groups of the prepared products. For this purpose, the synthesized product (CQD and CQD/Ag/Chit) was placed in a dark place at room temperature for 48 hours to ensure the absence of any water in the environment. Then, 0.1 g of the powdered sediment with 1.0 g of potassium bromide was pounded and homogenized into a tablet and the spectra were recorded.
In order to identify functional groups on the surface of CQDs, the absorption of synthesized nanoparticles in the IR spectral region was taken into consideration. CQDs are composed of a core of two-dimensional spherical structures whose surface are covered by hydrophilic groups in the raw materials and cause the optimal solubility of CQDs in the aqueous environment (Lv et al., 2017). After the synthesis and filtration, the CQDs sample of the sediment obtained was dried and the FT-IR spectrum was recorded (see Fig. 3). Using FT-IR, the spectral analysis shows a strong and relatively broad peak in the range of 3400 $cm^{-1}$ related to the stretching vibrations of the O-H groups. The stretching vibrations of the C-H group ($CH_2$/$CH_3$) also appeared around the wavenumber of 2930 $cm^{-1}$. The peaks appearing or the range of 1600-1700 $cm^{-1}$ belong to C=C and C=N bonds, and the peaks related to N=O and C-O of ester groups were observed at 1400 and 1200 $cm^{-1}$, respectively. The peak at 1730 $cm^{-1}$ is related to the C=O groups of the carboxylic acid in the relevant structures.
In the infrared spectrum of chitosan, the peak appearing at 3440 $cm^{-1}$ is related to the O-H group and intramolecular hydrogen bonds, while that at 2900 $cm^{-1}$ shows the prisons of C-H group of the carboxylic acid group in chitosan. On the other hand, the peak at $cm^{-1}$ 1600 is related to N-H bending vibrations of the amine group in this polymer. Also, peaks in the range of 1040-11000 $cm^{-1}$ are attributed to the C-O amine and ring vibrations (Dimzon and Knepper, 2015; Negrea et al., 2015).
In the infrared spectrum containing CQDs, silver and chitosan, the peak at the wavelength of 11650 cm indicates the C=O stretching vibrations of the amide bond and the possibility of forming a bond between the carboxyl group of CQD and the amine group of chitosan. The two peaks appearing in the range of 3400-3500 $cm^{-1}$ have shown the stretching vibrations of N-H and O-H group compared to the spectrum of chitosan alone (Dimzon and Knepper, 2015; Negrea et al., 2015) (Fig. 3).

### 3.4. XRD pattern of that prepared CQDs

X-ray diffraction (XRD) spectroscopy has been proven as a powerful technique for characterizing the structural properties of carbon quantum dots (CQDs). XRD analysis can provide information about the crystalline structure, phase composition, and average crystallite size of CQDs (Shaikh et al., 2019; Hamid Abd and Ibrahim, 2022; Sharma et al., 2022). The XRD pattern of CQDs typically exhibits a broad peak around 20-30° 2θ, indicating the presence of amorphous or nanocrystalline carbon (Shaikh et al., 2019; Sharma et al., 2022). The position and width of this peak can be used to estimate the average size of the CQD core, with smaller CQDs exhibiting broader peaks due to quantum confinement effects (Hamid Abd and Ibrahim, 2022; Das et al., 2024). Additionally, the presence of sharp peaks in the XRD pattern may suggest the formation of graphitic or crystalline domains within the CQDs (Das et al., 2024). By comparing the experimental XRD data with

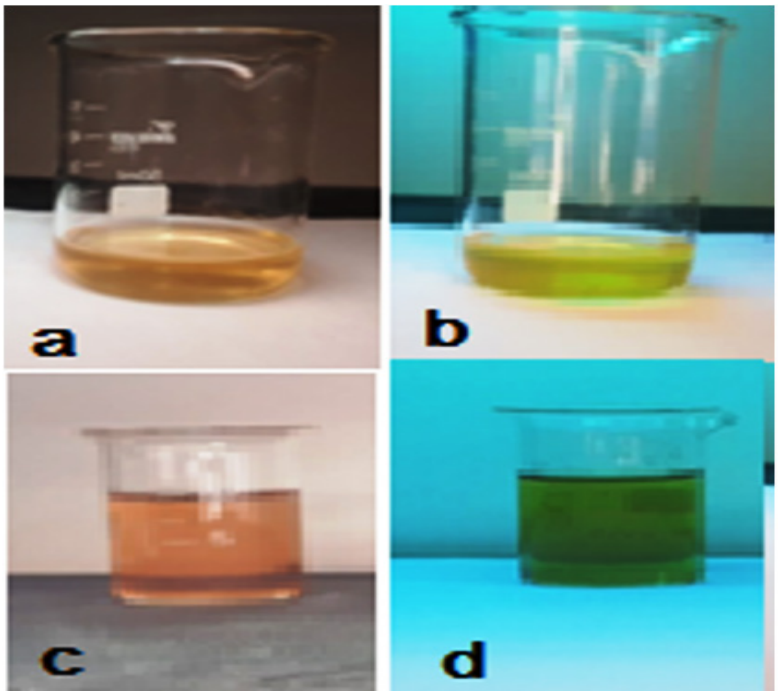



**Fig. 1. a:** CQD/Ag/Chit with 4 mL of CQD/Ag . **b:** Sample a under UV lamination. **c:** CQD/Ag/Chit with 10 mL of CQD/Ag . **d:** Sample c under UV lamination.

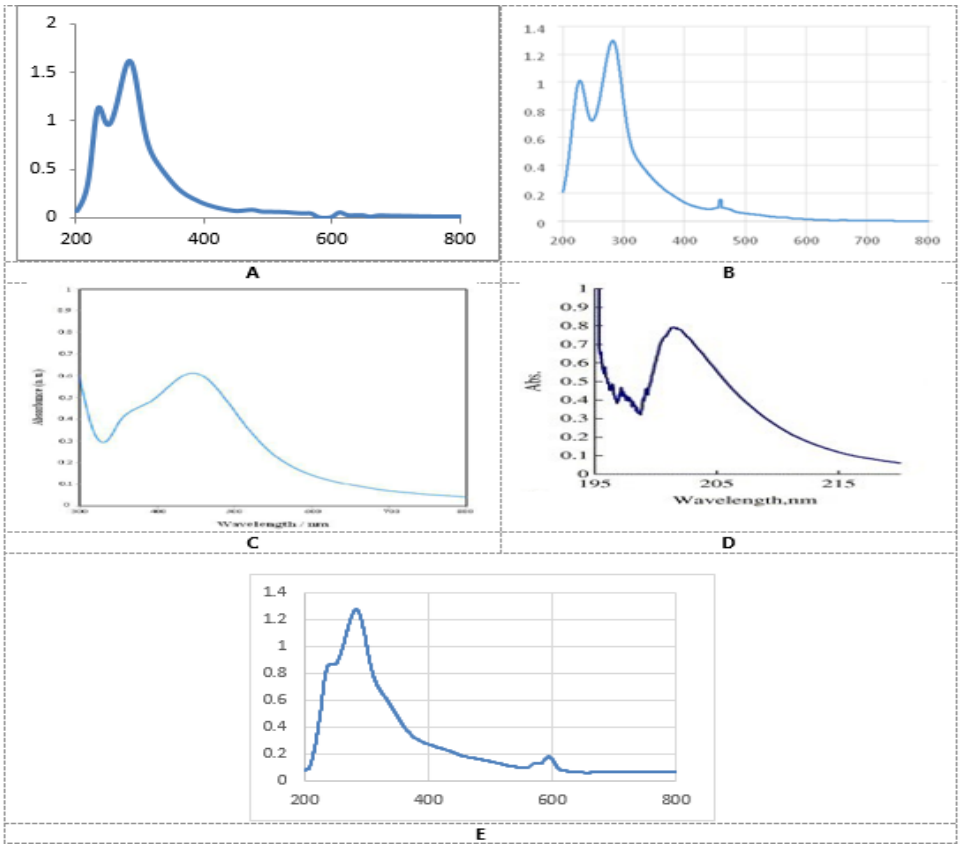



**Fig. 2.** UV-Visible spectra of **A:** CQDs, **B:** CQDs/Ag composite, **C:** AgNPs, **D:** Chitosan and **E:** CQDs/Ag/Chit.

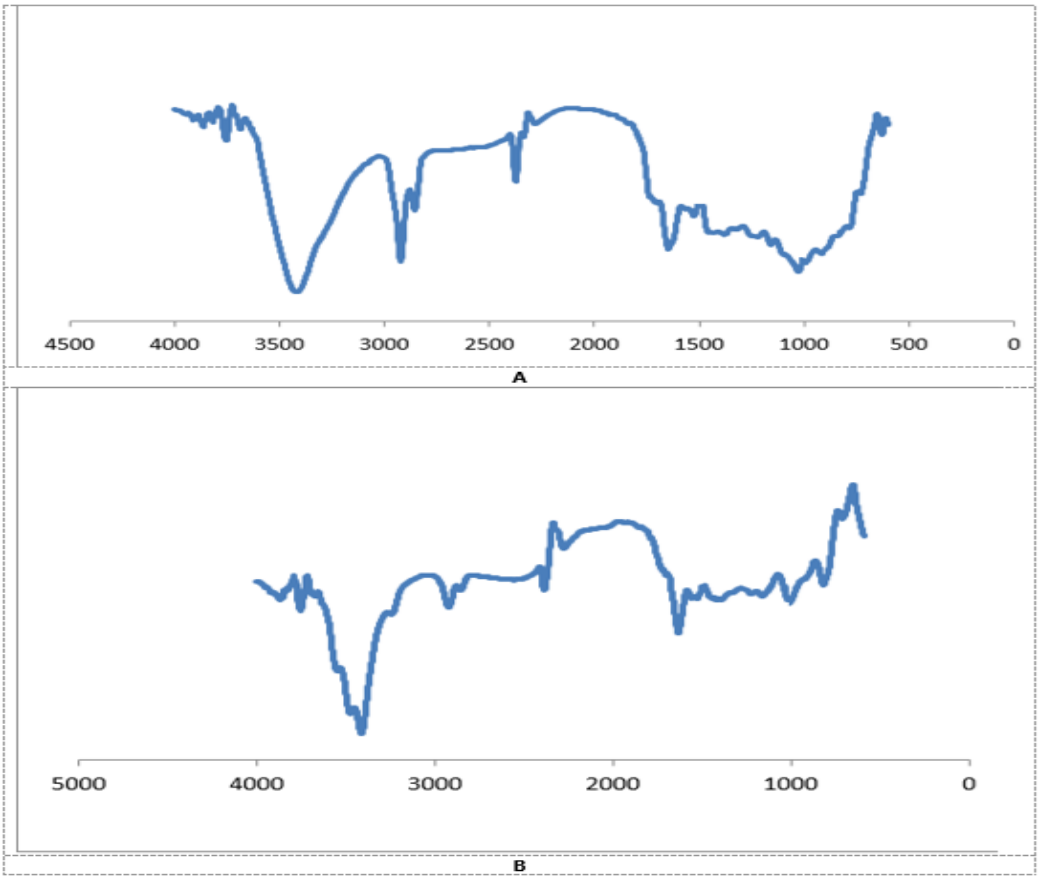



**Fig. 3.** IR absorption spectra of **A**: CQDs; **B**: CQDs/Ag/Chit.

reference patterns, it is possible to identify the presence of specific carbon allotropes or impurities in the CQD sample (Shaikh et al., 2019; Hamid Abd and Ibrahim, 2022; Sharma et al., 2022). In fact, XRD spectroscopy is a valuable tool for probing the structural characteristics of CQDs and understanding their formation mechanisms and properties.

As shown in Fig. 4, according to the XRD pattern of activated carbon, which has a strong peak in the range of $\Theta_2$ = 20˚ and the characteristic of the presence of activated carbon, it can be attributed to CQDs. This type of carbon has an irregular and mostly amorphous structure.

### 3.5. TEM images of the prepared CQDs

Transmission electron microscopy (TEM) is often considered as an efficient technique for characterizing nanoparticles, offering detailed structural and chemical insights at the nanoscale (Anandharamakrishnan and Anandharamakrishnan, 2014; Mast et al., 2020). TEM enables the analysis of 2D projections of individual particles, providing information on their size, shape, and composition (Jain et al., 2023). This technique is ideal for nanoscale studies, offering high-resolution imaging, electron diffraction, and chemical analysis in a single instrument. With advancements like highly coherent field emission electron sources, TEM enhances its analytical capabilities, allowing for the study of diverse nanomaterials such as carbon nanocages, metal nanoparticles, and soft materials used in drug delivery systems. The versatility of TEM in providing comprehensive information about nanoparticles makes it an indispensable tool in nanomaterial characterization (Kumar, 2013; Anandharamakrishnan and Anandharamakrishnan, 2014; Mast et al., 2020; Vladár and Hodoroaba, 2020; Jain et al., 2023).

To check the morphology and determine the average size of the particles, transmission electron microscopy (TEM) is used. As can be seen in the Fig. 5, the synthesized CQDs have shown a spherical crystal structure that is well dispersed in water and the particles do not have significant molecular aggregation. Also, the average diameter of each spherical particle is 25 nm, which is comparable to other works. In this context, the particles have the right size.

## 4. Concluding remarks

CQDs were prepared under hydrothermal conditions (in an aqueous environment at a temperature of 160 °C) using an easily available citrus species as a precursor. This method is an easy and environmentally friendly method that may be possible for large-scale production. The obtained CQDs contain a large amount of oxygen-containing groups, which were detected by infrared spectroscopy. According to the analyzed results, hydrothermal forms points close to polyaromatic

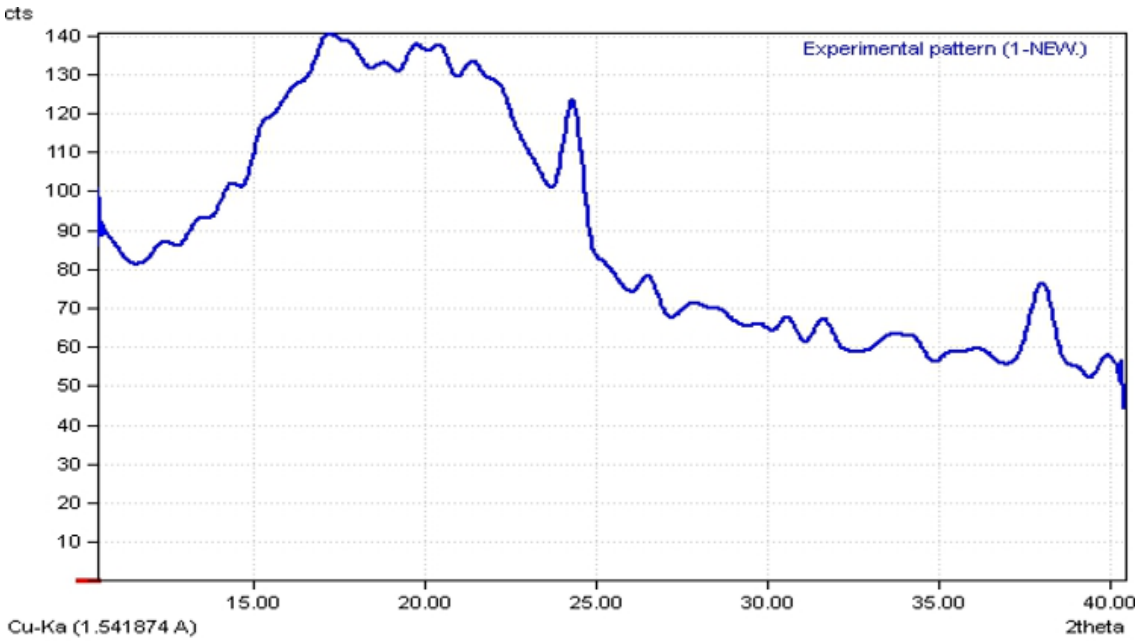



**Fig. 4.** XRD diagram of CQDs.

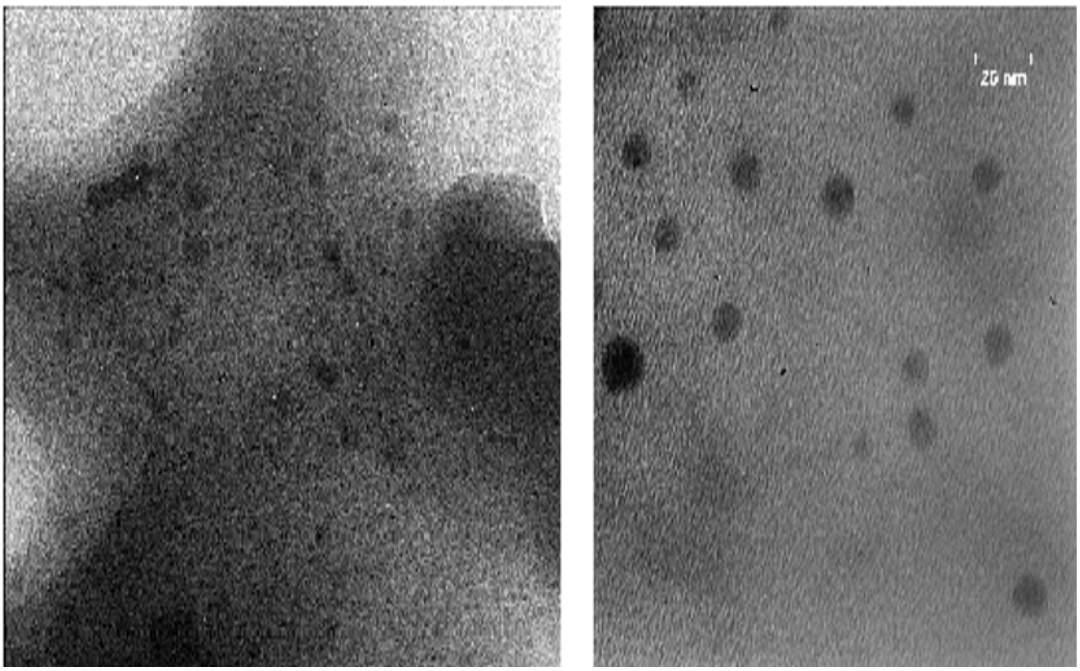


**Fig. 5.** TEM images of CQDs.

hydrocarbon clusters, such as furanocarbon-based polymers, and $sp^2$ and $sp^3$ points are formed with attractive luminescence properties. The prepared CQDs are worthy of attention due to their advantages in green synthesis, high solubility in aqueous environment and luminescence properties, and they can have potential applications as fluorescent markers and efficient catalysts in life and energy sciences. Also, the final product of this study is CQD-Ag-polymer biocomposite. Chitosan has also kept its optical and photoluminescence properties. This composite can be suitable in order to biological aspect, because according to literature, compounds such as chitosan and silver have strong antibacterial properties, and their combination with active CQDs can

intensify their antibacterial properties.

## Abbreviations

**AIE:** Aggregation-Induced Emission; **CQD/Ag/Chit:** Carbon Quantum Dots/Ag/Chitosan; **CQDs:** Carbon Quantum Dots; **FT-IR:** Fourier-Transform Infrared; **HOMO:** Highest Occupied Molecular Orbital; **LUMO:** Lowest Unoccupied Molecular Orbital; **TEM:** Transmission Electron Microscopy; **UV-Vis.:** Ultraviolet-Visible; **XRD:** X-Ray Diffraction.

## Data availability

The data can be provided from the authors upon reasonable request.

## Author contribution statement

Conceptualization and literature search were performed by Ehsan Koushki and Behnam Mahdavi. The first and final drafts of the manuscript were prepared by Behnaz Noori Dolouie and Majid Mohammadhosseini. Ehsan Koushki and Behnam Mahdavi critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare that there is no conflict of interest.

## References


Abazar, F., Noorbakhsh, A., 2020. Chitosan-carbon quantum dots as a new platform for highly sensitive insulin impedimetric aptasensor. Sensors Actuators B: Chem. 304.

Anandharamakrishnan, C., Anandharamakrishnan, C., 2014. Characterization of Nanoparticles, Techniques for Nanoencapsulation of Food Ingredients, pp. 65-67.

Bhunia, S.K., Saha, A., Maity, A.R., Ray, S.C., Jana, N.R., 2013. Carbon nanoparticle-based fluorescent bioimaging probes. Sci. Rep. 3.

Danawala, T.M., Trivedi, K.A., Lad, U.M., Desai, B.N., Singh, S.K., Suthar, D.H., Modi, C.K., 2024. Luminescent carbon quantum dots derived from syzygium cumini seeds with endogenous anti-oxidant and cytotoxic potency including *in vitro* photoluminescence and live cell imaging. New J. Chem. 48(7), 3045-3054.

Das, S., Mondal, S., Ghosh, D., 2024. Carbon quantum dots in bioimaging and biomedicines. Front. Bioeng. Biotechnol. 11, 1333752.

De, B., Karak, N., 2013. A green and facile approach for the synthesis of water soluble fluorescent carbon dots from banana juice. RSC Adv. 3(22), 8286-8290.

Demming, A., 2010. Editorial: King of the elements? Nanotechnology 21(30).

Dimzon, I.K.D., Knepper, T.P., 2015. Degree of deacetylation of chitosan by infrared spectroscopy and partial least squares. Int. J. Biol. Macromol. 72, 939-945.

Egbedina, A.O., Bolade, O.P., Ewuzie, U., Lima, E.C., 2022. Emerging trends in the application of carbon-based materials: A review. J. Environ. Chem. Eng. 10(2).

Esmaeilzadeh, M., Sadjadi, S., Salehi, Z., 2020. Pd immobilized on hybrid of magnetic graphene quantum dots and cyclodextrin decorated chitosan: An efficient hydrogenation catalyst. Int. J. Biol. Macromol. 150, 441-448.

