LC-MS/MS profiling, antibiofilm, antimicrobial and bacterial growth kinetic studies of *Pluchea dioscoridis* extracts

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ABSTRACT

The therapeutical applications of ornamental plants have been categorized to be of a great effectiveness in multiple industries from ancient times until present days. *Pluchea dioscoridis* is widely known Egyptian wooden plant that has been extensively applied for different medicinal purposes. In this study, LC-ESI-MS/MS analysis of the potent antimicrobial ethyl acetate and *n*-butanol extracts of *P. dioscoridis* leaves led to identification of 28 and 21 compounds, respectively. The identified compounds were categorized as phenolic acids, phenolic acids derivatives, organic acids, flavonoids (aglycones and glycosides), secoiridoids, coumarin derivatives, and gallotannins derivatives. Among them, caffeic acid 3-sulfate was the most predominate in the investigated extracts followed by ferulic acid and dicaffeoyl-quinic acid. Also, the antimicrobial potentiality of different extracts was evaluated against different pathogenic microbes including *Enterobacter cloacae*, *Micrococcus leutus*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Clostridium* species. Furthermore, different concentrations of the most potent extract were assayed for antibacterial efficacy on growth curve kinetics against the susceptible bacteria along 4 days incubation period. Our gathered data confirmed that, the antimicrobial activity against tested bacteria was different according to the solvent used in the extraction process. Mostly, all the extracts showed a wide spectrum antibacterial activity except the plant water extract which shows a mild activity against *Clostridium* sp. only. Based on the highest inhibition zone diameter, the ethyl acetate extract followed by butanol extract exhibited the highest inhibition zone with *Micrococcus luteus* and *B. subtilis* (20.0 and 18.5 mm) respectively. Determining the effect of ethyl acetate extract at different concentration (0, 0.66, 1.66, 3.33, 6.67, 13.34 and 20.01 mg mL⁻¹) on *M. luteus* growth kinetics, the data assured that the antibacterial activity shows concentration dependent manner with the highest antibacterial activity at 20.01 mg mL⁻¹ culture. The data also confirmed that, none of the selected concentration showed bactericidal activity in the prepared cultures, and with the prolonged incubation period the bacteria acquire resistance against the extract beginning from second or third day of incubation.

KEYWORDS

*Pluchea dioscoridis*, LC-ESI-MS/MS, polyphenolics, bacterial growth kinetic, antibiofilm, antimicrobial

INTRODUCTION

Recently, the questions raising the existence of a link between globalization, urbanization and human health, is inevitably presented especially related to the infectious disease’s transmission and prevalence. Infectious diseases could be air, water or food borne which usually
resulted in the dissemination of several biological agents such as pathogenic bacteria, fungi, viruses and protozoa [1]. Microbial infection had been the main cause of different diseases as chronic and acute gastrointestinal, respiratory and auto-immune diseases. Microbial pathogens that have deleterious effects on public health include but not limited to, bacteria such as *Escherichia coli* O157:H7, *Campylobacter* spp., *Clostridium botulinum*, *Salmonella* spp., all of which cause hundreds of thousands of infections annually, reported by Hassanain et al. (2021) [1]. Not to mention, the emerging of worse scenario related to multi-drug resistance in various bacterial pathogens, such as *Enterobacteriaceae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* sp. etc. . . [2]. The wide application of synthetic antibiotics as antimicrobial substances caused resistance behavior among the microbial communities which in turn raises the need to find alternatives or substitutes with less possible harmful effects to be tolerated with the human cells [3]. Moreover, the bacterial biofilm formation has been found to play a critical role in the persistence of bacterial nosocomial infections. This phenomenon facilitates bacterial colonization on living or non-living surfaces and is associated with 65–80% of all clinical infections [4–6]. Due to such adaptive changes; biofilm-forming bacteria are 10- to 1000-fold more resistant to conventional antibiotics, which thus present a great challenge to develop antimicrobials specifically to treat biofilms [7].

The medicinal usage of natural plants and herbs to control pathogens infections had been widely known from the old centuries without knowing the exact reason for such healing effect of the applied plants. Recent studies detected that, there are major bioactive compounds presented in different amount in the various plants' parts (leaves, stem, flower, etc...) such as polyphenols and flavonoids that are mainly responsible for the antimicrobial effect presented on controlling the pathogens infections to multiple diseases [8–10].

*Pluchea dioscoridis* is a common perennial, evergreen herb that is belonging to Asteraceae (Compositae) family. This plant is naturally growing in moist, humid regions and grows spontaneously in Egypt along the Nile river banks [11]. *P. dioscoridis* had been known for centuries for its remedy benefits and due to this fact; the plant was used by different human cultures as therapeutic and medicinal natural plant. The therapeutical activity of *P. dioscoridis* based on that it contains various bioactive components that possess antioxidant and antimicrobial activity against multiple human and animal pathogens. Various bioactive compounds had been isolated and identified from different extracts of *P. dioscoridis* including oils, triterpenoids, flavonoids, pluchecin sesquiterpenoids, and thiophenes. These compounds were extracted from different plant parts with a highly potent antimicrobial activity against different gram negative and gram-positive bacteria [12, 13]. Although literatures had exhibited the antimicrobial potentiality of *P. dioscoridis* extracts, none or very few studies have compared the microbial response of different strains against various extracts types neither less studying the growth kinetics of the microbe in the presence of different concentration of the extract. Based on this approach, the research study was applied to investigate the effect of different extracts on various pathogenic bacteria including *Enterobacter cloacae*, *Micrococcus leutus*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Clostridium* sp., and the response of the selected bacteria to the presence of different concentrations from the most potent extract alongside chemical profiling of the most active extracts via using LC-ESI-MS/MS in negative ion mode.

