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Evaluation of Antioxidant, Antibacterial and Cytotoxic Effects of Pink Pepper Fruit Essential Oil

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Abstract: Pink pepper is classified into the Californian/Peruvian type (*Schinus molle*) and the Brazilian type (*S. terebinthifolia*). According to studies, pink pepper extracts and essential oils have shown anti-inflammatory, antioxidant, genotoxic and antidiabetic properties in *in vitro* and *in vivo* studies. The results of bioactivity tests vary depending on the geographical origin of the pepper. In this paper, the cytotoxic, antioxidant and anti-bacterial effects of the essential oil of the commercial pink pepper fruit from the Tuzla market were investigated. To assess the cytotoxic potential, a tetrazolium salt reduction (MTT) viability assay was performed. The antioxidant potential was examined spectrophotometrically, using DPPH and FRAP methods. Diffusion techniques were used to evaluate the antibacterial activity of the essential oil. Using GC/MS, 24 components of red pepper essential oil were identified, of which α-pinene, α-phellandrene, δ-3-carene and D-limonene dominate. The studied pink pepper essential oil inhibited the cell proliferation in the HeLa cell line, causing a dose-dependent cytotoxic effect (IC_{50}=389.46 µg/mL). The essential oil inhibits DPPH radicals. The reducing ability is relatively weak. For the essential oil, an extremely good ability to inhibit the growth of the bacteria used in this study was confirmed. These results indicate a very high potential of essential oil of pink pepper fruit as an inhibitor of pathogenic organisms.

INTRODUCTION

Essential oils (EOs) are natural aromatic products with volatile and lipophilic properties. They can be isolated from leaves, flowers, seeds and other plant parts. As secondary plant metabolites, they are important in the resistance of plants to adverse external conditions such as climatic variations, mechanical damage, insects, etc. (Da Silva Dannenberg et al., 2016; Fonseca et al., 2021). EOs are widely used in the pharmaceutical, cosmetic and other industries, but also proved to be promising for use in the food industry. The use and studies of EOs are based on their diverse medicinal properties, primarily antimicrobial, anti-inflammatory and antioxidant.

Several studies have shown adequate antimicrobial function of EOs (Fonseca et al., 2021; Locali-Pereira et al., 2022). The medicinal properties of EOs are attributed to biologically active substances. EOs can induce pore formation in bacterial cell walls, resulting in disruption of bacterial cell walls and changes in permeability, thereby allowing the release of cellular components. Other modes of action include a decrease in intracellular pH and changes in the intracellular concentration of adenosine triphosphate (ATP) (Da Silva Dannenberg et al., 2016).

The biological function of these molecules in plants indicates that there is a possibility of applying EOs in different systems, with the aim of achieving effects similar to those that the compounds have in the plant (Dannenberg et al., 2019). Essential oils act as natural preservatives, as they have shown positive inhibitory results against some microorganisms in *in vitro* assays (Kavoosi and Rowshan, 2013). The antimicrobial activity of an EO can be affected by several factors, causing changes to occur. Some of them include the type and strain of microorganisms used, the chemical composition of the oil, the concentration of specific molecules, the climatic and soil conditions in which the plant was grown, etc (Fonseca et al., 2021).

The pink pepper (PP), *Schinus terebinthifolius* Raddi is a plant native to Brazil, Paraguay, Argentina. This species belongs to the Anacardiaceae family (Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021; Locali-Pereira et al., 2022; Merlo et al., 2019; Silva et al., 2017). It is used as a sophisticated condiment in the international cuisine. Recent research has shown that pink pepper extracts are an interesting alternative as natural food preservatives and aromatic spices (Almeida et al., 2022; Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021). The pharmaceutical industry uses PP essential oil (PPEO) in cosmetics for its aroma, with the advantage of being classified as GRAS (generally recognized as safe) (Ghabraie et al., 2016; Uliana et al., 2009). However, to ensure safety for effective commercial use in the food industry, detailed toxicological testing is required. Thanks to its medicinal, cosmetic, and pharmaceutical properties, pink pepper has great commercial potential. The astringent, antidiarrheal, depurative, diuretic and febrifugal properties of this species are attributed to various chemical compounds (Bittencourt Fagundes et al., 2020; Figueiredo et al., 2021; Fonseca et al., 2021; Locali-Pereira et al., 2022; Merlo et al., 2019).

