

Structural Elucidation, Antioxidant, Antibacterial, and Anticancer Activities of *Carduus pycnocephalus* L Active Constituents

Luay El Hayyany¹, El-Sayed El-Halawany¹, Hoda Soliman¹, and Yasser El-Amier^{1,*}

Department of Botany, Faculty of Science, Mansoura University, Mansoura 35516, Egypt,

* Correspondence: yasran@mans.edu.eg; +201017229120

Abstract

The current research aims to identify the chemical constituents of the essential oils of the extracted *Carduus pycnocephalus* by Gas Chromatography-Mass Spectroscopic analysis (GC-MS) and to assess the biological profiles of the plant and its botanical ingredients as a precise antioxidant, and anticancer, as well as, antimicrobial agents. The extraction process of the medicinal plant by methanol provided a possibility to extract and identify the polar chemical constituents that have the most effective categories of components. The DPPH antioxidant potency of the flower extract is the most potent with $IC_{50} = 30.69 \text{ mg L}^{-1}$ followed by leaves ($IC_{50} = 32.78 \text{ mg L}^{-1}$), stem ($IC_{50} = 41.31 \text{ mg L}^{-1}$), and root ($IC_{50} = 46.84 \text{ mg L}^{-1}$). Leaf, and flower extracts revealed the most potent activities than the antibiotic standards against *E. coli*, *S. typhimurium*, and *B. cereus* species with inhibition zones ranged from 20-26 mm. Also, the extracted *C. pycnocephalus* revealed a moderate cytotoxic effect against hepatocellular carcinoma (HepG2) tumor cell line using MTT assay with $IC_{50} = 46.2 \text{ } \mu\text{g mL}^{-1}$. The experimental interpretations inveterate the potential of *C. pycnocephalus* extract indicated its biological impacts as antioxidant, antibacterial, and moderate cytotoxic agents that provided the ease of using it in cancer therapy.

Keywords: *Carduus pycnocephalus*; GC/MS chromatography; antioxidant; cytotoxic, antibacterial activities.

1. Introduction

People's needs for food, nutrition, and medicines are increasing as the world's population expands. In this context, researchers and specialists from all over the world are interested in using natural eco-friendly materials derived from plants (Hafaz et al., 2022; Abd-ElGawad et al., 2020). The Asteraceae (sunflower family) is one of the largest flowering plant families, with a global distribution. Although the family includes a substantial number of shrubs, vines, and trees, the majority of the members are herbaceous. Asteraceae is extensively represented in Egypt's flora, with 98 genera and 228 species (Boulos

2009). The therapeutic capabilities of plants in this family have been widely employed in the past and are being used now. Many biological actions have been described for members of this family, including antibacterial, antifungal, antihelminthic, antitussive, antidiabetic, immunostimulatory, immunosuppressive, and cytotoxic properties (Abd-ElGawad et al., 2020; Elshamy et al., 2019; Cazella et al., 2019; Salazar et al., 2018; Sharma et al., 2018).

The *Carduus* genus is related to Astraceae family, which included roughly 100 species worldwide according to Chaudhary (2000). that are extensively distributed from place to place in the Mediterranean. Only three species of *Carduus* genus were recorded in the flora of Egypt, *C. pycnocephalus*, *C. getulus* and *C. argentatus*. The latter is a spiny annual herb growing wildly in the coastal desert of Egypt. The stem usually branches and has spiny wings, the plant reaches up to 60 cm with the taproot, which gives greyish-tomentose on the lower surface, with spinose lobes, and its flowering time extends from March to May (Boulos, 1983). Flu, stomach ache, and rheumatism of such human diseases were treated by *Carduus* genus in Chinese folk medicine (Esmaeili et al., 2005). In addition, the genus of *Carduus* revealed many biological characteristics, for example, antibacterial, antiviral, anticancer, antispasmodic, anti-inflammatory, and liver tonicity activities (Al-Shammari et al., 2015; Orhan et al., 2009; Esmaeili et al., 2005; Jordon-Thaden and Louda, 2003). *Rhinocyllus conicus* (Coleoptera: Curculionidae), as a type of *Carduus* species (Asteraceae) that grown in Argentina, was applied as a control for thistles (de Briano et al., 2013). Many types of research since antiquity and in recent times have focused on the study of *Carduus* species such as the study of the reproductive system (Olivieri et al., 1983). ecology (Doing et al., 1969). the accumulation of heavy metals in plants growing in contaminated soils (Brunetti et al., 2009). recovery of the colonization of the rootstock of mycorrhizal fungi (Jaunatre et al., 2016). and comparative studies electrophoresis (Olivieri, 1985).

The phytochemical analysis of several types of plants related to this *Carduus* genus indicated the characterization of diverse classes of chemical constitutes such as flavonoids, and coumarin (Jordon-Thaden and Louda, 2003). as well as earlier reports, reported the identification of other classes of secondary metabolites such as lignans, alkaloids, sterols, and triterpenes. In this due course, *C. pycnocephalus* was well-known in the literature as an anti-inflammatory, antispasmodic, and hypotensive agent, in which the isolated components from the different parts of this plant were characterized by spectroscopic analyses (Al-Shammari et al., 2015). The literature survey in earlier reports has

also identified the isolation of many other classes from *C. pycnocephalus* such as essential oils (Esmaeili et al., 2005). sterols, and triterpenes (Al-Shammari et al., 2015; Gallo and Sarachine 2009). have characterized ten compounds from the aerial parts of this plant that were related to flavonoid class, and anthraquinone, anthraquinone-linked carbohydrate, steroids, and steroids-linked carbohydrate derivatives as subclasses including kaempferol-7-methoxy-3-O- α -L-rhamnoside, diosmetin-7-O- β -D-xylosyl- β -D-glucopyranoside, diosmetin-7-O- α -L-arabinosyl- β -D-glucopyranoside, and kaempferol-3-O- α -L-rhamnosyl- α -L-rhamnoside along with the structures that were depicted in Figure 1.

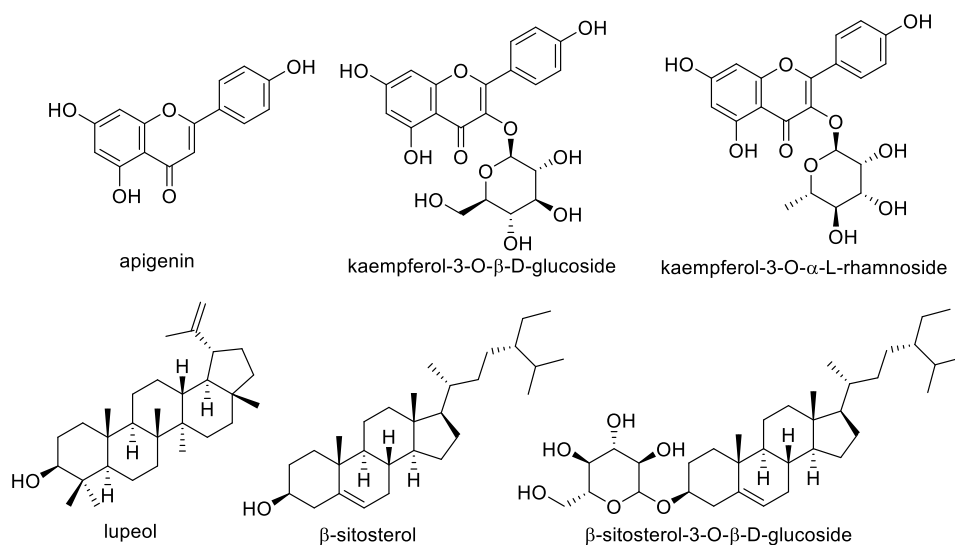


Figure 1. The effective chemical constituents that were isolated from *C. pycnocephalus*.

Esmaeili et al. (2005) have reported the analysis of the essential oils isolated from *C. pycnocephalus* that were grown in Iran by Gas Chromatography-Mass Spectroscopic analysis (GC-MS) with the identification of twenty-nine components demonstrating hexadecanoic acid (23.3%) as the main constituent. Marengo et al. (2017) have reported the characterization of four *Carduus* species, namely (*C. argyrea* Biv., *C. nutans* subsp. *macrocephalus* (Desf.) Nyman, *C. pycnocephalus*, *C. cephalanthus* Viv) that grown in the region of the Mediterranean by phytochemical and biomolecular analyses. The current research aimed to investigate the chemical constituents of the essential oils of the extracted *C. pycnocephalus* by Gas Chromatography-Mass Spectroscopic analysis, and to utilize the extracted sample to explore its biological aptitude as cytotoxic, and antimicrobial potency, as well as, the antioxidant characters of the extracted plant parts.