**EXPERIMENTAL**

**Plant material**

*P. dioscoridis* leaves were collected from Al-Qaliobia Governorate, Egypt during March 2020. The identification and authentication of the collected plant was performed by Dr. Tearse Labib, Department of Flora and Taxonomy, El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (No. P.d/le/2020) is kept in the Medicinal Chemistry Department, Theodor Bilharz Research Institute.

**Extraction and fractionation**

Dry powdered leaves of *P. dioscoridis* (1.7 kg) were soaked for four days in 80% methanol (4 × 3 L) at room temperature. The combined extracts were filtered and evaporated under vacuum using Rotatory evaporator to afford 80% methanol extract 287.77 g. The dried aqueous methanol extract was defatted via using petroleum ether (60–80 °C). Then, the defatted methanol extract was undergoing successive fractionation using organic solvents (e.g., dichloromethane, ethyl acetate, and n-butanol) to afford 31.50, 40.5, 15.34, 88.63, and 100.45 g respectively for petroleum ether, dichloromethane, ethyl acetate, and n-butanol, and water extracts. 500 mg of each extract were suspended and homogenized in 1.5 mL DMSO (Dimethyl sulfoxide) to be assessed for the antimicrobial screening experiment.

**Chemicals and reagents**

All solvents, standards and reagents were of highly analytical grade. Methanol, petroleum ether, dichloromethane, ethyl acetate and n-butanol were obtained from El-Nasr Pharmaceutical Chemicals Company (Cairo, Egypt). Nutrient agar and Nutrient Broth media were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India).

**Cultures’ preparation and screening of the antimicrobial activity**

Twenty four hour old, cultures were prepared in Nutrient broth medium from the following bacteria: Gram positive bacteria (*M. leutus*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *Clostridium* sp. and *S. aureus*), Gram negative bacteria (*E. cloacae*, *A. hydrophila*, *Proteus vulgaris*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*) and yeast (*Candida albicans*), the
cultures density were optimized to reach 0.5 to 1 MacFarland (equivalent absorbance at 600 nm: 0.1–0.3). Nutrient agar plates were poured and swapped with sterilized cotton swapper after immersed in the bacterial cultures separately. 7.00 mm sterilized puncher was used to form a maximum of four wells in each plate as designed by agar diffusion assay protocol according to Valgas et al. (2007) thereafter, 50 μL of each extract was added to the wells separately (=16.67 mg/50 μL). The plates were incubated for 24 h at 37 °C and the data were measured after incubation by ruler [14].

Effect of different concentrations of *P. dioscoridis* ethyl acetate extract on *M. luteus* growth kinetics

This experiment was designed to investigate the effect of *P. dioscoridis* ethyl acetate extract on *Micrococcus luteus* growth during 96 h incubation period. Concentrations of 0, 10, 25, 50, 100, 200 and 300 μL were added to 5 mL cultures inoculated with 250 μL of *M. luteus* culture (O.D.600: 1.5 abs). Microbial growth was measured spectrophotometrically by optical density determination at 600 nm at incubation intervals of (0, 24, 48, 72 and 96) respectively [4].

Antibiofilm activity evaluation

The biofilm inhibitory activity of tested extracts was measured using 96-well flat polystyrene plates toward four clinical microbes comprising gram-positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacteria (*P. aeruginosa* and *E. coli*). Briefly, each well was filled with 180 μL lysogeny broth (LB broth) then inoculated with 10 μL of pathogenic bacteria followed by addition of 10 μL (final concentration of 500 μg mL⁻¹) of samples along with control (without test sample). The plates incubated at 37 °C for 24 h and after incubation, content in the wells were removed and wells washed with 200 μL of phosphate buffer saline (PBS) pH 7.2 to remove free floating bacteria and left to dry at sterilized laminar flow for 1 h. For staining, 200 μL/well of crystal violet (0.1%, w/v) were added for 1 h then excessive stain removed and plates kept for drying. Further, dried plates were washed with 95% ethanol and optical density was determined at optical density 570 nm by using a Spectrostar Nano Microplate Reader (BMG LABTECH GmbH, Allmendgrun, Germany [4, 15].

**LC-ESI-MS/MS analysis**

The chemical constituents of the tested extracts were tentatively identified using a Thermo Finnigan (Thermo electron Corporation, OK, USA), coupled with an LCQ Duo ion trap mass spectrometer with an ESI source in negative ionization mode (ThermoQuest Corporation, Austin, TX, USA) [16].

**RESULTS AND DISCUSSION**

**In vitro antimicrobial activity**

Results presented in Table 1, Figs 1 and 2 indicate the effect of *P. dioscoridis* different extracts on various pathogenic bacteria. Our data confirmed that, all the plant extracts show a significant antibacterial activity against different bacterial species.

