Phytochemical analysis, anticancer screening and antimicrobial activity of *Ballota undulata* Fresen (Benth.) from Al- Baha region Saudi Arabia

Khulud Mohammed Alshehri*

Department of Biology, AL-Baha University, Al Bahah 65527, Baljurashi, Saudi Arabia

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**Abstract**

Plants of medicinal value play vital roles in prevention of disease and their use and promotion is fitting into all current prevention strategies. Nevertheless, diligent effort is required to properly identify, recognize and locate medicinal plants in the design and implementation of these strategies. The medicinal plant *Ballota undulata* Fresen (Benth.) that belonging to family Lamiaceae has been reported to exhibit antispasmodic, anti-allergic, anti-inflammatory and antimicrobial properties. In this context, this work aimed to assess the biological activity of its three different extracts (chloroform, ethyl acetate and methanol) as antitumor against different cancer cell lines as hepatocellular (HEPG-2) carcinoma, breast (MCF-7) carcinoma and colon (HCT-116) carcinoma cell lines. Further determine its activity as antimicrobial against a diverse human pathogen (five Gram negative bacteria, two Gram positive bacteria and yeast) and its chemical composition using Gas Chromatography–Mass Spectrometry (GC-MS) analysis and HPLC. Our results showed that the different extracts of *B. undulata* have antitumor activity against tested cell lines. In addition, the chloroform and methanol extracts showed marked antimicrobial activity against the tested strains. Furthermore, the percentage of the MIC activity of *B. undulata* methanolic extract can help as sources for treatment compounds assay. So, this results of *B. undulata* shed the light on the antimicrobial ability of extracts from Saudi Arabia medicinal flora, which can be used as antimicrobial natural agents in pharmaceutical drugs and food preservation strategy.

*Corresponding Author

Name: Khulud Mohammed Alshehri  
Phone:  
Email: dr.k2015@hotmail.com

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**INTRODUCTION**

Cancer, which is known to be one of the deadliest diseases in the world, is estimated at about 10 million carcinomas globally, half of which are in the developing nations (Khalil *et al.*, 2015; Khafagi and Dewedar, 2000). The prevalence of this disease has a greater impact in underdeveloped countries. Recently, in many countries, cancer is the second leading cause of death after heart diseases (Kau *et al.*, 2005; Adamczak *et al.*, 2019). A combination of surgery or radiation with chemotherapy is generally used for the treatment of this disease, however, it is not excused from adverse effects or resistance of the tumor to this type of treatment, for this reason the search for new drugs is still continuous. The plants are a promising source to attain this. The use of complementary and alternative medicinal products as herbal medicinal products, expressed mainly by plants, ranges from 30 to 75% among cancer patients in many countries (Khafagi and Dewedar, 2000). This also supports the interest in searching
for anti-cancer agents derived from natural products from different countries particularly from the Arab Peninsula.

As an antimicrobial medicine are commonly and sometimes indiscriminately used, many microorganisms have developed resistance to different antibiotic treatments and these strains are particularly evident in the hospital setting. This has developed a major clinical issue in infectious diseases care. Furthermore, synthetic chemical antibiotics are often associated with adverse effects on host, including hypersensitivity, degradation of healthy intestines and mucous membranes, immuno suppression and allergic reactions (Al-Bakri and Afifi, 2007).

Various plants with alkaloids, polyphenols and volatile oils as active ingredients are used as traditional medicinal products, while others are more traditional as finished products known as phytomedicines. The growth of microbial antibiotic resistance in the second half of the 20th century led scientists to explore multiple antimicrobial activity in many medicinal plants as an alternative form of health care and traditional medicine (Kau et al., 2005). Plants bioactive components contain a variety of compounds (e.g. lignans, quinones, coumarins, tannins, flavones, catechins, phenolic acids, anthocyanins, proanthocyanins, and stilbenes) that may increase life expectancy and slow or inhibit the development of degenerative diseases (Al-Bakri and Afifi, 2007).

The world’s leading cause of death is infectious diseases. Worldwide, about 50,000 people die from infectious illnesses every day. Antimicrobials originated from plants have huge therapeutic potential. They are effective in infectious diseases treatment, while at the same time mitigating numerous side effects that are often related to synthetic antimicrobials. In modern drug production, the bioactive compounds produced from wild plants play a key role. The development of many human diseases is driven by oxidative damage. Generally, the redox enzymes of aerobic bacteria mediate oxidative phosphorylation with an oxygen molecule, they produce reactive oxygen species (ROS) which have a pathological role to play in different infections. Plants are well known for generating a large number of biologically active metabolites with highly unusual structures because of their distinctive environments, cultivation and physiological behavior (Abdallah et al., 2017). This promotes the opportunity to explore new class chemicals with the prospect of therapy. Several studies have reported that these metabolites exhibit various bioactivities, such as antimicrobial, antiviral, anti-inflammatory, and antimicrobial activity (Abdallah et al., 2017; Hsieh et al., 2001). However, for herbal medicine, natural derivatives from wild plants are used as therapeutically agents. It is a rich source of many medicines which are strong and effective.