Several bioactive compounds have been extracted from these plants, many of which are the main compounds of PP essential oil (PPEO). PPEO can be isolated by different extraction methods, but hydrodistillation is widely used due to its simplicity (Almeida et al., 2022; Magalhaes et al., 2021). The most commonly used plant parts for PPEO isolation are green and ripe fruits, seeds and leaves. Some studies have shown that the PP peel also has a high bioactive potential (Soares Carneiro et al., 2022). The reddish fruit is small and round and has sparked the interest of researchers due to the properties of its essential oil. The results of previous studies indicate antimicrobial, antifungal and antitumor effects. Antimicrobial activity has been studied against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens that can cause outbreaks of foodborne diseases (Da Silva Dannenberg et al., 2016; Dannenberg et al., 2017, 2019; Fonseca et al., 2021).

Studies on the PPEO are limited, but various chemical compounds, such as terpenes, alcohols, ketones, ascorbic acid, phenols, flavonoids and carotenoids are present in the fruit, stem and leaves. Antioxidant, antibacterial, antitumor and anti-inflammatory activity can be related to the content of flavonoids, anthocyanins and carotenoids. Antibacterial activity is mainly attributed to terpenes, terpenoids and phenylpropenes. Regarding the cytotoxic activities on tumor cells, some studies have shown that the essential oil collected from the fruits of PP is more effective than the oil from the leaves. The presence of α-pinene is associated with apoptosis of cancer cells in melanoma therapy, while germacrene D from PP has a potential effect against prostate and ovarian cancer (Figueiredo et al., 2021; Merlo et al., 2019; Soares Carneiro et al., 2022).

MATERIAL AND METHODS

The pepper sample was obtained commercially. Pink pepper originates from Vietnam. The sample was crushed using an electric mill and kept in a dark and dry place until distillation. Double-distilled deionized water or culture medium were used for solution preparations and dilutions. Thiazolyl Blue Tetrazolium Bromide (MTT) cell viability reagent and methanol used for the DPPH radical neutralization assay were obtained from Sigma-Aldrich. 2,4,6-Tripyridyl-s-triazine, iron(III) chloride, hydrochloric acid, sodium acetate (for preparation of FRAP reagent) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA). Spectroscopic measurements were performed on a Perkin Elmer Lambda 25 spectrophotometer. The HeLa (Sigma-Aldrich) cell line was cultured in Minimum Essential Medium Eagle medium (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma Aldrich), 1% non-essential Amino Acids (Sigma- Aldrich), 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin antibiotics (Sigma-Aldrich).

Hydrodistillation

The crushed pink pepper fruit was subjected to hydrodistillation for four hours on a Clevenger apparatus. The obtained essential oil was separated, dried on anhydrous sodium sulfate and stored at -20°C until analysis.

GC/MS analysis

The essential oil of the red pepper fruit was analyzed by the gas chromatography with mass detector (GC-MSD) technique. This method was used to determine the composition of the EO, in which individual peaks of the chemical components within the obtained chromatogram were identified by comparing their retention indices with the indices of the compounds in the databases, and by matching the mass spectrum of the compounds in the sample with the mass spectrum in the databases. GC/MS analysis of the essential oil sample of pink pepper fruit was performed at Agilent Technologies, Inc. gas chromatograph (7820A) with capillary HP5-ms ultra-inert column (-60 to 325°C, 30m × 250µm, film thickness 0.25µm). The gas chromatograph was equipped with an Agilent mass selective detector (MSD-5977E). Helium gas (purity 5.0) was used as carrier gas at a constant flow rate of 1.0 mL/min. The sample was injected in a volume of 1 µL. The oven temperature was programmed from 60°C (hold 1 min) to 246 C (hold 0 min) at a rate of 3°C/min and then to 280°C at a rate of 10°C/min., Three washes of the needle with solvent (*n*-hexane) were used before and after each injection. The program resulted in a total duration of 86.40 minutes. The mass detector (MSD)

was operated in the 40-400 m/z range scan mode. The MSD transfer line temperature was 250°C, and the ion source temperature was 230°C. ChemStation software was used for instrument control and data analysis. The results are expressed as a percentage concentration (% (V/V)) of each component in relation to the entire area of the obtained chromatogram.