2. Materials and Methods

2.1. Plant material and extraction process

The plant materials of *C. pycnocephalus* were collected from the sandy habitat near Gamasa City, northern Mediterranean coast, Egypt (31°30'22.24"N 31°22'4.70"E). The plant materials were cleaned and divided into small pieces. A weighed 20 gm of the previously prepared plant materials were placed in a conical flask (250 mL) contained 150 mL methanol and extracted by a shaker after two hours at room temperature. The extract was concentrated to a fixed volume after the complete extraction process. The produced methanol extract was filtered using qualitative Whatman filter paper no. 1 (125 mm, Cat No 1001 125, Germany) and stored at 4 °C (Abd-ElGawad et al., 2020; Souza et al., 2018).

2.2. Gas chromatography-mass spectroscopy (GC-MS) analysis

The chemical constituents of the plant were characterized by implementing the plant extract on Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG-5MS (30 m x 0.25 mm x 0.25 µm film thickness). The temperature of the column oven was firstly held at 50 °C, raised subsequently by a rate of 5 °C per minute to reach 250 °C, and hold for 2 min, and then the temperature was raised to the final temperature (300 °C) by 30 °C per minute and hold for 2 min. the injector and MS transfer line temperature were kept at 270, and 260 °C, respectively. Helium (He) was used as a carrier inert gas at a constant flow rate of 1 mL/min. The solvent was released after 4 min and the diluted samples of 1 µL were injected directly using Autosampler AS1300 coupled with GC in the split mode. EI mass spectroscopy was collected at 70 EV ionization voltage over a range of 50-500 for m/z in packed scan mode. The temperature of the ion source was fixed at 200 °C. The chemical components of the individual extracted plant materials were interpreted by a comparison of their mass spectral data with those of WILEY 09, and NIST 14 mass spectroscopic database.

2.3. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH[•]), ascorbic acid, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), RPMI-1640 medium, and DMSO were purchased from Sigma Aldrich (St. Louis, USA). Fetal bovine serum (FBS; Gibco Life Technologies, Paisley, UK). Cephradine, Tetracycline, Azithromycin, and Ampicillin "antibiotics" were purchased from Merck (Darmstadt, Germany). Nutrient Agar (Contained

Peptone, HM Peptone B #, Yeast extract, Sodium chloride, Agar, and Beef extract) were purchased from Himedia Laboratories Pvt. Ltd. LBS Marg, Mumbai-400086, India.

2.4. Instruments

The horizontal water bath shaker (Memmert WB14, Schwabach, Germany), for the plant extraction process. GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA), and UV/Vis spectrophotometer apparatus for colorimetric measurements (Spekol 11 spectrophotometer, analytic Jena AG, Jena, Germany). For antimicrobial tests: Laminar (HPH12, 1/PE AC, 50 Hz, 1P20, SN. 40328380) (Heraeus, Kendro, Langenselbold, Germany), and Incubator (Type: FRIOCELL 111, Medcenter Einrichtungen GmbH, D-82166, Grafelfing, Germany).

2.5. Potential Biological Characteristics

2.5.1. Antioxidant DPPH assay

The extracts of root, stem, leaves, and flower of *C. pycnocephalus* plant were in vitro screened as antioxidant agents by a colorimetric DPPH free radical assay. The method depended on measuring the intensity in the violet color of the DPPH solution. The assay was applied exactly as applied by the procedure reported by Kitts et al. (Kitts et al., 2000). In this course, different concentrations of each sample were prepared in serial dilutions (5-50 mg L⁻¹) in six testing tubes by mixing with methanol. 0.135 mM of DPPH[•] solution was prepared and subsequently add to each tube of the serial dilution of the investigated samples of the plant species. The tubes were next incubated in dark at room temperature for approximately 30 min. The intensity in the violet color was measured on Spectrophotometric apparatus at a maximum wavelength of 517 nm. The linear regression analysis was applied to calculate the inhibitive concentrations of each tested sample by plotting the exponential curve (Parejo et al., 2000). expressing the concentrations of the investigated samples against the percent of the remaining DPPH[•] radical. The percent of inhibitions were calculated by applying the equation:

$$\% \text{ Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

(A control) expressed the absorbance of the control sample.

(A sample) expressed the absorbance of each sample concentration.

2.5.2. Procedure of Anticancer Activity

2.5.2.1. Tumor cell lines

HePG-2 (Hepatocellular carcinoma) was selected as a human tumor cell line was purchased from ATCC via a holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

2.5.2.2. Preparation of MTT solution

The MTT solution was prepared by mixing a solution of MTT in water (10 mg mL^{-1}), ethanol (20 mg mL^{-1}), and buffered salt solutions and media (5 mg mL^{-1}). The mixture was mixed by vortex or sonication, then filtered, and stored at -20°C .

2.5.2.3. Protocol of MTT assay (Zhu et al., 2017)

The cytotoxic activity of the investigated sample *C. pycnocephalus* was evaluated by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay utilizing an altered process termed by Terblanche et al. (Terblanche et al., 2017). For the determination of the IC_{50} of each particular drug, the adherent HepG2 cells were seeded onto 96-well plates at an initial density (3×10^3 cells/well suspended in $100 \mu\text{L}$ of complete medium). The crude plant extracts were prepared and used to stimulate the cells using five concentrations (31.3 , 62.5 , 125 , 500 , and $1000 \mu\text{g mL}^{-1}$) in culture media. The experimental techniques were performed in duplicates, then plates were incubated for 24 hours in a $5\% \text{ CO}_2$ at 37°C for settle down and adherence. Next, a serial dilution of each prepared concentration of the drug was applied onto the cells for 48 hours after adherence. The medium was removed by aspiration and a weighed MTT (0.5 mg mL^{-1}) was dissolved in a culture fresh medium and practically applied onto cells and the plates were incubated at 37°C and $5\% \text{ CO}_2$ for 4 hours. Ultimately SDS ($100 \mu\text{L}$) was added into each well. The Reduction at cell growth was measured at ($\lambda_{\text{max}} = 570 \text{ nm}$) (BioTek, Elx800, US) and the results were expressed as a percentage of control. The IC_{50} values of the drug, articulated the concentration that affects roughly 50% death of tumor cells, were estimated through a straight linear regression, type sigmoidal, analyzed using Origin 8.0@ software (OriginLab Corporation). The IC_{50} values were calculated by the fit line i.e. $Y = a \cdot X + b$, in which $\text{IC}_{50} = (0.57 - b)/a$. The percentage of inhibition in cell growth was calculated from the following equation, in which A is the absorbance of each control, and the tested sample:

$$\% \text{ Inhibition} = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \times 100$$

The relative cell viability percentage was subsequently calculated from the following equation, in which A expressed the control, and sample absorbance at $\lambda_{\text{max.}} = 570 \text{ nm}$.

$$\% \text{ Cell viability} = \frac{A \text{ treated samples} - A \text{ blank}}{A \text{ control} - A \text{ blank}} \times 100$$

2.5.3. Assessment of the antibacterial activity

2.5.3.1. Microbial Tendency

The antimicrobial aptitudes of *C. pycnocephalus* extract were estimated using the agar well diffusion assay (Valgas et al., 2007; Magaldi et al., 2004). Cephradine, Tetracycline, Azithromycin, and Ampicillin were used as standard antibiotics for comparison scales.

2.5.3.2. Bacterial species

The microbial species were purchased from the Cairo Microbiological Resources Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University. Gram-negative bacteria: *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 25566), *Staphylococcus epidermidis* (ATCC 12228). Gram-positive bacteria: *Bacillus cereus* (EMCC number 1080), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus haemolyticus* (ATCC 29970), *Staphylococcus xylosus* (NCCP 10937), and *Klebsella pneumoniae* (ATCC 10031).

