

Antibacterial Activity of *Onopordum Espinae*: Identification of Hispidulin and Dehydromelitensin-8-(4'-Hydroxy-Methacrylate)

Fatma Njeh^{a*}, Dhekra Mhalla^b, Ibtissem Ben Hammouda^a, Mohamed Trigui^b and Raoudha Mezghani-Jarraya^a

^aLaboratory of Organic Chemistry LR17ES08, Natural Substances Chemistry Team, Faculty of Sciences, 3000 Sfax, University of Sfax, Tunisia. ^bLaboratory of Biopesticides, Center of Biotechnology, 3018 Sfax, University of Sfax, Tunisia.

Abstract

Hexane, ethyl acetate and methanol extracts of *Onopordum espiniae* leaves were investigated for their antibacterial activities against *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis*. Hexane and ethyl acetate extracts were toxic to Gram-positive bacteria with inhibition zone diameters ranging between 12 and 30 mm. Two products, hispidulin **1**, and dehydromelitensin-8-(4'-hydroxy-methacrylate) **2** were isolated from the ethyl acetate extract of leaves and identified by 2D-NMR for the first time from this species. Ethyl acetate extract's total flavonoid content was the highest, as 78.73 mg quercetin equivalents (QE)/g. The methanol extract showed the highest total phenolic content as 243.43 mg gallic acid equivalents (GAE)/g and gave the most important 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity ($EC_{50} = 86 \mu\text{g/mL}$).

Keywords: total flavonoid content; total phenolic content; DPPH radical-scavenging assay; hispidulin; dehydromelitensin-8-(4'-hydroxy-methacrylate); antibacterial activity.

Introduction

Bacteria continue developing expanding defense against recent antibiotics manufactured lately by pharmaceutical companies. They become capable of gaining resistance to drugs that are used as curative agents. This is an interesting matter because of the large number of patients who have lost immunity and as a result of new bacterial strains multidrug-resistant. Therefore, new diseases can appear in hospitals resulting in high fatality.

Plants have always been an important source of natural phytochemicals for preserving health of humans, with more concern for natural

therapies (1).

Some species of the genus *Onopordum* were used as antipyretic agents and to treat diabetes, hepatic diseases, gastric disorders and gynecological diseases (2-4). Others exhibited antimicrobial and antioxidant activities (5, 6). *Onopordum espiniae* Bonnet (*Asteraceae*) locally known as "Bokk" is a cotton thistle endemic to Tunisia and Libya (7).

There are no previous studies on the chemical composition or on the antibacterial activity of this plant. Besides, there is no information about its traditional uses.

Thus, this study was carried out with the aim of evaluating the free radical-scavenging properties and the antibacterial activity of this plant, as well as identifying natural chemicals from it.

* Corresponding author:

E-mail: fatma.njeh@gmail.com

Experimental

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Butyl Hydroxy Toluene (BHT), α -tocopherol and gallic acid reagents were purchased from Sigma (Germany). Aluminum chloride and Folin-Ciocalteu reagents were purchased from Fluka (Germany). Sodium carbonate solid was from Normapur (France). All other reagents were of analytical grade.

Plant material

Onopordum espiniae was harvested in June 2013 from Sfax, Tunisia and identified by Pr. Mohamed Chaieb, a botanist at the faculty of Sciences of Sfax. The voucher specimen (LCSN121) was deposited at the Laboratory of Chemistry of Natural Substances, Faculty of Sciences, University of Sfax, Tunisia.

Preparation of extracts

Leaves of *Onopordum espiniae* were air dried, powdered using a mechanical grinder and successively macerated using increasing polarity solvents (hexane, ethyl acetate, and methanol) during 48 h each. The extracts were filtered, concentrated using a rotary evaporator and kept at 4 °C until use.

Isolation and identification of products 1 and 2 by NMR

Successive purifications of the leaf ethyl acetate extract using silica gel column chromatography were performed using a gradient elution technique and led to the identification of two pure products. ¹H and ¹³C NMR spectral data were compared to the previous reports (8, 9).