Ghasedi, A., Koushki, E., Baedi, J., 2022. Cation-π aggregation-induced white emission of moisture-resistant carbon quantum dots: A comprehensive spectroscopic study. Phys. Chem. Chem. Phys. 24(38), 23802-23816.

Guo, R., Li, T., Shi, S., 2017. Electron transition pathways of graphene oxide quantum dots unraveled by emission wavelength dependent photoluminescence lifetime. RSC Adv. 7(32), 19701-19706.

Haji, B.S., Barzinjy, A.A., 2023. *Citrullus colocynthis* fruit extract mediated green synthesis of silver nanoparticles: The impact of pH, temperature, and silver nitrate concentration. e-J. Surf. Sci. Nanotechnol. 21(1), 61-71.

Hamid Abd, A., Ibrahim, A., 2022. Synthesis of carbon quantum dot by electro-chemical method and studying optical electrical and structural properties. Chem. Methodol. 6, 823.

Huang, C., Dong, H., Su, Y., Wu, Y., Narron, R., Yong, Q., 2019a. Synthesis of carbon quantum dot nanoparticles derived from byproducts in bio-refinery process for cell imaging and *in vivo* bioimaging. Nanomaterials (Basel) 9(3).

Huang, S., Li, W., Han, P., Zhou, X., Cheng, J., Wen, H., Xue, W., 2019b. Carbon quantum dots: Synthesis, properties, and sensing applications as a potential clinical analytical method. Anal. Methods 11(17), 2240-2258.

Jain, S., Kumar, N., Tahir, M., Garg, S., 2023. Nanoparticle Synthesis, Characterization and Applications, The Impact of Nanoparticles on Agriculture and Soil. Elsevier, pp. 13-40.

Kakhki, M.R.V., Koushki, E., Khalilzadeh, S., Pouya, M.M., 2024. Green synthesis and characterization of silver nanoparticles using *Echium amoenum* Fisch. &C.A.Mey. extract: Colorimetric assay and nonlinear optical absorption. Plasmonics 1-10.

Khajavi, Z., Ghasedi, A., Mahdavi, B., Koushki, E., 2023. On the role of UV-generated ROS in the desorption of cephalexin from CQDs-based drug-loadable platform. J. Photochem. Photobiol. A Chem. 442.

Kou, X., Jiang, S., Park, S.J., Meng, L.Y., 2020. A review: Recent advances in preparations and applications of heteroatom-doped carbon quantum dots. Dalton Trans. 49(21), 6915-6938.

Kumar, C.S., 2013. Transmission Electron Microscopy Characterization of Nanomaterials. Springer Science & Business Media.

Kumar, P., Dua, S., Kaur, R., Kumar, M., Bhatt, G., 2022. A review on advancements in carbon quantum dots and their application in photovoltaics. RSC Adv. 12(8), 4714-4759.

Lai, S., Jin, Y., Shi, L., Zhou, R., Zhou, Y., An, D., 2020. Mechanisms behind excitation- and concentration-dependent multicolor photoluminescence in graphene quantum dots. Nanoscale 12(2), 591-601.

Li, P., Liu, S., Cao, W., Zhang, G., Yang, X., Gong, X., Xing, X., 2020. Low-toxicity carbon quantum dots derived from gentamicin sulfate to combat antibiotic resistance and eradicate mature biofilms. Chem. Commun. 56(15), 2316-2319.

Lv, H., Gao, X., Xu, Q., Liu, H., Wang, Y.G., Xia, Y., 2017. Carbon Quantum Dot-Induced MnO2 Nanowire Formation and Construction of a Binder-Free Flexible Membrane with Excellent Superhydrophilicity and Enhanced Supercapacitor Performance. ACS Appl. Mater. Interfaces 9(46), 40394-40403.

Magesh, V., Sundramoorthy, A.K., Ganapathy, D., 2022.

Recent advances on synthesis and potential applications of carbon quantum dots. Front. Mater. 9.
Mast, J., Verleysen, E., Hodoroaba, V.-D., Kaegi, R., 2020. Characterization of Nanomaterials by Transmission Electron Microscopy: Measurement Procedures, Characterization of nanoparticles. Elsevier, pp. 29-48.
Mat Zaid, M.H., Abd Manan, F.A., Mohd Bakhori, N., Mohd Razip Wee, M.F., 2023. Biocompatibility and cytotoxicity studies of zero-dimensional carbon nanomaterials, Zero-Dimensional Carbon Nanomaterials. Elsevier, pp. 611-640.
Montazeri, F., Ghasedi, A., Mahdavi, B., Koushki, E., 2024. The effect of *in-situ* cellulosic matrix on the photophysical properties of white emissive CQDs. Carbon Lett. 34(1), 399-406.
Nammahachak, N., Aup-Ngoen, K.K., Asanithi, P., Horpratum, M., Chuangchote, S., Ratanaphan, S., Surareungchai, W., 2022. Hydrothermal synthesis of carbon quantum dots with size tunability via heterogeneous nucleation. RSC Adv. 12(49), 31729-31733.
Negrea, P., Caunii, A., Sarac, I., Butnariu, M., 2015. The study of infrared spectrum of chitin and chitosan extract as potential sources of biomass. Dig. J. Nanomat. Biostr. 10(4), 1129-1138.
Rawat, P., Nain, P., Sharma, S., Sharma, P.K., Malik, V., Majumder, S., Verma, V.P., Rawat, V., Rhyee, J.S., 2023. An overview of synthetic methods and applications of photoluminescence properties of carbon quantum dots. Lumin. 38(7), 845-866.
Shaikh, A.F., Tamboli, M.S., Patil, R.H., Bhan, A., Ambekar, J.D., Kale, B.B., 2019. Bioinspired carbon quantum dots: An antibiofilm agents. J. Nanosci. Nanotechnol. 19(4), 2339-2345.
Shan, X., Lu, J., Li, C., Wu, Q., Li, H., Yang, S., Guo, Y., Song, Y., Li, R., Tian, L., 2024. Ultrasensitive solid-state electrochemiluminescence sensor based on lotus root shaped carbon fiber, CdSe QDs and $Fe_3O_4$ synergically amplify $Ru(bpy)_3^{2+}$ luminophore signal for detection of cyfluthrin. Microchim. Acta 191(4).
Shan, X., Lu, J., Wu, Q., Li, C., Li, H., Yang, S., Tian, L., 2023. Solid-state electrochemiluminescence sensor of CQDs based on ZIFs electrospun carbon fiber for malathion detection. Microchem. J. 195.
Sharma, N., Sharma, I., Bera, M.K., 2022. Microwave-assisted green synthesis of carbon quantum dots derived from calotropis gigantea as a fluorescent probe for bioimaging. J Fluoresc 32(3), 1039-1049.
Sharma, V.D., Kansay, V., Chandan, G., Bhatia, A., Kumar, N., Chakrabarti, S., Bera, M.K., 2023. Solid-state fluorescence based on nitrogen and calcium co-doped carbon quantum dots @ bioplastic composites for applications in optical displays and light-emitting diodes. Carbon 201, 972-983.
Soumya, K., More, N., Choppadandi, M., Aishwarya, D.A., Singh, G., Kapusetti, G., 2023. A comprehensive review on carbon quantum dots as an effective photosensitizer and drug delivery system for cancer treatment. Biomed. Technol. 4, 11-20.
Tajik, S., Dourandish, Z., Zhang, K., Beitollahi, H., Le, Q.V., Jang, H.W., Shokouhimehr, M., 2020. Carbon and graphene quantum dots: A review on syntheses, characterization, biological and sensing applications for neurotransmitter determination. RSC Adv. 10(26), 15406-15429.
Tang, J., Zhang, J., Zhang, Y., Xiao, Y., Shi, Y., Chen, Y., Ding, L., Xu, W., 2019. Influence of group modification at the edges of carbon quantum dots on fluorescent emission. Nanoscale Res. Lett. 14(1), 241.
Tungare, K., Bhori, M., Racherla, K.S., Sawant, S., 2020. Synthesis, characterization and biocompatibility studies of carbon quantum dots from *Phoenix dactylifera*. 3 Biotech 10(12), 540.
Vladár, A.E., Hodoroaba, V.-D., 2020. Characterization of Nanoparticles by Scanning Electron Microscopy, Characterization of nanoparticles. Elsevier, pp. 7-27.
Yang, L., Qin, A., Chen, S., Liao, L., Qin, J., Zhang, K., 2018. Manganese(II) enhanced fluorescent nitrogen-doped graphene quantum dots: A facile and efficient synthesis and their applications for bioimaging and detection of $Hg^{2+}$ ions. RSC Adv. 8(11), 5902-5911.
Zhang, Y., Xiao, J., Zhuo, P., Yin, H., Fan, Y., Liu, X., Chen, Z., 2019. Carbon dots exhibiting concentration-dependent full-visible-spectrum emission for light-emitting diode applications. ACS Appl. Mater. Interfaces 11(49), 46054-46061.
Zhao, F., Li, X., Zuo, M., Liang, Y., Qin, P., Wang, H., Wu, Z., Luo, L., Liu, C., Leng, L., 2023. Preparation of photocatalysts decorated by carbon quantum dots (CQDs) and their applications: A review. J. Environ. Chem. Eng. 11(2).
Zhu, S., Song, Y., Wang, J., Wan, H., Zhang, Y., Ning, Y., Yang, B., 2017. Photoluminescence mechanism in graphene quantum dots: Quantum confinement effect and surface/edge state. Nano Today 13, 10-14.
Zulfajri, M., Gedda, G., Chang, C.-J., Chang, Y.-P., Huang, G.G., 2019. Cranberry beans derived carbon dots as a potential fluorescence sensor for selective detection of $Fe^{3+}$ ions in aqueous solution. ACS Omega 4(13), 15382-15392.

Trends Phytochem. Res. 8(2) 2024 106-121

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Characterization of pharmacological properties and isolation of two bioactive compounds (ursolic acid and palmitoleic acid) from the stem bark extract of *Lannea coromandelica*

Ramisa Anjum[1], A.H.M. Nazmul Hasan[1]✉*, Mst. Al Asma Hawa[1], Md. Naimur Rahman[1], Md. Khokon Miah Akanda[1], Tania Binte Wahed[2], Fayad Bin Abdus Salam[3], Md. Rabiul Islam[4], And Mohammad Shahriar[1]

[1]Department of Pharmacy, University of Asia Pacific, 74/A Green Road, Farmgate, Dhaka 1205, Bangladesh
[2]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh
[3]Pharmacy Discipline, Khulna University, Khulna 9208, Bangladesh
[4]School of Pharmacy, BRAC University, Kha 224, Bir Uttam Rafiqul Islam Avenue, Merul Badda, Dhaka 1212, Bangladesh

**ABSTRACT**

The current research aimed to conduct bioassay-guided isolation of bioactive compounds from the stem bark of *Lannea coromandelica*. Using column chromatography and NMR spectroscopy, two compounds -ursolic acid and palmitoleic acid- were isolated from the plant's stem bark. The methanolic stem bark extract possessed higher total phenolic, flavonoid, and antioxidant capacity content than the *n*-hexane extract. The $IC_{50}$ value of the methanol and *n*-hexane stem bark extracts for DPPH free radical scavenging potential was found to be 37.37 and 27.726 µg/mL, respectively, while for nitric oxide, the $IC_{50}$ value was 14.615 and 22.136 µg/mL, respectively. The *n*-hexane extract exhibited higher antidiabetic effectiveness (46% blood glucose reduction) than the methanolic extract (37.8% blood glucose reduction) after 3 hours of glucose administration in mice model at a dose of 400 mg/kg body weight ($p < 0.001$). The methanolic extract demonstrated the highest anti-diarrheal action at a dose of 400 mg/kg body weight (88.70% inhibition of defecation, $p < 0.001$). Furthermore, the *n*-hexane extract showed the highest analgesic activity (70.5% inhibition of writhing, $p < 0.01$).

**ARTICLE HISTORY**

Received: 13 January 2024
Revised: 28 May 2024
Accepted: 15 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Antidiabetic
Antioxidants
Bioactive compound isolation
*Lanna coromendelica*
Palmitoleic acid
Ursolic acid

## 1. Introduction

Medicinal plants, often known as herbs, have been utilized to cure a wide range of illnesses since primitive times (Mulat et al., 2019). In order to treat a variety of human diseases, many newly developed therapeutic methods make use of medicinal plants. These natural plant resources are truly valuable for the human beings as they are enriched with beneficial phytochemical constituents, which are economically available and have potential pharmacological effects that may play a pivotal role to treat human illness (Martins and Brijesh, 2018). The therapeutic potential of medicinal plants has shown promising impacts in the treatment of various diseases, offering a natural and effective alternative to conventional medications (Makenzi et al., 2023; Singh et al., 2023). Plant materials are rich in a diverse array of valuable natural bioactive compounds, including phenolic compounds, sesquiterpenoids, coumarins, alkaloids, carotenoids, and dietary fibers, which possess antioxidant, antimicrobial, and anti-inflammatory properties, offering significant potential for applications in food, pharmaceutical, and cosmetic industries (Mohammadhosseini, et al., 2019; Mohammadhosseini, et al., 2021a; Mohammadhosseini, et al., 2021b).

*Lannea coromandelica* is a topical deciduous tree with broad leaves that is included in the Anacardiaceae family. It is frequently applied by tribal people such as Teli, Pahan, Garo in tropical countries like Bangladesh, India, etc. The juice prepared from the leaves of this plant can be used to treat ulcer-related discomfort. The sap of this plant is applied as a cough and cold remedy, while the bark is used for dysentery, dyspepsia, skin lesions and pain from ulcers and toothache (Alam et al., 2017). Diabetes, hepatitis, heart conditions, and digestive issues can also be treated using its stem bark. According to the literature, the twigs of this plant have

✉Corresponding author: A.H.M. Nazmul Hasan
Tel: +8801756199375; Fax: +8802-58157097
E-mail address: nazmul_0155@yahoo.com, **doi:**

shown apoptosis induction in the cancerous human liver cells, while the leaves have shown anti-diarrheal, antinociceptive and potential antioxidant capacity (Kaur et al., 2013, Weerapreeyakul et al., 2016). Furthermore, the barks of the plant have shown zoo sporicidal activity, anti-inflammatory, anti-hyperglycemic, hypotensive and antimicrobial activity (Islam et al., 2002). However, there is no extensive research on the pharmacological properties of stem bark of this plant.

In the current report, the stem bark of *L. coromandelica* was tested pharmacologically *in vitro* and *in vivo*, and the bioactive components of the plant were also successfully isolated and characterized.

## 2. Experimental

### 2.1. Collection and identification of plant sample

Mr. Abdur Rahim, a taxonomist from the Department of Botany at Jahangirnagar University in Savar, Dhaka, Bangladesh, identified *Lannea coromandelica* (Accession number: UAP_Herb/10541) and collected the plant material from the Khulna district in Bangladesh, which is located at latitude 22.820000 and longitude 89.550003.

### 2.2. Extraction of the plant sample

The approaches outlined by Shinwari (2011), Saradhajyothi and Subbarao (2011) as well as Welters et al. (2006) were used for the extraction process. Initially, the plant was dried naturally under the sunlight for 15 days, then oven-dried at 40 °C for a week before being ground into a coarse powder. In order to facilitate filtration, 600 g of the obtained powder was dissolved in 2.5 L of methanol and *n*-hexane separately and subjected to repeated shaking for 2 weeks. Next, the supernatant was obtained using cotton filtering. The extract, which included both methanol and *n*-hexane, naturally evaporated at ambient temperature.

### 2.3. Phytochemical screening

The existence of various phytoconstituent groups in the plant extract, such as carbohydrate, steroids, phenolics, flavonoids, alkaloids, saponin and so on, were determined using the established protocols (Trease and Evans, 1989, Tiwari et al., 2011).

### 2.4. Antioxidant potentials

#### 2.4.1. Determination of total phenolic content

Using Folin-Ciocalteu reagent (FCR), total phenolic content of the methanolic and *n*-hexane extracts of *L. coromandelica* was calculated. FCR actually determines total phenolic content of a plant extract (Stanojević et al., 2009). Test tubes containing 1 mL extracts and standard were mixed with 5 mL of FCR solution (1.9-2.1 N, diluted tenfold), followed by the addition of 4 mL of sodium carbonate solution (7.5% *w/v*). Then, the test tubes were incubated at 20.0 °C for 30 minutes for standard solutions and one hour at 20.0 °C for the plant extracts. The solutions' absorbances were measured at 765 nm using a spectrophotometer and compared to a control. Gallic acid equivalents (GAE) were used to determine the total amount of phenolic compounds in plant methanol extracts, as described by Harbertson and Spayd (2006).

#### 2.4.2. Determination of total flavonoid content

Flavonoids found in fruits and vegetables may enhance mental and physical performance, while lowering the risk of infection (Davis et al., 2009; Mehjabin et al., 2024). In this report, a colorimetric technique using aluminum chloride was employed to determine the flavonoids, with quercetin serving as the standard (Bao et al., 2005). Briefly, a mixture of 5.6 mL of distilled water, 3 mL of methanol, 0.2 mL of aluminum chloride (10.0%), and 0.2 mL of potassium acetate (1.0 M) was prepared with 1 mL of the sample and the standard at different concentrations. After 30 minutes at room temperature, the reaction mixture's absorbance was measured at 415 nm using a UV/Visible spectrophotometer. For the construction of the relevant calibration curve, various concentrations of quercetin solutions were considered, and the level of flavonoids was expressed as mg/g equivalent of the sample (Lin and Tang, 2007; Mahboubi et al., 2015).

#### 2.4.3. Determination of total antioxidant capacity

According to the steps outlined by Prieto et al. (1999), total antioxidant potential of both extracts was evaluated spectrophotometrically using the phosphomolybdenum technique. The test tubes contained a mixture of the sample extracts and a reagent solution. The reagent solution was prepared by combining 3.3 mL of concentrated $H_2SO_4$ (98%), 0.381 g of sodium phosphate and 0.494 g of ammonium molybdate and the final volume adjusted upto 100 mL with distilled water in a volumetric flask. The resulting mixture was then incubated at 95 °C for about 90 minutes and the corresponding absorbance was measured at 695 nm. A typical blank solution, consisting of 3 mL of reagent solution, was used in place of the required amount of sample solution (300 µL) of the same solvent.

#### 2.4.4. DPPH free radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay is a commonly used assay to evaluate an antioxidant's ability to scavenge free radicals. This technique enables the measurement of an antioxidant's antiradical power, as described by Choi et al. (2000). Each test tube received 2 mL of the reagent solution (DPPH: 0.004%) and 200 µL of the plant extracts or standards, and was then incubated for half an hour. The solution's absorbance was measured using a spectrophotometer at 517 nm, compared to a blank. The inhibitory activity was estimated as a percentage (%).

$$\text{Inhibition (\%)} = (A_o - A_1)/A_o \times 100 \quad \text{(Eqn. 1)}$$

Eqn. 1 was used to calculate the percentage (%) inhibitory activity. Next, inhibition percentages were plotted against log concentration, and the $IC_{50}$ was determined from the graph.

#### 2.4.5. Nitric oxide free radical scavenging capacity assay

It is generally known that at physiological pH (7.2), sodium nitroprusside degrades in aqueous solution, producing NO-. Nitric oxide (NO-) and oxygen interact to generate stable products (nitrate and nitrite) in an aerobic environment. The quantities of nitric oxide that react with oxygen to form stable products can be calculated using the Griess reagent. A chromophore was created when nitrite was diazotized with sulfanilamide and subsequently linked with naphthyl ethylene diamine dihydro chloride. At 550 nm, the absorbance of this chromophore was taken (Sreejayan and Rao, 1997; Haenen and Bast, 1999). 1 mL of sodium nitroprusside solution (5 mM) was added to each test tube along with 4 mL of each plant extract or standard solution of various concentrations. To finish the reaction, the test tubes were incubated for two hours at 30 °C. Then, 2 mL of solutions from each test tube containing the standards and extracts were withdrawn and mixed with 1.2 mL of Griess reagent (0.5% *w/v*) and the absorbances of the solutions were measured at 550 nm using a spectrophotometer against blank.

### 2.5. Determination of *in vitro* thrombolytic activity

Uddin et al. (2016) and Prasad et al. (2006) have described the method used to assess thrombolytic activity *in vitro*. Beforehand, each of the eight vials was weighed. One milliliter of blood was then added to each vial, and the vials were incubated at 37 °C for 45 minutes. After incubation, blood clots remained at the bottom of each vial, and the serum was removed from the upper part. The weight of the vial-containing clots was then measured. Four vials (vials 1, 2, 3, and 4) were filled with 100 µL of sample extract solutions, two vials (vials 5 and 6) were filled with 100 µL of distilled water, and the final two vials (vials 7 and 8) were filled with 100 µL of streptokinase (30,000 IU). After 90 minutes of incubation at 37 °C, the serum was removed from the upper part of each vial, leaving the clots at the bottom. The vials' weight was then measured once more and percent of clot lysis was estimated by using the following equation:

Clot lysis (%) = (Weight of the clot after lysis/Weight of the clot before lysis) × 100 (Eqn. 2)

### 2.6. Determination of membrane stabilizing potential

The Omale and Shahriar methods were used to test the extracts' hypotonic and heat-induced stabilizing membrane effects on human erythrocytes (Omale and Okafor, 2008; Shahriar et al., 2015). The test sample was made up of a 30 mL stock suspension of erythrocytes (RBCs) in a hypotonic solution containing sodium chloride (50 mM), sodium phosphate saline buffer (10 mM, pH 7.4), plant extracts, and acetyl salicylic acid (0.10 mg/mL, each). After the mixture was centrifuged for 10 minutes at 1500 rpm and preheated for 10 minutes to room temperature, the absorbance was measured at 540 nm. The following calculation was used to compute the percentage inhibition of membrane stabilization or hemolysis:

Hemolysis inhibition (%) = (OD1 - OD2)/OD1 × 100 (Eqn. 3)

Two falcon tubes were filled with isotonic buffer containing plant extracts and erythrocyte suspension along with reagents. The tubes were kept in an ice bath and incubated in water for 20 minutes. Percentage hemolysis inhibition was determined using the following equation (Omale and Okafor, 2008; Shahriar et al., 2015).