![Antibacterial effect of *P. dioscoridis* extracts on various pathogenic bacteria after 24 h incubation period.](image)

**Fig. 1.** Antibacterial effect of *P. dioscoridis* extracts on various pathogenic bacteria after 24 h incubation period. (C: Control, 1-5: extracts codes, concentration of the extracts (=16.67 mg/50 μL)

### Table 1. Antibacterial effect of *P. dioscoridis* extracts against bacteria illustrated by zone of inhibition measurements in mm

| Microorganisms          | H₂O | EtOAc | MeOH | Pet. ether | Butanol | CH₂Cl₂ |
|------------------------|-----|-------|------|------------|---------|--------|
| *E. cloacae*            | ND  | 10.5  | 4.75 | ND         | 9.50    | 1.00   |
| *M. luteus*             | ND  | 20.0  | 3.50 | 5.75       | 10.3    | 8.50   |
| *A. hydrophila*         | ND  | 1.00  | ND   | 1.00       | 16.5    | 3.00   |
| *B. cereus*             | ND  | ND    | ND   | 4.00       | 10.3    | ND     |
| *B. subtilis*           | ND  | 1.25  | 1.00 | 6.25       | 18.5    | 5.75   |
| *B. licheniformis*      | ND  | ND    | ND   | ND         | 14.0    | ND     |
| *Clostridium sp.*       | 8.00| 8.50  | 4.25 | 7.50       | 16.5    | 8.50   |
| *S. aureus*             | ND  | 15.5  | ND   | ND         | 16.0    | ND     |
| *P. vulgaris*           | 12.5| 13.0  | 14.5 | ND         | 16.0    | 11.5   |
| *E. coli*               | 5.0 | ND    | 7.50 | 10.5       | 12.0    | 13.5   |
| *P. aeruginosa*         | ND  | 7.50  | 4.50 | ND         | 5.50    | 10.0   |
| *C. albicans*           | 4.50| 3.50  | 15.5 | 4.00       | 17.0    | 15.0   |
| *K. pneumoniae*         | 10.0| 16.5  | 16.0 | ND         | 17.0    | 18.0   |

Antimicrobial activity was determined by well-agar diffusion method [14]. The initial concentration of the extracts in each well (50 μL).
pathogens except water extract that possess a reasonable antibacterial activity only against *P. vulgaris* and *K. pneumoniae*. The highest antibacterial activity detected for ethyl acetate extract against *M. luteus* with inhibition zone diameter of 20.0 mm. Butanol extract also shows a stringent, wide spectrum, antibacterial activity against various bacteria like *B. subtilis*, *A. hydrophila*, *Clostridium sp*, *S. aureus*, *P. vulgaris* and *B. Lichneformis* with inhibition diameters of (18.5, 16.5, 16.5, 16.0, 16.0 and 14.5 mm) respectively. Our data were similar to the results obtained by Ghorab et al. (2015) who exhibited that ethanolic extract of *P. dioscoridis* had a potent antimicrobial activity against different pathogenic microbes: Gram positive (*E. fecalis*, *S. aureus*, *L. monocytogenes* and *B. cereus*), and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae* and *Salmonella*) and *C. albicans* [11]. Moreover, our results also confirmed that *P. dioscoridis* methanol fraction have a potent antimicrobial effect against *K. pneumoniae*, *C. albicans* and *P. vulgaris*. On the other hand, EtOAc fraction also displayed moderate antibacterial activity towards *K. pneumoniae*, *S. aureus* and *P. vulgaris*, while petroleum ether fraction only showed antibacterial activity toward *E. coli* (10.5 mm). Also, the CH₂Cl₂ showed abroad antimicrobial activity against *K. pneumoniae*, *C. albicans* *E. coli*, *P. vulgaris* and *P. aeruginosa* with inhibition zone diameters (18.0, 15.0, 13.5, 11.5 and 10 mm). Obeidat et al. (2012) reported that leaves aqueous extracts of *P. dioscoridis* possess the highest antibacterial potency against all tested bacteria including, *E. coli*, *Staphylococcus typhimurium*, *P. aeruginosa*, *S. aureus* and *C. albicans* fungi [17]. Zalabani et al. (2013) reported that leaf and root extracts of the plant caused the highest growth inhibitory activity against *B. subtilis*, *Mycobacterium phlei*, *Listeria innocua*, and moderate inhibitory effect was detected against *C. albicans* and *S. aureus* [18]. Paerkh and Chanda (2008) reported high antimicrobial potency of *P. dioscoridis* leaf extract against yeasts and the tested Gram-positive bacteria [19].

**Effect of different concentrations of *P. dioscoridis* ethyl acetate extract on *M. luteus* growth kinetics**

Through assessing the effect of *P. dioscoridis* ethyl acetate extract on *M. luteus* growth kinetics, the data confirmed that all the concentrations applied showed antibacterial activity against *M. luteus* growth with concentration dependent manner in comparison to control culture, illustrated in Table 2 and Fig. 3. Data also poses that the highest

| Conc. (mg mL⁻¹) | 0   | 24  | 48  | 72  | 96  |
|-----------------|-----|-----|-----|-----|-----|
|                 | 0.02| 1.36| 1.47| 1.60| 1.71|
| 0.66 (10)       | 0.02| 0.88| 1.15| 1.26| 1.47|
| 1.66 (25)       | 0.02| 0.86| 0.86| 0.95| 0.95|
| 3.33 (50)       | 0.02| 0.88| 0.82| 0.85| 1.00|
| 6.67 (100)      | 0.02| 0.54| 0.65| 0.80| 0.85|
| 13.34 (200)     | 0.02| 0.24| 0.29| 0.33| 0.48|
| 20.01 (300)     | 0.02| 0.27| 0.28| 0.36| 0.50|