Compound 1: Yellow needles, C₁₆H₁₂O₆. R_f 0.54 (hexane/ethyl acetate, 3:7, v/v). ¹³C NMR (100 MHz, CDCl₃/CD₃OD) δ 59.96 (C-1), 164.72 (C-2), 102.16 (C-3), 182.68 (C-4), 153.06 (C-5), 131.22 (C-6), 156.94 (C-7), 94.02 (C-8), 152.29 (C-9), 104.46 (C-10), 121.74 (C-1'), 127.93 (C-2',6'), 115.60 (C-3',5'), 160.81 (C-4'). ¹H NMR (400 MHz, CDCl₃/CD₃OD) δ 3.90 (3H, s, OCH₃), 6.53 (1H, s, H-3), 6.54 (1H, s, H-8), 7.79 (2H, d, 8.9 Hz, H-2', 6'), 6.93 (2H, d, 8.9 Hz, H-3', 5').

Compound 2: Yellow amorphous powder,

C₁₉H₂₂O₆. R_f 0.36 (hexane/ethyl acetate, 3:7, v/v). ¹³C NMR (100 MHz, CDCl₃/CD₃OD) δ 146.24 (C-1), 111.30 (C-2), 111.81 (C-3), 143.99 (C-4), 50.32 (C-5), 78.46 (C-6), 51.58 (C-7), 69.33 (C-8), 44.38 (C-9), 41.39 (C-10), 137.39 (C-11), 169.89 (C-12), 118.12 (C-13), 17.49 (C-14), 65.12 (C-15), 164.91 (C-1'), 140.08 (C-2'), 124.34 (C-3'), 60.00 (C-4'). ¹H NMR (400 MHz, CDCl₃/CD₃OD) δ 5.06 (1H, dd, 17.5 Hz, 10.8 Hz, H-1), 4.23 (α , 1H, dd, 17.5 Hz, 0.7 Hz, H-2) 4.18 (β , 1H, dd, 10.8 Hz, 0.7 Hz, H-2), 4.59 (α , 1H, d, 0.8 Hz, H-3), 4.62 (β , 1H, d, 0.8 Hz, H-3), 1.73 (1H, d, 11.9 Hz, H-5), 3.69 (1H, t, H-6), 2.28 (1H, m, H-7), 4.52 (1H, m, H-8), 0.96 (α , 1H, dd, 2.3 Hz, 13.2 Hz, H-9), 1.18 (β , 1H, dd, 4.3 Hz, 13.2 Hz, H-9), 4.81 (α , 1H, d, 3.0 Hz, H-13), 5.26 (β , 1H, d, 3.0 Hz, H-13), 1.22 (3H, s, H-14), 3.16 (α , 1H, d, 15 Hz, H-15), 3.26 (β , 1H, d, 15 Hz, H-15), 5.15 (α , 1H, d, 1.5 Hz, H-3'), 5.49 (β , 1H, d, 1.5 Hz, H-3'), 3.50 (2H, s, H-4').

DPPH radical-scavenging assay

Free radical-scavenging activities of hexane, ethyl acetate, and methanol extracts were measured according to literature (10). The DPPH free radical-scavenging activity is based on one-electron reduction. Briefly, 1 mL of a DPPH solution (4% (w/v) in methanol) was mixed with 1 mL of the plant material at varying concentrations. The reaction mixture was well shaken and incubated in the dark for 30 min at room temperature. A control sample was prepared without any plant material. The absorbance of the solution was measured at 517 nm against a blank using a spectrophotometer (JENWAY 6320, United Kingdom). Percentage of DPPH scavenged by extracts or pure products at different concentrations was calculated as follows

$$\% \text{ inhibition} = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the DPPH control solution and A_{sample} is the absorbance of the sample. The concentration of this latter providing 50% inhibition (IC₅₀) of DPPH was calculated from the graph of percentage inhibition against extract concentration. α -Tocopherol and BHT were used as reference

DPPH - scavengers.

Total flavonoid content

The flavonoid content was determined based on the formation of a flavonoid–aluminum complex (11). 1 mL of the sample (containing 1 mg of each tested extract) was mixed with 1 mL of a 2% aluminum chloride methanol solution. The absorbance of the reaction mixture was measured at 415 nm after incubation for 15 min at room temperature. Quercetin was used as a standard to make the calibration curve. The amount of flavonoids was expressed as quercetin equivalents (mg QE/g dry weight of the samples). All the tests were carried out in triplicate.

Total phenolic content

Total phenolic content was determined by using Folin-Ciocalteu reagent (12). Briefly, 0.5 mL of the diluted sample (1 mg in 1 mL) was reacted with 0.5 mL of Folin-Ciocalteu reagent for 3 min, and then 0.5 mL of a saturated sodium carbonate solution (75 g/L) were added into the reaction mixture. Finally, 3.5 mL of water were added. The absorbance readings were taken at 725 nm after incubation at room temperature for 90 min. Gallic acid was used as a standard reference and results were expressed as milligram gallic acid equivalents (mg GAE)/g dry weight of samples. All the tests were carried out in triplicate.