Examination of the reducing ability (FRAP method)
The test of the reducing ability of the pepper oil was tested using the FRAP (ferric reducing antioxidant power) method, according to the published procedure (Benzie and Strain, 1999). 3 mL of the prepared FRAP reagent (a mixture of acetate buffer, iron(III) chloride hexahydrate and TPTZ reagent in a volume ratio of 10:1:1) was mixed with 0.1 mL of EO. Absorbance at 593 nm was recorded after 30 min of incubation at 37°C.

Inhibition of DPPH radicals
The DPPH radical inhibition assay was performed according to the published method (Horozić et al., 2019). Pink pepper fruit oil was mixed with absolute methanol and then mixed with a DPPH radical solution. Absorbance measurements were performed at 517 nm, after which DPPH radical inhibition was calculated according to the equation:

$$I = Ac - As / Ac \times 100 \ [\%]$$

where As is the absorbance of the solution containing the sample at 517 nm, and Ac is the absorbance of the DPPH solution.Results are expressed as IC_{50} value. Vitamin C was used as a positive control.

In vitro culture of the cell lines
Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. For each experiment, cells were grown to % confluence in cell culture flasks.

Analysis of Cell cytotoxicity by MTT
The cytotoxic effects of pink pepper fruit oil were assessed using the MTT assay. For each experiment, cells were seeded ($2x10^4$ cells/well) in 96 well plates and incubated overnight. The following day, cells were treated with increasing final concentrations of essential oil (80-800 µg/mL) and incubated for an additional 48 hours. After incubation, 10% MTT solution 5 mg/mL was added to each well, and the plates were incubated for another 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, resulting MTT-formazone crystals were dissolved by adding 200 µl of DMSO to each well with continuous shaking for 15 minutes. The absorbance was read using a microplate reader (Tecan, Sunrise) at a wavelength of 570 nm. The concentration of the essential oil leading to 50% inhibition of viability (IC_{50}) was assessed from the dose response curve plot. The experiment was repeated twice and each experiment was performed in triplicate. Untreated cells were used as a negative control, and cells treated with 30% DMSO in the culture medium were used as a positive control. The prepared stock solutions of essential oil were sterilized by filtration through 0.2 µm sterile syringe filters.

In vitro antibacterial activity
The antibacterial activity was investigated by the diffusion method on reference strains *E. faecalis* (ATCC 51299), *S. aureus* (ATCC 25923), *L. monocytogenes*(ATCC 19118), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. enterica* (ATCC 13076) and *B. subtilis* (ATCC 6633). Sterile drill-shaped holes (diameter 6 mm) were made in the agar into which 50 and 100 µL of EO were added. The plates were incubated at 37°C for 24 hours. After the incubation period, the size of the inhibitory zone was measured.

RESULTS AND DISCUSSION

Chemical composition
Essential oil was isolated from red pepper by hydrodistillation with a yield of 1.77%. The chemical composition of the essential oil obtained from the pink pepper is presented in Table 1. The identification of 24 components by GC/MS enabled for 97.1% of the total pink pepper fruit oil to be identified. Monoterpenes constitute 80.1% of the essential oil, while identified sesquiterpenes correspond to 17.1%. These percentages are close to the values reported for mono and sesquiterpenes fractions by other authors (Locali-Pereira et al., 2020; Dannenberg et al., 2019). The most abundant components were four monoterpenes δ-3-carene (22.0%), D-limonene (16.5%), α-phellandrene (16.1%) and α-pinene (13.4%) followed by two sesquiterpenes germacrene D (4.9%) and caryophyllene (4.1%). These components were also identified by other authors, but with different ratios. The results of the essential oil profile revealed the dominance of β-myrcene (Dannenberg et al., 2019), α-pinene (Cavalcanti et al., 2015) or α-phelandrene (Danila et al., 2019). The differences compared to data reported in the literature for pink pepper fruit oil can be attributed to location, climatic conditions, genetic variability (chemotype), pre-treatment of the raw material and extraction process.