**Table 2. *M. luteus* growth dynamics in response to different concentrations of *P. dioscoridis* ethyl acetate extract**

![Fig. 2. Antibacterial activity of *P. dioscoridis* extracts against pathogenic microbial strains](image1)

**Fig. 2. Antibacterial activity of *P. dioscoridis* extracts against pathogenic microbial strains**

![Fig. 3. *M. luteus* growth curve kinetics at different concentrations of *P. dioscoridis* ethyl acetate extract](image2)

**Fig. 3. *M. luteus* growth curve kinetics at different concentrations of *P. dioscoridis* ethyl acetate extract**
antibacterial activity detected in concentration 300 µL at which the growth rate was lowered by approximately 70.1% at the end of the incubation period. The antimicrobial potency of *P. dioscoridis* various extracts as well as any plant extract depends on the interactions between the extracted, bioactive substance and one or more constituents (target sites) of the microbial cell membrane. The succession of the interaction resulted in further penetration of the active material into the cellular cytoplasm and the surrounding protein, nucleic acids that ultimately caused death and execution of the microbial cell [20]. Different studies had isolated the antimicrobial material form *P. dioscoridis* various extracts, Metwally et al. (2015) isolated flavonoids, phenolics, and tannins in ethanolic extract of *P. dioscoridis* [21].

**Antibiofilm activity of *P. dioscoridis* extracts against four pathogenic microbial strains**

Despite numerous attempts to eradicate bacterial biofilm, there is still an urgent need to find an effective way to inhibit the biofilm formation by bacteria. Nosocomial infections caused by bacterial pathogens include *S. aureus*, Bacillus subtilis, *P. aeruginosa* and *E. coli* are common in hospitals and other health-care facilities. These pathogens have become resistant to antibiotics due to biofilm formation. Nosocomial infections are common in hospitals and other health-care facilities. These pathogens have become resistant to antibiotics due to biofilm formation. Despite numerous attempts to eradicate bacterial biofilm, these pathogens have become resistant to antibiotics due to biofilm formation.

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| Extract  | Biofilm inhibitory (%) |
|----------|------------------------|
|          | *S. aureus* | *B. subtilis* | *E. coli* | *P. aeruginosa* |
| H2O      | 0           | 5.55         | 0         | 0               |
| CH2Cl2   | 16.66       | 30.07        | 16.98     | 27.61           |
| BuOH     | 79.76       | 43.49        | 15.95     | 22.61           |
| Pet. ether | 0          | 0            | 0         | 0               |
| EtOAc    | 53.65       | 2.54         | 0         | 22.93           |
| MeOH     | 56.58       | 17.93        | 16.58     | 24.04           |

**LC-ESI-MS/MS profiling of ethyl acetate extract**

**Phenolic acids, organic acids and their derivatives.** Compound (1) showed a molecular ion peak [M–H]+ at m/z 111 with daughter ions at m/z 67 [M–H–44]+, corresponding to the neutral loss of a CO2 moiety, 49, and 41. This compound could be identified as furoic acid [22]. Compound (2) exhibited a molecular ion peak [M–H]+ at m/z 179 and the fragment ion at m/z 135 [M–H–44]– due the neutral loss of CO2 moiety, MS2 ions were also detected at m/z 125, and 107; it was tentatively identified as caffeic acid [23, 24]. Compound (3) showed a molecular ion peak [M–H]+ at m/z 147 with daughter ions at m/z 129 [M–H–18], corresponding to the neutral loss of a H2O moiety, and 103 [M–H–44], corresponding to the neutral loss of a CO2 moiety. This compound could be identified as cinnamic acid [25]. Compound (4) showed a molecular ion peak [M–H]+ at m/z 137 with daughter ions at m/z 109 [M–H–28], corresponding to the neutral loss of a CO moiety, and 93 [M–H–44], corresponding to the neutral loss of a CO2 moiety. This compound could be identified as salicylic acid [22]. Compounds (5, 6) showed molecular ion peaks [M–H]+ at m/z 515 with diagnostic fragments at m/z 353 [M–H–162]– corresponding to caffeoylquinic acid and due to neutral loss of caffeic acid moiety, 191 [M–H–162–162]– corresponding to quinic acid and due to further neutral loss of caffeic acid moiety, 179 [caffeic acid–H+]0, 173, and 135 [caffeic acid–CO2–H+]0. The two isomers could be identified as dicaffeoylquinic acid isomers [26]. Compound (7) showed a molecular ion peak [M–H]+ at m/z 259 with daughter ions at m/z 241 [sulfocaffeic acid–H2O–H+]0, 215 [sulfocaffeic acid–CO2–H+]0, 179 [sulfocaffeic acid–SO3–H+]0, and 135. This compound could be identified as Caffeic Acid 3-sulfate (Sulfocaffeic acid) [27]. Compound (8) showed a molecular ion peak [M–H]+ at m/z 193 and MS1 fragment ions at m/z 178 [M–H–CH3]+, 149 [M–H–CO3]+, and 134 [M–H–CO2–CH3]+. This compound could be identified as ferulic acid [28]. Compound (9) showed a molecular ion peak [M–H]+ at m/z 529 and MS1 fragment ions at m/z 427, 367 [M–H–caffeic acid moiety]+, 179 [M–H–ferulic acid moiety–quinic acid]0.
Table 4. Tentative identification of secondary metabolites in the ethyl acetate extract of *P. dioscoridis* leaves via negative LC-ESI-MS/MS