Phytochemical studies are of great importance commercially for development of new herbal medicines by pharmaceutical companies. The growing use of pharmaceutical extracts shows further that a systematic study of medicinal plants is essential in order to identify active compounds (Majdi et al., 2020; Bader et al., 2003). One of these medicinal plants Ballota undulata which belonging to family Lamiaceae and has been reported to exhibit anti-allergic, antispasmodic, anti-inflammatory and antimicrobial properties (Bader et al., 2003). The genus Ballota comprises around 33 species growing mostly in the Arabian Peninsula and Mediterranean regions. Ballota undulata is a woody perennial herb with an ovate-cordate and reticulate leaves, distributed in Arabian Peninsula, mainly in high elevated regions and mountains of Saudi Arabia (Majdi et al., 2020; Siciliano et al., 2005).

Bedouins use this plant for the treatment of wounds, bee, wasp, and scorpion stings. B. undulata aqueous extract has been investigated in several literatures for antimalarial and antitumor activities. In addition, plants from family lamiaceae, which are common in the world, have historically been used for the treatment of various diseases and have recently undergone tests for anti-cancer activities and have been used as a source of anticancer drugs. Essential oils also act as anti-multidrug resistant agents, tumor reduction and metastases inhibition (Mesquita et al., 2019). Methods included in the essential oils-mediated antiproliferative activity comprise apoptosis, cell cycle arrest, and DNA repair mechanisms (Zingue, 2014).

This research focuses on extracting essential oils from B. undulata as a bioactive component and evaluating its biological activity as an antitumor against various cancer cell lines. Further, determine its activity as antimicrobial against a diverse human pathogen. The current research was carried out to track the different B. undulata plant sample extracts from Saudi Arabia flora in keeping with this international pattern. Phytochemical analysis, separation and identification of the plant extracts must be done.

MATERIALS AND METHODS

Chemicals and Kits
Chemicals and reagents were high analytical grade
namely Aldrich-Sigma Chemical (St. Louis MO, USA) & ADWIC, Egypt. Fetal Bovine serum, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza (Belgium).

**Plant collection and preparation of the extracts**

The fresh aerial parts of *B. undulata* were collected at its growth period of spring season from Wadi Turbah Zahrani, Albaha region, southwestern Saudi Arabia. The plant was air dried at lab-temperature till constant weight, then ground to fine powder and kept being used for different plant analysis. Two hundred grams of plant powder were successively extracted by soxhlet apparatus using different organic solvents with analytical reagent (AR) quality. These solvents were chloroform, ethyl acetate, and finally methanol for 10 h. Each extract collected separately into dry clean beakers, after that they were evaporated under reduced pressure using rotavapor apparatus at 60 °C, then were dried in desiccators for 1 h and finally all the dried residues were stored in refrigerator at 5 ºC until the use.

**In vitro Cytotoxic Activity by MTT assay**

**Mammalian cell lines**

HepG-2 cells (human hepatocellular carcinoma cell line), HCT-116 cells (human colon carcinoma cell line) and MCF-7 cells (human breast carcinoma cell line) were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

**Cell line Propagation**

The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50μg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were sub-cultured two to three times a week.

**Cytotoxicity evaluation using viability assay**

The antitumor activity for different extracts and Cisplatin drug as positive control evaluated according to the method described by (Mosmann, 1983). By MTT assay the number of viable cells were determined and the percentage of viability was calculated as [(ODt/ODc)] x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The survival curve of each tumor cell line after treatment with the specified drug was plotted from the relation between surviving cells and drug concentration. By GraphPad Prism software (San Diego, CA, USA) the 50% inhibitory concentration (IC₅₀) was estimated from graphic plots of the dose response curve for each concentration.

**Antimicrobial activity assays**

**Bacterial and fungal pathogens**

**Five Gram negative bacteria**

*Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella typhi*, two Gram positive bacteria: *Streptococcus mutans*, *Staphylococcus aureus*, and yeast: *Candida albicans*, were isolated from microbiological lab of King Khaled Hospital, Al- Baha region, Saudi Arabia.

**Culture media**

Nutrient broth (NB) and agar (NA) media for both Gram-negative, Gram-positive bacteria Mueller-Hinton broth & agar medium (MHB & MHA) and potato dextrose agar (PDA) for yeast fungi. All media were purchased from (Sigma-Aldrich) California, USA. Gentamicin 32 μg as antibacterial and Fluconazole 32 μg as antifungal were purchased from (Sigma-Aldrich) California, USA. Incubation period for all bacteria was at 37°C for 18 hours and for fungi at 28°C for 48 hours.

**Antibacterial Activity Assay**

The antibacterial activity of all plant extracts was determined by disk diffusion method (Balouiri et al., 2016). Antibiotic Gentamicin was used as positive control and sterilized Miller Hinton broth were used as negative controls.

**Antifungal Activity assay**

The same for antibacterial activity assay except that the medium used is PDA and the incubation period is 48 hr. and using the fluconazole as a positive antifungal antibiotic control.

**Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and the Minimum Fungicidal Concentration (MFC)**

MIC, MBC and MFC of the all studied extracts were carried out according to Murray et al., (Balouiri et al., 2016; Murray, 1999) using modified Broth dilution assay with the help of Spectrophotometer at 595 nm in mg/ml.

Minimum Fungicidal Concentration (MFC) were done according to Mosmaan (Mosmann, 1983) by using *Candida albicans* as a pathogen and Fluconazole 32 μg antifungal as positive control, on PDA medium.

**Chemical Composition Evaluation**

**Gas Chromatography–Mass Spectrometry (GC-MS) analysis**

The GC-MS analysis of various crude extracts were performed using Trace GC-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct
capillary column TG–5MS (30 m x 0.25 mm x 0.25 \(\mu\)m film thickness) with the same condition as recorded by (Hashmi et al., 2013). The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral database.

**Qualitative Determination of Flavonoids and phenolics Using HPLC**

High performance liquid chromatography (HPLC) technique using Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector, set at flow 1 ml/min. Autosampler, degaser, column compartment set at 35°C and variable wavelength detector set at 280 nm, column: Hypersil C18 thermo 5\(\mu\)m, 250x4.6 mm was used and Buffer (0.1 % phosphoric acid in water) and Methanol as the mobile phase. We use 8 different standards were Kampleturn 0.4mg/ml, Gallic acid 1.2 mg/ml, Ellagic acid 0.4mg/ml, Chlorogenic acid 0.7mg/ml, Catechin 0.7mg/ml, Quercitin 0.3mg/ml, caffeic acid 1mg/ml and rutin 1mg/ml.

**Statistical analysis**

The results were analyzed using two-way analysis of variance (ANOVA). All statistical investigations were carried out using SPSS 18.0 software. The findings were reported as standard error (SE) ± of three replicates, and statistical significance was set as \(p\) value≤0.05.

**RESULTS AND DISCUSSION**

**Cytotoxic activity**

Our results showed that, the chloroform extract of *B. undulata* achieved cytotoxic effect against HEPG2, MCF7, HCT 116 With (IC\(_{50}\)=76.2, 113.9 and 93.6 \(\mu\)g/ ml, respectively), ethyl acetate extract was (IC\(_{50}\)=91.7, 188.4 and 101.8 \(\mu\)g/ ml, respectively) and methanol extract was (IC\(_{50}\)=101.2, 148.1 and 120.9 \(\mu\)g / ml, respectively) all these data were recorded at Table 1 and illustrated at Figures 1, 2 and 3 with cisplatin as control. The results of the previous protocols of the American National Cancer Institute NCI (Boyd, 1997) expressed strong when IC\(_{50}\) less than 20 \(\mu\)g/ml and moderate activities when IC\(_{50}\) 21-50 \(\mu\)g/ml. So our plant showed weak cytotoxic activity against HEPG2, MCF7, HCT 116 cell lines.

**Antimicrobial assay of Ballota undulata (Sieb. ex Fres.)**

The various extracts (chloroform, ethyl acetate, and methanol) of *B. undulata* shown antibacterial and an antifungal activity toward the selected strains as following (Gram-negative bacteria: *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Salmonella typhi*, Gram-positive bacteria: *Streptococcus mutans*, *Staphylococcus aureus*, and fungi: *Candida albicans*) as presented in Table 2.

**Minimum inhibitory concentration and minimum bactericidal concentration assay**

Minimum inhibition concentration and minimum bactericidal concentration (MIC and MBC) of various extracts of *Ballota undulata* (Sieb. ex Fres.), are registered in the Tables 3, 4 and 5. *B. undulata*, is a genus of family lamiaceae and responsible for a diverse mode of action, however the bioactivities of this plant have not yet been exploited before in Arabian Peninsula. The collected wild plants were extracted by (chloroform, ethyl acetate and methanol) exhibit powerful antimicrobial action more than the antagonistic values of chloroform and ethyl acetate. From our results and the MIC values indicated that the methanol fraction was reported as a significant activity toward tested microorganisms lacking for *C. albicans* including *E. coli*, *Klebsiella pneumoniae*, *S. aureus*, and *S. mutans* both of which are important for human health. This finding like the results documented by Majdi et. al. (Majdi et al., 2020), where the important activity against *S. mutans* 28 mm (inhibition zone) and lesser activity on *P. mirabilis* 9 mm. Chloroform extract shows moderate action on tested strains, extract gives high effect on *S. aureus* about 25 mm, *E. coli* 17 mm, and *K. pneumoniae* 13 mm diameter of inhibition zone respectively. On the other hands *P. mirabilis*, and *P. aeruginosa* exhibiting resistance to chloroform extract. Ethyl acetate extract giving the below activity on examined microorganisms, where the greatest effect of the extract on *S. aureus* 20 mm of clear zone, while *P. mirabilis*, *P. aeruginosa*, and *S. typhi* exhibit insensitivity for extract (no inhibition zone). *S. mutans*, *S. aureus*, and *E. coli* have not resistance for all extracts and on another hand, *P. mirabilis* has no sensitivity for all extracts. All examined isolates show stability for standard antibiotics as recorded in a Table 2.