Hemolysis Inhibition (%) = [1 - (OD2-OD1/OD3-OD1)] × 100 (Eqn. 4)

### 2.7. Cytotoxic activity: Brine shrimp lethality bioassay

This investigation utilized *Artemia salina*, or brine shrimp, as the test organism. Cysts were hatched in saline water (38 g/L NaCl) for 48 hours, transforming into living nauplii. Both methanolic and *n*-hexane extracts were prepared in various concentrations using dimethyl sulfoxide (DMSO) as the solvent. Each test tube was inoculated with ten nauplii, and the volume was adjusted with saline water. Vincristine sulfate, a highly cytotoxic alkaloid, served as the positive control, tested at concentrations of 25, 12.5, 5, 1, 0.5, 0.25, 0.125, and 0.06 µg/mL.Three pre-marked test tubes holding 4.9 mL of simulated sea water each received 50 µL of DMSO. Ten shrimp nauplii were added to serve as negative control groups. After a 24-hour rest period, all test tubes were counted to determine the number of living and dead nauplii using a magnifying glass against a black background, as described by Price et al. (1974). The percent of lethality was determined accordingly.

### 2.8. *In vitro* α-amylase inhibitory activity

This investigation utilized a modified starch-iodine technique, involving different plant extracts or standard dosages. Test tubes were incubated with amylase, starch solution (1.0%), and distilled water. Absorbance was determined at 565 nm and the relevant $IC_{50}$ values were calculated by the use of linear regression analysis. Three experiments were conducted, resulting in a comprehensive understanding of the process (Uddin et al., 2014).

### 2.9 Antimicrobial activity

A common test for antimicrobial susceptibility is the disc diffusion method. This strategy was first introduced by Bauer et al. (1966), who tested it on a large variety of microorganisms. This technique can also be used to test phytochemicals and plant extracts. In this method, the test material is placed in Mueller-Hinton Agar media after being soaked in 6 mm filter paper discs. There will be obvious places where there is enough of the test agent to stop bacterial growth. A drug's antibacterial activity can be estimated using the clear zone's diameter (Nascimento et al., 2000).

### 2.10. *In vivo* pharmacological activities

#### 2.10.1. Experimental animal

This study employed male Swiss albino mice that weighed 25-30 g (Akanda and Hasan, 2021). The International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB) provided the animals for all of the experiments. All animals were kept under standard environmental conditions: Relative humidity 55-65%, ambient temperature 22-25 °C, and a 12-hour light-dark cycle. The experiment was conducted in the Phytopharmacology Laboratory of the Department of Pharmacy of University of Asia Pacific as per the guideline.

### 2.10.2. Evaluation of anti-diarrheal activity

A typical digestive ailment, diarrhea is brought on by bacterial, viral, and parasite species. To test the effectiveness of *L. coromandelica* stem bark extracts as a diarrhea preventative, castor oil-induced diarrhea in mice was used. The control, positive, and test groups each had four mice. Loperamide was administered orally to the positive control group at a dose of 50 mg per kg. *n*-Hexane and methanolic extracts were administered to the test group at doses of 200 and 400 mg/kg body weight, respectively. 60 minutes after obtaining test samples, the mice were also given 0.5 mL of castor oil orally (Amabeoku, 2009; Umer et al., 2013; Araújo et al., 2015; Jabri et al., 2016).

### 2.10.3. Peripheral analgesic activity

The acetic acid-induced writhing method is Koster and Taber's favored method for determining *in vivo* peripheral analgesic activity (Koster, 1959; Taber et al., 1969). In the current experiment, diclofenac was used as a typical medicine. The pain-relieving potential of two distinct dosages of the crude extracts from the stem bark of *L. coromandelica* was examined to be 200 and 400 mg/kg of body weight (Koster, 1959; Taber et al., 1969).

### 2.10.4. Antidiabetic activity

This study investigated hypoglycemic action of plant extracts in hyperglycemic mice given glucose. Among six mice groups, group I served as the control and merely got DMSO mixed with regular saline water and Tween-80 (a suspending agent). Glibenclamide was given orally to Group II as the standard group at a dose of 5 mg/kg body weight. Groups III to VI were given oral doses of the crude methanolic and *n*-hexane extract in the amounts of 200 and 400 mg/kg body weight. After 60 minutes, mice were given a glucose solution. Blood samples were collected from the tail vein and tested using a glucometer and reactive strips (Upadhya et al., 2004).

### 2.10.5. Antipyretic activity

This study assessed the plant extract's ability to reduce fever caused by Brewer's yeast in mice (Tomazetti et al., 2005). A digital thermometer was used to gauge basal rectal temperature at zero hours. Animals with an increase of at least 0.6°C over 18 hours were chosen for the investigation. Pyrexia was produced by administering 10 mL/kg of body weight of a suspension of Brewer's yeast (15 *w/v*%) in distilled water subcutaneously. Four groups were formed, with control being Tween-80 (1.0%), paracetamol administered orally, temperatures were obtained from each group at various intervals, and mice from the control and standard groups were compared (Gupta et al., 2005).

### 2.10.6. Acute toxicity test

In this investigation, Swiss albino male mice were used to test the acute toxicity of *L. coromandelica* stem bark extracts. The crude extracts in various doses (500, 1000, 2000, and 4000 mg/kg) were given orally to 36 mice divided into 9 groups. After 24 hours, mortality rates were evaluated (Mosnaim et al., 2020).

### 2.11. Isolation of bioactive substances

Column chromatography can be used to identify bioactive compounds from plant extracts, followed by TLC, and ultimately NMR to determine and characterize the structure of the isolated compounds. The idea behind silica column chromatography is that the solvent (mobile phase) is passed through the stationary phase, while the mixture's molecules are applied to the surface of solid silica (stationary phase) (Coskun, 2016).
The plant's methanol extract was then placed on top of the stationary phase and run by a mobile phase composed of *n*-hexane, chloroform, and ethyl acetate in various ratios from non-polar to semi-polar. Eluted samples were stored in previously numbered (1-300) test tubes, and the eluted samples were then tested over TLC plates with a specified solvent system as part of the initial screening procedure to identify test tubes containing comparable chemicals. The test tube contents which had the same rf value were mixed together for further separation by TLC. Following that, the contents of the test tubes were run over a TLC plate with a mobile phase composed of *n*-hexane, chloroform, and ethyl acetate in various proportions, and the separated compounds were suitably spotted under UV lamps of long- and short wavelength. According to this procedure, the sample of 140-154 numbered test tubes were isolated with ethyl acetate (10.0%) in chloroform separated with preparative TLC, and compounds were spotted under UV lamp, eluted and coded as LC-002-10-EA-LS and sample of 188-200 numbered test tubes are eluted with ethyl acetate (25%) in $CHCl_3$ in a similar way, coded as LC-001-25-EA-L and referred for $^{1}$H-NMR and $^{13}$C-NMR study to BCSIR (Bangladesh Council of Scientific and Industrial Research). After comparing the NMR data with the references, structure of our isolated compounds was determined.

### 2.12. Statistical analysis

The data were expressed using the standard error of the mean (SEM). The results were statistically evaluated using Microsoft Excel 2010. T-test of two equal variances was done and results with less than $*p < 0.05$, $**p < 0.01$

and ***$p < 0.001$ are considered statistically significant. The $LC_{50}$ values for cytotoxicity as well as the $IC_{50}$ values for scavenging free radicals were calculated using the dosage response curve.

## 3. Results and Discussion

### 3.1. Phytochemical screening

The plant contains some secondary metabolites like carbohydrate, glucoside, saponin, steroid, flavonoid, and alkaloid, but it also shows the absence of glycoside and tannin. The secondary metabolites glucose, glucoside, saponin, steroid, flavonoid, and alkaloid are all present in the methanolic extract. The *n*-hexane extract contained large amounts of carbohydrates, glycosides, steroids, flavonoids, saponins, and alkaloids, as summarized in Table 1.

The initial phytochemical screening of *L. coromandelica* identified the presence of several crucial secondary metabolites. This finding aligns with previous research, where the methanolic extract of *L. coromandelica* leaves was reported to contain alkaloids, glycosides, cardiac glycosides, saponins, carbohydrates, and flavonoids (Manik et al., 2013).

According to a recent study, it was found that the ethanolic extract of pulp contained tannins, flavonoids, saponins, cyanogenic glycosides, terpenoids, but lacked phenolics, alkaloids, steroids, and anthraquinones (Islam et al., 2022).

### 3.2. Antioxidant potentials

#### 3.2.1. Determination of total phenolic content

In this study, the methanolic stem bark extract of *L. coromandelica* had a higher total phenolic content than the *n*-hexane fraction, estimated by using the standard curve of gallic acid ($y = 0.0069x + 0.0357$, $R^2 = 0.9958$). The total phenolic content of the methanolic and *n*-hexane fractions were found to be 52.30 ± 0.141 and 23.671 ± 0.101 mg/g of GAE, respectively (Table 2).

#### 3.2.2. Determination of flavonoid content

The equation $y = 0.0084x - 0.1082$, $R^2 = 0.9878$, derived from the reference standard of quercetin, was used to compute the total flavonoid concentration. The flavonoid content for methanolic and *n*-hexane fractions were found to be 47.450 ± 0.636 and 23.475 ± 1.025 mg/g of quercetin equivalent (Table 2). As seen, the total flavonoid content of *n*-hexane stem bark fractions of *L. coromandelica* was lower than the total phenolic content of methanolic fraction.

#### 3.2.3. Determination of total antioxidant capacity

Using the equation $y = 0.0054x-0.0926$, $R^2 = 0.9509$ derived from the reference standard of ascorbic acid, the total antioxidant capacity of various stem bark extracts was computed and expressed as mg/g ascorbic acid equivalent. In this study, the amount of total antioxidant capacity of *L. coromandelica* showed that methanolic stem bark extract was more potent than *n*-hexane extract. Total antioxidant capacity of methanolic and *n*-hexane stem bark extracts of *L. coromandelica* were found to be 12.048 ± 0.102 and 6.582 ± 0.025 mg/g of ascorbic acid equivalent (Table 2).

#### 3.2.4. DPPH free radical scavenging potential

The results of this study showed that the extracts exhibited dose-dependent scavenging activity against DPPH free radicals. Compared to the standard ascorbic acid concentration of 35.854 µg/mL, the $IC_{50}$ values for the methanolic and *n*-hexane fractions of the stem bark extract were 37.37 and 27.726 µg/mL, respectively (Table 2). Both fractions demonstrated scavenging activity comparable to that of ascorbic acid (Fig. 1).

#### 3.2.5. Nitric oxide free radical scavenging capacity assay

According to the experimental findings of this report, both stem bark extracts of *L. coromandelica* inhibited nitric oxide in a dose-dependent way (Fig. 2). This might be attributed to the fact that the extracts contained antioxidant elements that compete with oxygen to react with nitric oxide. According to our current study, methanolic extract was found more potent NO free radical scavenger than *n*-hexane extracts as compared with ascorbic acid (Table 2).

Oxidative stress may give rise to various disorders in the body. Antioxidants may prove to be an inhibitor of oxidative stress (Islam et al., 2022). A method referred as total antioxidant capacity (TAC) is widely applied for the assay of antioxidant properties present in the biological samples. It can also assess the antioxidant property against the free radicals generated in a particular disease (Marques et al., 2014). Compared to the *n*-hexane fraction (23.671 mg/g GAE), the methanolic fraction of the stem bark showed a greater total phenolic content (52.30 mg/g GAE). Comparably, the flavonoid content of the methanolic fraction was greater (47.450 mg/g) than that of the *n*-hexane fraction (23.475 mg/g). The total antioxidant capacity of the methanolic extract was higher (12.048 mg/g ascorbic acid equivalent) than that of the *n*-hexane extract (6.582 mg/g ascorbic acid equivalent).

The review of the literature demonstrates that the total antioxidant capacity of the stem bark of *L. coromandelica* using phosphomolybdenum method was not previously reported and numerous polyphenols and flavonoids are essential for plants overall antioxidant activity (Wahid, 2012).

Free radicals damage cells through reactions with membrane lipids, nucleic acids, proteins, enzymes, contributing to aging and degenerative diseases like cardiovascular and cancer. According to a recent study, methanolic extract of stem bark has a higher potential for nitric oxide scavenging ability than ascorbic acid, which is consistent with the results of the present investigation (Sztanke and Sztanke, 2017). A free radical regarded as DPPH has a deep-violet or purple color and has the capacity to accept an electron or hydrogen

**Table 1**
Phytochemical screening of *Lannea coromandelica* extractives.

| Phytochemical Tests | Methanolic extract | *n*-Hexane extract |
|---|---|---|
| Carbohydrate | + | + |
| Glycoside | - | - |
| Glucoside | + | + |
| Saponin | + | + |
| Steroid | + | + |
| Tannin | - | - |
| Flavonoid | + | + |
| **Alkaloid test by different reagent** | | |
| Hager's reagent | + | + |
| Wagner's reagent | + | + |
| Dragendroff's reagent | + | + |

Here, [- = Not present, + = Present].

**Table 2**
Antioxidant activity of stem bark extract of *L. coromandelica*.

| Sample | Total Phenolic Content, mg/g GAE ± STD | Total Falvonoid Content, mg/g QE ± STD | Total Antioxidant Capacity, mg/g AAE ± STD | Scavenging capacity assay, $IC_{50}$ (µg/mL) | |
|---|---|---|---|---|---|
| | | | | DPPH | NO |
| Methanolic extract | 52.30 ± 0.141 | 47.450 ± 0.636 | 12.048 ± 0.102[a] | 37.373[a] | 14.615 |
| *n*-Hexane extract | 23.671 ± 0.101 | 23.475 ± 1.025 | 6.582 ± 0.025[a] | 27.726[a] | 22.136 |
| Ascorbic acid | - | - | - | 35.854[a] | 16.03 |

[Note:Values are represented as mean ± SEM, t-test of two equal variance was done to analyze the data set. Values in the same column with different superscripts are significantly different from another, $p < 0.05$].

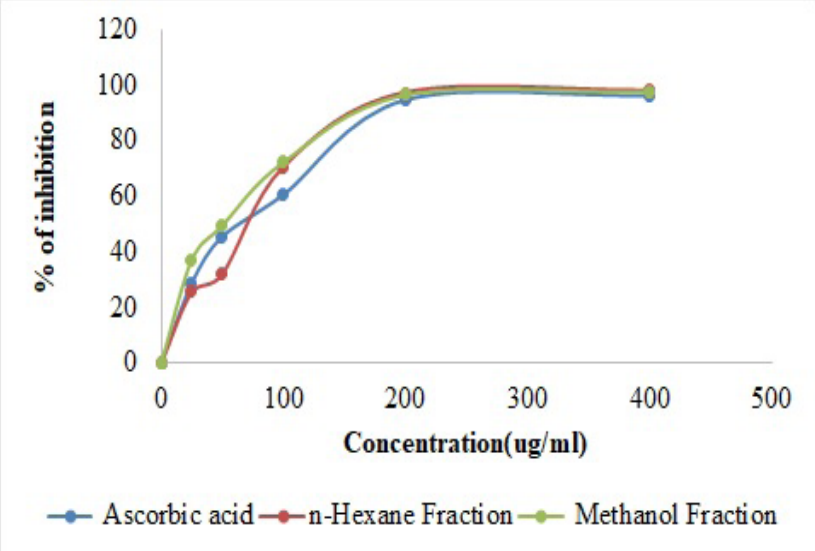



**Fig. 1.** DPPH free radical scavenging curve of stem bark extracts of *L. coromandelica* at different concentrations.

radical to get stabilized as a diamagnetic molecule that is yellow in color (DPPH) followed by the reaction with antioxidants, whether of natural or synthetic origin (Kedare and Singh, 2011). The DPPH free radical scavenging activity of the methanolic and *n*-hexane extracts was dose-dependent, with $IC_{50}$ values of 37.37 and 27.726 µg/mL, respectively. Ascorbic acid, *n*-hexane and methanolic extract also exhibited dose-dependent scavenging of NO free radicals.

### 3.3. Determination of *in vitro* thrombolytic activity

A positive control of 100 mL of streptokinase (SK) for fibrinolytic medications (30,000 IU) demonstrated 66.381% clot destruction, whereas a negative control of 50.038% clot lysis was observed using sterile distilled water. Methanolic stem bark extract exhibited 26.196% and *n*-hexane stem bark extract showed 43.46%, respectively, in an *in vitro* thrombolytic activity investigation (Table 3). The results of this investigation demonstrated poor thrombolytic activity of *L. coromandelica* stem bark extract, as *n*-hexane extract was potent than methanolic extract (Table 3).
Streptokinase, a fibrinolytic medication, lowers mortality rates in myocardial infarction patients by triggering a plasminogen enzyme that dissolves fibrin. This medication retains blood flow to the ischemic myocardium and reduces necrosis (Manik et al., 2013). A previous research has shown that the *n*-hexane fraction of *L. coromandelica* bark exhibits weaker activity compared to other extracts, which is consistent with the findings of this study (Manik et al., 2013). It has been documented that the average clot lysis activity of the dichloromethane leaf extract was 23.98 ± 2.71%, while the *n*-hexane leaf extract showed an average activity of 7.19 ± 2.09%. In contrast, the dichloromethane partitionate of *L. coromandelica* bark demonstrated the highest activity, with an average clot lysis value of 34.81 ± 3.02% (Manik et al., 2013).

### 3.4. Determination of membrane stabilizing potential

In comparison to the standard acetylsalicylic acid (ASA), *L. coromandelica* was tested for the hemolysis of RBC induced by hypnotic solution and heat induced hemolysis. When compared to the standard and test sample, hemolysis is caused by both a hypotonic solution and heat, ASA decreased RBC hemolysis in hypotonic solution-induced cases by 64.235 ± 0.629%, methanolic extract by 31.178 ± 0.291%, and *n*-hexane fraction by 24.674 ± 1.465%. On the other hand, the inhibitions of heat induce hemolysis showed higher compared with inhibition of hypotonic solution hemolysis. The percent of inhibition of heat induce hemolysis standard acetylsalicylic acid (ASA) was 67.591 ± 2.06%, where test sample methanolic fraction of stem bark was 36.177 ± 4.60% and *n*-hexane fraction of the stem bark was found to be 51.729 ± 1.53%, respectively (Table 3). Both extracts exhibited moderated inhibition of percent of hemolysis compared to the standard ASA. The results can be used to hypothesize that the anti-inflammatory activity of *L. coromandelica* may have a mechanism of action involving its ability to stabilize membranes and its suppression of erythrocyte lysis.

### 3.5. Cytotoxic activity: Brine shrimp lethality bioassay

Percentage of lethality of methanol (y = 53.388x-83.038, $R^2$ = 0.8959) and *n*-hexane (y = 56.8x-80.9, $R^2$ = 0.9167) were calculated by monitoring the number of alive nauplii after 24 hours and the corresponding $LC_{50}$ values were determined by using their respective regression equations and compared with the standard cytotoxic, vincristine sulphate (y = 54.48 + 28.5, $R^2$ = 0.964). Both the methanolic and *n*-hexane extracts exhibited similar cytotoxic properties. The $LC_{50}$ (lethal concentration that kills 50% of cells) values were 2.35 µg/mL for the methanolic extract and 2.303 µg/mL for the *n*-hexane extract, indicating that both extracts possessed potent cytotoxic potential (Table 3). *Artemia larva*e mortality rate indicates potential anticancer activity. Toxicity tests correlate with cytotoxicity of anticancer compounds, but brine shrimp lethality bioassay is not specific. The methanolic fraction of the plant has $LC_{50}$ of 50-60 µg/mL, suggesting potential anticancer, antibacterial, and antiviral properties (Shoeb et al., 2014). In the brine shrimp lethality assays, both the methanolic and *n*-hexane extracts demonstrated strong cytotoxicity, with $LC_{50}$ values of 2.35 and 2.303 µg/mL, respectively. This shows that the stem bark of *L. coromandelica* can have the potential use as an anticancer herb.

### 3.6. *In vitro* α-amylase inhibitory activity

When compared to the standard acarbose, both of the *L. coromandelica* stem bark extracts displayed almost similar and comparable α-amylase inhibitory potential (Table 3). The methanolic stem bark extract of *L. coromandelica* had an $IC_{50}$ value of 1.743 µg/mL for antidiabetic activity, while the *n*-hexane extract had an $IC_{50}$ of 1.968 µg/mL. In comparison, the standard drug acarbose had an $IC_{50}$ of 1.665 µg/mL. These results indicate that both the methanolic and *n*-hexane extracts of *L. coromandelica* stem bark possess potent antidiabetic activity, comparable to the standard.