2.5.3.3. Preparation of the culture media

28.0 grams of the nutrient agar were placed in a 2 L conical flask and suspended in 1 L distilled water. The conical flask contents were heated to boiling point to a completely dissolved medium. The mixture was sterilized by autoclave at 15 lbs pressure, 121 °C for 15 minutes. The medium was left to cool to 45-50 °C. The medium can be enriched with 5-10% blood or biological fluids. The mixture was well-shaken and poured sterile Petri plates.

2.5.3.4. Microbial Susceptibility Testing

The antimicrobial activity of the investigated plant extract was estimated by filter paper disc technique (Kim et al., 2019; Murray et al., 1995). using inoculums containing 106 bacterial cells / mL to spread on nutrient agar plates. Sterilized filter paper discs (Whatman no.1, 6 mm in diameter) were saturated with the extracted sample and another

set of filter paper discs were soaked in methanol and served as controls. The discs were placed on the surface of agar plates seeded with the test bacterial species. The plates were incubated at 37 °C. The inhibition zone diameters (mm) were measured after 18-24 hours (Sardari et al., 1998).

2.6. Data Analysis

Using the COSTAT 6.3 programme (CoHort Software, Monterey, CA, USA, <http://www.cohort.com>), the antioxidant and antimicrobial activities were submitted to one-way ANOVA and the mean values were separated based on Duncan's test at the 0.05 probability level.

3. Results and Discussion

In this research, we aim to explore the reactive chemical components of the essential oil of the methanol extracted *C. pycnocephalus* that are considered as the major factors affected on the biological aptitudes such as antioxidant, cytotoxic, and antibacterial activities. Each type of biological character is affected by a significant class of compounds that control its biological behavior i.e. the phenolic contents are crucial for potent antioxidant characters.

3.1. Gas-Chromatography Mass Spectroscopy (GC-MS)

The components of the essential oil of the extracted *C. pycnocephalus* were elucidated by Gas-Chromatography Mass Spectroscopy "GC-MS" (Figure 2 and Table 1). The results revealed that the extract of *C. pycnocephalus* includes about mainly six characterized components. In general, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one located the major component with 57.43% composition which was identified after 9.11 min. Accordingly, other constituents were characterized with high composition % such as (E)-4-(((2-methoxyoctadec-4-en-1-yl)oxy)methyl)-2,2-dimethyl-1,3-dioxolane (11.53%), methyl (E)-octadec-11-enoate (10.98%), and ethyl iso-allocholate (11.89%), which were identified after 4.92, 29.18, and 35.71 minutes. Additionally, 2-(hept-6-yn-1-yl)malonic acid (3.53%), and methyl 11-((2R,3S)-3-pentyloxiran-2-yl)undecanoate (4.64%) were recorded after 4.15, and 25.89 minutes of retention time. The compounds were classified under several types of naturally occurring components, in which the class of ester of fatty acid has the majority of these components. Therefore, the esters of fatty acids includes (E)-4-(((2-methoxyoctadec-4-en-1-yl)oxy)methyl)-2,2-dimethyl-1,3-dioxolane, methyl

11-((2R,3S)-3-pentyl-oxiran-2-yl)undecanoate, and methyl (E)-octadec-11-enoate. Another class of compounds was identified as hydrocarbons including two components namely, 2-(hept-6-yn-1-yl)malonic acid, and 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one. Ethyl iso-allocholate followed the steroid class was found with 11.89% of composition (Supplementary file).

Alternatively, Al-Shammari et al. (Al-Shammari et al., 2012). have interpreted the chemical constituents isolated from the essential oil of *C. pycnocephalus* that grown in Saudi Arabia as basically nineteen components. Thus, the interpreted GC-MS of the plant extract indicated tetradecanoic acid (2.74%), hexadecanoic acid ethyl ester (4.81%), hexadecanoic acid (39.62%), phytol isomer (6.31%), linolenic acid ethyl ester (4.84%), 9,12-linoleic acid (19.46%), 1,2-benzendicarboxylic acid iso-octyl ester (7.11%), and heptacosane (2.34%). Therefore, the major component of this plant was related to hexadecanoic acid (39.62%), which followed the fatty acid class of compounds. On the other hand, the GC-MS spectroscopic analysis of *C. pycnocephalus* that grown in Iran revealed that the essential oil of this extract contains twenty-nine components such as hexadecanoic acid (23.3%), dibutyl, 1,2-benzene dicarboxylate (8.2%), 6,10,14-trimethyl-2-pentadecanone (7.4%), 1-pentyl octylbenzene (3.7%), and tetradecanoic acid (4.3%). The major component was found to be hexadecanoic acid (23.3%), which is related to the fatty acid class. The established components are terpenes with low composition percentages, fatty acids, and their esters, hydrocarbons, and alkylbenzenes.

Additionally, twenty components of the esters of fatty acids isolated from *C. pycnocephalus* that grown in Saudi Arabia including 1,2-benzene dicarboxylic acid, dimethyl ester (31.08%), azelaic acid dimethyl ester (7.6%), and palmitic acid methyl ester (20.08%) as the major constituents of this plant extract. Other researches have been reported the composition of the components of the volatile oils of *C. pycnocephalus* grown in Iran (Esmaeili et al., 2005). and the identification of the terpenes, and flavone glycoside of *C. pycnocephalus* (Al-Shammari et al., 2015).

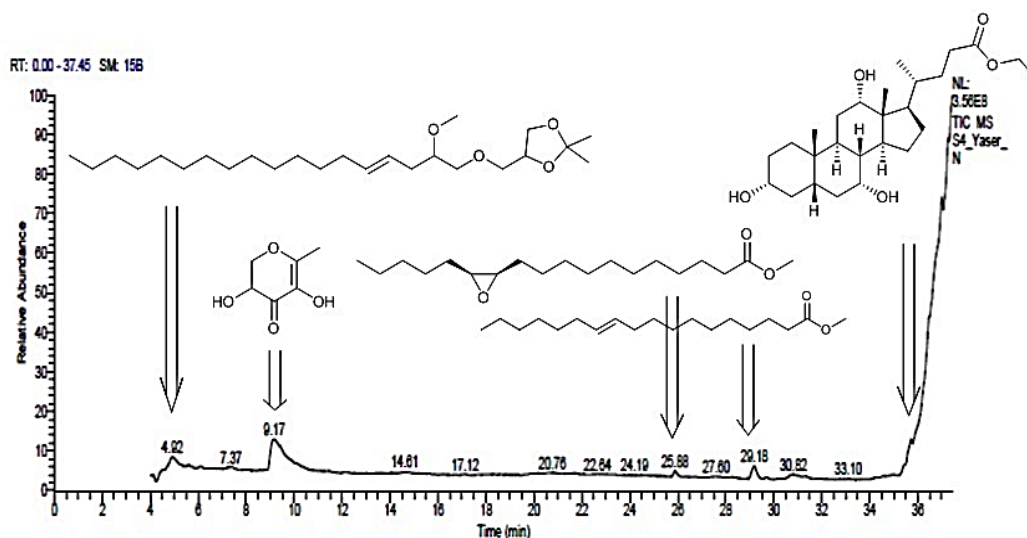


Figure 2. Chromatogram and structures of basic components of the essential oil of the extracted *C. pycnocephalus* by GC-MS.

Table 1. The interpreted components of the essential oil of the extracted *C. pycnocephalus*.

Entry	Chemical name	Classification	Retention time (RT, min)	Molecular Weight	Molecular formula	Composition %
1	2-(Hept-6-yn-1-yl)malonic acid	Hydrocarbon "dicarboxylic acid"	4.15	198.22	C ₁₀ H ₁₄ O ₄	3.53
2	(E)-4-(((2-Methoxyoctadec-4-en-1-yl)oxy)methyl)-2,2-dimethyl-1,3-dioxolane	Ester of fatty acid	4.92	412.66	C ₂₅ H ₄₈ O ₄	11.53
3	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	Oxygenated hydrocarbon	9.11	144.13	C ₆ H ₈ O ₄	57.43
4	Methyl 11-((2R,3S)-3-pentyloxiran-2-yl)undecanoate	Ester of fatty acid	25.89	312.49	C ₁₉ H ₃₆ O ₃	4.64
5	Methyl (E)-octadec-11-enoate	Ester of fatty acid	29.18	296.50	C ₁₉ H ₃₆ O ₂	10.98
6	Ethyl iso-allocholate	Steroid	35.71	436.63	C ₂₆ H ₄₄ O ₅	11.89
						Σ= 100.0

3.2. The biological characteristics of the plant extracts

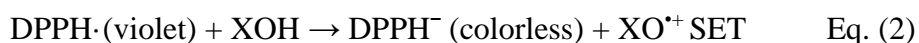
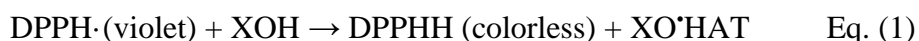
Once upon a time, medicinal plant extracts have been applied in the treatment of various diseases, where the discovery of medicines lies in the presence of their active essential components in the contents of the plant extract, which led to an advance in pharmacology. The *C. pycnocephalus* plant has been widely used in traditional medicine for the treatment of many diseases.