Antioxidant activity
The results of the analysis of polyphenol content and antioxidant activity in *in vitro* conditions are shown in Table 2. Red pepper fruit oil showed effectiveness in inhibiting DPPH radicals, with an IC_{50} value of 41 mg/mL. The EO showed extremely weak reducing ability. Vitamin C was used as a control, which was found to have a significantly higher reducing capacity, as well as a higher efficiency in neutralizing DPPH radicals.The mechanism of the antioxidant effect is associated with the presence of oxygen-containing groups. The antioxidant capacity also depends on the presence of an aromatic core in the structure of the compounds rich in essential oil, whereby the free radical can be neutralized by direct reduction by electron transfer or radical quenching by hydrogen atom transfer.
Using the FRAP method, an extremely low conversion efficiency of Fe(III) ions into Fe(II) ions using red pepper essential oil was determined. The essential oil showed low reducing ability compared to the positive control.

Cytotoxic Activity

To evaluate the cytotoxic effect of pink pepper fruit oil, HeLa cells were treated for 24 hours with increasing concentrations of the essential oil (80-800 µg/mL). The obtained results indicate a dose-dependent cytotoxic effect at all tested concentrations (Figure 1).

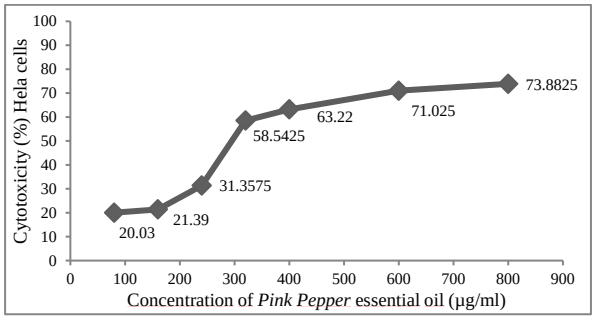

Figure 1: Cytotoxic effects on the HeLa cancer cell line, assessed by MTT after 24 hours exposure to increasing concentrations of pink pepper fruit essential oil

The highest cytotoxic effect was observed in the sample treated with 800 µg/mL essential oil, where 73.88% cytotoxicity was measured. The lowest cytotoxic effect of 20.03% was measured in cells treated with 80 µg/mL essential oil. The calculated IC_{50} value is 389.46 µg/mL. The obtained results are in accordance with previously published results in which the cytotoxic activity of the essential oil extracted from the leaves and fruits of the pink pepper on lung, breast and melanoma cancer cells was determined (Magalhaes et al., 2021). Anti-proliferative effects of *Schinus mole* L. extracts rich in sesquiterpene hydrocarbons and elemol were confirmed on neuroblastoma SH-SY5Y cells and leukemia HL60 cells (Ovidi et al., 2021).

Table 1: Chemical composition of pink pepper fruit essential oil

Test parameter / Component	Retention index	Retention time	Result v/v (%)
α-thujene	929	5.639	0.3
α-pinene	937	5.842	13.4
sabinene	974	6.971	1.6
β-pinene	979	7.077	2.6
β-myrcene	991	7.500	3.0
α-phellandrene	1005	7.973	16.1
δ-3-carene	1011	8.176	22.0
o-cymene	1022	8.650	2.9
D-limonene	1030	8.819	16.5
α-terpinolene	1088	11.043	1.4
5-isopropenyl-2-methyl-7-oxabicyclo[4.1.0]heptan-2-ol	1169	17.915	0.3
δ-elemene	1338	21.577	0.7
β-elemene	1391	23.877	0.3
caryophyllene	1419	24.968	4.0
trans-α-bergamotene	1435	25.658	0.2
humulene	1454	26.351	0.4
germacrene D	1481	27.484	4.9
bicyclogermacrene	1495	28.098	0.4
aciphyllene	1499	28.212	0.3
δ-cadinene	1524	29.184	0.7
elemol	1549	30.186	3.5
germacrene B	1557	30.444	0.2
caryophyllene oxide	1581	31.442	0.3
rosifoliol	1600	32.432	0.3