### 3.7. Antimicrobial activity

The antimicrobial activities were evaluated on two-Gram positive bacteria, namely *Staphylococcus aureus*, *Bacillus megaterium,* two-Gram negative bacteria, namely *Escherichia coli, Salmonella typhi* along with a fungus*,* namely *Aspergillus niger*. No zone of inhibition was found in any of the five species examined in the methanol or *n*-hexane stem bark extract of *L. coromendelica,* as the plant extracts lack antimicrobial activities on the tested microorganisms.
Infectious disease, which accounts for more than 50% of all mortality in tropical countries, is one of the main causes of death worldwide (Shetty and Shetty, 2009). Studies suggest that certain bacteria are susceptible to *L. coromandelica*, with some showing moderate efficacy. The ethanolic extract of *L. coromandelica* bark's aqueous partitionate showed moderate efficacy against several bacteria *e.g.*, *Shigella dysentery* 9 mm (800 g) and 8 mm (400 g), *Pseudomonas aeruginosa* 10 mm

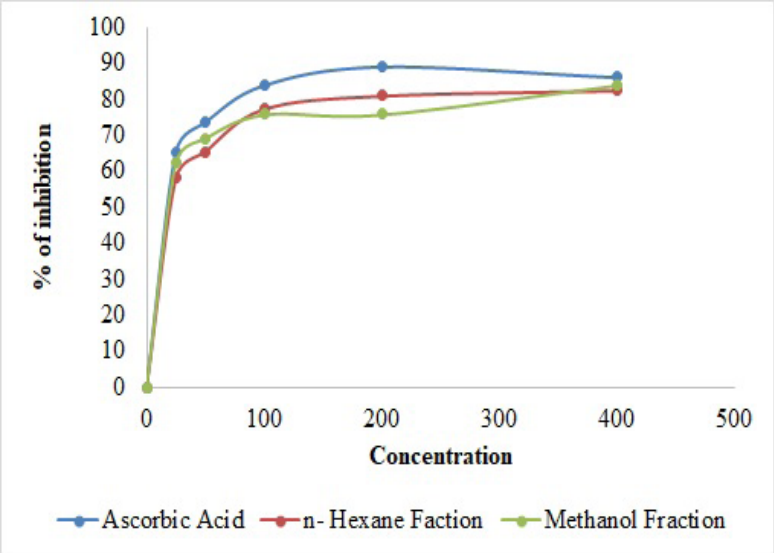



**Fig. 2.** Nitric oxide free radical scavenging curve of stem bark extracts of *L. coromandelica* at different concentrations.

**Table 3**
Thrombolytic activity, membrane stabilizing potential, brine shrimp lethality bioassay and α-amylase inhibitory activity of *L. coromandelica* and standard.

| Extracts /Std | Thrombolytic activity | % Inhibition of hemolysis ± STD (membrane stabilizing potential) | | Brine shrimp lethality bioassay (µg/mL) | α-Amylase inhibitory activity (µg/mL) |
|---|---|---|---|---|---|
| | Clot lysis (%) | Hypotonic Solution | Heat Induce | $LC_{50}$ | $IC_{50}$ |
| Methanol extract | 26.196 ± 6.393 | 31.178 ± 0.291 | 36.18 ± 4.6 | 2.35 | 1.743 |
| *n*-Hexane extract | 43.462 ± 1.507 | 24.674 ± 1.465 | 51.729 ± 1.5 | 2.303 | 1.968 |
| Streptokinase | 66.381 ± 0.896 | - | - | - | - |
| Distilled water | 50.038 ± 0.966 | - | - | - | - |
| Acetyl salicylic acid | - | 64.235 ± 0.629 | 67.591 ± 2.0 | - | - |
| Vincristine sulfate | - | - | - | 0.392 | - |
| Acarbose | - | - | - | - | 1.665 |

(800 g) and 8 mm (400 g). However, or other examined bacteria, the same extract did not show any zone of inhibition, indicating no antibacterial effect (Ramadhan et al., 2022).

### 3.8. Evaluation of anti-diarrheal activity

In this study, the defective pellet was reduced by 74.78% and 86.70% ($p < 0.001$) at 200 and 400 mg/kg body weight dose, respectively, from the methanolic extracts (ME). However, the *n*-hexane (*n*-HE) extract showed a statistically significant decrease in defection of 51.30% and 64.35% ($p < 0.001$). The reduction of the defective pellet of the standard loperamide was 73.475% at a dose of 50 mg/kg body weight (Fig. 3-a). The stem bark methanolic extract was more potent than the *n*-hexane extract at the same dosage.

Ricinoleate salts in the intestine cause inflammation and permeability, affecting intestinal absorptive cells. Castor oil inhibits prostaglandin E2 formation, which induces intestinal secretion. This inhibition may contribute to its antidiarrheal effects on stem bark. Reduced ricinoleic acid secretion stimulates $Na^+/K^+$ ATPase activity, promoting the release of $Na^+$ and $K^+$ from the intestinal mucosa. Terpenoids, tannins, and flavonoids in seed and bark extracts may improve colon absorption of water and electrolytes (Mbahi et al., 2018).

### 3.9. Peripheral analgesic activity

At dosages of 200 and 400 mg/kg body weight, respectively, the methanolic extract achieved 49.02%

and 68.63% (Fig. 3-b) writhing inhibition in the acetic acid-induced writhing model in mice, which is used to evaluate peripheral analgesic activity. *n*-Hexane extract suppressed writhing by 49.02% and 70.59% (Fig. 3-b), respectively at the same dose. The positive standard diclofenac sodium inhibited 60.78% of at a dose of 50 mg/kg body weight. Both extracts exhibited almost similar analgesic activity at higher dose and found statistically significant ($p < 0.01$). Both the extracts exhibited dose-dependent inhibition of percent of writhing.

Analgesic activity was assayed in mice through the application of acetic acid induced writhing model. As the extract concentration was raised, the writhing inhibition grew stronger. The extract caused impressive writhing inhibition at the higher dose tested (400 mg/kg body weight), which was comparable and, in some cases, even greater than the positive standard, diclofenac sodium. Acetic acid-induced writhing has produced algesia by releasing endogenous chemicals that in turn stimulate the pain nerve terminals (Ricciotti and FitzGerald, 2011). Acetic acid administered intraperitoneally has been shown to induce pain, which is associated with elevated levels of PGE2 and PGF2 in peritoneal fluid. Given this result, it is reasonable to assume that a peripheral mechanism may be involved in the mode of action. As alkaloids, flavonoids, and saponins have been proven to be responsible for analgesic and anti-inflammatory effects, the results of phytochemical analysis from previous studies of *L. coromandelica* support the antinociceptive activity (Rajesh and Selvakumar, 2022). The methanolic extract demonstrated marginally greater potency at higher dosages than the other extract, although both showed dose-dependent peripheral analgesic action.

### 3.10. Antidiabetic activity

Both extracts exhibited antidiabetic efficacy dose-dependently and the highest activity was found at the 3rd hour after glucose administration and found statistically significant. Methanolic (ME) and *n*-hexane (*n*-HE) stem bark extract of *L. coromandelica* reduced 37.8% and 46.0% (Fig. 4-a, $p < 0.001$) of blood glucose in mice model at 400 mg/kg body weight dose after 3 h administration of glucose. According to this study, *n*-hexane extract exhibited higher antidiabetic efficacy than methanolic stem bark extract.

The complex disease known as diabetes mellitus is characterized by a significant breakdown in the metabolism of proteins, carbohydrates, and lipids. It is a long-term metabolic disorder of glucose metabolism that eventually alters the vasculature and has challenging side effects (American Diabetes Association, 2010). In a glucose management trial, insulin production took two to three hours to return blood sugar levels. The plant extracts have antihyperglycemic properties, likely due to peripheral glucose ingestion or increased beta cell sensitivity to glucose (Aziz et al., 2021). In conclusion, our research reveals that the stem bark extract of *L. coromandelica* might contain a number of active phytochemicals that may likely have hypoglycemic and antihyperglycemic effects via a variety of pathways.

### 3.11. Antipyretic activity

The initial temperature of 6 different groups of Swiss albino mice was recorded. Then, the mice were fed the sample extracts of *L. coromendeica* and temperature was recorded for the next 3 h. It is seen that both the methanolic extract decreased body temperature by 7.5% ($p < 0.05$) and *n*-hexane fraction by 5.7% ($p < 0.01$) at 400 mg/kg BW dose after 3 h (Fig. 4). Hence, it can be concluded that the methanolic stem bark of *L. coromendelica* demonstrated higher anti-pyretic efficacy (Fig. 4-b), and then it could be inferred that this organic extract has moderate anti-pyretic effectiveness. A high body temperature has always been a cause for concern because of its negative consequences, which includes intracranial hemorrhage, sepsis, Kawasaki syndrome, thyroid storm, and serotonin syndrome (Mercier et al., 2021). In conclusion, it can be claimed that *L. coromandelica's* stem bark exhibits some mild antipyretic effect. The antidiabetic effects of both extracts were dose-dependent, with the *n*-hexane extract showing the greatest effectiveness. The methanolic extract was more effective than the *n*-hexane fraction in terms of antipyretic efficacy.

### 3.12. Acute toxicity test

In the present study, even a higher dose of plant extract which is 4000 mg/kg did not show any signs of toxicity or mortality for animals. Thus, plant extract of stem bark even at 4000 mg/kg may be considered for further investigation. Toxicology studies the adverse effects of substances on organisms, focusing on their potential to harm people and animals. Factors like host characteristics, species, entry point, dose, and exposure length can impact the effects. Yuet Ping et al. (2013) have reported that at a single dose of of 5000 mg/kg, none of the rats observed during the observation period exhibited any signs of acute toxicity or mortality. A dose of 1000 mg/kg significantly reduced the rats' body weight gain during the sub chronic test, The plant extract did not exhibit any toxicity or mortality symptoms, even at a greater dose of 4000 mg/kg, indicating that it is safe for more research.

### 3.13. Isolation of bioactive substances

The eluted two samples coded as LC-001-25-EA_L and LC-002-10-EA_LS were studied by NMR spectroscopy and NMR data were compared with the phytochemistry report to finally determine their structures. One major molecule has been found from each sample (LC-001-25-EA_L and LC-002-10-EA_LS, respectively), according to the report obtained from BCSIR.

One significant chemical was discovered in the first sample. Sample 1's $^1$H NMR spectrum (400 MHz, $CDCl_3$) showed a doublet at H 2.09, 0.81, and 0.91, respectively, which corresponds to the locations of H-18, H-29, and H-30 in the structure. The spectra also showed a peak associated with the hydroxyl group, H 3.63, which indicates H-3, along with numerous additional singlets at H 1.58, 1.72, 1.30, 2.04, etc.

All of these indications suggested that LC-001-25-EA_L

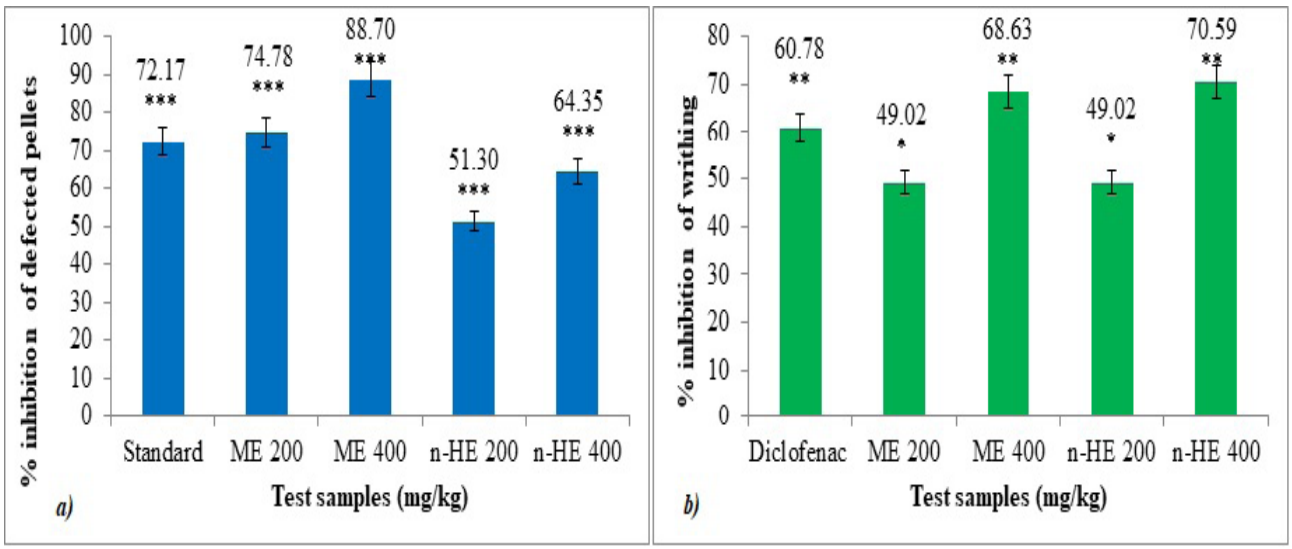



**Fig. 3.** Inhibition (%) of ***a)*** defecation and ***b)*** % of Inhibition writhing of *L.coromandelica* extractives of plant extract and standard at two different dose.

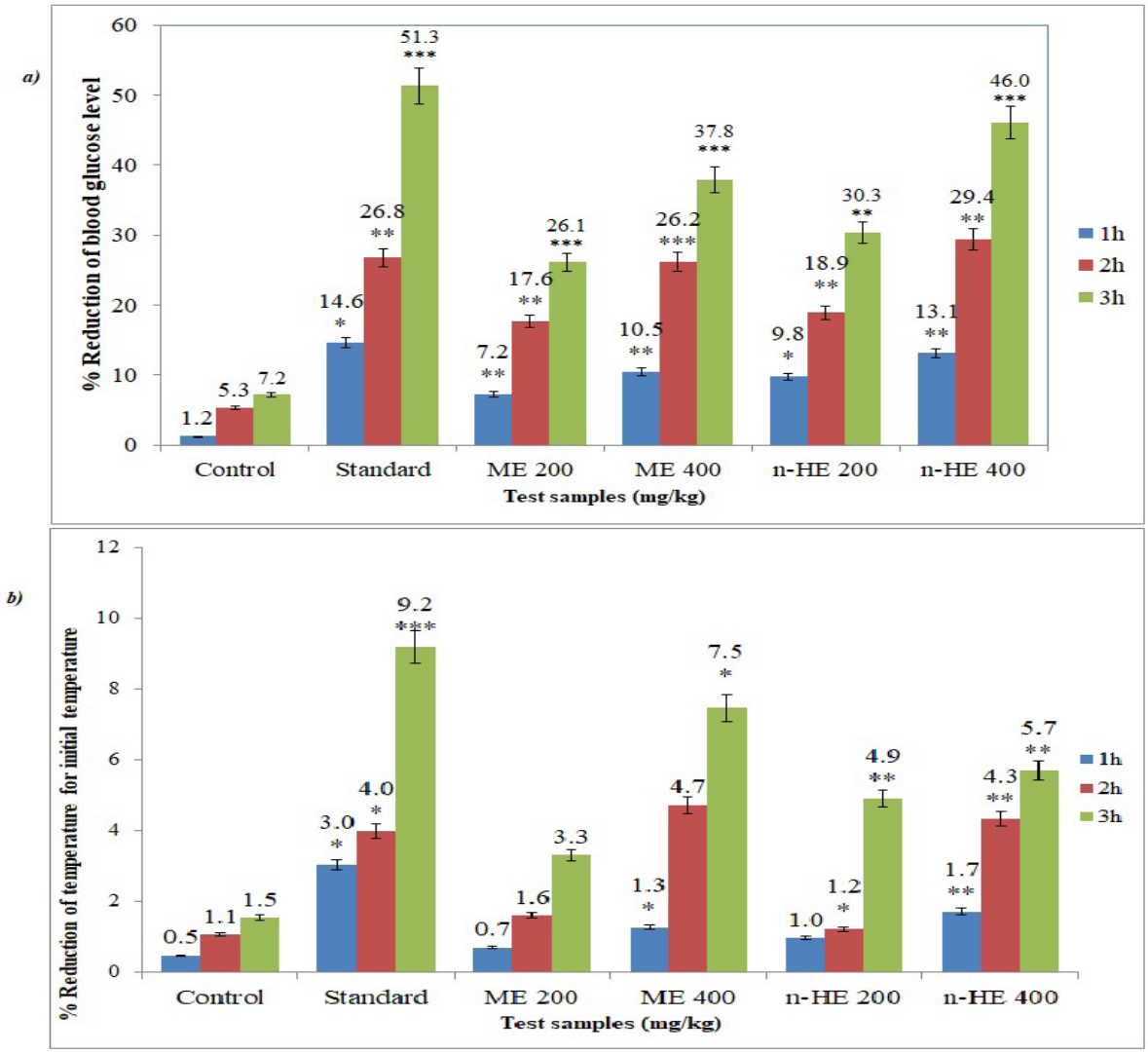



**Fig. 4. a)** Percent decrease in glucose level and b) Percent decrease in body temperature after three hours of feeding different *Lannea coromendelica* stem bark samples to Swiss albino mice.

includes ursolic acid, a pentacyclic triterpenoid moiety that is a natural triterpene chemical, and when compared to published ursolic acid data, it was determined that the pentacyclic moiety is ursolic acid as shown in Fig. 5 and Fig. 6. The ursolic acid values from were compared to the $^{1}$H NMR data (Labib et al., 2016). Table 4 shows the comparison between the standard and the isolated sample 1.

On this basis, the identity of sample 1 was confirmed as ursolic acid shown in Fig. 7(a). The $^{1}$H and $^{13}$C NMR spectrum of the isolated compound LC-001-25-EA_L is illustrated in Fig. 5.

The positions of the doublet at H 2.338 (d, *J = 7.5*) in LC-002-10-EA_LS, $^{1}$H NMR spectrum (400 MHz, $CDCl_3$) are suggestive of H-4 in the structure. The spectrum also gave rise to a triplet at $\delta_H$ 0.882 (3H, t) which indicates Me-18 proton. and multiplate at $\delta_H$ 1.684, 1.379, 2.087, 2.043, 2.007, 1.305, 0.906 that revealed H-5, H-9, H-10, H-13, H-15, H-16 and H-17 proton. All of these signals pointed to the presence of a chemical with a monounsaturated fatty acid moiety in sample LC-002-10-EA_LS, and a comparison to previously published data confirmed the compound as palmitoleic acid (Table 5 and Table 6, Fig. 4-b) (Knothe and Kenar, 2004). The $^{1}$H and $^{13}$C NMR spectrum of isolated compound LC-001-25-EA_L is illustrated in Fig. 5.

Ursolic acid, found in plants like apple peels and holy basil, is a compound used in cosmetics and anticancer drugs. It may increase muscle mass, while reducing fat mass, but no human trials have been conducted. Consuming ursolic acid may reduce fat accumulation, increase muscle growth, promote fat burning, and maintain muscle mass (Saraswati et al., 2013). This acid has been isolated from *L. coromandelica* stem bark for the first time. It is a popular anti-inflammatory, antioxidant, anti-apoptotic, and anti-carcinogenic compound found in fruits and vegetables. Ursolic acid is a crucial component of the human diet, with nearly 700 research articles published in the past decade (Khwaza et al., 2020). It has demonstrated antioxidant and anti-cancer properties. Due to the fact that it scavenges reactive oxygen species, it might play a crucial part in the high glucose-mediated apoptosis. The therapeutic use of ursolic acid affects the growth and death of malignant cells (Saraswati et al., 2013).

Palmitoleic acid, a monounsaturated fatty acid, is found in small amounts in the human diet and blood plasma. Macadamia oil is a significant potential source, making it crucial to understand its importance. *trans*-Palmitoleate is mostly an exogenous source in ruminant fat and dairy products, while it can also be produced in humans. Recent research suggests that palmitoleate is a lipokine that is released from adipose tissue and can influence the metabolism of distant organs. The metabolic effects and mechanisms of palmitoleate have been investigated, which may have potential anti-thrombotic properties in medicinal applications (Bermúdez et al., 2022).

Palmitoleic acid is a monounsaturated omega-7 fatty acid that is primarily found in plants and marine sources (Yang et al., 2019). It has been well documented that palmitoleic acid improves insulin sensitivity, lipid metabolism, and hemostasis. Beta-cell apoptosis that is brought on by glucose or saturated fatty acids may be prevented by palmitoleic acid (Welters et al., 2006). Ultimately, a thorough analysis of *L. coromandelica* extracts has shown a high level of bioactivity, including cytotoxic, anti-diarrheal, antioxidant, and antidiabetic effects. Ursolic acid and palmitoleic acid's separation provides important new insights into the plant's possible bioactive constituents. However, to investigate the molecular processes behind these actions and evaluate the plant's medicinal potential in a range of medical diseases, more research is necessary.

## 4. Concluding remarks

This research highlights the considerable therapeutic potential of *L. coromandelica* stem bark, which has been confirmed by an extensive analysis that used both *in vitro* and *in vivo* techniques. The plant's medicinal richness is further supported by the extraction of bioactive components, such as ursolic and palmitoleic acid, using the relevant extraction techniques and column chromatography. Although both extracts were effective, the *n*-hexane extract showed slightly stronger antidiabetic effects along with better thrombolytic, membrane stabilizing, and cytotoxic qualities. According to this study's findings, brine shrimp are effectively killed by extracts from *L. coromandelica* that have good to moderate cytotoxic and antioxidant activity. The antioxidant and cytotoxic potential of the plant may be due to its polyphenol components, flavonoids, and other phytochemicals, and it can be an excellent source of new, natural antioxidants. Additionally, it is obvious that variations in the thrombolytic and membrane stabilizing abilities of the various *L. coromandelica* extracts point to a high potential for efficient anti-inflammatory activity both *in vitro* and *in vivo*. Due to the abundance of flavonoids, it may be believed that these extracts are a great source of thrombolytic and membrane-stabilizing substances. Although further research is required to completely understand the underlying mechanisms, it's feasible that the effects of *L. coromandelica* will have a big impact on medicine.

## Author contribution statement

Conceptualization and literature search was conducted by Ramisa Anjum, A.H.M. Nazmul Hasan, and Mst. Al Asma Hawa. Data curation was handled by Ramisa Anjum, A.H.M. Nazmul Hasan, and Mst. Al Asma Hawa. Formal analysis was carried out by Md. Khokon Miah Akanda, Fayad Bin Abdus Salam, Tania Binte Wahed, Md. Rabiul Islam, and Mohammad Shahriar. The investigation was performed by Mst. Al Asma Hawa, Ramisa Anjum, Md. Naimur Rahman, and Fayad Bin Abdus Salam. Methodology was developed by Mst. Al Asma Hawa, A.H.M. Nazmul Hasan, and Ramisa Anjum. Supervision was provided by A.H.M. Nazmul Hasan. The original draft was written by A.H.M. Nazmul Hasan and Ramisa Anjum, while the review and editing were completed by A.H.M. Nazmul Hasan and Tania Binte Wahed. All authors read and approved the final manuscript.