Antibacterial activity

Bacterial strains

Extracts were tested against six bacterial strains from American Type Culture Collection (ATCC): *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella enteritidis* (food isolate). Bacterial strains were grown on Mueller-Hinton broth (Bio-Rad, France) at 37 °C for 12–14 h. The turbidity of the overnight broth was adjusted to 0.5 McFarland standards ($1-1.5 \times 10^8$ CFU/mL) by Densimat spectrophotometer (BioMérieux, Italy) and then diluted in Mueller-Hinton broth to a final inoculum concentration of 10^7 CFU/mL.

Agar well-diffusion assay

Antibacterial tests were performed by agar well diffusion method (13) using sterile Mueller-Hinton medium (Bio-Rad, France). A fresh cell suspension (100 µL) was inoculated onto the surface of agar plates. Thereafter, 6 mm diameter wells were punched in the inoculated agar medium using sterile Pasteur pipettes and the extracts were added to each well. Negative controls consisted of 20% DMSO and 50% ethanol which were used to dissolve the plant extracts. Gentamicin (15 µg/ well) was used as a positive control to determine the sensitivity of each bacterial strain. The plate was allowed to stand for 2 h at 4 °C to permit the diffusion of the extracts followed by incubation at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone diameter IZD (clear zone around the well) against the tested microorganisms. All tests were repeated three times.

Results

Phenolic and flavonoid contents

Total phenolic contents of the tested extracts/fraction varied from 30.5 to 243.43 mg of GAE/g of sample, using the standard curve of gallic acid ($R^2 = 0.9990$).

Using the standard curve generated by quercetin ($R^2 = 0.9999$), the total flavonoid contents of extracts/fraction varied from 5.24 to 78.73 mg of QE/g of sample (Table 1).

DPPH radical-scavenging activity

The results of DPPH radical-scavenging activity are also summarized in Table 1. The IC_{50} values ranged from 86 to 463 µg/mL. The methanol extract exhibited the highest activity.

Antibacterial activity

Onopordum espiniae extracts were tested against both Gram-positive and Gram-negative bacteria and the data are shown in Table 2. Results are given by means of IZDs. The hexane and ethyl acetate extracts showed strong antibacterial activity against Gram-positive bacteria with inhibition zones ranging between 12 and 30 mm. The methanol extract was inactive against the tested microorganisms. The

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and EC₅₀ values of DPPH free radical scavenging of *Onopordum espiniae* leaf extracts

Samples	Yield (%)	TPC* mg of GAE/g	TFC† mg of QE/g	DPPH‡ EC ₅₀ ¶ (µg/mL)
Hex E	5.08	30.50 ± 1.1	5.24 ± 0.9	Inactive
EtOAc E	6.13	156.20 ± 0.6	78.73 ± 0.2	199 ± 1.1
MeOH E	13.23	243.43 ± 0.8	60.63 ± 0.7	86 ± 0.6
Fraction 4	-§	40.42 ± 0.3	24.41 ± 1.6	463 ± 0.9
1	-	-	-	310 ± 0.2
2	-	-	-	Inactive
Positive control				
α-tocopherol	-	-	-	26 ± 0.3
BHT	-	-	-	17 ± 0.2

Hex E: Hexane extract; **EtOAc E:** Ethyl acetate extract; **MeOH E:** Methanol extract.

*: Total phenolic content (TPC) expressed as gallic acid equivalent (mg GAE/g of tested sample).

†: Total flavonoid content (TFC) expressed as quercetin equivalent (mg QE/g of tested sample).

‡: Results of free radical scavenging activity (DPPH assay).

¶: EC₅₀ (µg/mL) values corresponding to the amount of extract/fraction required to scavenge 50% of radicals present in the reaction mixture.

§: not done.

1: Hispidulin.

2: Dehydromelitensin-8-(4'-hydroxy-methacrylate).

antibacterial activity was observed only against Gram-positive bacteria, Gram-negative ones were resistant to the tested extracts. The best activity was exhibited by ethyl acetate extract against *Staphylococcus aureus*, one of the most common Gram-positive bacteria causing food poisoning, followed by *Bacillus cereus*.