3.2.1. Antioxidant activity - DPPH assay

As commonly reported, the antioxidant activities of *C. pycnocephalus* extracts were evaluated by DPPH• (2,2'-diphenyl-1-picrylhydrazyl radical) colorimetric assay as the

most commonly used assay for the extracted plants. The procedure is usually applied for the hydrophilic antioxidant constituents, while their application for evaluating the antioxidant capacity of the hydrophobic components is limited. In this method, the reactive components that provided potent radical scavenging activities should contribute with a weak A-H bonding, which will increase the possibility for stabilizing or trapping the free radicals of DPPH• at a maximum wavelength 517 nm resulting in a discoloration of the DPPH molecule. The violet color of the DPPH radical will change subsequently to colorless by increasing the concentration of the scrutinized samples. The importance of antioxidants arises from the ability of these components to inhibit the oxidation of lipids. The process followed by scavenging or trapping the free radicals of DPPH, and hence determining the radical scavenging activity, as well as the assay in another route is an indication for reducing the capacity of the antioxidants in their reactions with DPPH radical.

The inexpensive reagents applied to enable to run of the investigated sample by this proficient colorimetric assay (Bondet et al., 1997; Brand-Williams et al., 1995; Blois 1958). The mechanism of DPPH assay is postulated to underwent by a mean of a HAT (Litwinienko and Ingold, 2005). SET (Huang et al., 2005; Fotiet al., 2004), or mixed (Schaich et al., 2015). mechanisms as rendering to the succeeding reaction sequences:



The phytochemical constituents (Kozyra et al., 2019). such as phenolic, and flavonoid contents are proficient components that act as strong scavengers for the DPPH radical increasing the antioxidant potential of these samples to protect the lipoperoxy, and lipid peroxidative from oxidation progression. The plausible mechanism of action was controlled by several beneficial effects. Thus, the presence of antioxidant agents enables the scavenging of the free radicals of DPPH depending on the nature of the source of the free radical components, and the concentration of the inspected sample. Therefore, by increasing the sample concentration, the percent of radical scavenging activity will increase resulting in a release in the color intensity of the DPPH (Siripatrawan and Vitchayakitti, 2016).

In this work, the antioxidant properties of root, stem, leaves, and flower extracts of *C. pycnocephalus* were in vitro evaluated by DPPH assay. The radical scavenging activity percent increased by increasing the sample concentration in a proportional relationship (Table 2). The samples were tested for six diversified concentrations (5, 10, 20, 30, 40,

and 50 mg L⁻¹). At the concentration of 10 mg L⁻¹, the root extract was found with the most potent antioxidant activity (% RSA= 27.44±1.61%), and the stem extract has the second-order of antioxidant capacity (% RSA= 23.76±1.40%). The concentration of 20 mg L⁻¹ of the investigated sample is more applicable for the comparison of the antioxidant scavenging radical activities for all the tested samples relative to the results of ascorbic acid. Thus, the flower extract revealed the potent antioxidant capacity of the other plant extracts with % RSA at 45.65±2.69%, this result is comparable with that of ascorbic acid (% RSA= 64.97%). Leaves extract situated the second-order of antioxidant potency (% RSA= 41.77±2.46%), then stem extract (% RSA= 38.29±2.25%), and finally the root extract (% RSA= 30.52±1.80%) at a concentration of 20 mg L⁻¹. In the same sequence, flower extract is the most potent antioxidant agent at the concentrations of 30, and 40 mg L⁻¹ with % scavenging radical activity at 56.43±3.32, and 61.38±3.61%, respectively. The order of activity of the other plant constitutes is followed the extract of leaves (% RSA= 48.80±2.87 & 54.62±3.21%), stems (% RSA= 43.37±2.55 & 47.26±2.78%), and to end with the root (% RSA= 37.62±2.21 & 44.78±2.63%). Unexpectedly, at the higher concentration (50 mg L⁻¹), we noticed that leaves extract has the most potent antioxidant activity with % scavenging radical activity at 71.82±4.22% than the flower extract with % scavenging radical activity at 68.47±4.03%. The behavior of stem and root extracts at the concentration of 50 mg L⁻¹ followed the same sequence of the lower concentration (20, 30, and 40 mg L⁻¹) with % scavenging radical activity at 55.22±3.25%, and 51.27±3.02% (Table 2).

Table 2. The antioxidant results of the extracted root, stem, leaves, and flower of *C. pycnocephalus* plant.

Treatment	Concentration (mg mL ⁻¹)	Radical Scavenging Activity (%)			
		Root	Stem	Leaves	Flower
<i>C. pycnocephalus</i>	5	12.58 ± 0.74	17.14±1.01	10.37±0.61	8.70±0.51
	10	27.44±1.61	23.76±1.40	19.28±1.13	21.69±1.28
	20	30.52±1.80	38.29±2.25	41.77±2.46	45.65±2.69
	30	37.62±2.21	43.37±2.55	48.80±2.87	56.43±3.32
	40	44.78±2.63	47.26±2.78	54.62±3.21	61.38±3.61
	50	51.27±3.02	55.22±3.25	71.82±4.22	68.47±4.03
LSD _{0.05}		10.51***			
IC ₅₀ (mg L ⁻¹)		46.84	41.31	32.78	30.69
Ascorbic acid	1		2.73		
	2.5		10.99		
	5		38.2016		
	10		45.1916		
	15		56.2316		
	20		64.9716		

LSD _{0.05}	0.65***
IC ₅₀ (mg L ⁻¹)	13.29

*** significant at 0.001 after Duncan's post hoc test.

The values of inhibitive concentrations "IC₅₀" is the half-maximal inhibitory concentration expressed the potential concentration of the tested sample that achieves scavenging for the radicals of DPPH by 50%. The values of IC₅₀ were calculated from the exponential curve (Parejo et al., 2000). plotting the sample concentrations against the remaining percent of DPPH• radical applying linear regression analysis.

The results in Table 2 verified that flower extract is the most potent antioxidant agent with IC₅₀ = 30.69 mg L⁻¹ relative to the results of ascorbic acid (IC₅₀ = 13.29 mg L⁻¹). The leaves extract of *C. pycnocephalus* located the second-order of radical scavenging capacity with IC₅₀ = 32.78 mg L⁻¹, succeeding by the stem extract (IC₅₀ = 41.31 mg L⁻¹), and root extract (IC₅₀ = 46.84 mg L⁻¹). The significant antioxidant potency of the samples depended in this scale of comparison on the nature of the chemical components contained in the distinct extract as the presence of electron sources such as reactive oxygen or nitrogen sorts (Singh et al., 2021; El-Amier et al., 2019). such as hydroxyl, phenoxyl, alkyl peroxy, linoleic acid, peroxy, and glutamyl radicals that can stabilize or trap the DPPH free radical improve the antioxidant aptitude (Mishra, 2016; Kwak et al., 2009).

3.2.2. Potential antibacterial activity

The antibacterial activity of the extracted botanical ingredients of *C. pycnocephalus* from methanol was assessed by a disc diffusion technique as an in vitro antimicrobial susceptibility testing. The tested samples were prepared in a concentration of 10 mg L⁻¹ from the root, stem, leaf, and flower extracts. The results are shown in Table 3, and Figure 3 revealed that the four samples are potent antibacterial agents against *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *B. cereus* bacterial species. Particularly, potent antibacterial activities were recorded for leaf, and flower extracts against *E. coli* species with inhibition zones equivalent to that of antibiotic standards (20 mm). The root and stem extracts revealed remarkable antibacterial activities against *P. aeruginosa* species with inhibition zones 22, and 20 mm, respectively, with high potency than the antibiotic standard "Azithromycin" (13 mm), along with good activities of the leaf, and flower extracts with inhibition zones at 10 mm.

