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Chiliadenus montanus (Vahl.) Brullo which grows wild in the Jordanian environment shows distinguished Terpinen-4-ol levels and antibacterial powers

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Abstract

Chiliadenus montanus(Vahl.) Brullo is a wild herb that grows in narrow parts of Jordan and is locally named Hneedeh. Despite being used for treating mild abdominal and respiratory ailments, Hneedeh is still locally known on a very small scale, and its healing capabilities are still waiting for a solid scientific justification. No previous studies were made to explore the antibacterial potential of Jordanian Hneedeh as well as its chemical composition despite being researched by scientists from neighboring countries. For this reason, this research aimed to unveil the active compounds behind the medicinal properties of wild *C. montanus* collected from Garissa-Jordan by analyzing the essential oil using the GC MS system. Also, the antibacterial activity was explored in two extracts (methanolic and ethanolic) collected from the succulent branches of *C. montanus* was examined using Microdilution and Disc Diffusion Assays. Data revealed the presence of 23 compounds grouped into eight classes and oxygenated monoterpenes were the predominant class (40.36%).Terpinen-4-ol, which is known for its super curative antibacterial and antitumoral powers, was indicated to be the major chemical compound in the essential oil of Hneedeh as it comprised (23.3%) of the essential oil, which disagreed with other previous studies made on the same plants growing in neighboring counties. Moreover, both extract types restricted growth in most tested bacterial types. The results recorded in the Microdilution Assay showed that the inhibitory effect of both extract types was stronger thanthose obtained in the antibiotic treatment (control) in *E. coli* and *Staphylococcus aureus*. Meanwhile, both methanolic and ethanolic extracts were as effective as the antibiotic when administrated to *Bacillus subtilis and Salmonella* sp. at similar minimal inhibition concentration (MIC) values. Moreover, Disc Diffusion Assay showed that both extract types could prevent the development of six bacteria types while no effect was recorded in *Klebsiella pneumoniae*. However, *E coli* bacteria was the most affected strain. The presence of the oxygenated monoterpenes, especially Terpinen-4-ol at premium levels, might be the causal agent behind the remarkable antibacterial powers of *C. montanus* extract. To our knowledge, this is the first time that *C. montanus*which grows wild in the village of Ghareesa is reported to containTerpinen-4-ol as the main active ingredient, which is unlike results reported in other studies conducted on similar plant species growing in other countries.

Keywords: Antibacterial activity, *Chiliadenus montanus,*Garisa, Jordan, oxygenated monoterpenes, Terpinen-4-ol.

1. Introduction

Controlling ailments worldwide is threatened nowadays by the antimicrobial resistance phenomenon which would put the whole of humanity under serious challenge with microbes(WHO, 2019). Unfortunately, earnest actions towards such a global threat have not been taken yet, although the World Health Organization (WHO) has announced most antibiotics are useless due to antimicrobial resistance (Masi et al., 2021).Finding alternative weapons against microorganisms is a priority to overcome the growing resistance to antibiotics (Tahtamouni et al., 2018).

Herbal medicine is mostly practiced by people in developing countries as they have found in plants an effective, natural, safe, and cheap source to cure diseases. Numerous plants have been documented to exhibit medicinal properties effective against various types of microorganisms. (Jaganthan et al., 2015). So, plant extracts can be used as natural antibiotics against pathogens that might in some cases outperform synthetic ones (Masi et al., 2021).

Many endemic plants in Jordan were screened for their healing properties. Abu Odeh et al. (2023) have listed many examples of medicinal plants that are endemic to the Jordanian environment and were researched by Jordanian scientists for their healing properties such as *Ocimum*

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basilicum (Hudaib et al,. 2008), *(Achillea santolina* L. and *Origanum syriacum* L. (Oran and Al-Eisawi, 2015), *Salvia ceratophylla* L. (Abu-Darwish et al., 2020) and *Viscum cruciatum* Sieb (Abo-Elghiet et al., 2022), and many more, but many of Jordan's medicinal plants are still veiled.

Ghareesa is an ancient village located in the Jordanian governorate of Zarqa, Al-Hashimiya District where 52% of the Jordanian industry is located and concentrated like the oil refinery, the Al-Hussein thermal station, multiple iron factories, along with the existence of the Khirbet Al-Samra station for the processing of household sewage. Consequently, Ghareesa suffers from air pollution due to emissions of air pollutants from these factories, such as sulfur dioxide, hydrogen sulfide, and carbon monoxide(Saffarinia and Odat, 2008).Air pollution, climate change, and excessive collection have put the unique plant biodiversity of Garissa under threat of extinction.