| No. | RI (min) | m/z | M.wt. | M.F. | MS/MS fragments (m/z)* | Tentatively identified compounds | Class |
|-----|----------|-----|-------|------|------------------------|--------------------------------|-------|
| 1   | 0.73     | 111 | 112   | C₇H₄O₃ | 111, 67, 49, 41        | Furoic acid                    | Heterocyclic carboxylic acid    |
| 2   | 0.77     | 179 | 180   | C₆H₄O₄ | 179, 135, 125, 107     | Caffeic acid                   | Phenolic acids                  |
| 3   | 0.98     | 147 | 148   | C₆H₄O₃ | 147, 129, 115, 103     | Cinnamic acid                  | Phenolic acids                  |
| 4   | 2.11     | 137 | 138   | C₆H₄O₃ | 137, 109, 93           | Salicylic acid                 | Phenolic acids                  |
| 5   | 6.22     | 515 | 516   | C₂₅H₂₄O₁₂ | 515, 353, 335, 191, 179, 173, 135 | Dicaffeoyl-quinic acid isomer | Phenolic acids                  |
| 6   | 6.33     | 515 | 516   | C₂₅H₂₄O₁₂ | 515, 353, 335, 191, 179, 173, 135 | Dicaffeoyl-quinic acid isomer | Phenolic acids                  |
| 7   | 6.58     | 259 | 260   | C₈H₇O₅ | 259, 241, 215, 179, 173, 161, 135 | Caffeic acid 3-sulfate         | Phenolic acid derivatives       |
| 8   | 6.83     | 193 | 194   | C₁₀H₁₀O₄ | 193, 178, 149, 134     | Ferulic acid                   | Phenolic acids                  |
| 9   | 6.98     | 529 | 530   | C₂₆H₂₆O₁₂ | 529, 367, 193, 179, 173, 151 | Feruloyl caffeoyl-quinic acid | Phenolic acid derivatives       |
| 10  | 7.09     | 571 | 572   | C₁₀H₁₀O₁₂ | 571, 285               | Dihydrophilinotisflavone       | Flavonoid derivatives           |
| 11  | 7.24     | 529 | 530   | C₁₀H₁₀O₆ | 529, 511, 419, 289, 273 | (epi)-Guibourtinidol-(epi)-afzelechin | Flavonoid derivatives |
| 12  | 7.32     | 311 | 312   | C₂₀H₂₀O₈ | 311, 296, 293, 242     | Eicosanoic acid                | Saturated fatty acids           |
| 13  | 7.49     | 529 | 530   | C₁₀H₁₀O₆ | 529, 511, 419, 289, 273 | (epi)-Guibourtinidol-(epi)-afzelechin | Flavonoid derivatives |
| 14  | 7.68     | 309 | 310   | C₁₅H₁₆O₈ | 309, 291, 263, 240, 211, 197 | Linolenic acid 13-hydroperoxide | Unsatuated fatty acids          |
| 15  | 7.98     | 529 | 530   | C₁₀H₁₀O₆ | 529, 511, 419, 289, 273 | (epi)-Guibourtinidol-(epi)-afzelechin | Flavonoid derivatives |
| 16  | 8.30     | 359 | 360   | C₁₅H₁₄O₉ | 359, 344, 329           | Rosmarinic acid                 | Phenolic acid derivatives       |
| 17  | 8.71     | 315 | 316   | C₁₀H₁₀O₇ | 315, 300, 283, 271, 164, 163, 151 | *isorhamnetin                  | Flavonoids                      |
| 18  | 9.19     | 359 | 360   | C₁₀H₁₀O₆ | 359, 344, 329           | Rosmarinic acid                 | Phenolic acid derivatives       |
| 19  | 9.53     | 315 | 316   | C₁₀H₁₀O₆ | 315, 153, 109           | Protopatechuic acid glucoside  | Phenolic acid derivatives       |
| 20  | 9.81     | 313 | 314   | C₁₀H₁₀O₆ | 313, 298, 285           | 3’,7-dimethoxyluetin           | Flavonoid derivatives           |
| 21  | 9.93     | 247 | 248   | C₁₀H₁₀O₆ | 247, 219, 191           | Brevifolin [Geranium]          | Benzopyrone derivatives         |
| 22  | 10.59    | 373 | 374   | C₁₀H₁₀O₆ | 373, 358, 343, 328      | Methylsudachitin               | Flavonoid derivatives           |
| 23  | 10.85    | 421 | 422   | C₂₀H₂₀O₁₀ | 421, 313, 271, 211, 169, 125 | Benzy1-O-galloyl glucose      | Gallotannins derivatives        |
| 24  | 11.32    | 363 | 364   | C₁₀H₁₀O₇ | 363, 345, 313, 299, 281, 255 | Tetra-O-methylcatechin derivatives | Flavonoid derivatives |
| 25  | 11.59    | 247 | 248   | C₁₀H₁₀O₆ | 247, 219, 191           | Brevifolin [Geranium]          | Benzopyrone derivatives         |
| 26  | 14.99    | 559 | 560   | C₂₅H₂₆O₁₃ | 559, 381                | 1-O-caffeoyl-3-O-sinapoylquinic acid | Phenolic acid derivatives       |
| 27  | 15.49    | 555 | 556   | C₂₅H₂₆O₁₄ | 555, 403, 393           | Hydroxyleuropein               | Secoiridoids                    |
| 28  | 22.01    | 327 | 328   | C₁₅H₁₈O₅ | 327, 314, 299, 229, 211 | Oxiranedioctanoic              | Fatty acids                     |
| 29  | 31.07    | 100 | 101   | –         | –                      | Unknown                         | -                              |