By studying the results of the antibacterial activities of samples as inhibitors of bacterial growth, we found that the four samples had distinct activities in the process of inhibiting the growth of bacterial species of the type *S. typhimurium* by 14, 13, 26, and 25 mm, respectively, relative to the results of the antibiotic "Tetracycline" (10 mm). Medium to high activities of the four samples was observed against *S. epidermidis* bacterial strains compared to the results of the four antibiotics, while the highest result of inhibiting the growth of those bacterial species was for the flower extract with an inhibition zone at 15 mm. Looking at the results of the four samples as components of *C. pycnocephalus*, we found that the activity of methanolic extracts against Gram-positive bacterial species is not good compared to their results with Gram-negative bacteria. This does not indicate the lack of quality of those extracts to inhibit the growth of Gram-positive bacteria, as some extracts have higher results than antibiotics such as extracts of leaves and flowers towards inhibiting the growth of bacterial species of type *B. cereus* by an amount of 23 and 25 mm, respectively, and these results are higher than that obtained by all the antibiotics (5-20 mm) (Table 3).

Table 3. The antimicrobial activities are represented by the inhibition zone diameter (mm) of the methanol extract of *C. pycnocephalus* and standard antibiotics.

Microbes	Inhibition zone in mm							
	<i>C. pycnocephalus</i> (10 mg L ⁻¹)				Standard antibiotic (10 mg L ⁻¹)			
	Root	Stem	Leaf	Flower	Cephadrin	Tetracycline	Azithromycin	Ampicillin
Gram-negative bacteria								
<i>E. coli</i>	13	10	20	20	15	20	20	20
<i>P. aeruginosa</i>	22	20	10	10	0	0	13	0
<i>S. typhimurium</i>	14	13	26	25	0	10	0	0
<i>S. epidermidis</i>	10	5	5	15	10	20	23	10
Gram-positive bacteria								
<i>B. cereus</i>	11	13	23	25	20	10	20	5
<i>S. aureus</i>	12	10	10	14	20	20	20	30
<i>S. haemolyticus</i>	0	0	5	5	25	23	23	20
<i>S. xylosus</i>	0	0	0	5	20	20	20	25
<i>K. pneumoniae</i>	10	10	13	13	10	20	13	5
LSD _{0.05}	1.61	1.32	1.47	1.50	1.71	1.84	1.41	2.31

LSD_{0.05} expressed the calculated least of the smallest significance between two means as each test was run on those two means (calculated by Factorial ANOVA).

Among the good results are also a high activity of inhibiting the growth of *S. aureus* of the flower methanolic extract by an amount of 14 mm and the good activity of leaf and flower extracts as inhibitors of *K. pneumoniae* growth with an amount of 13 mm compared to the highest inhibitory activity of the antibiotic "Tetracycline" with an inhibition efficiency of 20 mm. The results also demonstrated that the lowest efficiency to inhibit

the growth of bacterial microorganisms was found against *S. haemolyticus* and *S. xylosus* using any of the four extracts with inhibition efficiency from inactive to 5 mm (Table 3).

The mechanism of action for the bacterial infections (Sawa, et al., 2019; Bascones-Martínez et al., 2009). is controlled by six main factors: (1) the interface with the bacterial cell wall (inhibit cell wall synthesis) (Mohammad et al., 2017). (2) depolarize the cell membrane (Higgins et al., 2005). (3) inhibition of the protein synthesis (De Vriese et al., 2006). (4) inhibition of nucleic acid synthesis (Srivastava et al., 2011). and (5) metabolic pathway inhibition (Li et al., 2019). Additionally, five basic mechanisms of action for antibiotics (Etebu & Ariekpar, 2016). are known including the inhibition of cell wall synthesis, protein synthesis (translation), nucleic acid synthesis, cell membranes alteration, and antimetabolite activity. The action of the tested samples as antibacterial agents is to continually disrupt and prevent the growth of bacterial species. The interpretation of these results is consistent with previous results in deducing the efficacy of the plant extract as an antimicrobial agent (Hafaz et al., 2022; Sultan et al., 2022).

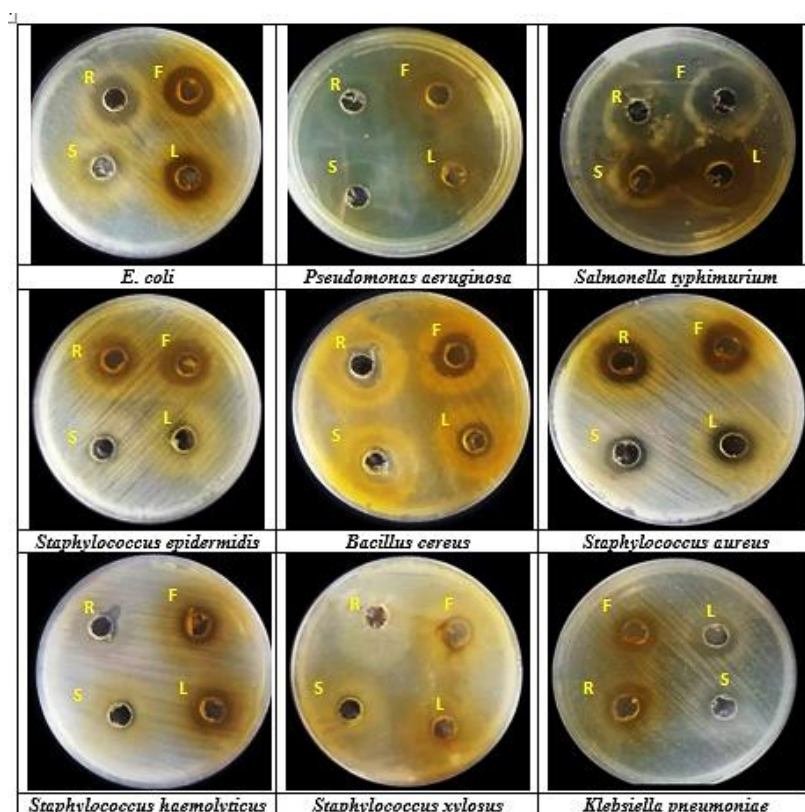


Figure 3. The inhibition zones were raised from the effects of the extracted root, stem, leaf, and flower of *C. pycnocephalus* on the plates seeded with different bacterial species by disc diffusion technique. R≡ Root extract; S ≡ stem extract; L ≡ Leaf extract; F ≡ Flower extract.

3.2.3. Cytotoxic activity

The cytotoxic activity of *C. pycnocephalus* methanol extract was assessed using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay under distinct conditions. The method is identical to a cell number growth curve. The MTT reagent as a reliable indicator is so sensitive to the light, this engaged the run of the experiments in the dark. Hepatocellular carcinoma (HepG2) tumor cell line was selected to assess the anticancer potency of the investigated extracted medicinal plant. The method was applied for determining the cell metabolic activity based on the aptitude of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the MTT tetrazolium dye to its formazan "insoluble" that has a purple color. The number of viable cells should increase with growth, decrease with cytotoxic treatments, and remain the same (or plateau) with cytostatic treatments. The IC₅₀ values expressed the concentration that represented 50% of the inhibition of cell growth was calculated by applying the curves obtained from plotted the percentages of cell survival versus drug concentration (μM). Thus, the potency of cytotoxicity will rise by the decrease in the extract concentration and IC₅₀ values. The MTT solution may be affected by the results of cytotoxicity, so we have run a control sample "blank" that was a few "empty" wells containing MTT solution without any of the cell lines. The control sample is a benefit for calculating the cell viability percent, as it produces 100% viability of healthy cells. The experiments were run using five concentrations of each plant extract (31.3, 62.5, 125, 500, and 1000 μg mL⁻¹) prepared in a serial dilution (Table 4).

Table 4. Cytotoxic results of the extracted *C. pycnocephalus* against HepG2 tumor cell line.