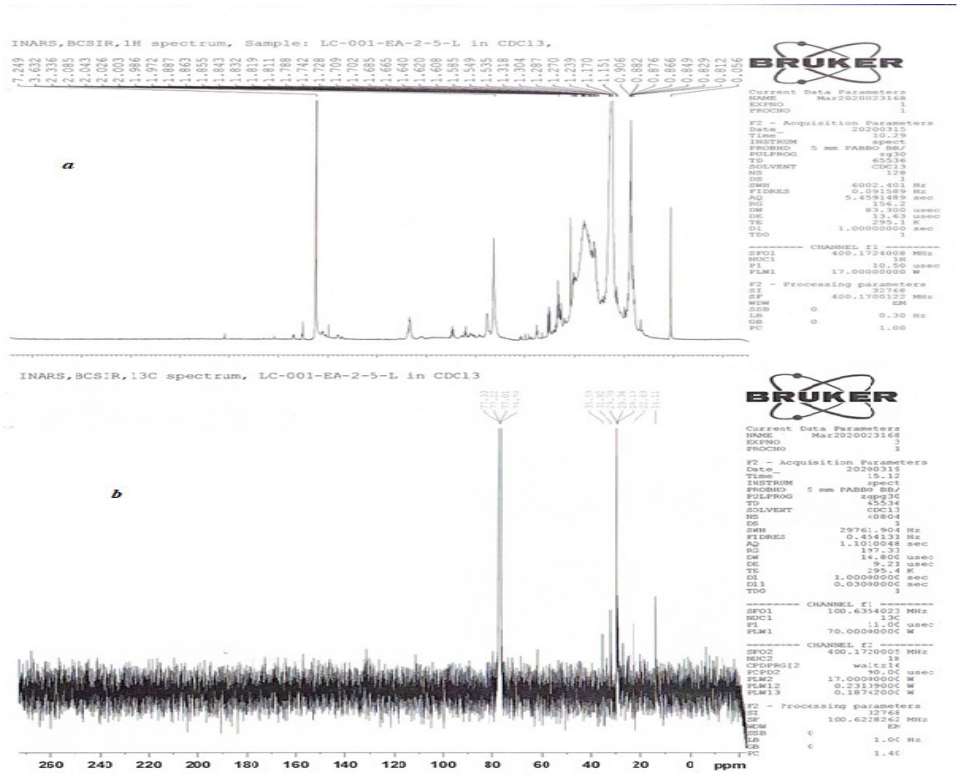



**Fig. 5.** a) $^{1}H$ and b)$^{13}C$ NMR spectrum of LC-001-25-EA_L.

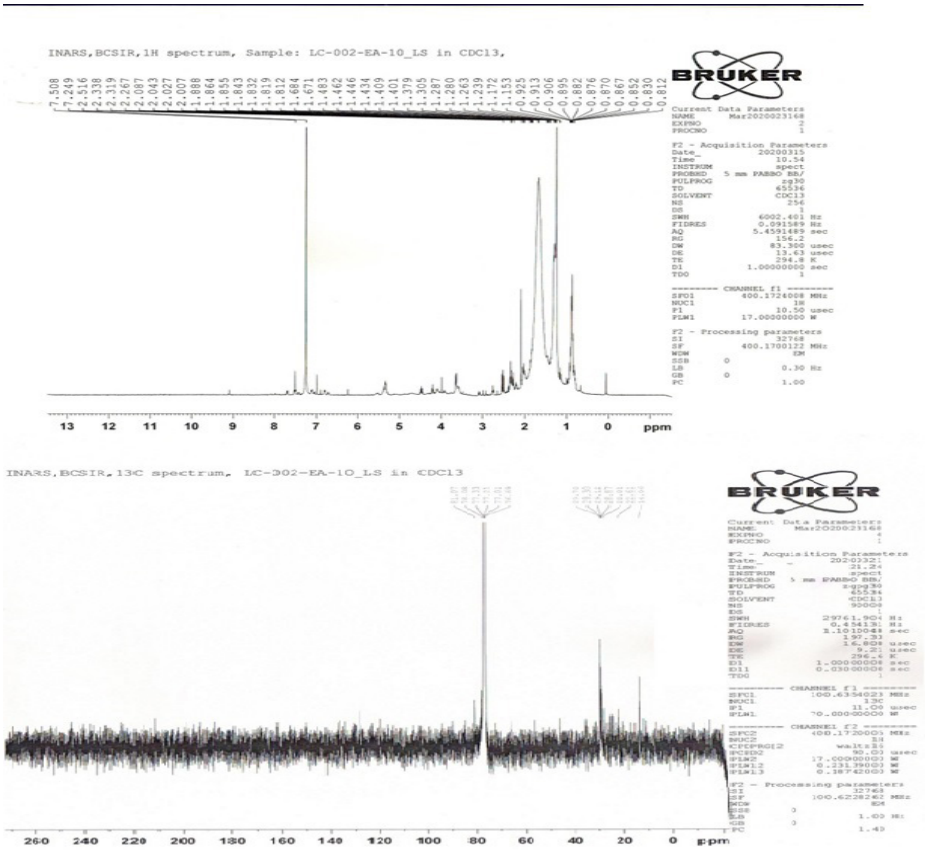



**Fig. 6.** $^{1}H$ and $^{13}C$ NMR spectrum of LC-002-10-EA_LS.

**Table 4**
$^{1}$H-NMR (400 MHz) spectral data of LC-001-25-EA_L and Ursolic acid in $CDCl_3$.

| Si No. | LC-001 | | Ursolic acid | |
|---|---|---|---|---|
| | $^{13}$C NMR | $^{1}$H NMR | $^{13}$C NMR | $^{1}$H NMR |
| 1 | | 1.58 | 38.8 | 1.56 |
| 2 | | 1.72 | 27 | 1.72 |
| 3 | 76.7 | 3.63 | 76.9 | 3.58 (OH, bs) |
| 4 | | | 38.4 | |
| 5 | | 1.32 | 54.8 | 1.39 |
| 6 | | 1.52 | 18 | 1.52 |
| 7 | 31.92 | 1.3 | 32.7 | 1.31 |
| 8 | | | 40.2 | |
| 9 | | | 47.1 | |
| 10 | | | 36.6 | |
| 11 | 22.69 | 2.04 | 22.9 | 2.04 |
| 12 | | | 124.6 | 5.12 (1H, bs) |
| 13 | | | 138.2 | |
| 14 | | | 41.7 | |
| 15 | | | 27.6 | |
| 16 | | | 23.9 | |
| 17 | | | 46.9 | |
| 18 | | 2.09 (1H, d) | 52.4 | 2.10 (1H, d) |
| 19 | | 1.64 | 38.6 | 1.63 |
| 20 | | 1.61 | 38.5 | 1.6 |
| 21 | 29.7 | 1.27 | 30.2 | 1.27 |
| 22 | 35.59 | | 36.4 | |
| 23 | 29.13 | | 28.3 | 0.67 (3H, s) |
| 24 | 14.11 | 0.88 (3H, s) | 15.3 | 0.89 (3H, s) |
| 25 | | 0.87 (3H, s) | 16.1 | 0.86 (3H, s) |
| 26 | | | 17 | 0.74 (3H, s) |
| 27 | | | 23.3 | 1.03 (3H, s) |
| 28 | | | 178.3 | |
| 29 | | 0.81 (3H, d) | 16.9 | 0.81 (3H, d) |
| 30 | | 0.91 (3H, d) | 21.1 | 0.90 (3H, d) |

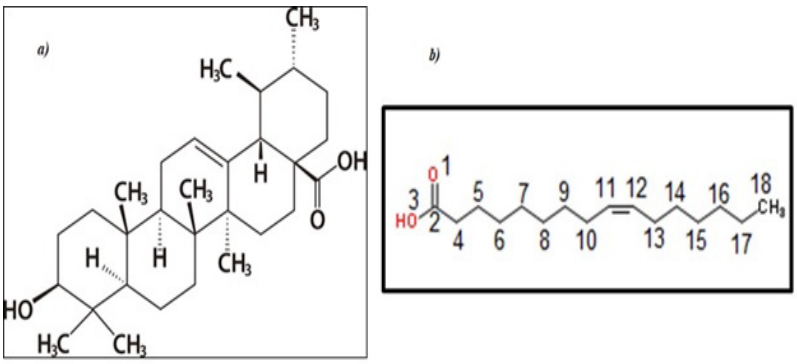



**Fig. 7.** a) Structure of ursolic acid and b) Palmetoleic acid.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgement

The authors are thankful to ICDDR'B for providing laboratory animals and Analytical Service Division, BCSIR to facilitate NMR study of the isolated compounds.

## Ethical guidelines

A research proposal was submitted to the Research Ethics Committee (REC) prior to the study for approval and approved in a meeting (Ref: UAP/REC/20231212) by the REC of the Department of Pharmacy, University of Asia Pacific. Standard protocol was followed in handling of laboratory animal (Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council, Washington DC, USA). Blood sample was collected by taking written inform consent from the volunteers and blood was collected by taking all necessary precautions.

## References

Akanda, M.K.M., Hasan, A.N., 2021. Characterization of pharmacological properties of methanolic seed and stem bark extracts of *Ziziphus mauritiana* (BAU Kul) using *in-vitro* and *in-vivo* animal (Swiss albino male mice) model. Clin. Phytosci. 7, 1-13.

Alam, M.B., Kwon, K.R., Lee, S.H., Lee, S.H., 2017. *Lannea coromandelica* (Houtt.) Merr. induces heme oxygenase 1 (ho-1) expression and reduces oxidative stress via the p38/c-jun n-terminal kinase-nuclear factor erythroid 2-related factor 2 (p38/jnk-nrf2)-mediated antioxidant pathway. Int. J. Mol. Sci. 18(2), 266.

Amabeoku, G., 2009. Antidiarrhoeal activity of *Geranium incanum* Burm. f. (Geraniaceae) leaf aqueous extract in mice. J. Ethnopharmacol. 123, 190-193.

American Diabetes Association, 2010. Diagnosis and classification of diabetes mellitus. Diabetes care, 33(Supplement_1), S62-S69.

Araújo, T.S., Costa, D.S., Sousa, N.A., Souza, L.K., De Araújo, S., Oliveira, A.P., Sousa, F.B.M., Silva, D.A., Barbosa, A.L., Leite, J.R.S., 2015. Antidiarrheal activity of cashew GUM, a complex heteropolysaccharide extracted from exudate of *Anacardium occidentale* L. in rodents. J. Ethnopharmacol. 174, 299-307.

Aziz, I., Saqib, Q.N., Younus, M., Ali, E., Qadir, M.I., Siddique, F.A., Wazir, M.A., Shaheer, M. T., Adnan, Q., Shirazi, J.H., 2021. Physicochemical, antidiarrheal and antidiabetic potential of super food (*Moringa oleifera* Lam.). Pak. J. Pharm. Sci. 34, 723-729.

Bao, J., Cai, Y., Sun, M., Wang, G., Corke, H., 2005. Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. J. Agric. Food. Chem. 53, 2327-2332.

Bauer, A., Kirby, W., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493-496.

Bermúdez, M.A., Pereira, L., Fraile, C., Valerio, L., Balboa, M.A., Balsinde, J., 2022. Roles of palmitoleic acid and its positional isomers, hypogeic and sapienic acids, in inflammation, metabolic diseases and cancer. Cells 11, 2146.

Choi, H.Y., Jhun, E.J., Lim, B.O., Chung, I.M., Kyung, S.H., Park, D.K., 2000. Application of flow injection-chemiluminescence to the study of radical scavenging activity in plants. Phytother. Res. 14, 250-253.

Coskun, O., 2016. Separation techniques: Chromatography. North. Clin. Istanb. 3, 156.

Davis, J.M., Murphy, E.A., Carmichael, M.D. 2009. Effects of the dietary flavonoid quercetin upon performance and health. Curr. Sports Med. Rep. 8, 206-213.

Gupta, M., Mazumder, U., Kumar, R.S., Gomathi, P., Rajeshwar, Y., Kakoti, B., Selven, V.T., 2005. Anti-inflammatory, analgesic, and antipyretic effects of methanol extract from *Bauhinia racemosa* stem bark in animal models. J. Ethnopharmacol. 98, 267-273.

Haenen, G.R., Bast, A., 1999. Nitric oxide radical scavenging of flavonoids. Methods Enzymol. 301, 490-503.

Harbertson, J.F., Spayd, S., 2006. Measuring phenolics in the winery. Am. J. Enol. Vitic. 57, 280-288.

Islam, F., Mitra, S., Nafady, M.H., Rahman, M.T., Tirth, V., Akter, A., Emran, T.B., Mohamed, A. A.R., Algahtani, A., El-Kholy, S.S., 2022. Neuropharmacological and antidiabetic potential of *Lannea coromandelica* (Houtt.) Merr. leaves extract: An experimental analysis. J. Evid. Based Complementary. Altern. Med. 2022.

Islam, M.T., Ito, T., Sakasai, M., Tahara, S., 2002. Zoosporicidal activity of polyflavonoid tannin identified in *Lannea coromandelica* stem bark against phytopathogenic oomycete *Aphanomyces cochlioides*. J. Agric. Food. Chem. 50, 6697-703.

Jabri, M.A., Rtibi, K., Ben-Said, A., Aouadhi, C., Hosni, K., Sakly, M., Sebai, H., 2016. Antidiarrhoeal, antimicrobial and antioxidant effects of myrtle berries (*Myrtus co mmunis* L.) seeds extract. J. Pharm. Pharmacol. 68, 264-274.

Kaur, R., Jaiswal, M.L., Jain, V., 2013. Protective effect of *Lannea coromandelica* Houtt. Merrill. against three co mmon pathogens. J. Ayurveda. Integr. Med. 4, 224-228.

Kedare, S.B., Singh, R., 2011. Genesis and development of DPPH method of antioxidant assay. J. Food. Sci. Technol. 48, 412-422.

Khwaza, V., Oyedeji, O.O., Aderibigbe, B.A., 2020. Ursolic acid-based derivatives as potential anti-cancer agents: An update. Int. J. Mol. Sci. 21, 5920.

Knothe, G., Kenar, J.A., 2004. Determination of the fatty acid profile by $^{1}$H-NMR spectroscopy. Eur. J. Lipid. Sci. Technol. 106, 88-96.

Koster, R., Anderson, M., De, B.E., 1959. Acetic acid for analgesic screening. Fed. Proc. 18, 412-417.

Labib, R.M., Ebada, S.S., Youssef, F.S., Ashour, M.L., Ross, S.A., 2016. Ursolic acid, a natural pentacylcic triterpene from *Ochrosia elliptica* and its role in the management of certain neglected tropical diseases. Pharmacogn. Mag. 12, 319.

Lin, J.Y., Tang, C.Y., 2007. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 101, 140-147.

Mahboubi, A., Asgarpanah, J., Sadaghiyani, P.N.,

Faizi, M., 2015. Total phenolic and flavonoid content and antibacterial activity of *Punica granatum* L. var. *pleniflora* flowers (Golnar) against bacterial strains causing foodborne diseases. BMC. Complement. Altern. Med. 15, 366.
Mehjabin, S., Akanda, M.K.M., Hoque, N., Hasan, N.A.H.M., Parvez, M.G.M., Mosaddik, A. 2024. Flavonoid Intake and Risk of Toxicity in Chronic Metabolic Disease. In; Mishra, N., Ashique, S., Gowda, J., Garg, A. Role of Flavonoids in Chronic Metabolic Diseases: From Bench to Clinic. Scrivener Publishing LLC, Wiley. pp. 511-534.
Makenzi, A.M., Manguro, L.O.A., Owuor, P.O., 2023. Sesquiterpenoids with antiplasmodial activities from *Warburgia stuhlmanii* Engl. root bark. Trends Phytochem. Res. 7(2), 89-94.
Manik, M., Wahid, M., Islam, S., Pal, A., Ahmed, K., 2013. A comparative study of the antioxidant, antimicrobial and thrombolytic activity of the bark and leaves of *Lannea coromandelica* (Anacardiaceae). Int. J. Pharm. Sci. Res. 4, 2609.
Marques, S.S., Magalhães, L.M., Tóth, I.V. Segundo, M.A., 2014. Insights on antioxidant assays for biological samples based on the reduction of copper complexes-The importance of analytical conditions. Int. J. Mol. Sci. 15, 11387-11402.
Martins, J., Brijesh, S., 2018. Phytochemistry and pharmacology of anti-depressant medicinal plants: A review. Biomed. Pharmacother. 104, 343-365.
Mbahi, M.A., Mbahi, M.A., Amos, P.I., 2018. Phytochemical screening and anti-diarrheal activity of the pulp and seed extracts of *Ziziphus mauritiana* in rats. Int. J. Biochem. Res. Rev. 24, 1-9.
Mercier, J.C., Ouldali, N., Melki, I., Basmaci, R., Levy, M., Titomanlio, L., Beyler, C., Meinzer, U., 2021. Severe acute respiratory syndrome coronavirus 2-related multisystem inflammatory syndrome in children mimicking Kawasaki disease. Arch. Cardiovasc. Dis. 114, 426-433.
Mohammadhosseini, M., Frezza, C., Venditti, A., Sarker, S., 2021. A systematic review on phytochemistry, ethnobotany and biological activities of the genus *Bunium* L. Chem. Biodivers. 18(11), e2100317.
Mohammadhosseini, M., Venditti, A., Akbarzadeh, A., 2019a. The genus *Perovskia* Kar.: Ethnobotany, chemotaxonomy and phytochemistry: A review. Toxin Rev. 40(4), 484-505.
Mohammadhosseini, M., Venditti, A., Frezza, C., Serafini, M., Bianco, A., Mahdavi, B., 2021b. The genus *Haplophyllum* Juss.: Phytochemistry and bioactivities—A review. Molecules 26(15), 4664.
Mosnaim, A.D., Wolf, M.E., III. O'donnell, J.J., Hudzik, T., 2020. β-Phenylethylamine and various monomethylated and para-halogenated analogs. Acute toxicity studies in mice. Drug. Chem. Toxicol. 43, 369-372.
Mulat, M., Pandita, A., Khan, F., 2019. Medicinal plant compounds for combating the multi-drug resistant pathogenic bacteria: A review. Curr. Pharm. Biotechnol. 20, 183-196.
Nascimento, G.G., Locatelli, J., Freitas, P.C., Silva, G.L., 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Braz. J. Microbiol. 31, 247-256.
Omale, J., Okafor, P.N., 2008. Comparative antioxidant capacity, membrane stabilization, polyphenol composition and cytotoxicity of the leaf and stem of *Cissus multistriata*. African. J. Biotech. 7(17).
Prasad, S., Kashyap, R.S., Deopujari, J.Y., Purohit, H.J., Taori, G.M., Daginawala, H.F., 2006. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thrombosis J. 4, 1-4.
Price, K.S., Waggy, G.T., Conway, R.A., 1974. Brine shrimp bioassay and seawater BOD of petrochemicals. J. Water Pollution Cont. Fed. 63-77.
Prieto, P., Pineda, M., Aguilar, M., 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal. Biochem. 269, 337-341.
Rajesh, R., Selvakumar, K., 2022. Evaluation of *in vitro* anthelmintic activity of *Lannea coromandelica* (Houtt.) Merr. against *Pheretima posthuma* and *Ascardia galli*. Res. J. Pharm. Technol. 15, 2539-2542.
Ramadhan, A., Herman, H., Sutrisnawati, S., 2022. Ethanolic extract of *Lannea coromandelica* stem bark: Histopathology and infiltration of inflammatory cells in the rat's gastric. J. Adv. Pharm. Technol. Res.13, 296.
Ricciotti, E., Fitzgerald, G.A., 2011. Prostaglandins and inflammation. Arterioscler. Thromb. Vasc. Biol. 31, 986-1000.
Saradhajyothi, K., Subbarao, B., 2011. Antibacterial potential of the extracts of the leaves of *Azadirachta indica* Linn. Not. Sci. Biol. 3.
Saraswati, S., Agrawal, S., Alhaider, A.A., 2013. Ursolic acid inhibits tumor angiogenesis and induces apoptosis through mitochondrial-dependent pathway in *Ehrlich ascites* carcinoma tumor. Chem. Biol. Interact. 206, 153-165.
Shahriar, M., Mannan, M., Akhter, R., Bhuiyan, M., 2015. Evaluation of *in vitro* membrane stabilizing activity and thrombolytic activity of *Averrhoa bilimbi* leaf extracts. Int. J. Pharm. 5, 357-60.
Shetty, N.P., Shetty, P.S., 2009. Epidemiology of Disease in the Tropics. Manson's Tropical Diseases, Vol. 19.
Shinwari, Z., 2011. Antibacterial activity in herbal products used in Pakistan. Pak. J. Bot. 43, 155-162.
Shoeb, H.A., Madkour, H.M., Refahy, L.A., Mohamed, M.A., Saad, A.M., Ghareeb, M.A., 2014. Antioxidant and cytotoxic activities of *Gmelina arborea* ROXB. leaves. Br. J. Pharm. Res. 4, 125.
Singh, A., Singh, D., Sharma, S., Mittal, N., 2023. A review on biosynthesis, regulation, and applications of terpenes and terpenoids. Trends Phytochem. Res. 7(4), 228-245.
Sreejayan, X., Rao, M., 1997. Nitric oxide scavenging by curcuminoids. J. Pharm. Pharmacol. 49, 105-107.
Stanojević, L., Stanković, M., Nikolić, V., Nikolić, L., Ristić, D., Čanadanovic-Brunet, J., Tumbas, V., 2009. Antioxidant activity and total phenolic and flavonoid contents of *Hieracium pilosella* L. extracts. Sensors 9, 5702-5714.
Sztanke, M., Sztanke, K., 2017. Biologically important hydrazide-containing fused azaisocytosines as antioxidant agents. Redox Rep. 22, 572-581.
Taber, R.I., Greenhouse, D.D., Rendell, J.K., Irwin, S., 1969. Agonist and antagonist interactions of opioids on acetic acid-induced abdominal stretching in mice. J. Pharmacol. Exp. Ther. 169, 29-38.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., Kaur, H., 2011. Phytochemical screening and extraction: A review. IPS 1, 98-106.
Tomazetti, J., Ávila, D.S., Ferreira, A.P.O., Martins, J.S., Souza, F.R., Royer, C., Rubin, M.A., Oliveira, M.R., Bonacorso, H.G., Martins, M.A.P., 2005. Baker yeast-induced fever in young rats: Characterization and validation of an animal model for antipyretics screening. J. Neurosci. Methods. 147, 29-35.
Trease, G., Evans, W. 1989. Pharmacognosy. 13th. ELBS/ Bailliere Tindall, London, pp. 345-346.
Uddin, J., Islam, M.N., Ali, M.H., Khan, S.A., Labu, Z.K., 2016. Correlation of thrombolytic and membrane stabilizing activities with total flavonoid content aerial parts of *Spondias pinnata*. Bangladesh Pharm. J. 19, 48-52.
Uddin, N., Hasan, M.R., Hossain, M.M., Sarker, A., Hasan, A.N., Islam, A.M., Chowdhury, M.M. H., Rana, M.S., 2014. *In vitro* α-amylase inhibitory activity and *in vivo* hypoglycemic effect of methanol extract of *Citrus macroptera* Montr. fruit. Asian Pac. J. Trop. Biomed. 4, 473-479.
Umer, S., Tekewe, A., Kebede, N., 2013. Antidiarrhoeal and antimicrobial activity of *Calpurnia aurea* leaf extract. BMC. Complement. Altern. Med. 13, 1-5.
Upadhya, S., Shanbhag, K.K., Suneetha, G., Balachandra Naidu, M., Upadhya, S., 2004. A study of hypoglycemic and antioxidant activity of *Aegle marmelos* in alloxan induced diabetic rats. Indian. J. Physiol. Pharmacol. 48, 476-480.
Wahid, A., 2012. *In vitro* Phytochemical and Biological Investigation of Plant *Lannea coromandelica* (Family: Anacardiaceae). East West University.
Weerapreeyakul, N., Junhom, C., Barusrux, S., Thitimetharoch, T., 2016. Induction of apoptosis in human hepatocellular carcinoma cells by extracts of *Lannea coromandelica* (Houtt.) Merr. and *Diospyros castanea* (Craib) Fletcher. Chin. Med. 11, 19.
Welters, H.J., Diakogiannaki, E., Mordue, J.M., Tadayyon, M., Smith, S.A., Morgan, N.G., 2006. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β-cells exposed to palmitate. Apoptosis 11, 1231-1238.
Yang, Z.H., Pryor, M., Noguchi, A., Sampson, M., Johnson, B., Pryor, M., Donkor, K., Amar, M., Remaley, A.T., 2019. Dietary palmitoleic acid attenuates atherosclerosis progression and hyperlipidemia in low density lipoprotein receptor deficiency mice. Mol. Nutr. Food Res. 63(12), 1-22.
Yuet Ping, K., Darah, I., Chen, Y., Sreeramanan, S., Sasidharan, S., 2013. Acute and subchronic toxicity study of *Euphorbia hirta* L. methanol extract in rats. Biomed. Res. Int. 2013.