Chiliadenus montanus (Vahl.) Brullo is a type of healing herb that is classified under the Asteraceae family. This shrublet grows wild along the southern edge of the Mediterranean, especially in Egypt, Sinai, and Jordan (Bengtson and. Anderberg, 2018). In Jordan, it is commonly known as (Hneedeh). This plant grows wild in Garissa, especially on hillsides, rocky valleys, and roadsides. Recently, this plant was put under the microscope by scientists from nearby countries, especially Egypt, for its remarkable healing properties, as it was found to possess antioxidant, antidiabetic and anti-obesity powers(Hegazy et al., 2014; Helal et al., 2015) besides its curative potential against Alzheimer's disease and tumors (Ahmad et al., 2018; Elhadyet al., 2020). In Jordan, this valuable medicinal plant is not well-researched yet. However, according to the findings of Eissa et al. (2014), the essential oil derived from *C. montanus* aerial parts collected from Sinai, Egypt, contained cis-verbenol as its primary component. Cis-Verbenol has been reported for its anti-inflammatory and anti-ischemic characteristics, as well as its ability to promote healthier neuronal growth (Nadeem et al., 2022).

Disc diffusion and Microdilution assays were routinely used in testing the antimicrobial powers of plant extracts in many research studies. The official method employed in numerous clinical microbiology laboratories is Disk Diffusion Assay, which was developed in 1940 (Heatley, 1944), where discs of agar are treated with the tested microorganism, then filter paper discs with a desired concentration of the tested extract are positioned on the agar surface before being kept under appropriate circumstances, and the diameters of the regions where growth inhibition takes place were evaluated(Balouiri et al., 2016).

Microdilution Assay is also considered very suitable in antimicrobial research, as it was described to allow the estimation of the concentration of the antimicrobial agent which was found to stop the growth of the microbes (Balouiri et al., 2016).

The GC/MS was commonly utilized for characterizing and uncovering new compounds and metabolites, and it was reported to be an exceptional and potent technology as it has offered a distinct chance to analyze novel compounds (Al-Rubaye et al., 2017).

So, based on the lack of information about *C. montanus* which grows in the Jordanian environment and is used by limited local people without solid scientific proof, the purpose of our study was to determine the active components of the extract of *C. montanus* (Vahl.) Brullo growing wild in Garissa, Jordan, using GC/MS technique ,and to assess the extract's antimicrobial efficacy against specific bacterial strains using Disc diffusion and Microdilution assays. The results of this study might encourage other research work toward the conservation and development of this neglected plant.

2. Materials and Methods

2.1. Extraction and identification of active ingredients

The areal branches of *C. montanus* (Vahl.) Brullo were taken from a single shrublet growing wild in Ghareesa, Zarka- Jordan(32°10'13.0"N; 36°04'30.8"E) in March 2019(Figure 1). Extraction and identification of the active ingredients were performed according to Abu Rjai et al. (2020) where plant material was hydro-distilled to extract oil by Clevenger apparatus. The weight of the collected oil served as a benchmark for determining the proportionate amount of oil obtained from the dried plant material (w/w %).Next, the aliquot of oil-diluted samples(1 µl) was injected into the GC-MS system utilizing an automated injection device. The GC-MS was performed using a modified Chrompack CP3800 GC/MS/MS-200 (Satum, Netherlands) which was outfitted with a split- splitless injector and DB-5 capillary column (5 % diphenyl 95 % dimethyl polysiloxane, 30 m×0.25 mm ID, 0.25 µm film thickness)(Abu Rjai et al. 2020). The linear temperature program was utilized, with the injector temperature set at 250° and a split ratio of 1:30, starting with a column temperature of 60° for 1 minute then was gradually increased to 250° at a rate of 3°/min and maintained at 250° for 2 minutes, resulting in a total runtime of approximately 66 minutes(Abu Rjai et al. 2020). Helium was the carrier gas, and the ions were scanned according to the protocol established by Abuja et al. (2020), with a DB-5 column utilized in GC-MS. The oil components were calculated by identifying peaks through the linear retention index (Arithmetic-Kovats index), and subsequently separated by GC-MS based on retention times and n-alkanes retention time references according to Van Den Dool Equation (1963).The computer software libraries NIST, WILEY, and ADAMS were utilized to separate the essential oil components (Abuja et al., 2020). Different oils of eugenol, linalool, and eucalyptol were analyzed to confirm their constituents. Standards were acquired from (Sigma-Aldrich) and employed to determine the oil components using identical chromatography (GC) parameters, while the chemical constituents of the oil were identified through utilization of GC-FID analysis using an FID detector and a split less injector in a thermos quest gas chromatograph and a column of HP-5 (Abu Rjai et al., 2020). The standard curve was formulated, and the quantities of the compounds were determined by calculating the area under each peak.