Samples	Conc. (μg mL ⁻¹)	R1 ^[a]	R2 ^[a]	IC ₅₀ (μg mL ⁻¹) ^[b]
<i>C. pycnocephalus</i> extract	1000	0.714	0.755	46.2
	500	1.4	1.32	
	125	2.4	1.7	
	62.5	1.6	1.7	
	31.3	1.8	1.8	
	0	1.3	1.3	

^[a] R1, R2 are the absorbance read of the extracts at diverse concentrations.

^[b] IC₅₀ values are the inhibitive concentration expressed sample concentration that contributes roughly 50% of the death of cancer cells.

Table 4 signified the results of the cytotoxic effects specified by the inhibitive concentration values of the extract of *C. pycnocephalus* on HepG2 tumor cell line. The results of cytotoxicity revealed that the extract of *C. pycnocephalus* has a potent cytotoxic effect on HePG-2 cell line with an IC₅₀ value at 46.2 μg mL⁻¹. It is worth mentioning that the

mechanism of cytotoxicity of the extracted samples as cytotoxic agents on HepG2 tumor cell line is commonly dependent on the structural nature of the components of each extract, and the nature of the cancer cell line. Additionally, the nature of the particles of the extracted plants such as surface morphology, size, and aggregation of the particles might control the results of cytotoxicity. The efficiency of the plant extract as an efficient anti-cancer agent for tumor cell growth depended on several factors as previously noted, including the nature of the chemical components of this extract, the type of cancer cell, and the concentration of the extracted plant used in this assess (Khacha-Ananda et al., 2013).

3.2.3.1. EC_{50} of *C. pycnocephalus* extract

The dose-response relationship of the assessed *C. pycnocephalus* extract is plotted in Figure 6. The dose-response curve in Figure 6a was normalized in the X-axis direction by its EC_{50} value (Figure 6b). The value of EC_{50} of the methanol extract of *C. pycnocephalus* was initially calculated by plotting the sample absorbance against the log of doses at different concentrations of the serial dilution (Figure 6). The low concentrations of the extract are not enough to produce a response, while the high doses produce a maximal response, and the vertical point of the curve resembles an EC_{50} value. The data analysis specified that the higher concentration (dose = $1000 \mu\text{g mL}^{-1}$) as calculated for EC_{50} value ($2.82 \mu\text{g mL}^{-1}$) has a cytotoxic effect on HepG2 cell lines.

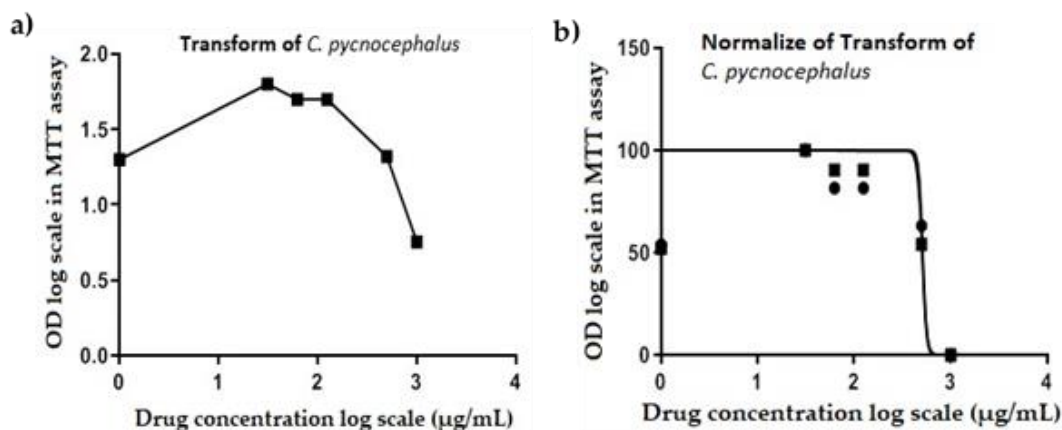


Figure 6. Dose response curve. (a) Transform of *C. pycnocephalus* extract. (b) Normalization of transform of *C. pycnocephalus* extract. (OD) expressed the measured absorbance value at a definite concentration of the investigated extracted sample.

4. Conclusion

C. pycnocephalus plant is related to Asteraceae family displayed a wide spectrum of biological activities (Rustaiyan and Faridchehr, 2021). In this work, we used the extract of *C. pycnocephalus* as a medicinal plant or any of its plant parts such as the stem, leaves, flowers, and roots to identify the chemical components that were isolated from the essential oils of the methanolic extract to study the effect of these components on the different biological results. We found that the four parts of the plant have high antioxidant capacities compared to the results of ascorbic acid as a result of these extracts containing a high percentage of phenolic components. Higher than antibiotics. Through that study, we discovered the efficiency of the plant extract as a cytotoxic agent with a moderate potency against the growth of hepatocellular carcinoma (HepG2), which indicated that the drug will not affect the growth of normal cells. The results also specified that the extracts of different parts of the plant, especially the that of leaves and flowers, have high and distinctive anti-bacterial activities to kill or inhibit the growth of the bacterial species such as *E. coli*, *S. typhimurium*, and *B. cereus* strains with higher efficiency than antibiotics.

Supplementary Materials: Figures S1-S7 were included in a supplementary file including the mass spectral charts of the identified chemical components isolated from the essential oils of the extracted *C. pycnocephalus*, and the inhibition zones formed by the action of the tested antibiotics, and the control sample against all the bacterial species. A caption was specified for each table as follows: Figs. S1-S6. The chemical structure of the chemical constituents identified after 4.15-35.71 min of retention time for the essential oil isolated from the extracted *C. pycnocephalus* Fig. S7. The inhibition zones raised from the effects of the control sample, and the different antibiotic standards (Cephadrin, Tetracycline, Azithromycin, and Ampicillin) on the plates seeded with diverse bacterial species by disc diffusion technique.

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Supplementary data

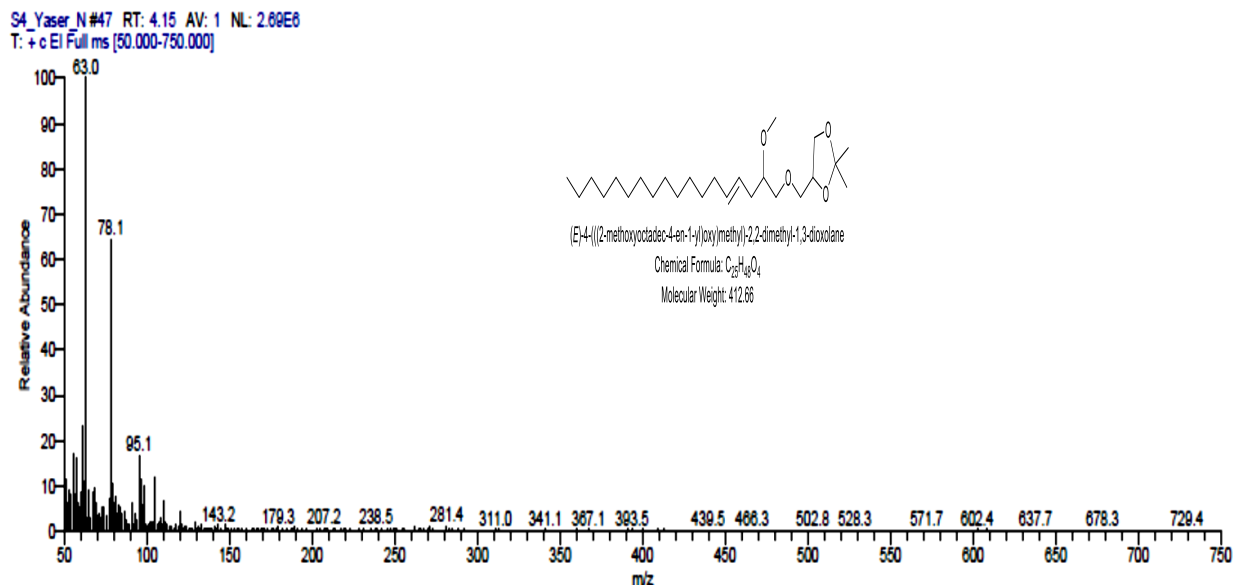


Fig. S1. The chemical structure of the chemical component that was identified after 4.15 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