Trends Phytochem. Res. 8(2) 2024 93-105

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Phytochemical characterization and biological properties of *Ocimum sanctum* L. and its active phytocomponents: Eugenol and β-caryophyllene

Krupali Trivedi[1], Nilam Parmar[2], Khairah Ansari[1], Vaibhavi Srivastava[1], Nishi Modi[1], And Devendrasinh Jhala*[1]

[1]Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad, Gujarat 380009, India
[2]Department of Life Science, University School of Sciences, Gujarat University, Ahmedabad, Gujarat 380009, India

**ABSTRACT**

This study explored the pharmacognostical and biological properties of *Ocimum sanctum* L., focusing on its hydromethanolic (HME) and aqueous (AQE) extracts, as well as the bioactive compounds eugenol (EUG) and β-caryophyllene (BCP). Phytochemical analysis revealed a diverse range of compounds, with HME showing higher levels of phenolics, flavonoids, and tannins compared to AQE. Both extracts exhibited antioxidant activity, while EUG and BCP displayed significant cytotoxicity against MCF-7 cells. Molecular docking studies indicated that EUG has potential binding to catalase. The findings of this study underscore *O. sanctum* as a valuable source of bioactive compounds with potential antioxidant and anticancer properties. Notably, the extraction method played a crucial role in determining the phytochemical profile. However, further investigations are necessary to fully understand the mechanisms and therapeutic applications of EUG and BCP, particularly as potential catalase inhibitors.

**ARTICLE HISTORY**

Received: 07 November 2023
Revised: 27 May 2024
Accepted: 14 June 2024
ePublished: 28 June 2024

**KEYWORDS**

Antioxidant activity
β-Caryophyllene
Cytotoxicity
Eugenol
Molecular docking
*Ocimum sanctum* L.

## 1. Introduction

The Lamiaceae (Labiatae) family, commonly known as the mint family, is a diverse group of flowering plants that includes over 7,000 species and 236 genera (Hashemi-Moghaddam et al., 2015; Mohammadhosseini et al., 2019a; Mohammadhosseini et al., 2019b; Kianasab et al., 2023). This family is characterized by its distinctive four-sided stems, aromatic leaves, and tubular flowers. Many members of the Lamiaceae family are widely cultivated for their culinary and medicinal properties, such as basil, rosemary, lavender, and thyme, and are found all over the world (Anita Margret et al., 2022; Sarkar et al., 2023). Cancer is a growing global health concern. Conservative estimates suggest that liver, breast, lung, cervical, stomach, and colorectal cancers account for approximately 13% of annual deaths worldwide (Organisation mondiale de la santé, 2018; Omara et al., 2020). Oxidative stress resulting from chemotherapy-induced reactive oxygen species (ROS) contributes to or is primarily responsible for many adverse effects of the treatment. Cancer cells compared to normal cells, have higher levels of ROS and more likely to have mitochondrial dysfunction (Acuña et al., 2012). Overproduction of oxygen-centered free radicals or any ROS such as hydroxyl and superoxide free radicals can cause oxidative damage in biomolecules, *e.g.*, lipids, proteins and DNA which is also evident in some kinds of cancers such as cancer of the liver, lung, stomach, colon, breast, and so forth (Poulsen et al., 1998; Bhalla et al., 2013). The most rational approach to prevent carcinogenesis is by interfering with the basic modulation steps such as initiation, promotion, progression and the associated signal transduction pathways (Fresco et al., 2006; Bhalla et al., 2013). Antioxidants such as vitamin C remove damaging oxidizing agents like hydrogen peroxide from living

Corresponding author: Devendrasinh Jhala
Tel: 9824240644; Fax: 9824240644
E-mail address: ddjhala@gmail.com, **doi:**

organisms and neutralize free radicals that harm our body cells (Pehlivan, 2017). The modern world's socio-cultural life is heavily reliant on allopathic medicine, including chemotherapy. However, much contemporary research has focused on addressing this disease using ayurvedic herbs as a complementary or alternative approach, driven by the need to mitigate side effects such as alopecia, chemotherapy-induced peripheral neuropathy, nausea and vomiting, cardiotoxicity, diarrhea, infertility, and chemo brain (Pandey et al., 2013; Brianna and Lee, 2023). Therefore, it is crucial to develop an effective strategy and novel drugs with minimal side effects or toxicity that can mitigate the sequelae of cancer chemotherapy.

Numerous herbs have been scientifically shown to possess anti-carcinogenic properties, which are used to treat various cancers (Farzaneh and Carvalho, 2015). Among a broad spectrum of medicinal plants, *Ocimum sanctum* L. has been widely used in traditional Indian medicine for centuries to treat a range of ailments and diseases (Kaushal et al., 2018). *O. sanctum* L., "The Queen of herbs" known as 'Tulsi' in Hindi and 'Holy Basil' in English, belongs to the Angiospermae and family Lamiaceae (Prakash and Gupta, 2005). This herbal species . has two assortments, namely dark (Krishna Tulsi) and green (Rama Tulsi), and their chemical constituents are comparable. Basil or Tulsi leaves contain a high percentage of eugenol (EUG) (57.9%) (4-allyl-2-methoxyphenol) and β-caryophyllene (BCP) (15.3%) [(1*R*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0] undec-4-ene] (9.1%) (Mirdha et al., 2007).

Eugenol (EUG) is a methoxyphenol with a short hydrocarbon chain and an aromatic ring, featuring a hydroxyl group that enables it to act as a hydrogen atom donor and inhibit oxidation. This structural-activity relationship contributes to its antioxidant properties (Gülçin, 2011), anti-genotoxic (Rompelberg et al., 1996), anti-carcinogenic (Zheng et al., 1992; Gülçin, 2011), etc. β-Caryophyllene (BCP) is a sesquiterpene, which has interesting chemopreventive properties such as anti-inflammatory, genoprotective and anti-proliferative (Di Giacomo et al., 2016, 2017).

This investigation aims to:

i) Evaluate the pharmacognostic and antioxidant properties of hydromethanolic (HME) and aqueous (AQE) extracts of *O. sanctum* L., as they contain most of the active phytocomponents.

ii) Study the *in vitro* cytotoxicity of HME, AQE, eugenol (EUG), and β-caryophyllene (BCP) against the human breast cancer cell line MCF-7.

iii) Predict the binding affinity of EUG and BCP with the antioxidant enzymes catalase (CAT) and glutathione reductase (GR) using in silico docking studies.

This work underscores the crucial impact of extraction methods on the phytochemical profile and bioactivity of *O. sanctum*, providing new insights into its antioxidant and anticancer properties and paving the way for further research into the therapeutic applications of its bioactive compounds.

## 2. Experimental

### 2.1. Pharmacognostic profile

#### 2.1.1. Collection, authentication of plant material and preparation of *O. sanctum* leaves extracts

Fresh leaves of *O. sanctum* L. or holy basil (Fig. 1a) were collected from the plant grown at Samaras girl's hostel, Navrangpura, Ahmedabad (Fig. 1b) and authenticated by Prof. and Head, Dr. Hitesh Solanki, Department of Botany, School of Sciences, Gujarat University, Ahmedabad. The herbarium number was GU/BOT/L/O18.

After washing thoroughly in distilled water, the green leaves were shade-dried at room temperature, then pulverized into fine powder, and stored in an airtight container for future use (Harborne, 1984). The dried leaf powder of *O. sanctum* L. (5.0 g) was subjected to extraction by soxhlet apparatus using hydromethanol solvent (70:30) to prepare hydromethanolic extract and filtered using charcoal filter paper to remove impurities. For aqueous extract, leaves powder (25 g) was refluxed with 300 mL of double distilled water for 3 h at 60-65 °C on the magnetic stirrer. It was then cooled and filtered using muslin cloth followed by evaporation for desired volume in a hot air oven at 37 to 40 °C.

#### 2.1.2. Qualitative analysis of *Ocimum sanctum* leaf extract

##### 2.1.2.1. Determination of pH and percentage yield

A pH meter (WW-35634-90, Cole-Parmer, USA) was used to determine the pH. HME and AQE were kept in an incubator for 3 days at 37 °C for drying and percentage yield was calculated by the following formula:

Percentage yield (%) = [Weight of product after evaporation in incubator (g)/ Weight of powder used in soxhelt system (g)] × 100 (Eqn. 1)

##### 2.1.2.2. FTIR analysis

*O. sanctum* L. (Tulsi) leaves extracts (HME and AQE) and their bioactive compounds EUG and BCP were used for FTIR analysis using Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy (Bruker, Germany) to determine functional groups present.

##### 2.1.2.3. Phytochemical analysis of *Ocimum sanctum*

Phytochemicals were analyzed to ascertain the presence or absence of various phytocomponents like saponins, alkaloids, tannins, phenolic compounds, flavonoids, glycosides, fats, steroids, and triterpenoids in *O. sanctum* L. by the method of Harborne (1984). Molisch's, Fehling's, Benedict's, and Barfoed's tests were respectively performed for the absence or presence of carbohydrates. Biuret's, Millon's, and xanthoprotein's tests were performed for proteins. Ninhydrin test was performed for amino acid presence. Salkowaski's and Liebermann-Burchard's tests were performed for steroids and triterpenoids presence. Shinoda and lead acetate tests were performed for flavonoid presence. $FeCl_3$, acetic acid and iodine tests were performed for tannin and phenolic compounds presence.

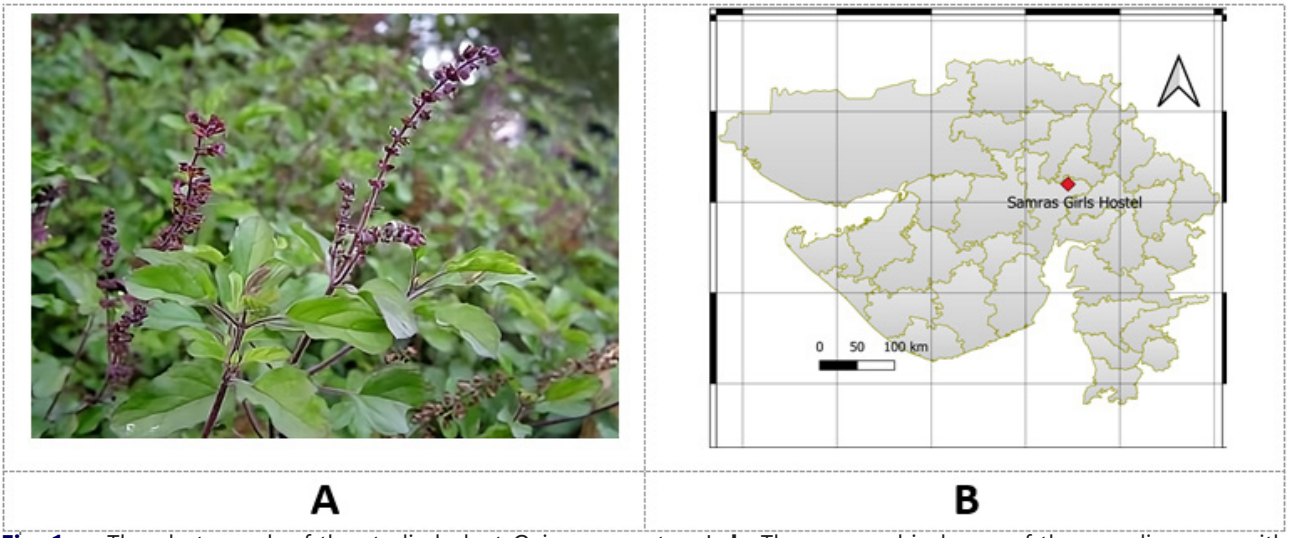



**Fig. 1. a:** The photograph of the studied plant *Ocimum sanctum* L. **b:** The geographical map of the sampling area with Longitude= 72.540678°, Latitude= 23.037545°.

Dragendorff's, Mayer's, Hager's and Wagner's tests were performed for alkaloids presence. A foam test was performed for fat and saponins presence.

#### 2.1.2.4. Thin layer chromatography (TLC)

Thin layer chromatography was performed as described by Hassan and coworkers (2015) to confirm the presence of EUG and BCP in the extracts of plant leaves using different solvents as mobile phase, namely toluene:ethyl acetate:acetic acid (10:1:0.32) for the presence of EUG and ethyl acetate: *n*-hexane (7:3) for the presence of BCP.

### 2.1.3. Quantitative analysis of *Ocimum sanctum* leaf extract

#### 2.1.3.1. Estimation of total phenolic content (TPC)

The total phenolic content of *O. sanctum* L. leaf extracts was determined using the method of Singleton and Rossi (1965) with minor modifications. A standard curve was prepared using different concentrations of gallic acid (5-50 µg/mL) to measure the comparative total phenolic level in the samples. The results were expressed in terms of gallic acid equivalent (µg of gallic acid per mg of extract) and calculated using the linear regression equation of the standard curve.

#### 2.1.3.2. Estimation of total flavonoid content (TFC)

With minor changes in the method of Harborne (1984), the total flavonoid content of *O. sanctum* L. leaf extracts was estimated. The concentration of flavonoids in HME and AQE extracts (1 mg/mL) was calculated using a regression equation derived from the graph of quercetin concentration versus absorbance at 470 nm. Quercetin (1-10 µg/mL) was used as a standard, and the results were expressed in terms of quercetin equivalent (µg of quercetin per mg of extract).

#### 2.1.3.3. Estimation of total tannin content (TTC)

The total tannin content in *O. sanctum* L. leaf extracts was analyzed using the method of Harborne (1984) with minor modifications. The concentration of total tannins in HME and AQE extracts (1 mg/mL) was calculated using a regression equation derived from the graph of tannic acid concentration versus absorbance at 550 nm. Tannic acid (5-30 µg/mL) was used as a standard, and the results were expressed in terms of tannin equivalent (µg of tannic acid per mg of extract).

### 2.1.4. Determination of antioxidant activity

#### 2.1.4.1. DPPH scavenging assay

To assess the antioxidant activity of the plant extracts, the method adapted from Blois (1958) was used with slight modifications. A stock solution of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) (0.1 mM) stock solution was prepared in methanol and stored in anamber bottle, kept in the dark at 4 °C between the measurements. For the assay, 50 µL solution of DPPH methanol was added to 50 µL plant extracts solutions at varying concentrations, (3.9 to 500 µg/mL), prepared by serial dilution. The mixture was incubated in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm against methanol as blank. Ascorbic acid, at the same concentrations as the samples, was used as a standard. The $IC_{50}$ value was determined by linear regression analysis of the dose-response curve, plotting percentage inhibition against concentration.

#### 2.1.4.2. ABTS scavenging assay

ABTS (2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging activity was assessed as described by Re and coworkers (1999) with slight modifications. The $ABTS^{\cdot+}$ radicals were generated

by mixing ABTS solution (7.0 mM) and potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark overnight. Samples and standards, with concentrations ranging from 4.88 to 625 µg/mL, were then added (100 µL each) to a 12-well plate, followed by the addition of ABTS solution (900 µL) to each well. After 5 min of incubation at room temperature, the decrease in absorbance was quantified spectrophotometrically at 540 nm. Ascorbic acid was used as standard to prepare the standard inhibition curve and the $IC_{50}$ value of *O. sanctum* L. extracts was calculated based on this curve.

#### 2.1.4.3. Superoxide anion scavenging assay

The superoxide radical scavenging activity of *O. sanctum* L. leaf extract was evaluated according to the method of Yen and Hsieh (1995) using nitro blue tetrazolium (NBT) dye with some minor modifications. The reaction mixture contained 0.1 mL of NBT (1 mM), 0.1 mL of NADH (0.1 mM), 0.5 mL of the sample with the concentration range between 100-1000 µg/mL and 0.25 mL of phosphate buffer saline. After 1 min of incubation, 0.1 mL phenyl methosulfate (PMS) (120 µM) was added and absorbance was quantified spectrophotometrically at 560 nm. Ascorbic acid was taken as standard to prepare the standard inhibition curve and the $IC_{50}$ value of leaf extracts was determined based on this curve.

#### 2.1.4.4. Nitric oxide scavenging assay

Nitric oxide radical scavenging capacity was estimated using Griess-Ilosvay's reagent (Green et al., 1982; Badami et al., 2003). The reaction mixture containing 1 mL of sodium nitroprusside (10 mM), 0.25 mL of phosphate buffer saline, and 0.25 mL of sample or standard solution of ascorbic acid at various concentrations ranging between 1-10 µg/mL were incubated at 25 °C for 150 min. After incubation, 0.5 mL of the Griess reagent was added. A pink-colored chromophore was formed in diffused light. The absorbance of these solutions was measured spectrophotometrically at 540 nm. The inhibition curve was plotted and the $IC_{50}$ value of *O. sanctum* L. leaf extracts was determined.

### 2.2. Cytotoxicity study: MTT assay

#### 2.2.1. Cell Culture

MCF-7, a human breast cancer cell line was purchased from the National Centre for Cell Science (NCCS), Pune, and was cultured in RPMI 1640 media, supplemented with fetal bovine serum (FBS, 10%), 2 mM L-glutamine and 0.1 mM non-essential amino acids. The cells were maintained at 37 °C in a 5% $CO_2$ incubator (Biocenter, Salvis Lab). Once each cell line became confluent (80%), cells were trypsinized (0.25% trypsin-EDTA), counted, and seeded in a 96-well plate according to the experimental protocol.

#### 2.2.2. Experimental groups

To check the anticancer effects of EUG, BCP, HME and AQE, the study was divided into six different groups as listed in Table 1.

**Table 1**
Experimental groups for MTT assay.

| Group | Compound | Dose |
|---|---|---|
| I | Control | - |
| II | Vehicle control (DMSO) | 1.00% |
| III | Eugenol (EUG) | 50-600 nM |
| IV | β-Caryophyllene (BCP) | 100-1200 nM |
| V | Hydromethanolic extract (HME) | 1-20 µg/mL |
| VI | Aqueous extract (AQE) | 1-20 µg/mL |

#### 2.2.3. MTT assay

The MTT [3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to measure the cytotoxicity and cell proliferation based on the conversion of MTT into formazan crystals by mitochondrial dehydrogenase in living cells (Mosmann, 1983). Cells were harvested and seeded into a 96-well plate ($5\times10^3$ cells/well) and incubated for 24 h. The cells were treated with different doses of compounds as mentioned in Table 1, for 24 h. 20 µL MTT (5 mg/mL in PBS) was added to each well of the 96-well culture plate at the end of various treatments and were incubated for 4 h at 37 °C in a 5% $CO_2$ incubator. After incubation, 50 µL of dimethyl sulfoxide (DMSO) was added to solubilize formazans. After incubation of at 37 °C for 10 min, the absorbance was recorded at 570 nm using a double beam spectrophotometer (Epoch microplate spectrometer, Biotek, USA). DMSO was used as a vehicle control (1.0%), only media was taken as blank and media along with the cells, without any treatment having maximum cell viability was taken as control.