Figure 1. *C. montanus* (Vahl.) Brullo was collected from Ghaeesa, Zarka- Jordan (32°10'13.0"N; 36°04'30.8"E) in March 2019.

2.2. Antibacterial Activity

For studying the antibacterial activity of *C. montanus* (Vahl.) Brullo, samples of the aerial components were subjected to drying in the oven at a temperature of 45°C.beforegrounded into fine powder. Subsequently, samples of the powder derived from plant material were treated with either ethanol or methanol in a 1:10 ratio. The resulting extracts underwent filtration and evaporation until they reached a state of dryness, after which they were dissolved in DMSO. The Disc diffusion and Microdilution techniques were employed to assess the antibacterial activity of every extract type. Seven bacterial strains were tested (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Klebsiella pneumoniae* ATCC 31488, *Esherichia coli, Salmonella* sp., and *Erwinia carotovora*).

The Microdilution Method was used to find the minimum inhibitory concentration (MIC) based on guidelines set by the National Committee for Clinical Laboratory Standards(NCCLS 1997). To achieve this, methanolic and ethanolic extracts were diluted in a serial manner, with each dilution being doubled, using Muller Hinton and potato dextrose broth. As a control, the eleventh well contained nutrient broth plus Tetracycline (10 mg/mL). The resulting concentrations from both extracts were between 50 mg/mL to 0.098 mg/ ml. After incubating for 24 hours at 37 °C in a plate shaker incubator the results for (MIC) were recorded.

In the Disc Diffusion Assay, discs with a diameter of 6.0 mm were placed on Muller Hinton agar plates from Mast Group Ltd. U.K. A volume of 100 µL of each bacterial type fixed to 0.5 McFarland (Karlsmose, 2010) was streaked on the discs. Subsequently, 10 mg of both methanolic and ethanolic extracts were applied to each disc. Tetracycline antibiotic at a level of 10 mg/mL served as the reference standard. The zone of inhibition was recorded after 24hrs. of being kept at 37°C to determine antibacterial activity.

Treatments in the Disc Diffusion Assay were randomized completely and were repeated 5 times. Data of the inhibition zones were analyzed according to (ANOVA) using SPSS (17), and means were compared using Tukey's HSD test at a significance level of 0.05.

3. Results and Discussion

3.1. Chemical composition of the essential oil

Results obtained in (Figure 2, Table 1) illustrated that (23) compounds were identified in the extracted essential oil of *C. montanus* (Vahl.) Brullo aerial parts. The identified compounds were grouped into eight classes where oxygenated monoterpenes were the predominant class as they comprised (40.36%) (Table 1). The major chemical compound identified in the extract was Terpinen-4-ol as it comprised (23.3%) of the essential oil (Table 1). Our results matched the findings of Eissa et al. (2014) as they reported that oxygenated monoterpenes were the main class detected in the oil extracted from *C. montanus* growing wild in Sinai- Egypt. On the other hand, our data contrasted with Eissa et al. (2014) results when they reported Cis-Verbenol as the major compound of the essential oil of Hneedeh of Egypt. According to our results,Terpinen-4-ol was the major component (23.3%) of the essential oil extracted from *C. montanus*aerial parts collected from Ghareesa- Jordan. No Terpinen-4-ol was found in Eissa et al. (2014) results, as they reported cis-verbenol (22.2%) as the main constituent of the oil extracted from Hneedeh fresh branches collected from Sinai, Egypt. Such variation might be attributed to the different nature of climate, rainfall, soil, and time of collection. To our knowledge, this is the first time that Terpinen-4-ol has been announced as the major active ingredient in *C. montanus*. In another study, Terpinen-4-ol was detected as the major compound (42%) in the essential oil *Melaleuca alternifolia* (Tea Tree), a tree that was reported in many research articles for its super curative antibacterial, anti-inflammatory, and antitumoral powers (Hart et al., 2000). Recently, Terpinen-4-ol attracted the attention of scientists as it was found to stop the development of many tumors such as melanoma (Calcabrini et al., 2004), lung cancer (Wu et al., 2012), leukemia, (Banjerdpongchai et al., 2013), and colorectal cancer(Nakayama et al., 2017). Moreover, Terpinen-4-ol is extensively utilized in the food sector to prevent staling as it forms a microbial biofilm in food products instead of using salt to prevent food spoilage (Yong et al., 2022). Our results indicated that *C. montanus* can be used as a natural source of Terpinen-4-ol, and this plant might become easily a potential bioreactor for the production of Terpinen-4-ol if introduced to means of plant biotechnology.