Methanol and chloroform extracts show the high significant antagonistic results of MIC and MBC activity, while ethyl acetate extract gives the weakest results of MIC and MBC action. *S. aureus* and *S. mutans* recorded the highest degree of MIC and MBC action each, while *P. mirabilis* shows the lower value of MIC and MBC in ethyl acetate extract as recorded in a Tables 3, 4 and 5. This disagree with Khalil et al. (Khalil et al., 2009) who noted that *B. undulata* has some medical uses, as, Antioxidant, diuretic, hemostatic, and ethanolic extract has

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**References**

- Hashmi et al., 2013
- Boyd, 1997
- Khalil et al., 2009
Figure 1: Cytotoxic effect of successive extract of \textit{B. undulata} plant and Cisplatin on human hepatocellular carcinoma cell line (HepG-2)

Figure 2: Cytotoxic effect of different extract of \textit{B. undulata} plant and Cisplatin against human breast carcinoma cell line (MCF-7)

Figure 3: Cytotoxic effect of different extract of \textit{B. undulata} plant and Cisplatin against human colon carcinoma cell line (HCT-116)
Figure 4: GC-MS analysis of chloroform extract of *B. undulata* plant

Figure 5: GC-MS analysis of Ethyl acetate extract of *B. undulata* plant

Figure 6: GC-MS analysis of Methanol extract of *B. undulata* plant
Table 1: Cytotoxic activity of successive extracts of *Ballota undulata* plant against HepG-2, MCF-7 and HCT-116 cell lines

| No | Extract  | IC$_{50}$ (µg/mL) | HepG-2 | MCF-7 | HCT-116 |
|----|----------|--------------------|--------|-------|---------|
| 1  | Chlorofom| 76.2 ± 3.6         | 113.9 ± 6.3 | 93.6 ± 3.3 |
| 2  | Ethyl acetate | 91.7 ± 9.1      | 188.4 ± 6.9 | 101.8 ± 4.9 |
| 3  | Methanol | 101.2 ± 4.8        | 148.1 ± 5.7 | 120.9 ± 5.3 |
| 4  | Cisplatin | 3.68 ± 0.19        | 5.71 ± 0.53 | 4.51 ± 0.72 |
Figure 9: HPLC chromatogram of methanol extract of *B. undulata* plant

| Test microorganisms     | Diameter of inhibition zone of different extracts (mm) | Diameter of inhibition zone of control (antibiotics (mm)) |
|-------------------------|--------------------------------------------------------|----------------------------------------------------------|
|                         | Chloroform Ethyl acetate Methanol                     | Gentamicin Fluconazole 32 µg 32 µg                       |
| **Gram-negative bacteria** |                                         |                                                          |
| Proteus mirabilis       | NA NA 9.0 ± 0.3                                       | 20 ± 1.0 —                                               |
| Klebsiella pneumoniae   | 13 ± 0.3 12 ± 0.4 10 ± 0.2                           | 18 ± 1.3 —                                               |
| Escherichia coli        | 17 ± 0.5 11 ± 0.3 12 ± 0.3                           | 22 ± 1.1 —                                               |
| Pseudomonas aeruginosa  | NA NA 15 ± 0.3                                       | 20 ± 1.0 —                                               |
| Salmonella typhi        | 9 ± 0.2 NA 13 ± 0.4                                   | 19 ± 1.0 —                                               |
| **Gram-positive bacteria** |                                         |                                                          |
| Streptococcus mutans    | 9 ± 0.2 9 ± 0.2 28 ± 1.2                              | 22 ± 0.8 —                                               |
| Staphylococcus aureus   | 25 ± 1.0 20 ± 0.8 15 ± 0.6                            | 20 ± 1.2 —                                               |
| **Fungi**               |                                         |                                                          |
| Candida albicans        | 10 ± 0.4 NA —                                       | 20 ± 0.9 —                                               |

NA:no activity, ± SD; (Diameter on inhibition zone including well diameter of 6mm)
Table 3: Minimum Inhibition Concentration and Minimum Bactericidal Concentration of chloroform extract of *Ballota undulata*

| Test microorganisms          | MIC $\mu$g/ml | MBC $\mu$g/ml | MFC $\mu$g/ml |
|------------------------------|---------------|---------------|---------------|
| **Gram-negative bacteria**   |               |               |               |
| *Proteus mirabilis*          | NA            | NA            |               |
| *Klebsiella pneumoniae*      | 500 ± 0.05    | 500 ± 0.05    |               |
| *Escherichia coli*           | 125 ± 0.04    | 250 ± 0.06    |               |
| *Pseudomonas aeruginosa*     | NA            | NA            |               |
| *Salmonella typhi*           | 1000 ± 0.08   | 1000 ± 0.08   |               |
| **Gram -positive bacteria**  |               |               |               |
| *Streptococcus mutans*       |               |               |               |
| *Staphylococcus aureus*      | 75 ± 0.03     | 100 ± 0.03    |               |
| **Yeast**                    |               |               |               |
| *Candida albicans*           | 1000 ± 0.09   | 1000 ± 0.09   | 1000 ± 0.09   |