*Major ion in MS*, marked in bold numbers (100% relative abundance); *Compounds previously identified in the plant.*
acid moiety]", and 151. This compound could be identified as feruloyl-caffeoyl-quinic acid [25]. Compounds (16, 18) showed molecular ion peaks [M−H]− at m/z 359 and diagnostic fragment ions at m/z 344, and 329. These compounds could be identified as rosmarinic acid [26–28]. Compounds (19) showed molecular ion peaks [M−H]− at m/z 315 and diagnostic fragment ions at m/z 153 [M−H-162]− corresponding to protocatechuic acid moiety and due to neutral loss of glucose moiety, and 109 [M−H-162-44]− due to further neutral loss of CO2 moiety. This compound could be identified as protocatechuic acid glucoside [29]. Compounds (26) showed a molecular ion peak [M−H]− at m/z 559, and MS fragment ion at m/z 381. This compound could be identified as 1-O-caffeoyl-3-O-sinapoylquinic acid [30].

Flavonoids and their derivatives

Compound (10) showed a molecular ion peak [M−H]− at m/z 571 and a diagnostic fragment ion at m/z 285 [M−H-286]−. This compound could be identified as dihydrophilonotisflavone [30]. Compounds (11, 13, 15) showed molecular ion peaks [M−H]− at m/z 529 and diagnostic fragment ions at m/z 511, 419, and 273 [M-Guibourtinidol moiety-H]− corresponding to afzelechin moiety. These compounds could be identified as (epi)-Guiboutrinidol-(epi)-afzelechin [31]. Compound (17) showed a molecular ion peak [M−H]− at m/z 315 and diagnostic fragment ions at m/z 300 [M−H-CH3]−, 283, 271, 243, 164, 163, 151, and 107. This compound could be identified as isorhamnetin [32]. Compound (20) showed a molecular ion peak [M−H]− at m/z 595 and diagnostic fragment ions at m/z 285 and 273 [M-Guaihydropinochromone moiety-H]− corresponding to guaihydropinochromone moiety. This compound could be identified as guaihydropinochromone [33].

Fig. 4. Negative LC-ESI-MS/MS profile of phenolic compounds from ethyl acetate extract of P. dioscoridis leaves. Numbers at peaks refer to Table 4.

Fig. 5. Proposed fragmentation pattern of dicafeoyl-quinic acid using negative ionization mode.
peak [M−H]− at m/z 313 and diagnostic fragment ions at m/z 298 [M−H−CH3]−, and 283 [M−H−2 x CH3]−. This compound could be identified as 3,7-dimethoxyluteolin [25]. Compound (22) showed a molecular ion peak [M−H]− at m/z 373 and diagnostic fragment ions at m/z 358 [M−H−CH3]−, 343 [M−H−2 x CH3]−, 328 [M−H−3 x CH3]−. This compound could be identified as methyl-sudachitin [33]. Compound (24) showed a molecular ion peak [M−H]− at m/z 363 and fragment ions at m/z 345, 313, 299, 281, and 255. This compound could be identified as tetra-O-methylcatechin derivatives [34].

Other compounds

Compound (12) showed a molecular ion peak [M−H]− at m/z 311 and fragment ions at m/z 296 [M−H−CH3]−, 293 [M−H−H2O]−, and 195. This compound could be identified as eicosanoid acid [16]. Compound (14) showed a molecular ion peak [M−H]− at m/z 309 and fragment ions at m/z 291 [M−H−H2O]−, 277, 211, and 197. This compound could be identified as linolenic acid 13-hydroperoxide [26]. Compounds (21, 25) showed molecular ion peaks [M−H]− at m/z 247 and fragment ions at m/z 219 [M−H−CO]−, and 191 [M−H−CO−CO]−. These compounds could be identified as brevifolin [Geranium] [35]. Compound (23) showed a molecular ion peak [M−H]− at m/z 421 and fragment ions at m/z 331 [M−H−90]− corresponding to galloyl glucose moiety and due to neutral loss of benzyl moiety, 313 [M−H−90−18]− due to further neutral loss of H2O molecule, 271, 211, 169 [M−H−90−162]− due to further neutral loss of glucose moiety, and 125 [M−H−90−162−44]− due to further neutral loss of CO2 moiety. This compound could be identified as benzyl-O-galloyl glucose [16, 36]. Compound (27) showed a molecular ion peak [M−H]− at m/z 555 and fragment ions at m/z 537, 403, 393, and 323. This compound could be identified as hydroxyoleuropein [37]. Compound (28) showed a molecular ion peak [M−H]− at m/z 327 and fragment ions at m/z 314, 299, 229, and 211. This compound could be identified as oxiraneoctanoic acid [33].