The isolated compounds hispidulin **1** and dehydromelitensin-8-(4'-hydroxy-methacrylate) **2** were also tested for their antibacterial activities, but none of them showed any antibacterial activity against the tested bacteria within the tested concentration range (1 mg/ well)

Structural identification of compounds 1 and 2

The chemical structures of both isolated compounds were identified according to their ¹H and ¹³C NMR data (Figure 1). Compared to literature (8-9), they were identified as hispidulin **1** and dehydromelitensin-8-(4'-hydroxy-methacrylate) **2**.

Discussion

Pathogenic bacteria continue raising strong

resistance and after effects against drugs, mainly antibiotics. Consequently, massive attention has been taken to extracts and biologically active compounds isolated from the vegetable kingdom. Plants can give a new source of antibacterial agents for treatment. Phenolic substances have significant antimicrobial activity and it is estimated that their function in tissues where they accumulate might provide chemical barriers to invading microorganisms (14). A wide variety of antioxidants occurs in plants, with different compositions, physical and chemical properties, mechanisms and sites of action. Several species of the genus *Onopordum* showed antimicrobial and antioxidant activities. For instance, the antimicrobial activity of *Onopordum cynarocephalum* Boiss and Blanche (*Asteraceae*) polar extracts was discussed (5). The antimycobacterial properties of *Onopordum anatolicum* Boiss. (*Asteraceae*) were also studied (15). *Onopordum illyricum* L. was reported to contain antioxidant compounds (6). The present work is a continuation of research in this field.

In this paper, the ethyl acetate extract of *Onopordum espiniae* was the most active against

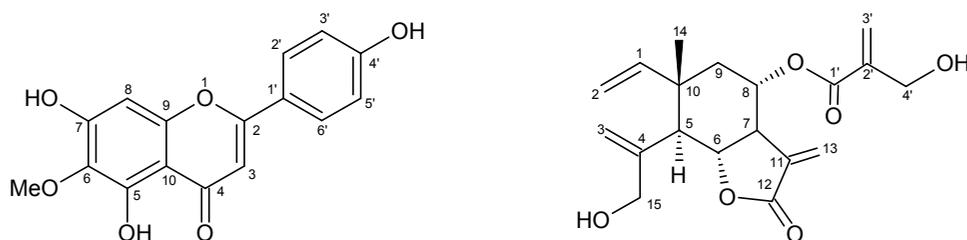


Figure 1. Compounds **1** and **2** isolated from *Onopordum espiniae* leaves.

Gram-positive bacteria. It was even more toxic to *Bacillus cereus* and *Enterococcus faecalis* than gentamicin, a known standard antibiotic. This significant activity is probably due to the high amount of flavonoids inside (78.73 mg QE/g), as these latter are known antibacterial agents (16). This extract was subjected to silica gel column chromatography to yield 10 fractions. The TLC profile of the fourth was the least complex; it showed two main spots with some minor compounds. Further chromatography of fraction 4 on a silica gel column too yielded 16 sub-fractions among which the sixth and the seventh were pure. NMR analysis led to identify these products as hispidulin **1** and dehydromelitensin-8-(4'-hydroxy-methacrylate) **2**.

The antibacterial activity is influenced

by phenolics and flavonoids composition. A significant correlation between phenolic composition and antimicrobial activity was reported in several studies. The phenolic compounds are antibacterial agents such as gallic and ferulic acids (17). In our study, we noticed an antibacterial activity using ethyl acetate as a solvent of extraction, but the methanol extract which contains the highest concentration of phenolics (243.43 mg of GAE/g) failed to show any antibacterial activity. This result demonstrates that the antibacterial activity of *Onopordum espiniae* cannot be attributed to phenolic compounds. On the other hand, flavonoids or other compounds seem to be implicated in antibacterial activity since the ethyl acetate extract contains the highest

Table 2. The antibacterial activity of *Onopordum espiniae* leaf extracts.

Strains	Inhibition zones diameter IZD (mm) [*]			
	Hex E	EtOAc E	MeOH E	Genta [†]
<i>Gram-positive</i>				
<i>Bacillus cereus</i> ATCC 14579	12 ± 0.5	30 ± 1.2	-‡	20.3 ± 0.4
<i>Staphylococcus aureus</i> ATCC 25923	12 ± 0.5	23 ± 1.1	-	25.5 ± 1.1
<i>Enterococcus faecalis</i> ATCC 29212	12 ± 0	15 ± 0.8	-	12 ± 0.2
<i>Gram-negative</i>				
<i>Escherichia coli</i> ATCC 25922	-	-	-	21 ± 1.2
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	-	-	18 ± 0.6
<i>Salmonella enteritidis</i> (food isolate)	-	-	-	18 ± 0.8

Hex E: Hexane extract; EtOAc E: Ethyl acetate extract; MeOH E: Methanol extract.