NA: no activity, ± SD; (Diameter on inhibition zone including well diameter of 6mm)

Table 4: Minimum Inhibition Concentration and Minimum Bactericidal Concentration of ethyl acetate extract of *Ballota undulata*

| Test microorganisms          | MIC $\mu$g/ml | MBC $\mu$g/ml | MFC $\mu$g/ml |
|------------------------------|---------------|---------------|---------------|
| **Gram-negative bacteria**   |               |               |               |
| *Proteus mirabilis*          | NA            | NA            |               |
| *Klebsiella pneumoniae*      | 1000 ± 0.07   | 1000 ± 0.07   | —             |
| *Escherichia coli*           | 250 ± 0.05    | 250 ± 0.06    | —             |
| *Pseudomonas aeruginosa*     | NA            | NA            | —             |
| *Salmonella typhi*           | NA            | NA            | —             |
| **Gram-positive bacteria**   |               |               |               |
| *Streptococcus mutans*       |               |               |               |
| *Staphylococcus aureus*      | 100 ± 0.04    | 150 ± 0.04    | —             |
| **Yeast**                    |               |               |               |
| *Candida albicans*           | 1000 ± 0.07   | 1000 ± 0.07   | 1000 ± 0.07   |

NA: no activity, ± SD; (Diameter on inhibition zone including well diameter of 6mm)

Table 5: Minimum Inhibition Concentration and Minimum Bactericidal Concentration of Methanol extract of *Ballota undulata*

| Test microorganisms          | MIC $\mu$g/ml | MBC $\mu$g/ml | MFC $\mu$g/ml |
|------------------------------|---------------|---------------|---------------|
| **Gram-negative bacteria**   |               |               |               |
| *Proteus mirabilis*          | 500 ± 0.03    | 600 ± 0.05    |               |
| *Klebsiella pneumoniae*      | 500 ± 0.05    | 500 ± 0.05    |               |
| *Escherichia coli*           | 500 ± 0.04    | 1000 ± 0.09   |               |
| *Pseudomonas aeruginosa*     | 250 ± 0.05    | 500 ± 0.05    |               |
| *Salmonella typhi*           | 300 ± 0.05    | 500 ± 0.05    |               |
| **Gram -positive bacteria**  |               |               |               |
| *Streptococcus mutans*       | 50 ± 0.02     | 150 ± 0.03    |               |
| *Staphylococcus aureus*      | 125 ± 0.04    | 250 ± 0.04    |               |
| **Yeast**                    |               |               |               |
| *Candida albicans*           | NA            | NA            | NA            |

NA: no activity, ± SD; (Diameter on inhibition zone including well diameter of 6mm)
Table 6: Chemical composition of chloroform extract of *Ballota undulata* plant by GC-MS

| Peak N. | R.T | Peak area (%) | Compound name | Formula | MF |
|--------|-----|--------------|---------------|---------|----|
| 1      | 21.27 | 0.98 | (-)-Spathulenol | C15H24O | 928 |
| 2      | 23.07 | 6.87 | 2-Furanmethanol, tetrahydro-à,à,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-, | [2S-[2à,5à(R*)]]-Nerolidol-Epoxyacetate | C17H28O4 | 777 |
| 3      | 23.18 | 1.00 | (S)-2,2,6-Trimethyl-6-((S)-4-methylcyclohex-3-en-1-yl)dihydro-2H-pyran-3(4H)-one | C15H24O2 | 935 |
| 4      | 23.70 | 5.17 | 2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, | | C15H26O2 | 955 |
| 5      | 25.11 | 83.77 | (Z)-2-(Hexa-2,4-diyn-1-ylidene)-1,6-dioxaspiro[4.4]non-3-ene | | C13H12O2 | 940 |
| 6      | 27.89 | 2.22 | Bicyclo[4.1.0]heptan-2-ol, 1à-(3-methyl-1,3-butadienyl)-2à,6à-dimethyl-3à-acetoxy- | | C16H24O3 | 759 |

Potent antibacterial activity against gram-negative bacteria: *P. aeruginosa* (about 19 mm diameter of inhibition zone), while no activity of extract toward gram-positive bacteria: *S. aureus*, and fungi: *C. albicans*. They recorded that MIC values from 80 ppm against *P. aeruginosa* and this result similar to our results for methanol extract. From a literature review found that *B. undulata* exhibit antioxidant activity which may be recommended for antimicrobial activity (Siciliano *et al.*, 2005). Also, Al-Bakri and Afifi (Al-Bakri and Afifi, 2007) published that antimicrobial activity using rapid XTT colorimetry of *B. undulata* collected from Jordan against selected microbes (*P. aeruginosa* (ATCC 9027), *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538P), and *B. subtilis* (ATCC 6633) and recommended that ethanolic extract show strong antimicrobial activity on *S. aureus* and *B. subtilis*, while *E. coli* show the weakest sensitivity, on the other hand, *P. aeruginosa* was resistant for ethanolic extract of *B. undulata* and these results match with our results (Al-Bakri and Afifi, 2007). On another hand, Khafagi and Dewedar (Khafagi and Dewedar, 2000) recorded that various extracts (hexane, ethyl acetate, and ethanol) of *B. undulata* (collected from Sinai, Egypt) have not any antimicrobial activity on tested microorganisms either Gram-positive bacteria: *Bacillus subtilis, Staphylococcus aureus*, or Gram-negative bacteria: *Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris*, and fungi: *Candida albicans, Microsporum canis*, and *Trichophyton mentagrophytes*. This may be due to antimicrobial activity is related to the presence of different secondary metabolites; alkaloids, phenolics and terpenoids reported or identified in these plant species and the habitat affects the production and activity of these metabolites (Khafagi and Dewedar,
Table 7: Chemical composition of ethyl acetat extract of *Ballota undulata* plant by GC-MS