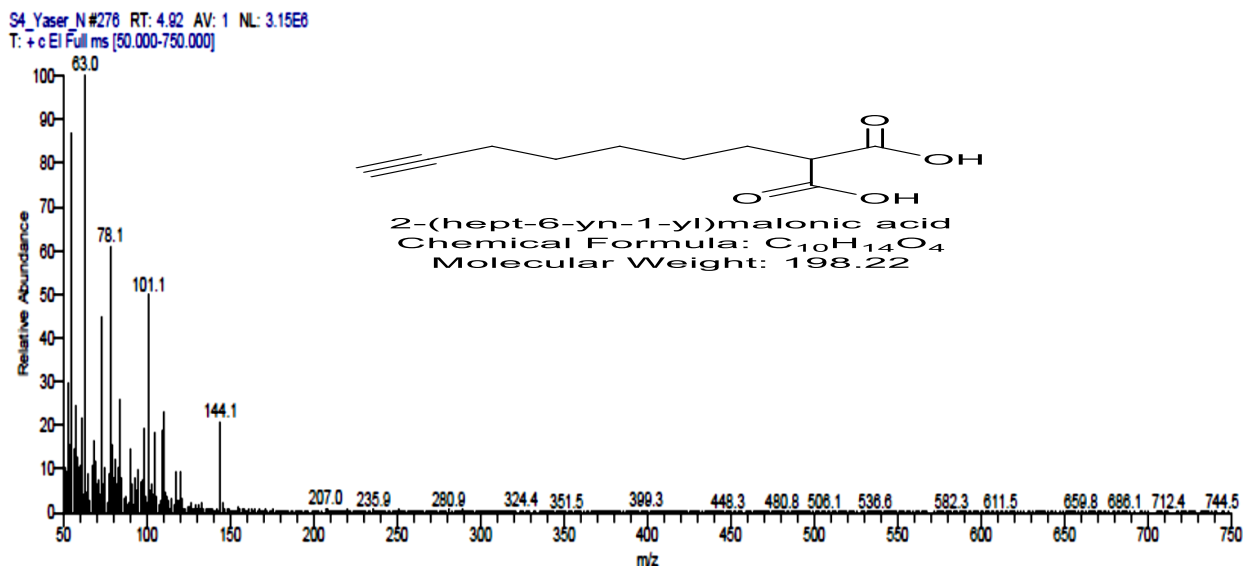


Fig. S2. The chemical structure of the chemical component that was identified after 4.92 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

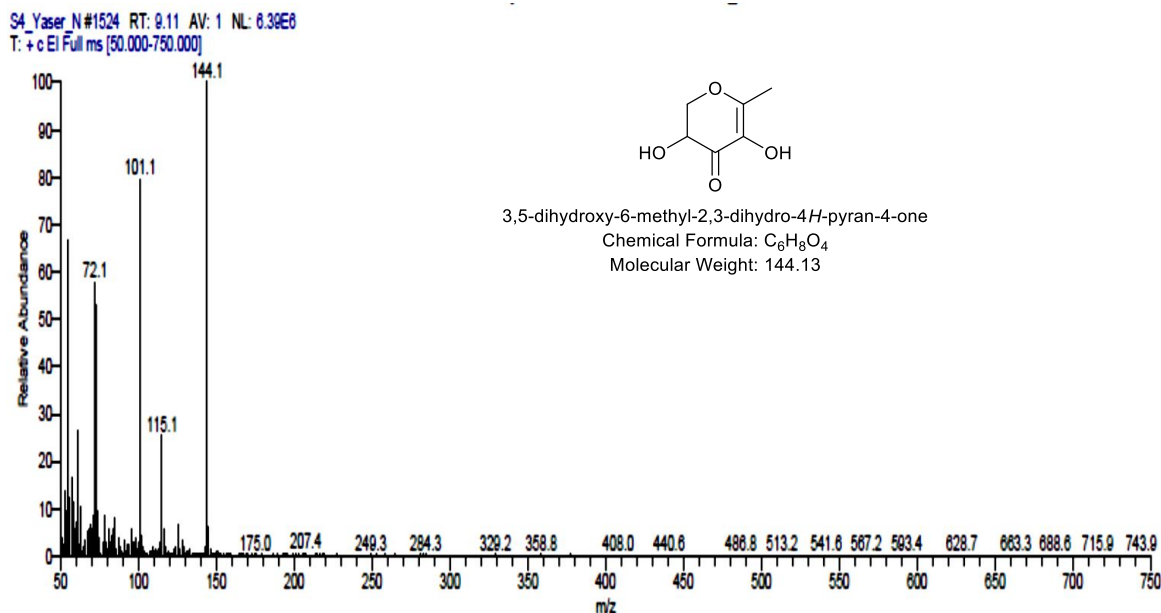


Fig. S3. The chemical structure of the chemical component that was identified after 9.11 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

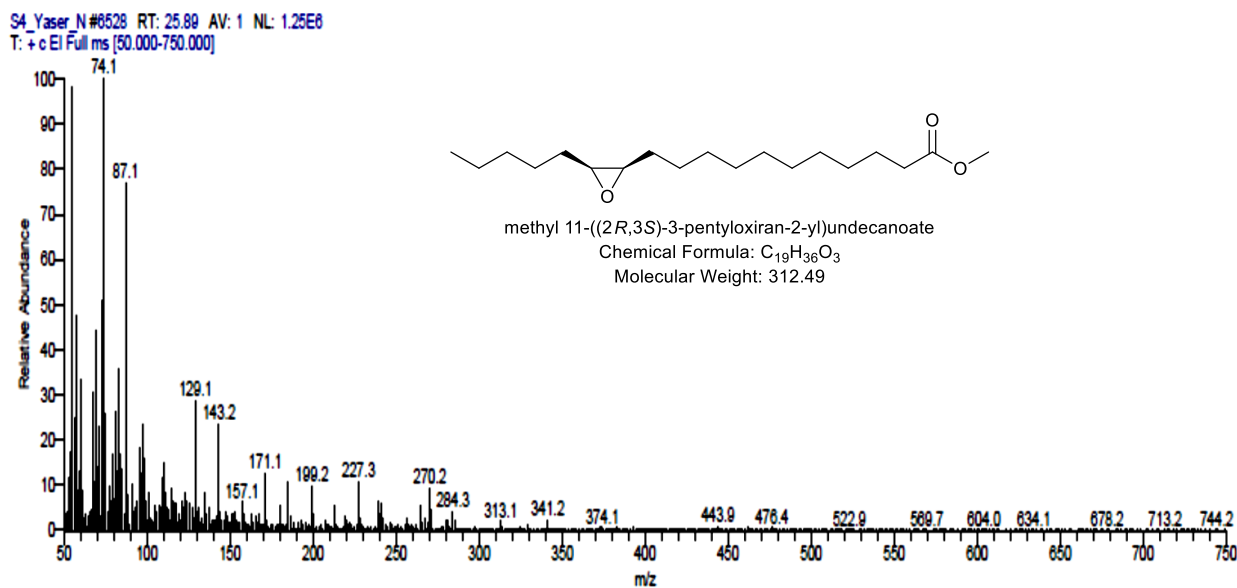


Fig. S4. The chemical structure of the chemical component that was identified after 25.89 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

S4_Yaser_N#7508 RT: 29.18 AV: 1 NL: 1.46E8
T: + c EI Full ms [50.000-750.000]