^{*}: Diameter of inhibition zones of various extracts including the diameter of the disc (6 mm).

[†]: Genta: Gentamicin used as a standard antibiotic at 15 µg/ well.

[‡]: Activity not detected.

Data are mean ± SD of three different experiments.

concentration of flavonoids (78.73 mg of QE/g) compared to the methanol extract. Quercetin, a known flavonoid, is an antibacterial molecule that can inhibit bacteria lipase production and inhibit D-alanine ligase activity which occurs in peptidoglycans production (18).

In this study, the antibacterial activity was detected only against Gram-positive bacteria, no activity was observed against Gram-negative ones. These differences could be probably due to cell membrane permeability or other genetic factors. The outer membrane of Gram-negative bacteria acts as a barrier to many environmental substances including antibiotics (19).

Hispidulin is a flavonoid identified for the first time from *Onopordum espiniae*, but it was already isolated from *Onopordum acaulon* L., *Onopordum ambiguum* Fresen, *Onopordum corymbosum* Willk, *Onopordum heteracanthum* C.A.Mey. (*Asteraceae*) and others (20). Its DPPH scavenging activity of 300 µg/ mL proves its DPPH radical-scavenging effect. The free radical-scavenging assay of this flavonoid and its antibacterial activity were also described in literature (21), but methods used were different. The antifungal activity of hispidulin against *Aspergillus tubingensis*, *Botrytis cinerea* and *Penicillium digitatum* was also investigated in another report (22). These studies emphasize the efficiency of this flavonoid aglycone against bacteria and fungi as well and assert its antioxidant activity. Other medicinal properties of hispidulin such as antihepatotoxic, analgesic, anti-inflammatory, and tumor inhibiting were also reported (23-25).

Dehydromelitsin-8-(4'-hydroxy-methacrylate) is identified for the first time from this species, but it was previously isolated from *Onopordum acaulon*, *Onopordum ambiguum*, *Onopordum corymbosum*, *Onopordum cyrenaicum* Maire & Weiller, *Onopordum laconicum* Heldr. & Sart. ex Rouy, *Onopordum leptolepis* DC. (*Asteraceae*) and others (20). This sesquiterpene lactone was not reported for any biological activity yet. In this study, it was not capable to scavenge the DPPH radical.

The methanol extract was the richest in phenolic compounds (243.43 mg of GAE/g) and was the best DPPH scavenger (86 µg/ mL). These data emphasize the role of phenolic compounds

as antioxidant agents.

In summary, this study has shown that hexane and ethyl acetate extracts of *Onopordum espiniae* are potentially good sources of antibacterial agents and demonstrates the importance of such a plant in medicine. The present study will be essential for selecting plant species for further investigation in the discovery of new natural bioactive substances. Further investigations should focus on the isolation and structure elucidation of antibacterial active constituents from *Onopordum espiniae*.

Acknowledgements

This work was supported by grants from the "Tunisian Ministry of Higher Education and Scientific Research".

References

- (1) Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science*. (1992) 257: 1050–5.
- (2) Cakılcıoğlu U, Türkoglu I and Kursat M. Ethnobotanical features of Harput (Elazığ) and its vicinity. *J. Res. East. Anatol. Reg.* (2010) 5: 22–8.
- (3) Al-Qura'n S. Taxonomical and pharmacological survey of therapeutic plants in Jordan. *J. Nat. Prod.* (2008) 1: 10–26.
- (4) Özgen U, Kaya Y and Houghton P. Folk medicines in the villages of Ilıca District (Erzurum, Turkey). *Turkish J. Biol.* (2012) 36: 93–106.
- (5) Barbour EK, Al Sharif M, Sagherian VK, Habre AN, Talhouk RS and Talhouk SN. Screening of selected indigenous plants of Lebanon for antimicrobial activity. *J. Ethnopharmacol.* (2004) 93: 1–7.
- (6) Montoro P, Braca A, Pizza C and de Tommasi N. Structure-antioxidant activity relationships of flavonoids isolated from different plant species. *Food. Chem.* (2005) 92: 349–55.
- (7) Guittonneau GG, Astie M, Begel P, Bismuth H, Chaffin C, Charpin A, Covillot J, Dakhli G, Delaigue M, Dreger F, Duclos A, Dupont JM, Georges C, Le Strat F, Leveugle A, Magnouloux M and Thiebault P. La flore et la végétation de la Tunisie méridionale. *Doc. Act. Soc. Bot. Fr.* (2011) 6: 281–359.
- (8) Salama MM, Ezzat SM and Sleem AA. A New hepatoprotective flavone glycoside from the flowers of *Onopordum alexandrinum* growing in Egypt. *Z. Naturforsch.* (2011) 66: 251–9.
- (9) Machado FB, Yamamoto RE, Zanoli K, Nocchi SR, Novello CR, Schuquel ITA, Sakuragui CM, Luftmann H, Ueda-Nakamura T, Nakamura CV and De Mello JCP. Evaluation of the antiproliferative activity of