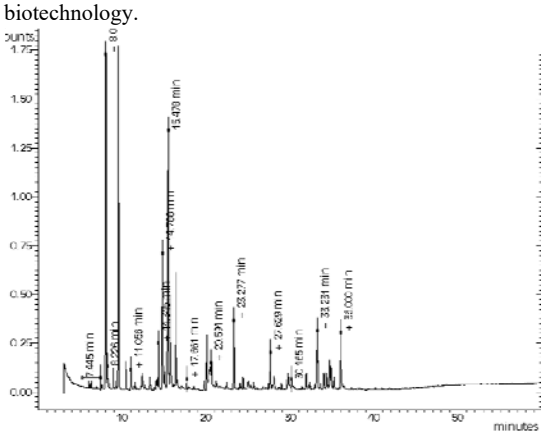

Figure2. Essential oil components of *C. montanus* as obtained by GC-MS chromatography.

Table 1. Components of essential oil extracted from *C. montanus* aerial parts.

NO.	Compound name	KI lit.	Content%	Classification group name
1.	Unknown	-	12.7	-
2.	2,5,5-trimethyl-3,6-heptadien-2-ol (Yomogi alcohol)	988	1.43	Monoterpenes alcohol
3.	α-Terpinene	1014	0.70	Monoterpenes hydrocarbons
4.	Eucalyptol (1,8Cineol)	1026	12.4	Oxygenated monoterpenes
5.	γ-Terpinene	1054	1.04	Monoterpenes hydrocarbons
6.	Unknown	1024	1.3	Monoterpenes hydrocarbons
7.	Trans-Sabinene hydrate	1098	1.51	Monoterpenes hydrocarbons
8.	Terpineol	1130	1.26	Oxygenated monoterpenes
9.	Neroloxide	1154	2.14	Oxygenated monoterpenes
10.	Lavandulol	1165	9.29	Monoterpenes alcohol
11.	Terpinen-4-ol	1174	23.3	Oxygenated monoterpenes
12.	Trans-Piperitol	1208	4.75	Monoterpenes alcohol
13.	Bornyl acetate	1287	2.4	Miscellaneous
14.	Chrysanthemumic acid	1296	3.82	Bronsted acid
15.	Neryl acetate	1359	2.9	Monoterpenes ester
16.	Unknown	-	0.53	-
17.	2-(2-butynyl)-cyclohexanone		1.78	Miscellaneous
18.	Germacerene D	1249	0.53	Oxygenated monoterpenes
19.	Delta cadinene	1522	1.78	Sesquiterpenes hydrocarbons
20.	Palustrol	1567	1.2	sesquiterpenes alcohol
21.	B-oplopenone	1607	0.73	Oxygenated monoterpenes
22.	Unknown	-	0.62	-
23.	Murrola-4,10(14) -dien-1-beta-ol	1630	1.73	sesquiterpenes hydrocarbons
24.	α-cadinol	1652	2.5	Sesquiterpenoid alcohol
25.	Torreyol	1645	1.12	Phenolic compound
26.	Apiol	1677	3.4	Phenolic compound

3.2. Antibacterial activity of C. montanus extract

3.2.1. Microdilution Assay

Obtained data showed that extract of *C. montanus* has inhibited growth in most selected types of bacteria (Table 2). Moreover, our results indicated that the growth inactivation effect of both extracts (methanolic and ethanolic) against some bacteria strains was auspicious as it was stronger than the results obtained in the control treatment (Tetracycline) (Table 2). For example, both methanolic or ethanolic extracts at a level of (0.39 mg/mL) were able to stop growth in *E. coli* bacteria, while Tetracycline concentration needed to be increased four times that of *C. montanus* extract (1.56 mg/mL) to stop *E. coli* growth (Table 2). This trend was also recorded in *Staphylococcus aureus* bacteria, while the effect of both extract types on the growth of *Bacillus subtilis* was similar to those obtained in the control (Table 2). On the other hand, higher levels of both extract types were needed to inhibit the growth of *Staphylococcus epidermidis, Erwinia carotovora, and Salmonella* sp. compared to the control, while both extract types were not able to prevent the development of *Klebsiella pneumoniae* at all levels (Table 2). Meanwhile, data indicated that the inhibitory effect of the methanolic extract of *C. montanus* was stronger than that obtained when ethanolic extract was administrated to *Staphylococcus epidermidis, Erwinia carotovora* and *Salmonella* sp. (Table 2). Extract type was reported in many research articles to affect extract efficiency against microbes. Eloff (1998) reported that the superiority of methanolic extract might be due to its polarity as it is higher than that in ethanol which would permit more active compounds to dissolve in the extract.