| Peak N. | R.T  | Peak area (%) | Compound name                                           | Formula  | MF  |
|--------|------|---------------|---------------------------------------------------------|----------|-----|
| 1      | 4.01 | 0.16          | 2H-pentaleno[1,6-BC]furan-2-one, 2A,4A,5,6,6A,6B-hexahydro,   | C₉H₁₀O₂  | 913 |
| 2      | 4.15 | 0.50          | 1-Butanol, 3-methyl-acetate                               | C₇H₁₄O₂  | 869 |
| 3      | 4.30 | 0.83          | Isopentyl propionate                                     | C₈H₁₄O₂  | 798 |
| 4      | 4.41 | 2.34          | 1-butanol, 3-methyl-acetate                               | C₇H₁₄O₂  | 853 |
| 5      | 4.59 | 0.50          | Antipain                                                | C₂₅H₄₄N₁₀O₆ | 721 |
| 6      | 4.83 | 1.88          | 1-Ethoxypropan-2-yl acetate                              | C₇H₁₄O₃  | 871 |
| 7      | 5.18 | 1.35          | Benzene, propyl-                                         | C₉H₁₂     | 897 |
| 8      | 5.36 | 7.48          | Benzene, 1-ethyl-3-methyl-                               | C₉H₁₂     | 943 |
| 9      | 5.50 | 1.82          | Benzene, 1,3,5-trimethyl-                                | C₉H₁₂     | 931 |
| 10     | 6.07 | 3.37          | Benzene, 1,2,3-trimethyl-                                | C₉H₁₂     | 935 |
| 11     | 6.17 | 36.46         | Butanoic acid, butyl ester                              | C₈H₁₆O₂  | 942 |
| 12     | 6.60 | 1.95          | Acetic acid,hexyl ester                                  | C₈H₁₆O₂  | 877 |
| 13     | 6.76 | 1.07          | 2(3H)-Furanone, dihydro-5-methyl-5-phenyl                | C₁₁H₁₂O₂  | 801 |
| 14     | 7.32 | 0.56          | Acetic acid, 5-methylhex-2-y1 ester                      | C₉H₁₆O₂  | 768 |
| 15     | 7.50 | 0.66          | 10,12-Octadecadiynoic acid                               | C₁₅H₂₈O₂  | 687 |
| 16     | 8.82 | 2.97          | Undecane                                                | C₁₁H₂₄    | 935 |
| 17     | 11.03| 0.64          | 9-Oxa-10 thiatrcyclo[3.3.1.1(2,7)]decan-6-ol             | C₈H₁₃O₃S  | 691 |
| 18     | 23.07| 1.94          | 2-Furanmethanol,tetrahydro-à,à,5-trimethyl-5-{(4-methyl-3-cyclohexen-1-y1)-[2S[2à,5à(R*)]]}-   | C₁₅H₂₀O₂  | 685 |
| 19     | 23.70| 0.88          | (S)-2,2,6-Trimethyl-6-((S)-4methylcyclohex-3-en-1-y1)dihydro-2H-pyran-3(4H)-one | C₁₅H₂₄O₂  | 825 |
| 20     | 25.07| 15.96         | 2H-Pyran-3-ol,tetrahydro-2,2,6-trimethyl-6-((4-methyl-3-cyclohexen-1-y1)-, [3S[3à,6à(R*)]]-Diisoocty phthalate | C₁₅H₂₆O₂  | 900 |
| 21     | 39.72| 16.69         |                                                          | C₂₄H₃₈O₄  | 964 |
### Table 8: Chemical composition of Methanol extract of *Ballota undulata* plant by GC-MS