- the leaves from *Arctium lappa* by a bioassay-guided fractionation *Molecules* (2012) 17: 1852–9.
- (10) Kirby AJ and Schmidt RJ. The antioxidant activity of Chinese herbs for eczema and of placebo herbs—I. *J. Ethnopharmacol.* (1997) 56: 103–8.
- (11) Djeridane A, Yousfi BM, Nadjemi B, Boutassouna D, Stocker P and Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food. Chem.* (2006) 97: 654–60.
- (12) Singleton VL and Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* (1965) 16: 144–58.
- (13) Tagg JR and McGiven AR. Assay system for bacteriocins. *Appl. Microbiol.* (1971) 21: 943–.
- (14) Martini ND, Katerere DRP and Eloff JN. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *J. Ethnopharmacol.* (2004) 93: 207–12.
- (15) Tosun F, Akyüz Kızılay Ç, Şener B and Vural M. The Evaluation of plants from Turkey for *in-vitro* antimycobacterial activity. *Pharm. Biol.* (2005) 43: 58–63.
- (16) Cushnie TPT and Lamb AJ. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents.* (2005) 26: 343–56.
- (17) Borges A, Ferreira C, Saavedra MJ and Simões M. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb. Drug Resist.* (2013) 19: 256–65.
- (18) Wu D, Kong Y, Han C, Chen J, Hu L, Jiang H and Shen X. D-Alanine: D-alanine ligase as a new target for the flavonoids quercetin and apigenin. *Int. J. Antimicrob. Agents* (2008) 32: 421–6.
- (19) Tuney I, Cadirci BH, Unal D and Sukatar A. Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). *Turkish J. Biol.* (2006) 30: 171–5.
- (20) Bruno M, Maggio A, Rosselli S, Safder M and Bancheva S. The metabolites of the genus *Onopordum* (Asteraceae): chemistry and biological properties. *Curr. Org. Chem.* (2011) 15: 888–927.
- (21) Wadah JAO, Mothana RA, Basudan O, Mohammed MS and Mohamed MS. Antibacterial effect and radical scavenging activity of hispidulin and nepetin; a two flavonoids from *Tarconanthus camphoratus* L. *World J. Pharm. Res.* (2015) 4: 424–33.
- (22) Exarchou V, Kanetis L, Charalambous Z, Apers S, Pieters L, Gekas V and Goulas V. HPLC-SPE-NMR characterization of major metabolites in *Salvia fruticosa* Mill. extract with antifungal potential: relevance of carnolic acid, carnosol, and hispidulin. *J. Agric. Food Chem.* (2015) 63: 457–763.
- (23) Ferrandiz ML, Bustos G, Paya M, Gunasegaran R and Alcaraz MJ. Hispidulin protection against hepatotoxicity induced by bromobenzene in mice. *Life Sci.* (1994) 55: 145–50.
- (24) Kavimani S, Mounissamy VM and Gunasegaran R. Analgesic and antiinflammatory activities of hispidulin isolated from *Helichrysum bracteatum*. *Indian Drugs (Bombay)* (2000) 37: 582–4.
- (25) He L, Wu Y, Lin L, Wang J, Wu Y, Chen Y, Yi Z, Liu M and Pang X. Hispidulin, a small flavonoid molecule, suppresses the angiogenesis and growth of human pancreatic cancer by targeting vascular endothelial growth factor receptor 2-mediated PI3K/Akt/mTOR signaling pathway. *Cancer. Sci.* (2011) 102: 219–25.