LC-ESI-MS/MS profiling of n-butanol extract

Phenolic acids, organic acids and their derivatives. Compound (1) showed a molecular ion peak [M−H]− at m/z 191 with daughter ions at m/z 173 [M−H−18]−, corresponding to the neutral loss of a H2O moiety, 127, and 111. This compound could be identified as quinic acid [23]. Compound (2) showed a molecular ion peak [M−H]− at m/z 153 with daughter ion as a base peak at m/z 109 [M−H−44]− due to the neutral loss of a CO2 moiety. This compound could be identified as protocatechuic acid [22]. Compounds (3, 4, 5) showed molecular ion peaks [M−H]− at m/z 353 with daughter ions at m/z 191 [M−H−162]− corresponding to quinic acid and due the neutral loss of a caffeic acid moiety, 179 [M−H−18]− corresponding to caffeic acid and due to further neutral loss of a H2O moiety, 173 [quinic acid-H-H2O]−, 161 [caffeic acid-H-H2O]−, and 135 [caffeic acid-H-CO2]−. These compounds could be identified as chlorogenic acid [38]. Compound (7) showed a molecular ion peak [M−H]− at m/z 341 with characteristic ions at m/z 179 [M−H−162]− corresponding to caffeic acid and due to further neutral loss of a glucose moiety, 161 [caffeic acid-H-H2O]−, and 135 [caffeic acid-H-CO2]−. This fragmentation pattern was typically assigned to caffeic acid-O-hexoside [39]. Compounds (9, 10) showed molecular ion peaks [M−H]− at m/z 515 with diagnostic fragments at m/z 353 [M−H−162]− corresponding to caffeoyl-quinic acid and due to neutral loss of a glucose moiety, 191 [M−H−162−62]− corresponding to quinic acid moiety and due to further neutral loss of caffeic acid moiety, 179 [M−H−162−74]− corresponding to caffeic acid moiety and due to further neutral loss of quinic acid moiety, 173 [M−H−162-162-18]−, 161 [M−H−162-174-
**Table 5. Tentative identification of secondary metabolites in the butanol extract of *P. dioscoridis* leaves via negative LC-ESI-MS/MS**

| No. | R_t (min) | m/z (M-\text{H})^- | M.wt. | M.F. | MS² fragments (m/z)* | Tentatively identified compounds | Class |
|-----|-----------|---------------------|-------|------|----------------------|--------------------------------|-------|
| 1   | 0.80      | 191                 | 192   | C₇H₁₂O₆ | 191, 173, 127, 111   | Quinic acid                     | Organic acids |
| 2   | 2.32      | 153                 | 154   | C₅H₆O₄ | 153, 109             | Protocatechuic acid             | Phenolic acids |
| 3   | 4.45      | 353                 | 354   | C₁₀H₁₄O₆ | 191, 179, 173, 161, 135 | *Chlorogenic acid               | Phenolic acids |
| 4   | 4.50      | 353                 | 354   | C₁₀H₁₄O₆ | 191, 179, 173, 161, 135 | Chlorogenic acid                | Phenolic acids |
| 5   | 5.25      | 353                 | 354   | C₁₀H₁₄O₆ | 191, 179, 173, 161, 135 | Chlorogenic acid                | Phenolic acids |
| 6   | 6.11      | 303                 | 304   | C₁₃H₁₂O₇ | 303, 285, 271, 259, 241, 177, 151 | Taxifolin                     | Flavonoids     |
| 7   | 6.27      | 341                 | 342   | C₁₃H₁₄O₈ | 341, 251, 233, 203, 179, 161, 135 | Caffeic acid-O-hexoside         | Phenolic acid glycosides |
| 8   | 6.94      | 421                 | 422   | C₂₀H₂₂O₁₀ | 421, 313, 271, 211, 169, 125 | Benzyl-O-galloyl glucose       | Phenolic acid derivatives |
| 9   | 7.61      | 515                 | 516   | C₂₃H₂₄O₁₂ | 515, 353, 335, 191, 179, 173, 135 | Dicaffeoyl-quinic acid         | Phenolic acid derivatives |
| 10  | 7.72      | 515                 | 516   | C₂₃H₂₄O₁₂ | 515, 353, 335, 191, 179, 173, 135 | Dicaffeoyl-quinic acid         | Phenolic acid derivatives |
| 11  | 7.99      | 259                 | 260   | C₅H₆O₅S | 259, 241, 215, 179, 173, 161, 135 | Caffeic acid 3-sulfate (Sulfo-caffeic acid) | Phenolic acid derivatives |
| 12  | 8.40      | 193                 | 194   | C₁₀H₈O₄ | 193, 176, 149, 147, 134 | Ferulic acid                   | Phenolic acids |
| 13  | 8.56      | 529                 | 530   | C₁₉H₁₉O₉ | 529, 511, 419, 289, 273 | (epi)-Guibourtinidol-(epi)-afzelechin | Flavanoid derivatives |
| 14  | 8.98      | 529                 | 530   | C₁₉H₁₉O₉ | 529, 367, 193, 179, 173, 151 | Feruloyl-cafeyrol-quinic acid  | Phenolic acid derivatives |
| 15  | 9.17      | 479                 | 480   | C₁₁H₁₈O₁₃ | 479, 317, 299, 284, 255 | Gossypetin-8-glucoside (Gossypin) | Flavonoids |
| 16  | 9.41      | 529                 | 530   | C₂₇H₂₇O₁₃ | 529, 367, 352, 329, 219, 335, 317 | Glyccoumarin derivatives       | Coumarin derivatives |
| 17  | 9.58      | 677                 | 678   | C₁₈H₁₈O₁₅ | 677, 515, 497, 353, 323, 191 | Tricaffeoylquinic acid         | Phenolic acid derivatives |
| 18  | 10.07     | 315                 | 316   | C₁₄H₁₈O₇ | 315, 300, 283, 272, 171, 164, 163, 151 | Isorhamnetin                  | Flavonoids |
| 19  | 11.89     | 571                 | 572   | C₂₀H₂₀O₁₂ | 285                  | Hegoalexane B                   | Flavonoids |
| 20  | 12.56     | 285                 | 286   | C₁₄H₁₄O₆ | 285, 267, 241, 217, 199, 175, 161, 125 | Luteolin                      | Flavonoids |
| 21  | 20.31     | 555                 | 556   | C₂₅H₁₉O₁₄ | 555, 403, 393          | Hydroxyoleuropein              | Secoiridoids |
and 135 [M–H–162–174–44]−. These compounds could be identified as dicaffeoyl-quinic acid [40]. Compound (11) showed a molecular ion peak [M–H]− at m/z 259 with daughter ions at m/z 241 [sulfocaffeic acid–H2O–H]−, 215 [sulfocaffeic acid–CO2–H]−, 179 [sulfocaffeic acid–SO3–H]−, 173, 161, 135, and 125. This compound could be identified as Caffeic acid 3-sulfate (Sulfo-caffeic acid) [26]. Compound (12) showed a molecular ion peak [M–H]− at m/z 193 and MSn fragment ions at m/z 178 [M–H–CH3]−, 149 [M–H–CO2]−, 147, and 134 [M–H–CO2–CH3]−. This compound could be identified as ferulic acid [27]. Compound (14) showed a molecular ion peak [M–H]− at m/z 529 and MSn fragment ions at m/z 367 [M–H–caffeic acid moiety]−, 193, 179 [M–H–ferulic acid moiety–quinic acid moiety]−, 173, and 151. This compound could be identified as feruloyl-caffeoyl-quinic acid [25]. Compound (17) showed a molecular ion peak [M–H]− at m/z 677 and MSn fragment ions at m/z 515 [M–H–caffeic acid moiety]−, 497 [M–H–caffeic acid moiety–H2O]−, 353 [M–H–2 × caffeic acid moiety]−, 335 [M–H–2 × caffeic acid moiety–H2O]−, and 191 [M–H–3 × caffeic acid moiety]−. This compound could be identified as 1,3,5-O or 1,4,5-O-Tricaffeolyquinic acid [41].