| Peak N | R.T  | Peak area (%) | Compound name                                      | Formula   | MF  |
|--------|------|---------------|----------------------------------------------------|-----------|-----|
| 1      | 4.08 | 4.12          | p-Xylene                                          | C₆H₁₀     | 957 |
| 2      | 4.64 | 0.57          | Cumol                                              | C₆H₁₂     | 884 |
| 3      | 4.77 | 0.21          | Tridecanoic acid, 3-methyl-, methyl ester          | C₁₃H₃₃O₂  | 683 |
| 4      | 5.21 | 0.53          | Benzene, propyl-                                  | C₆H₁₂     | 933 |
| 5      | 5.39 | 20.28         | Benzene, 1-ethyl-3-methyl-                         | C₆H₁₂     | 970 |
| 6      | 6.08 | 17.52         | Benzene, 1,2,3-trimethyl-                          | C₆H₁₂     | 944 |
| 7      | 6.46 | 0.36          | Cyclohexane, 1,2,4-tris(methylene)-                | C₆H₁₂     | 753 |
| 8      | 6.80 | 3.67          | Benzene, 1,2,4-trimethyl-                          | C₆H₁₂     | 928 |
| 9      | 7.12 | 0.48          | Benzene, 2-propenyl-                              | C₆H₁₀     | 908 |
| 10     | 7.54 | 1.37          | Benzene, 1-methyl-3-propyl-                        | C₁₀H₁₄    | 912 |
| 11     | 7.65 | 0.78          | Benzene, 1,3-diethyl-                              | C₁₂H₁₄    | 883 |
| 12     | 7.73 | 1.09          | Benzene, 2-ethyl-1,4-dimethyl-                     | C₁₀H₁₄    | 924 |
| 13     | 7.93 | 0.31          | Benzene, 1-methyl-2-propyl-                        | C₁₀H₁₄    | 863 |
| 14     | 8.21 | 1.39          | Benzene, 2-ethyl-1,4-dimethyl-                     | C₁₀H₁₄    | 922 |
| 15     | 8.41 | 1.29          | o-Cymene                                           | C₁₀H₁₄    | 924 |
| 16     | 8.84 | 1.26          | Undecane                                           | C₁₁H₂₄    | 904 |
| 17     | 8.96 | 0.29          | Benzene, 1-ethyl-2,3-dimethyl-                     | C₁₀H₁₄    | 862 |
| 18     | 9.22 | 0.73          | Benzene, 1,2,3,4-tetramethyl-                      | C₁₀H₁₄    | 903 |
| 19     | 9.33 | 1.09          | Benzene, 1,2,4,5-tetramethyl-                      | C₁₀H₁₄    | 908 |
| 20     | 10.18| 0.27          | 6,7-Dimethyl-3,5,8,8a-tetrahydro-1H-2-benzopyran   | C₁₁H₁₆O   | 891 |
| 21     | 11.57| 0.37          | Dodecane                                           | C₁₂H₂₀    | 788 |
| 22     | 16.93| 0.45          | Tetradecane                                        | C₁₄H₃₀    | 912 |
| 23     | 19.44| 0.38          | Tetradecane, 2,6,10-trimethyl-                     | C₁₄H₃₀    | 793 |
| 24     | 21.83| 0.87          | Nonadecane                                         | C₁₅H₄₀    | 937 |
| 25     | 23.07| 0.30          | 2-Furanmethanol, tetrahydro-à,à,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-, [2S-[2à,5á(R*)]]- | C₁₅H₂₀O₂  | 840 |
| 26     | 24.10| 0.87          | 1-Chlorooctadecane                                 | C₁₈H₃₇Cl  | 804 |
| 27     | 25.07| 2.19          | 2H-Pyran-3-ol,tetrahydro-2,2,6,trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, [3S[3à,6à(R*)]]- | C₁₅H₂₀O₂  | 892 |
| 28     | 26.27| 1.53          | Octadecane                                         | C₁₈H₃₈    | 944 |
| 29     | 27.08| 0.25          | Neophytadiene                                      | C₁₈H₃₈    | 907 |
| 30     | 28.87| 4.67          | Hexadecanoic acid, methyl ester                    | C₁₇H₃₄O₂  | 951 |
| 31     | 29.73| 0.42          | Dotriacontane                                      | C₂₀H₆₀    | 720 |
| 32     | 29.87| 0.17          | Erucic acid                                        | C₂₂H₄₂O₂  | 702 |
| 33     | 30.30| 3.97          | Triacontane                                        | C₂₀H₆₂    | 917 |
| 34     | 32.08| 2.06          | Ethyl (9Z,12Z)-9,12-octadecadienoate               | C₁₈H₃₆O₂  | 898 |
| 35     | 32.22| 17.78         | 9-Octadecenoic acid (Z)-, methyl ester             | C₁₈H₃₆O₂  | 941 |
| 36     | 32.70| 0.60          | Octadecenoic acid, methyl ester                    | C₂₀H₃₈O₂  | 781 |
| 37     | 33.73| 1.82          | Nerolidyl acetate                                  | C₁₇H₂₈O₂  | 798 |
| 38     | 35.72| 0.53          | Heptacosane                                        | C₂₀H₆₆    | 802 |
| 39     | 37.38| 1.90          | Docosane                                           | C₂₀H₄₆    | 837 |
| 40     | 39.73| 0.31          | 4H-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- | C₁₈H₁₀O₇  | 698 |
| 41     | 40.53| 0.92          | Dotriacontane                                      | C₃₂H₆₆    | 770 |
Table 9: Qualitative analysis of flavonoids and phenolic of different extracts of Ballota undulata by HPLC