Table 2. Minimal Inhibition Concentration (MIC) of the extracts of *Chiliadenus montanus* (Vahl.) Brullo against selected strains of bacteria.

Bacteria strain	Methanolic extract (mg/mL)	Ethanolic extract (mg/mL)	Tetracycline (control: mg/mL)
E. coli	0.39	0.39	1.56
Staphylococcus aureus	0.78	0.78	1.56
Staphylococcus epidermidis	3.12	6.24	1.56
Klebsiella pneumoniae	-	-	3.12
Bacillus subtilis	1.56	1.56	1.56
Erwinia carotovora	6.24	12.48	3.12
Salmonella sp.	1.56	3.12	1.56

3.2.2. Disc Diffusion Assay:

Disc Diffusion Assay test revealed that both extract types of *Chiliadenus montanus* (Vahl.) Brullo were able to inhibit growth in six bacteria strains while no effect was recorded in *Klebsiella pneumoniae* (Table 3).The inhibitory power of the methanolic extract against the development of *E. coli* bacteria was more effective than the antibiotic (control) as it was able to record an inhibition zone diameter of (28.0 mm) compared to (24.0 mm) obtained in the control (Table 3). Meanwhile, administration of methanolic extract to *Staphylococcus aureus* was as efficient as the control as it recorded an inhibition zone diameter of (24.0 mm) (Table 3). Growth of the rest of the bacteria strains was inhibited in response to exposure of the methanolic extract but to a lesser extent than those recorded in the control treatment (Table 3). Moreover, growth was inhibited in most bacteria strains when treated with the ethanolic extract, while no effect was recorded in *Klebsiella pneumoniae* and *Erwinia carotovora* (Table 3). However, it was noticed from the results that methanolic extract was more effective than ethanolic extract which would confirm the findings recorded earlier in the microdilution assay experiment.

Our results showed that both extract types of *Chiliadenus montanus* (Vahl.) Brullo has promising antibacterial powers against the tested strains, which could be attributed to the presence of high amounts of oxygenated monoterpenes (40.36%) (Table 1). The oxygenated monoterpenes were reported to possess variable antibacterial powers depending on bacteria strain (Kotan et al., 2007). Moreover, our data revealed that Terpinen-4-ol was the major compound in the extract (23.3%) (Table 1). This oxygenated monoterpene was repeatedly reported in many research articles for its antibacterial, antifungal, antiviral, and anticancer potentials (Barra et al., 2007; Cha et al., 2007; Shapira et al., 2016). Zhang et al. (2018) explained the mechanism by which Terpinen-4-ol attacks bacteria, as they found that Terpinen-4-ol was able to damage cell membranes and to affect negatively the synthesis of protein and DNA in *Streptococcus agalactiae* bacteria. Additionally, Bordini et al. (2018) investigated the effect of Terpinen-4-ol on bacteria at the gene level, as they reported that Terpinen-4-ol was able to modulate the production of the gbpA and slpA genes which are responsible for the adherence and biofilm development in *Streptococcus mutans* and *Lactobacillus acidophilus*.

Table 3. Inhibition zone diameters (mm) recorded in Disc Diffusion Assay of the extracts of *Chiliadenus montanus* (Vahl.) Brullo against selected strains of bacteria.

Bacteria strain	Methanolic extract	Ethanolic extract
Tetracycline (control)	24.0± 1.04 ab	20.0± 1.09 a
E. coli	28.0± 2.28 a	22.0± 2.42 a
Staphylococcus aureus	24.0± 1.67 ab	21.0± 1.97 a
Staphylococcus epidermidis	12.0± 1.54 cd	4.0± 0.89 c
Klebsiella pneumoniae	0.0± 0.0 e	0.0± 0.0 d
Bacillus subtilis	18.0± 1.97 b	15.0± 1.45 ab
Erwinia carotovora	8.0± 0.84 d	0.0± 0.0 d
Salmonella sp.	15.0± 1.97 bc	10.0± 1.37 b

* Means that have different letters were found to be significantly different based on Tukey's HSD test at a probability level of 0.05. Means within each column were analyzed separately.

4. Conclusions

From our data, it might be concluded that the oxygenated monoterpenes which were found at premium levels in the essential oil of *C. montanus* in addition to their high content of Terpinen-4-ol (57.7% of total oxygenated monoterpenes) might have contributed to the distinguished antibacterial powers of the extracts. More attention should be paid to this valuable threatened plant, and more research needs to be done to improve the production of these curative active ingredients from *C. montanus* (Vahl.) Brullo growing wild in the Jordanian environment.

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