### 2.3. Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean ± SD. The statistical significance was evaluated by two way-analysis of variance (ANOVA) using GraphPad Prism version 8. The value of $p < 0.05$ was considered to indicate significant difference.

### 2.4. *In silico* study: Molecular docking

EUG and BCP were docked with two enzymatic antioxidants, catalase (CAT) and glutathione reductase (GR). The 3D structure of the CAT (PDB ID: 1DGH), and GR (PDB ID: 1XAN) were downloaded from PDB database (https://www.rcsb.org/) and prepared using *protein preparation wizard*; later Molecular docking was performed using "GLIDE" Software of Schrödinger suite (Schrödinger, LLC, NY, 2023). The structure of EUG and BCP were retrieved from the PubChem database

https://pubchem.ncbi.nlm.nih.gov) and prepared using the *LigPrep* module, inducing potential ionization at pH 7.0. Protein structures were optimized and minimized, then subjected to grid generation using the receptor grid generation module of the software. The 3D structure of target proteins was retrieved in their complex forms with co-crystallized ligands from the PDB. These inhibitor ligands were used as a native ligand and selected during the receptor grid generation to determine the active site of the protein. All the docking calculations were performed using Glide's XP (Extra precision) mode. The visualization of the docked (protein-ligand complex was performed using Discover studio visualizer, Biovia.

## 3. Results and Discussion

### 3.1. Qualitative analysis of *O. sanctum* extracts

#### 3.1.1. pH and percentage yield

The pH of *O. sanctum* HME and AQE was 7.0 and 6.0, respectively (Table 2). The HME and AQE extracts were dried in an incubator at 37 °C for 3 days, yielding 16.07% of HME and 16.61% of AQE (Table 2). These results are consistent with the findings of Mandal et al. (1993).

**Table 2**
pH and percentage yield of hydromethanolic extract and aqueous extract.

| Tests | HME | AQE |
|---|---|---|
| pH | 7 | 6 |
| Percentage yield | 16.07% | 16.61% |

HME: Hydromethanolic extract, AQE: Aqueous extract.

#### 3.1.2. FTIR analysis

The functional groups in both HME and AQE extracts, as well as EUG and BCP phytocomponents, were analyzed using FTIR spectroscopy. The results are presented in Fig. 2 (a-d), which show the corresponding FTIR spectra. FTIR analysis revealed the presence of various functional groups in all samples, including alcohols, alkanes, aromatic hydrocarbons, and ethers, but alkynes were absent. The analysis also found that carboxylic acids, amines, amides, and halides were absent in EUG and BCP, while aldehydes, ketones, and esters were absent in HME, EUG, and BCP. AQE showed the presence of all functional groups except alkynes. The differences in spectral shape and absorbance intensity indicated variations in composition and quantity among the samples. The results confirmed the presence of EUG and BCP in both HME and AQE extracts. The wavenumbers and corresponding functional groups are presented in Table 3. The presence of various functional groups suggests the existence of multiple active phytochemicals that may interact with biological systems through multiple biochemical pathways, such as enzymatic inhibition, oxidation reactions, and altered biochemical formation processes (Ullah et al., 2020). These functional groups can interact with biological receptors, enzymes, or cellular structures, influencing the compound's effects on the body. For example, the presence of hydroxyl groups (-OH) is commonly found in phenolic compounds like flavonoids and is associated with antioxidant and anti-inflammatory activities (Gomathi et al., 2014). Previous reports indicate that peaks for *Ocimum sp*. are characteristic of EUG, linalool, and terpenes, which are abundant in these plant extracts (Ramteke et al., 2013).

#### 3.1.3. Phytochemical screening of *O. sanctum* L. leaves extracts (HME and AQE)

The phytochemical analysis was carried out for both HME and AQE of the *O. sanctum* leaves to identify the presence of carbohydrates, protein, amino acids, steroid, triterpenoids, flavonoids, tannin, phenol, saponin, fatty acids, cardiac glycosides, and alkaloids. The preliminary phytochemical analysis and characterization of bioactive compounds from plants is an important step in ascertaining their medicinal value (Sasidharan et al., 2011; Madike et al., 2017). The phytochemical analysis and qualitative estimation of HME and AQE of *O. sanctum* L. leaves showed the presence of carbohydrates, proteins, amino acids, steroids, triterpenoids, flavonoids, tannins, phenol, and fatty acids. Saponin and cardiac glycosides were absent in the extracts (Table 4). Similar results were observed by Devendran and Balasubramanian (2011) during qualitative phytochemical screening and GC-MS analysis of extracts of *O. sanctum* L. leaves. The presence of ROS scavenging phytochemical molecules (*e.g*., flavonoids, alkaloids) indicates that the extracts that contain these phytochemicals can be used as an antioxidant to forage free radicals and can also be used as a potent antioxidative agent to treat various diseases.

#### 3.1.4. Thin layer chromatography (TLC)

The presence of EUG and BCP was confirmed in both HME and AQE extracts using TLC. EUG was detected with a solvent system of toluene: ethyl acetate: acetic acid (10:1:0.32) and an Rf value of 0.49 (Fig. 3a) after derivatization with PMA. BCP was detected with a mobile phase of ethyl acetate: *n*-hexane (7:3) and an Rf value of 0.82 (Fig. 3b) after derivatization with $KMnO_4$. The bioactive components EUG and BCP were present in both extracts, as confirmed by TLC with retention factors of 0.49 and 0.82, respectively. This finding is consistent with previous research by Lalla et al. (2007). Additionally, a study by Anandjiwala et al. (2006) reported the presence of EUG in green and black Tulsi using high-performance liquid chromatography (HPLC).

#### 3.1.5. Quantitative analysis of *O. sanctum* L. leaves extracts (HME and AQE)

The quantitative analysis of phytochemicals of HME and AQE was carried out to determine total phenolic (TPC), flavonoid (TFC), and tannin (TTC) content. Results were obtained using a regression formula generated using gallic acid, quercetin, and tannic acid as standard for phenolic, flavonoid, and tannin content, respectively.

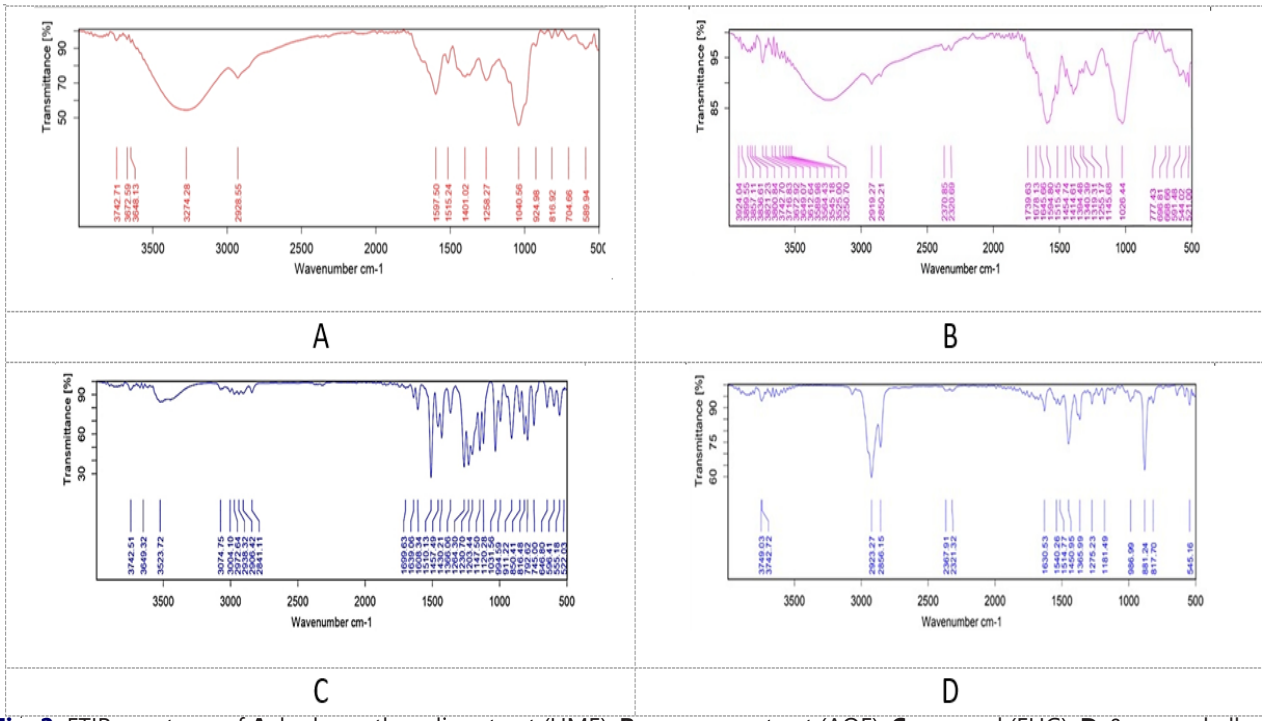



**Fig. 2.** FTIR spectrum of **A**: hydromethanolic extract (HME), **B**: aqueous extract (AQE), **C**: eugenol (EUG), **D**: β-caryophyllene (BCP).

| Fig. no. | 3a | 3b |
|---|---|---|
| Compound | EUG | BCP |
| Solvent system | Toluene: Ethyl acetate: Acetic acid (10: 1: 0.32) | Ethyl acetate: *n*-hexane (7: 3) |
| Derivatized with | PMA (propylene glycol monomethyl ether acetate) | $KMnO_4$ |

When both extracts (1 mg/mL) were compared based on the regression formula generated from the graph for total phenol (Fig. 4a), flavonoid (Fig. 4b), and tannin (Fig. 4c) content, their concentrations were relatively high in hydromethaolic extract compared with aqueous extract (Table 5). It also showed that total phenolic content was relatively higher in both extracts as compared to total flavonoid and tannin content (TPC > TFC > TTC). The quantitative analysis is important due to their potential health benefits as well as for free radical-associated oxidative damages. A previous reports also showed the presence of high phenolic and flavonoid content in *O. sanctum l*eaves (Mondal, 2014). It has also been implied that higher phenolic content is correlated with the relevant antioxidant capacity (Fernandez et al., 2014).

### 3.1.6. Determination of antioxidant activity of *O. sanctum* L. leaves extracts (HME and AQE)

In this study, four different assays were performed to evaluate the free radical scavenging activity of hydromethanolic (HME) and aqueous (AQE) extracts prepared from *O. sanctum* :
i) DPPH scavenging assay
ii) ABTS scavenging assay
iii) Superoxide anion scavenging assay
iv) Nitric oxide scavenging assay
The results are presented in Fig. 5 and Table 6. Both extracts successfully scavenged free radicals in a dose-dependent manner. Ascorbic acid was used as a standard in all assays. The $IC_{50}$ values were:
i) DPPH: 25.014 ± 0.176 µg/mL for HME and 39.102 ± 0.108 µg/mL for AQE
ii) ABTS: 218.946 ± 3.484 µg/mL for HME (not determined for AQE)
iii) Superoxide anion: 449.8 ± 7.267 µg/mL for HME and 752.31 ± 2.101 µg/mL for AQE
iv)Nitric oxide: 2.480 ± 0.012 µg/mL for HME and 4.478 ± 0.009 µg/mL for AQE
The lower $IC_{50}$ values for HME in scavenging DPPH and NO radicals may be attributed to its higher polyphenolic and flavonoid content compared to AQE (Table 2). Quantitative analysis showed that HME contained more phenols, flavonoids, and tannins than AQE. The higher phenolic content likely contributes to HME's more potent free radical scavenging effects (Pourmorad et al., 2006). This result is consistent with the finding of Gulluce et al. (2007), who reported that methanolic extracts are particularly strong free radical scavengers due to their total phenol and flavonoid content (Lam et al., 2018).

### 3.1.6. Cytotoxicity study: MTT assay

Dose-dependent cytotoxicity was observed on the

**Table 3**
Functional groups detected based on FTIR spectra for *Ocimum sanctum* L. leaves extracts (HME and AQE) and phytocomponents (EUG and BCP).

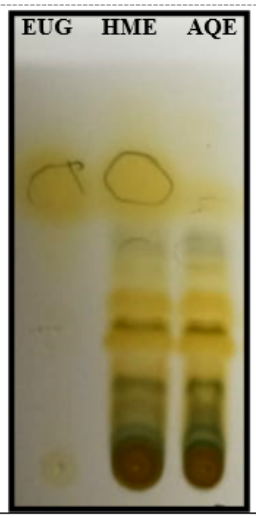
| Absorption frequency ($cm^{-1}$) | Characteristic bond | Functional group | HME | AQE | EUG | BCP |
|---|---|---|---|---|---|---|
| 3750-3200 | O-H stretch | Alcohol | + | + | + | + |
| 1300-1210 | C=O stretch | | | | | |
| 2960-2850 | C-H stretch | Alkanes | + | + | + | + |
| 1300-800 | C-C stretch | | | | | |
| 3100-3000 | C-H stretch | Alkenes | - | + | + | + |
| 1675-1650 | C=C stretch | | | | | |
| 3500-3300 | C-H stretch | Alkynes | - | - | - | - |
| 2150-2100 | C≡C stretch | | | | | |
| 3050-3000 | stretch | Aromatic hydrocarbon | + | + | + | + |
| 1120-1070 | R-O-R stretch | Ethers | + | + | + | + |
| 2940-2850 | C-H stretch | | | | | |
| 1740-1720 | CHO stretch | Aldehyde | - | + | - | - |
| 1725-1700 | C=O stretch | Ketone | - | + | - | - |
| 1715 | COOH stretch | Carboxylic acid | + | + | - | - |
| 1750-1735 | C-O-R stretch | Esters | - | + | - | - |
| 3500-3425 | N-H stretch | Amines | + | + | - | - |
| 3500-3300 | O=C-N-H stretch | Amides | + | + | - | - |
| 500-1000 | R-X | Halides | + | + | - | - |

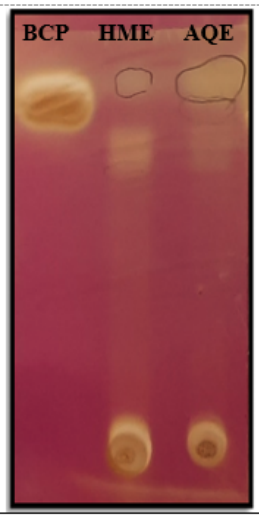
**Table 4**
Phytochemical screening of *Ocimum sanctum* L. leaves extracts (HME and AQE).

| No. | Phytocomponents | HME | AQE |
|---|---|---|---|
| 1 | Carbohydrates | + | + |
| 2 | Protein | + | + |
| 3 | Amino acids | + | + |
| 4 | Steroids | + | + |
| 5 | Triterpenoids | + | + |
| 6 | Flavonoids | + | + |
| 7 | Tannin | + | + |
| 8 | Phenol | + | + |
| 9 | Saponin | - | - |
| 10 | Fatty acids | + | + |
| 11 | Cardio glycosides | - | - |
| 12 | Alkaloids | + | + |

HME: Hydromethanolic extract, AQE: Aqueous extract.

MCF-7 breast cancer cell line after the treatment of various doses of EUG (0.05-0.6 µM) and BCP (0.1 to 1.2 µM) for 24 h in the culture medium (Fig. 6a and Fig. 6b). It was found that the incubation of MCF-7 cells with EUG at a dose of 387.691 ± 5.99 nM resulted in 50% cell viability (Fig. 6a). Whereas 577.856 ± 14.61 nM dose of BCP on MCF-7 showed 50% cell viability (Fig. 6b). Hydromethanolic and aqueous extracts of *O. sactum* leaves at concentrations of 1-20 µg/mL were checked for cytotoxicity on the MCF-7 cell line (Fig. 6c). These results showed that a significant decrease ($p < 0.01$ to $p < 0.001$) in viability was observed for MCF-7 with both




| Figure no. | 3a | 3b |
|---|---|---|
| Compound | EUG | BCP |
| Solvent system | Toluene: Ethyl acetate: Acetic acid (10: 1: 0.32) | Ethyl acetate: *n*-hexane (7: 3) |
| Derivatized with | PMA (propylene glycol monomethyl ether acetate) | KMnO4 |

AQE: Aqueous extract, BCP: β Caryophyllene, EUG: Eugenol, HME: Hydromethanolic extract.

**Fig. 3.** Thin layer chromatography of **a:** Eugenol and **b:** β-Caryophyllene.

the extracts in a dose-dependent manner (5 to 20 µg/mL) as compared with the control group. On MCF-7 cell line, the $IC_{50}$ value of hydromethanolic and aqueous extracts were found to be 23.137 ± 0.965 and 24.963 ± 0.00 µg/mL, respectively, EUG, BCP and both extracts were highly cytotoxic to MCF-7 cells as compared to AQE, the HME showed cell inhibition more effectively and hence, it may have good anticancer property. Vidhya and Devaraj (2011) showed that cells treated with a higher concentration of EUG (1, 2, and 4 µM) exhibited a significant decrease in the number of viable cells. The best cytotoxic activity against MCF-7 cells was obtained for the methanolic extract of *O. sanctum* L. leaves ($IC_{50}$ = 31 mg/mL) (Basak et al., 2014). Moreover, it was observed that HME has a rich source of antioxidant compounds which could be an essential protagonist against excess generation of free radicals which contributes to apoptotic cell death and hence, leads to the death of cancer cells.

### 3.1.6. Molecular docking study

Molecular docking analysis was used to determine the binding affinity of EUG and BCP with two different enzymatic antioxidants (Fig. 7; Table 7). High binding energy showed good binding affinity between the two molecules. Virtual screening for EUG showed the interaction in higher to lower order as catalase (-3.659 kcal/mol) > glutathione reductase (-4.38 kcal/mol). Whereas BCP showed a relatively high binding affinity for catalase (-2.271 kcal/mol) (Table 7). From both the studied compounds (EUG and BCP), the difference of binding energy between positive inhibitor and EUG was lowest for CAT and GR. An *In silico* study revealed that EUG and BCP showed relatively higher binding affinities for catalase than their binding affinities for GR. Catalase plays an important role in maintaining endogenous ROS production and it shows antioxidant and pro-oxidant activities in response to DNA damage (Kang et al., 2013). The binding of EUG with catalase suggests that it could be enhancing its antioxidant activity. These binding affinities and docking scores suggest that both compounds have strong antioxidant activity. With the possession of free radical scavenging activity, if plant-derived bioactives are non-toxic to normal cell lines and show cytotoxicity in cancer cell lines, such compounds can be advanced to clinical trials for further therapeutic development (Greenwell and Rahman, 2015).

## 4. Concluding remarks

This study provides insights into the bioactivities of *O. sanctum* L. leaves extracts (HME and AQE) and their bioactive components, EUG and BCP. Medicinal plants have been used for centuries to treat various illnesses worldwide. Phytochemical screening of *O. sanctum* L. leaves extracts revealed a rich presence of major phytochemicals, including phenols, flavonoids, tannins, terpenoids, and alkaloids. Our findings suggest that the hydromethanolic extract could be a valuable source of natural antioxidants with therapeutic potential, surpassing the aqueous extract. TLC results confirmed the presence of EUG, a phenolic compound, and BCP, a plant-derived sesquiterpene,

in both HME and AQE extracts. Notably, EUG, BCP, and both extracts showed strong cytotoxic properties against human breast cancer cells (MCF-7). Molecular docking studies revealed high binding efficacy of EUG and BCP with enzymatic antioxidants. This study contributes to the understanding of *O. sanctum* L. leaves' medicinal properties, anticancer activity, and interaction with antioxidant enzymes, potentially leading to the development of novel therapeutic interventions and natural antioxidant agents. However, further investigation is needed to prove the synergistic effects of EUG, BCP, and both extracts when used incombination with chemotherapeutic drugs, including *in vitro* free radical toxicity testing and *in vivo* experiments.

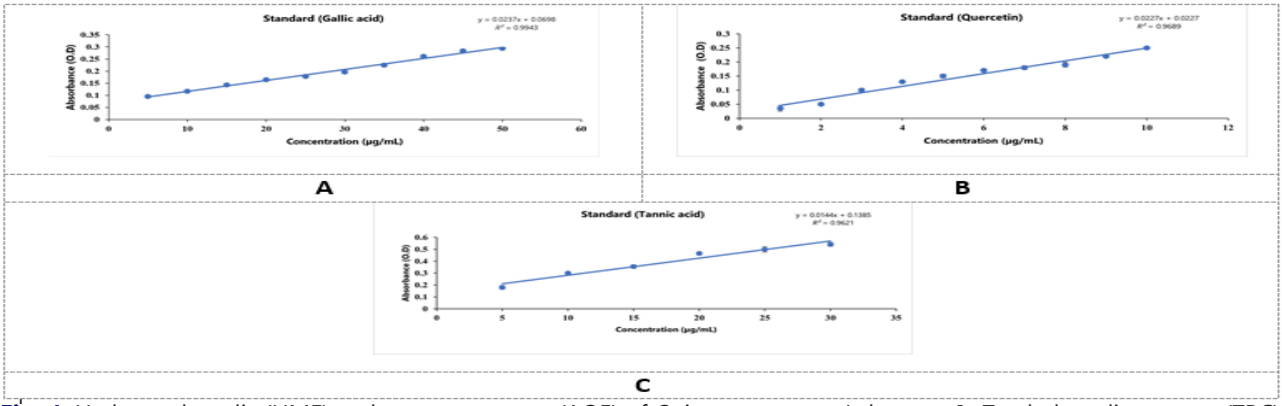



**Fig. 4.** Hydromethanolic (HME) and aqueous extract (AQE) of *Ocimum sanctum* L. leaves: **A:** Total phenolic content (TPC), **B:** Total flavonoid content (TFC), **C:** Total tannin content (Values are expressed as mean ± SD).