| NO. | Compounds       | Chloroform |   |   |   | Ethyl acetate |   |   |   | Methanol |   |   |
|-----|-----------------|------------|---|---|---|----------------|---|---|---|----------|---|---|
|     |                 | RT.        | Area % | RT. | Area % | RT. | Area % | RT. | Area % |
| 1   | Kampferol       | 48.586     | 100.00 | 48.651 | 12.29 | -   | -     | 26.120 | 63.26 |
| 2   | Quercitin       | -          | -      | 44.741 | 1.45  | -   | -     | -   | -      |
| 3   | Rutin           | -          | -      | 38.466 | 2.15  | -   | -     | -   | -      |
| 4   | Catechin        | -          | -      | -     | -     | -   | -     | -   | -      |
| 5   | Gallic acid     | -          | -      | -     | -     | -   | -     | -   | -      |
| 6   | Ellagic acid    | -          | -      | -     | -     | -   | -     | -   | -      |
| 7   | Chlorogenic acid| -          | -      | -     | -     | -   | -     | 26.120 | 63.26 |
| 8   | Caffeic acid    | -          | -      | 28.961 | 83.81 | 28.493 | 36.74 | -   | -      |

The antimicrobial activity of the tested plant extracts (chloroform & ethyl acetate) against the *P. mirabilis* S. typhi, *P. aeruginosa* was either low or inactive. In general, Gram negative bacteria show higher resistance towards antimicrobial agents of *B. undulata* extracts, and this result compatible with the results obtained by Majdi et al (Majdi et al., 2020).

The chemical constituents in *B. undulata* was qualitatively and quantitatively detected by GC-MS and HPLC.

GC-MS analysis of the chloroform extract revealed the presence of 7 compounds (Figure 4 and Table 6) the major components was 2H-Pyran-3-ol, tetrahydro – 2,2,6- trimethyl -6-(4-methyl-3-cyclohexen-1-yl)-, [3S-[3α, 6α (R*)]] (83.77%). GC-MS analysis of the ethyl acetate extract revealed the presence of 21 compounds (Figure 5 and Table 7) the major components were Butanoic acid, butyl ester (36.46%), Diisooctyl phthalate (16.69%) and 2H-Pyran-3-ol,tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, [3S [3α, 6α (R*)]]- (15.96%), while GC-MS analysis of the methanol extract revealed the presence of 41 compounds (Figure 6 and Table 4) the major components were Benzene, 1-ethyl-3-methyl- (20.28 %), 9-Octadecenoic acid (Z)-, methyl ester (17.78%) and Benzene, 1,2,3-trimethyl- (17.52%).

Luteolin 7-O-glucoside, apigenin 7-O-glucoside, and rutin were isolated from *B. undulata* by majdi et al (Majdi et al., 2020) and the composition of its essential oil has been reported recently. Further Sesquiterpenes were the main constituents, which caused the most antiproliferative essential oil against HepG2 cells with high percentage of inhibition. However, 22 compounds which represent more than 98% of the total essential oil were extracted and evaluated as strong to moderate antimicrobial agent to treat infections and as food preserver in many cases. These compounds are four phenylpropanoids, forsythoside B1, lysionotoside 2, verbascoside 3, betonyoside F4, an iridoid, verminoside 5, seven flavonoids, compounds 6-12, and two betaine derivatives 13 and 14 were isolated from the aerial parts of *B. undulata* (Mesquita et al., 2019). Several compounds have been reported in *Ballota* sps., including terpenes, flavonoids and tannins (Khalil et al., 2009). The chemical composition of the essential oil of *B. nigra* and *B. undulata* shows some similarities: monoterpenes are little represented, while sesquiterpenes are present in large amounts. In both cases germacrene D is the main compound. Previous phytochemical studies on the genus *Ballota* evidenced the presence of labdane diterpenoids, flavonoids, and phenylpropanoids (Rigano et al., 2017). The aqueous extract of *B. undulata* has different pharmacological properties with a diverse bioactivity as antitumor and antimalarial activities. *Ballota* species have been widely used in traditional medicine as sedative, antispasmodic, diuretic, choleretic, and ant hemorrhoidal agents (Mesquita et al., 2019; Rigano et al., 2017). Literature surveys suggest the important role played by plant-based drugs in treating infectious diseases (Khalil et al., 2009). Due to the diversity and complexity of natural phenolic compounds, it is difficult to characterize every compound present in the crude extract to elucidate its structure (Zou et al., 2015), qualitative estimation for some phenolic and flavonoids compounds for different successive extract of *B. undulata* was observed at Table 8 by HPLC. The chloroform extract contains kaempferol (Figure 7), the ethyl acetate extract contains caffeic acid, kaempferol, rutin and...
quercetin (Figure 8) and the methanolic extract contain chlorogenic acid and Caffeic acid (Figure 9).

**CONCLUSIONS**

Our study showed that all different extracts of *B. undulata* possess weak cytotoxic activity against different three cell line using MTT assay in addition, the antimicrobial activity of methanol and chloroform of *B. undulata* shown the most important value of action on most of the tested strains. Furthermore, the percentage of the MIC activity of *B. undulata* methanolic extract can help as sources for treatment compounds assay.

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**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

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