**Flavonoids and their derivatives**

Compound (6) showed a molecular ion peak [M–H]− at m/z 303 with daughter ions at m/z 285, 271, 259, 241, 177, and
151. This fragmentation pattern was typically assigned to taxifolin [34]. Compound (13) showed a molecular ion peak [M–H]⁻ at m/z 529 and diagnostic fragment ions at m/z 511, 419, 289, and 273 [M-Guibourtinidol moiety-H]⁻ corresponding to afzelechin moiety. This compound could be identified as (epi)-Guibourtinidol-(epi)-afzelechin [31]. Compound (15) showed a molecular ion peak [M–H]⁻ at m/z 479 with daughter ions at m/z 479, 317, 299, 284, and 255. This fragmentation pattern was typically assigned to Gossypetin-8-glucoside (Gossypin) [34]. Compound (18) showed a molecular ion peak [M–H]⁻ at m/z 571 and a diagnostic fragment ion at m/z 285. This compound could be identified as Hegoques, I. Characterization of antibiotic resistant and pathogenic Escherichia coli in irrigation water and vegetables in household farms. Int. J. Food Microbiol. 2017, 257, 192–200.

Other compounds

Compound (8) showed a molecular ion peak [M–H]⁻ at m/z 421 and fragment ions at m/z 331 [M–H–90]⁻ corresponding to galloyl glucose moiety and due to neutral loss of benzyl moiety, 313 [M–H–90–18]⁻ due to further neutral loss of H₂O molecule, 271, 211, 169 [M–H–90–162]⁻ due to further neutral loss of glucose moiety, and 125 [M–H–90–162–44]⁻ due to further neutral loss of CO₂ moiety. This compound could be identified as benzyl-O-galloyl glucose [36]. Compound (16) showed a molecular ion peak [M–H]⁻ at m/z 479 with daughter ions at m/z 529, 367, 352, 329, 219, 335, 317, and 299. This fragmentation pattern was typically assigned to glycycomar derivatives [34]. Compound (21) showed a molecular ion peak [M–H]⁻ at m/z 555 and fragment ions at m/z 537, 403, 393, and 323. This compound could be identified as hydroxyoleuropein [37].

CONCLUSIONS

The chemical profiling of P. dioscoridis leaf extracts led to identifying of 28 and 21 compounds, respectively from the ethyl acetate and n-butanol extracts, most of them are flavonoides and phenolic acid derivatives. Some compounds were identified for the first time in plant and also in the genus Pluchea. Moreover, the testes extracts showed a potent antimicrobial and antibiofilm activities against the selected bacteria except the water extract which showed a moderate activity on only two isolates from the selected bacterial strains. The variation between the antimicrobial activities of extracts against different bacteria related to bacterial type and microbial resistance behavior against the bioactive compounds presented in different concentrations in extracts used in the extraction process.

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