Table 2: Antioxidant activity of pink pepper fruit essential oil

Sample	FRAP [µmol/g of EO]	DPPH IC_{50} value [mg/mL]
Pink pepper fruit essential oil	0.99	41.2
Vitamin C	14 250	0.03

Antibacterial activity

The results of the antibacterial screening of pink pepper fruit EO for volumes of 50 and 100 µL are presented in Table 3. The EO showed significant antibacterial potential against gram positive and gram negative bacteria. The zones of inhibition are in the range of 12-17 mm for a volume of EO of 50 uL, or 16-23 mm for 100 uL of EO.

Table 3: Results of the antibacterial effect of pink pepper fruit EO

Bacterial strain	Inhibition zone [mm]	
	50 µL	100 µL
E. faecalis	13	16
L. monocytogenes	17	23
S. aureus	14	18
B. subtilis	15	20
E. coli	13	16
P. aeruginosa	-	-
S. enterica	12	17

The complete absence of antibacterial activity was recorded in *P. aeruginosa*. The highest efficiency of bacterial growth inhibition was recorded with *L. monocytogenes*, *B. subtilis* and *S. aureus*. In general, better inhibition results were reported for Gram positive bacteria. The better efficiency of EO in inhibiting the growth of Gram-positive bacteria can be related to the hydrophobicity of EO, whereby it easily penetrates into bacterial cells, leading to lysis and cell death (Sikkema et al., 1994; Kumar Patra and Baek, 2016). Ciprofloxacin at a concentration of 0.5 mg/mL, which was used as a control, showed a higher efficiency of inhibiting the growth of bacterial strains, with inhibition zones greater than 20 mm.

CONCLUSIONS

Commercial essential oil of pink pepper fruit, bought in a market in Tuzla, shows a cytotoxic effect on the HeLa cell line. The antibacterial potential of the oil is extremely high, and the mechanism of the inhibitory effect is connected to its hydrophobicity, which enables it to bind to cell membranes, which leads to disruption of cell integrity and cell death. The antioxidant potential of EO of pink pepper fruit is extremely weak compared to the results of antioxidant capacity obtained for vitamin C. The mentioned *in vitro* studies need to be further expanded in order to gain a better insight into the biological action of this essential oil.

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Summary/Sažetak

Ružičasti biber se svrstava u kalifornijski/peruanski tip (*Schinus molle*) i brazilski tip (*S. terebinthifolia*). Prema studijama, ekstrakti i eterična ulja ružičastog bibera pokazali su protuupalna, antioksidativna, genotoksična i antidijabetička svojstva u *in vitro* i *in vivo* studijama. Rezultati testova bioaktivnosti variraju u zavisnosti od geografskog porijekla ružičastog bibera. U ovom radu istraženo je citotoksično, antioksidativno i antibakterijsko djelovanje ploda eteričnog ulja komercijalnog ružičastog bibera sa tuzlanskog tržišta. Da bi se procijenio citotoksični potencijal, korišten je MTT test. Antioksidativni potencijal ispitan je spektrofotometrijski, DPPH i FRAP metodom. Za procjenu antibakterijske aktivnosti eteričnog ulja korištena je difuziona tehnika. Pomoću GC/MS identifikovane su 24 komponente eteričnog ulja crvenog bibera, od kojih dominiraju α-pinen, α-felandren, δ-3-karen i D-limonen. Proučavano eterično ulje ploda ružičastog bibera inhibira ćelijsku proliferaciju u HeLa ćelijskoj liniji, uzrokujući citotoksični efekat ovisan o dozi (IC_{50}=389,46 μg/mL). Eterično ulje inhibira DPPH radikale. Sposobnost redukcije Fe(III) iona je također visoka. Za eterično ulje potvrđena je izuzetno dobra sposobnost inhibicije rasta bakterija korištenih u ovoj studiji. Ovi rezultati ukazuju na vrlo visok potencijal eteričnog ulja ploda ružičastog bibera kao inhibitora patogenih organizama.