**Table 5**
Total phenolic, flavonoid and tannin content in in hydromethanolic (HME) and aqueous extract (AQE) of *Ocimum sanctum* L. leaves.

| Extracts of Ocimum sanctum L. leaves | TPC (µg GAE/ mg extract) | TFC (µg QE/ mg extract) | TTC (µg TAE/ mg extract) |
|---|---|---|---|
| HME | 176.24 | 145.82 | 4.84 |
| AQE | 103.68 | 72.34 | 2.28 |

AQE: Aqueous extract; GAE: Gallic acid equivalent; HME: Hydromethanolic extract; TAE: Tannic acid equivalent; TFC: Total flavonoid content; TPC: Total phenolic content; TTC: Total tannin content; QE: Quercetin equivalent.

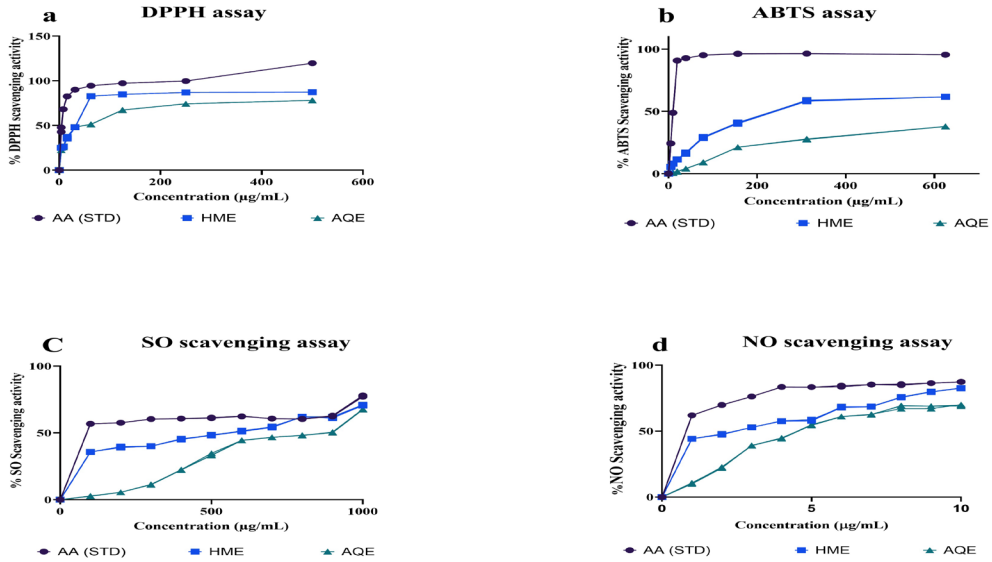



**Fig. 5.** Scavenging of free radicals at various concentrations of ascorbic acid (AA), hydromethanolic extract (HME) and aqueous extract (AQE); Values are expressed as mean ± SD. **a:** DPPH free radical scavenging activity; **b:** ABTS free radical scavenging activity; **c:** Superoxide (SO) free radical scavenging activity; **d:** Nitricoxide (NO) free radical scavenging activity.

**Table 6**
$IC_{50}$ values of AA (STD), HME and AQE for DPPH, ABTS, SO and NO free radical scavenging activity.

| Test compounds | $IC_{50}$ in DPPH free radical scavenging activity (µg/mL) | $IC_{50}$ in ABTS free radical scavenging activity (µg/mL) | $IC_{50}$ in Superoxide (SO) free radical scavenging activity (µg/mL) | $IC_{50}$ in Nitric oxide (NO) free radical scavenging activity (µg/mL) |
|---|---|---|---|---|
| AA (STD) | 8.193 ± 0.287 | 13.764 ± 0.003 | 176.50 ± 0.096 | 1.337 ± 0.002 |
| HME | 25.014 ± 0.176 | 218.946 ± 3.484 | 449.8 ± 7.267 | 2.480 ± 0.012 |
| AQE | 39.102 ± 0.108 | - | 752.31 ± 2.101 | 4.478 ± 0.009 |

Values are expressed as mean ± SD; AA (STD): Ascorbic acid (Standard); HME: Hydromethanolic extract; AQE: Aqueous extract; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

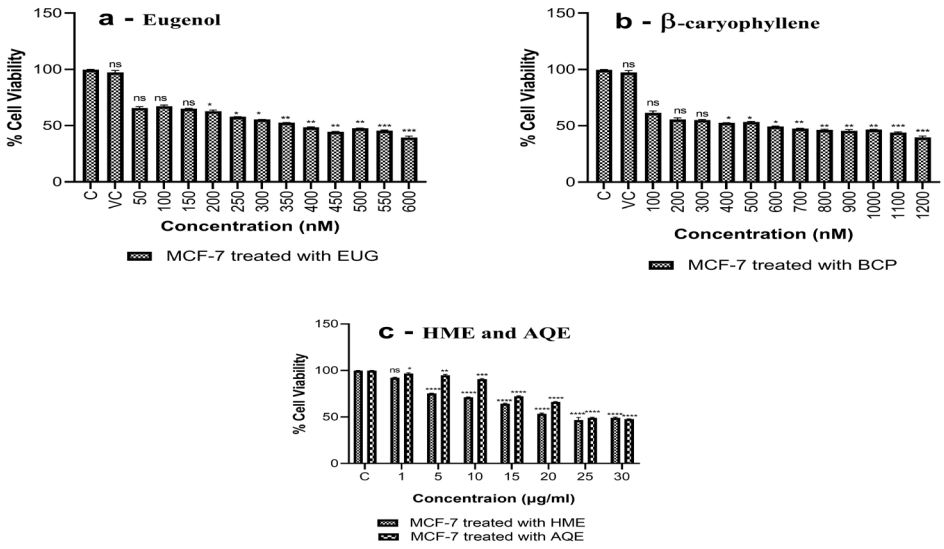



**Fig. 6.** Cytotoxicity study by MTT assay of **a:** Eugenol at different concentrations (50 nM to 600 nM) **b:** β-caryophyllene at different concentrations (100 nM to 1200 nM) **c:** HME and AQE at different concentrations (1 µg/mL to 20 µg/mL) on the MCF-7 cell line. CON: Control, VC: Vehicle Control (DMSO). Values are expressed as mean ± SD; * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, and ns: Non-significant when the different concentrations of eugenol and vehicle control compared with the control group.

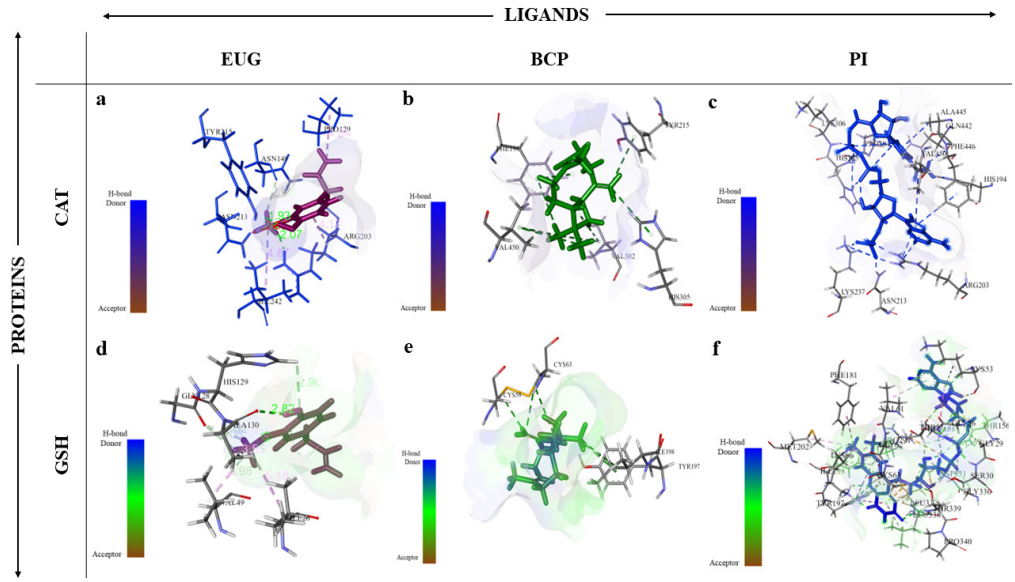



**Fig. 7.** 3D Docked protein- ligand interaction pose based on hydrogen bond acceptor (HBA) and donor (HBD) of Catalase (CAT) protein with **a:** Eugenol (EUG), **b:** β-Caryophyllene (BCP), and **c:** NADPH (PI-Positive Inhibitor); and Glutathione reductase (GR) proteins with **d:** Eugenol (EUG), **e:** β-Caryophyllene (BCP), and **f:** FAD (PI-Positive Inhibitor).

**Table 7**
Binding energy and contacting receptor residues in docking study of eugenol and β-caryophyllene with antioxidant enzymes.

| Sr no. | Macromolecule | Code | Method | Resolution | Binding energy (kcal/mol) | | |
|---|---|---|---|---|---|---|---|
| | | | | | EUG | BCP | PI |
| 1 | Catalase (CAT) | 1DGH | X-RAY DIFFRACTION | 2.00 Å | -3.659 | -2.271 | -7.791 |
| 2 | Glutathione reductase (GR) | 1XAN | X-RAY DIFFRACTION | 2.00 Å | -4.38 | -2.509 | -9.322 |

BCP: β-Caryophyllene, EUG: Eugenol, PI: Positive Inhibitor (NADPH for CAT; FAD for GR).

## List of abbreviations

**ABTS:** 2,2-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid); **AQE:** Aqueous Extract; **BCP:** β-Caryophyllene; **CAT:** Catalase; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **EUG:** Eugenol; **FTIR:** Fourier Transform Infrared Spectroscopy; **GR:** Glutathione Reductase; **HME:** Hydromethanolic Extract; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content; **TTC:** Total Tannin Content.

## Funding

This work was supported by SHODH-ScHeme of Developing High Quality Research, Knowledge Consortium of Gujarat, Education Department, Government of Gujarat with Student Ref No: 201901380008 (KCG/SHODH/2020-21).

## Author contribution statement

Devendrasinh Jhala: Conceptualization, methodology, experimental studies, formal analysis, investigation, resources, manuscript review and editing, funding acquisition; Krupali Trivedi: Conceptualization, software, methodology, experimental studies, data curation, data analysis, writing-original draft preparation, manuscript review and editing, funding acquisition; Nilam Parmar, Khairah Ansari, Nishi Modi and Vaibhavi Srivastava: Experimental studies, formal analysis, manuscript review.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgements

All the authors gratefully acknowledge Department of Botany, School of sciences, Gujarat University, Ahmedabad for authenticating the plant material and acknowledge Department of Chemistry, School of sciences, Gujarat University, Ahmedabad for providing the computational facility for executing molecular docking studies.

## References

Acuña, U.M., Wittwer, J., Ayers, S., Pearce, C.J., Oberlies, N.H., De Blanco, E.J.C., 2012. Effects of (5Z)-7-oxozeaenol on the oxidative pathway of cancer cells. Anticancer Res. 32(7), 2665-2671.

Anandjiwala, S., Kalola, J., Rajani, M., 2006. Quantification of eugenol, luteolin, ursolic acid, and oleanolic acid in black (Krishna Tulasi) and green (Sri Tulasi) varieties of *Ocimum sanctum* Linn. using high-performance thin-layer chromatography. J AOAC Int. 89(6), 1467-1474.

Anita Margret, A., Maheswari, R., Sherlin Rosita, A., Jesucastin, E., 2022. Discerning the regulated wound healing potential of *Ocimum americanum* by probing the rosmarinic acid content-a paradigm on zebrafish caudal fin regeneration. Trends Phytochem. Res. 6(2), 106-115.

Badami, S., Gupta, M.K., Suresh, B., 2003. Antioxidant activity of the ethanolic extract of *Striga orobanchioides*. J. Ethnopharmacol. 85(2), 227-230.

Basak, P., Mallick, P., Mazumder, S., Verma, A.S., 2014. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of Tulsi (*Ocimum sanctum*) leaves. Adv. Pharmacol. Toxicol. 15(1), 19-29.

Bhalla, Y., Gupta, V.K., Jaitak, V., 2013. Anticancer activity of essential oils: A review. J. Sci. Food Agric. 93(15), 3643-3653.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181(4617), 1199-1200.

Brianna, Lee, S.H., 2023. Chemotherapy: How to reduce its adverse effects while maintaining the potency? Med. Oncol. 181(4617), 1199-1200.

de Fernandez, M. de los A., SotoVargas, V.C., Silva, M.F., 2014. Phenolic compounds and antioxidant capacity of monovarietal olive oils produced in Argentina. J. Am. Oil Chem. Soc. 91(12), 2021-2033.

Devendran, G., Balasubramanian, U., 2011. Qualitative phytochemical screening and GC-MS analysis of *Ocimum sanctum* L. leaves. Asian J. Plant Sci. Res. 1(4), 44-48.

Di Giacomo, S., Di Sotto, A., Mazzanti, G., Wink, M., 2017. Chemosensitizing properties of β-caryophyllene and β-caryophyllene oxide in combination with doxorubicin in human cancer cells. Anticancer Res. 37(3), 1191-1196.

Di Giacomo, S., Mazzanti, G., Di Sotto, A., 2016. Mutagenicity of cigarette butt waste in the bacterial reverse mutation assay: The protective effects of β-caryophyllene and β-caryophyllene oxide. Environ. toxicol. 31(11), 1319-1328.

Farzaneh, V., Carvalho, I.S., 2015. A review of the health benefit potentials of herbal plant infusions and their mechanism of actions. Ind. Crops Prod. 65(2015), 247-258.

Fresco, P., Borges, F., Diniz, C., Marques, M.P.M., 2006. New insights on the anticancer properties of dietary polyphenols. Med. Res. Rev. 26(6), 747-766.
Gomathi, D., Ravikumar, G., Kalaiselvi, M., Devaki, K., Uma, C., 2014. Antioxidant activity and functional group analysis of *Evolvulus alsinoides*. Chin. J. Nat. Med. 12(11), 827-832.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal. Biochem. 126(1), 131-138.
Greenwell, M., Rahman, P., 2015. Medicinal plants: Their use in anticancer treatment. Int. J. Pharma. Sci. Res. 6(10), 4103-4112.
Gülçin, İ., 2011. Antioxidant activity of eugenol: A structure-activity relationship study. J. Med. Food. 14(9), 975-985.
Gulluce, M., Sahin, F., Sokmen, M., Ozer, H., Daferera, D., Sokmen, A., Polissiou, M., Adiguzel, A., Ozkan, H., 2007. Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. Food Chem. 103(4), 1449-1456.
Harborne, J.B., 1984. Methods of Plant Analysis, in: Harborne, J.B. (Ed.). Phytochemical Methods. Springer, Dordrecht. pp. 1-36.
Hashemi-Moghaddam, H., Mohammadhosseini, M., Basiri, M., 2015. Optimization of microwave assisted hydrodistillation on chemical compositions of the essential oils from the aerial parts of *Thymus pubescens* and comparison with conventional hydrodistllation. J. Essent. Oil-Bear. Plants 18(4), 884-893.
Hassan, I.A., Nasiru, I.A., Malut, A.M., Ali, A.S., 2015. Phytochemical studies and thin layer chromatography of leaves and flower extracts of *Senna siamea* Lam. for possible biomedical applications. J. Pharmacogn. Phytother. 7(3), 18-26.
Kang, M.Y., Kim, H.-B., Piao, C., Lee, K.H., Hyun, J.W., Chang, I.-Y., You, H.J., 2013. The critical role of catalase in prooxidant and antioxidant function of p53. Cell Death Differ. 20(1), 117-129.
Kaushal, N., Rao, S., Ghanghas, P., Abraham, S., George, T., D'souza, S., Mathew, J.M., Chavali, J., Swamy, M.K., Baliga, M.S., 2018. Usefulness of *Ocimum sanctum* Linn. in Cancer Prevention: An Update, in: Akhtar, M., Swamy, M. (Eds.) Anticancer Plants: Properties and Application. Springer, Singapore. Vol. 1, pp. 415-429.
Kianasab, M.R., Mohammadhosseini, M., Nekoei, M., Mahdavi, B., Baheri, T., 2023. Screening of the compositions of essential oils and volatiles of *Perovskia abrotanoides* Karel. along with antioxidant, antibacterial and cytotoxic impacts of its methanol extract. Nat. Prod. Res., 1-5.
Lalla, J., Hamrapurkar, P., Singh, A., 2007. Quantitative HPTLC analysis of the eugenol content of leaf powder and a capsule formulation of *Ocimum sanctum*. JPC-J. Planar Chromat.-Modern TLC. 20(2), 135-138.
Lam, S.N., Neda, G.D., Rabeta, M.S., 2018. The anticancer effect of *Ocimum tenuiflorum* leaves. Food Res. 2(2), 154-162.
Madike, L.N., Takaidza, S., Pillay, M., 2017. Preliminary phytochemical screening of crude extracts from the leaves, stems, and roots of *Tulbaghia violacea*. Int. J. Pharmacogn. Phytochem. Res. 9(10), 1300-1308.
Mandal, S., Das, D.N., De, K., Ray, K., Roy, G., Chaudhuri, S.B., Sahana, C.C., Chowdhuri, M.K., 1993. *Ocimum sanctum* Linn-A study on gastric ulceration and gastric secretion in rats. Indian J. Physiol. Pharmacol. 37(1993), 91-91.
Mohammadhosseini, M., Frezza, C., Venditti, A., Akbarzadeh, A., 2019. Ethnobotany and phytochemistry of the genus *Eremostachys Bunge*. Curr. Org. Chem. 23, 1828-1842.
Mohammadhosseini, M., Venditti, A., Akbarzadeh, A., 2019. The genus *Perovskia* Kar.: Ethnobotany, chemotaxonomy and phytochemistry: A review. Toxin Rev. 40(4), 484-505.
Mondal, P., 2014. Study of total phenolic and flavonoid content, antioxidant activity and antimicrobial properties of medicinal plants. J. Microbiol. Exp. 1(1), 1-6.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65(1), 55-63.
Omara, T., Kiprop, A.K., Ramkat, R.C., Cherutoi, J., Kagoya, S., Moraa Nyangena, D., Azeze Tebo, T., Nteziyaremye, P., Nyambura Karanja, L., Jepchirchir, A., 2020. Medicinal plants used in traditional management of cancer in Uganda: A review of ethnobotanical surveys, phytochemistry, and anticancer studies. Evid. Based Complementary Altern. Med. 2020(2020), 1-26.
Organisation mondiale de la santé (Ed.), 2018. World health statistics 2018: Monitoring health for the SDGs sustainable development goals. World health organization, Geneva.
Pandey, M.M., Rastogi, S., Rawat, A.K.S., 2013. Indian traditional ayurvedic system of medicine and nutritional supplementation. Evid. Based Complementary Altern. Med. 2013, 1-12.
Pehlivan, F. E, 2017. Vitamin C: An Antioxidant Agent. Vitamin C, 2, pp. 23-35.
Poulsen, H.E., Prieme, H., Loft, S., 1998. Role of oxidative DNA damage in cancer initiation and promotion. Eur. J. Cancer Prev. 7(1), 9-16.
Pourmorad, F., Hosseinimehr, S.J., Shahabimajd, N., 2006. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr. J. Biotechnol. 5(11), 1142-1145.
Prakash, P., Gupta, N., 2005. Therapeutic uses of *Ocimum sanctum* Linn. (Tulsi) with a note on eugenol and its pharmacological actions: A short review. Indian J. Physiol. pharmacol. 49(2), 125-131.
Ramteke, C., Chakrabarti, T., Sarangi, B.K., Pandey, R.-A., 2013. Synthesis of silver nanoparticles from the aqueous extract of leaves of *Ocimum sanctum* for enhanced antibacterial activity. J. Chem. 2013(2013), 1-7.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radic. Biol. Med. 26(9-10), 1231-1237.
Rompelberg, C.J.M., Evertz, S., Bruijntjesrozier, G., Van den Heuvel, P.D., Verhagen, H., 1996. Effect of eugenol on the genotoxicity of established mutagens in the liver. Food chem. Toxicol. 34(1), 33-42.
Sarkar, S., Hoda, M.U., Das, S., 2023. Anti-melanogenic, antioxidant potentialities and metabolome classification of six *Ocimum* species: Metabolomics and *in-silico*

approaches. Trends Phytochem. Res. 7(1), 30-50.
Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., Latha, L.Y., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J. Tradit. Complement. Altern. Med. 8(1), 1-10.
Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16(3), 144-158.
Ullah, A., Munir, S., Badshah, S.L., Khan, N., Ghani, L., Poulson, B.G., Emwas, A.-H., Jaremko, M., 2020. Important flavonoids and their role as a therapeutic agent. Molecules 25(22), 1-39.
Vidhya, N., Devaraj, S.N., 2011. Induction of apoptosis by eugenol in human breast cancer cells. Indian J. Exp. Biol. 49(11), 871-878.
Yen, G.-C., Hsieh, P.-P., 1995. Antioxidative activity and scavenging effects on active oxygen of xylose-lysine maillard reaction products. J. Sci. Food Agric. 67(3), 415-420.
Zheng, G.-Q., Kenney, P.M., Lam, L.K., 1992. Sesquiterpenes from clove (*Eugenia caryophyllata*) as potential anticarcinogenic agents. J. Nat. prod. 55(7), 999-1003.