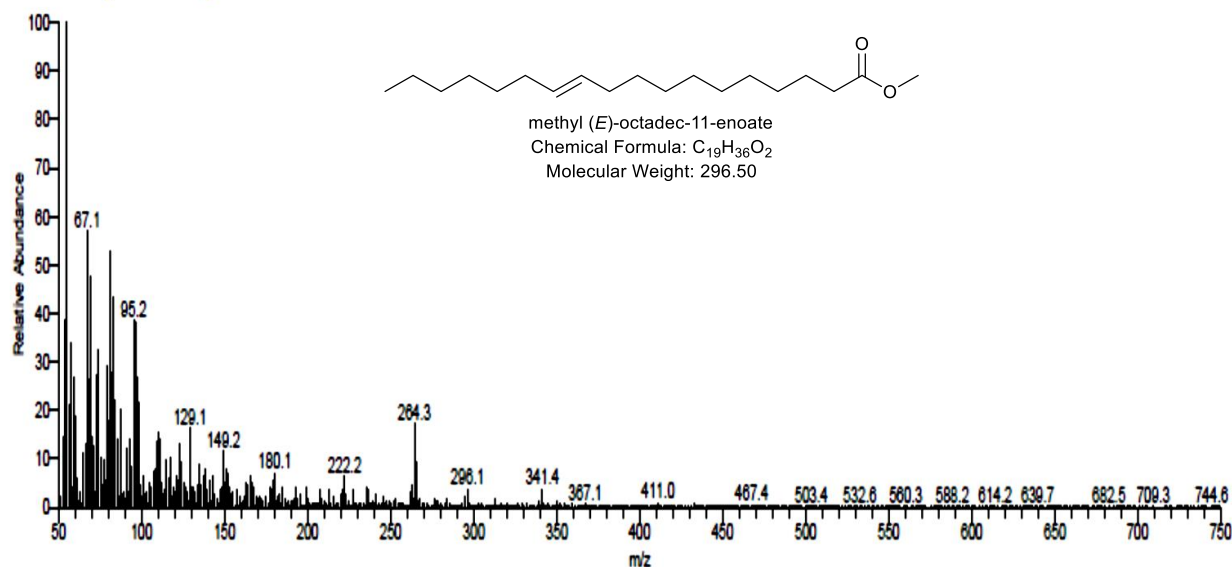


Fig. S5. The chemical structure of the chemical component that was identified after 29.18 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

S4_Yaser_N#9455 RT: 35.71 AV: 1 NL: 2.38E8
T: + c EI Full ms [50.000-750.000]

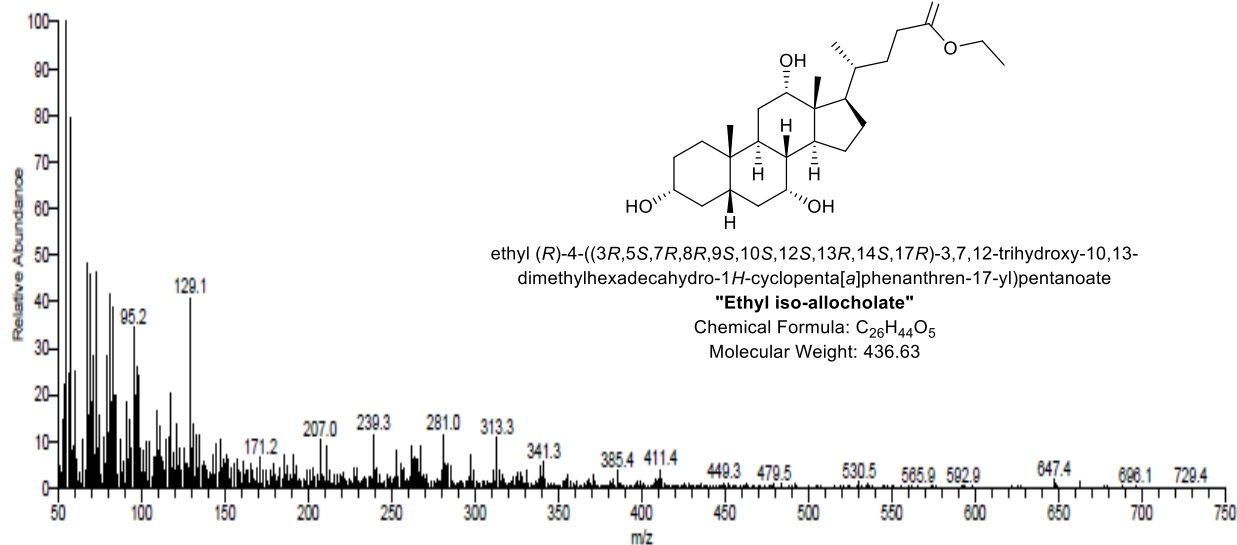


Fig. S6. The chemical structure of the chemical component that was identified after 35.71 min of retention time for the essential oil isolated from the extracted *Carduus pycnocephalus* L.

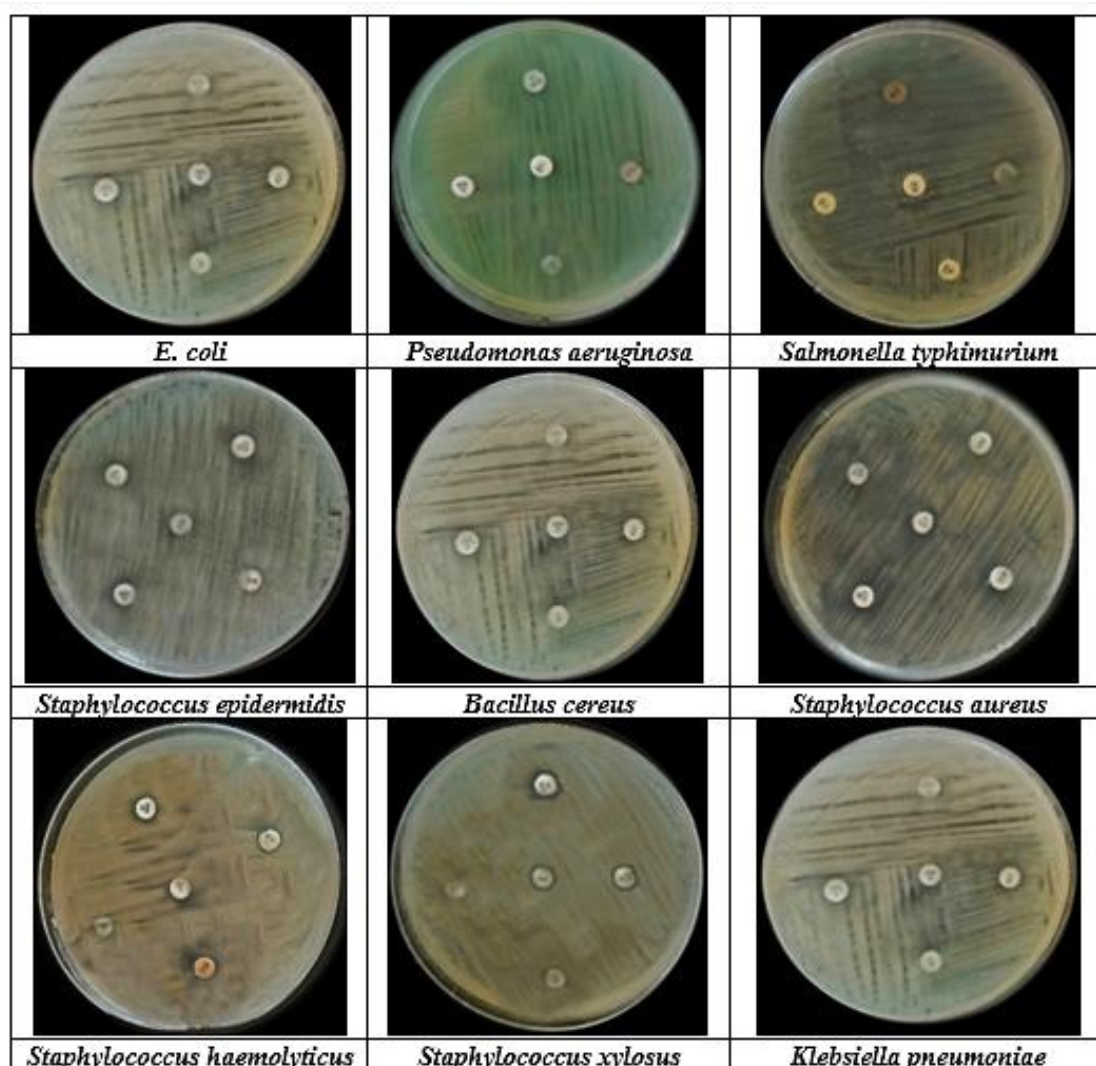


Fig. S7. The inhibition zones raised from the effects of the control sample, and the different antibiotic standards (Cephadrin, Tetracycline, Azithromycin, and Ampicillin) on the plates seeded with diverse bacterial species by disc diffusion technique.