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ANTIBACTERIAL EFFECT AND RADICAL SCAVENGING ACTIVITY OF HISPIDULIN AND NEPETIN; A TWO FLVAONES FROM *TARCONANTHUS CAMPHORATUS L*

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ABSTRACT

This study aimed to evaluate the antibacterial and radical scavenging activity effects of two flavones isolated from *Tarconanthus camphoratus L.* Bioactivity –guided isolation of the most active extract led to isolation and identification of two flavones Nepetin and Hispidulin. radical scavenging activity was evaluated by using DPPH (1,1-diphenyl-2-picryl hydrazyl and β-carotene bleaching method, while antibacterial test achieved by using agar dilution method. The two flavones showed strong radical scavenging activity similar in effect to the standard compounds ,Moreover the two flavones showed antibacterial activity only against Gram-+ve bacteria.

KEWWORDS: Radical scavenging activity, antibacterial, *Tarconanthus camphoratus* L**,** Hispidulin, Nepetin.

1. INTRODUCTION

Bacteria represent an important cause of many serious infections including the respiratory tract, skin and soft tissue, neonatal sepsis, endocarditis, meningitis, and bacteremia.[1] On the other hand, antibiotic use has resulted in the development of widespread resistance to commonly prescribed antibacterial agents. This affects the prevalence of infections caused by multiple antibiotic resistant organisms.[2] Therefore, development of new antimicrobial compounds with diverse chemical structures and novel mechanisms of action is of great importance. Plants remain an important source of diverse chemical entities which have been

used as drugs or provide scaffolds from which new drugs have been derived.[3] The selection of a suitable candidate species for investigations is based on the assumption that plants used for long-term by humans might be safer than plant species with no history of human use.[4]

Free radical reactions participating in reactive oxygen species to the overall metabolic perturbations that result in tissue injury and disease. Cellular damage, due to free radical causes serious derangements such as ischemia-reperfusion injury.[5] A number of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been developed, but their use has begun to be restricted due to their toxicity.[6] As a result, there is considerable interest in the food industry and in preventive medicine in the development of natural antioxidants from botanical sources.[7]

Tarchonanthus Camphoratus Herba family (Asteraceae) consists grows in southern regions of Saudi Arabia and Yemen and it's the only plant belonging to the genus *Tarconanthus* which found in the two countries [8, 9] and In this study antibacterial and antioxidant activities of two isolated favones from *Tarchonanthus Camphoratus* were evaluated.

Material and methods

2.1. Plant Material

The plant was collected from Sana'a-Yemen by Dr. Ramzi Mothana and identified at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. The identification was confirmed by Priv. Doz. Dr. Peter Koenig, at the botanical Garden, Ernst-Moritz-Arndt-University, Greifswald, Germany. A voucher specimen was deposited on the Pharmacognosy Department, Faculty of Pharmacy, Sana' University.

2. 3. Determination of the Radical Scavenging Activity

2. 3. 1. Determination of the Antioxidant Activity Using Dpph Method

The DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay was carried out for the evaluation of the antioxidant activity. This assay measures the free radical scavenging capacity of the investigated compounds. DPPH is a molecule containing a stable free radical, and in the presence of an antioxidants compounds which can donate an electron to the DPPH, the purple color typical for free DPPH radical decays and the change in absorbency at λ=517 nm was followed spectrophotometrically. This test was carried out as described by Brand *et al*. [10]. Various concentrations (1000, 500, 100, 50 and 10 µg/ml) of the extract, isolated compounds and the standard was mixed with DPPH solution and after 30 min incubations at

25°C the decrease in absorbance was measured at λ=517 nm, the radical scavenging activity was calculated from the equation.

$$\% \text{ of radical scavenging activity} = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right] \times 100$$

2. 3. 2. Determination of the Antioxidant Activity Using B-Carotene-Linoleic Acid Method

The antioxidant activity of the extract of *Tarconanthus camphoratus* were evaluated also, using β-carotene bleaching method as described by Norhaizan, *etal* by some modification.[11] One millimeter of 0.2 mg/ml β-carotene solution in chloroform was added to flask containing 0.02 ml of linoleic acid and 0.2 ml of Tween-20. The chloroform was removed at 40°C using a rotary evaporator for 10 min. The resultant mixture was immediately diluted with 100 ml of distilled water and mixed for min to form an emulsion. A mixture prepared similarly but without β-carotene, was used as blank. A control containing 0.2 ml of methanol instead of the tested substance was also prepared. A 5 ml aliquot of the emulsion was added to a tube containing 0.2 ml of the tested substance at 1 mg/ml. Rutin (1 mg/ml), in methanolic solution, was used as standard. The tubes were placed in a water bath at 40°C for 2 hr. Absorbance was read at 470 nm at zero time, 1 hr and 2 hr using a UV-Vis spectrophotometer. The antioxidant activity of each sample was calculated as percent inhibition relative to the control, using the following equation.

$$\text{Antioxidant activity (\%)} = 1 - [(A_o - A_t) \div (A^o_O - A^o_t)] \times 100$$

Where: A_o and A^o_O are the absorbance values measured at zero time of incubation for tested substance and control, respectively.

A_t and A^o_t are the absorbance values for tested substance and control, respectively, at t = 120 min.

2. 4. Determination of the Antibacterial Activity

The antimicrobial evaluation was carried out, using agar dilution method as described by Mitscher *et al.* [12]. For testing at 1000 µg/ml, 10 mg of the tested compounds and tested extracts were dissolved or suspended in 0.2 ml of acetone or dimethylsulfoxide, because these solvent preliminary studies showed no inhibition of the cultures at this level (2% final concentration). Trypticase-soy agar was prepared and sterilized in the usual fashion by

autoclaving. Before congealing, 10 ml of agar medium was added to each of the Petri dishes containing the pure compounds or plant extract and the Petri dishes were swirled carefully until the agar begins to set. The tested organisms (Gram-positive bacteria: *Bacillus subtilis* (ATCC 26633), *Staphylococcus aureus* (ATCC 25923), Gram – negative bacteria: *Escherichia coli* (ATCC 25922), *Psuedomonas aeruginosa* (ATCC 27853) and *Mycobacterium smegmatis* (ATCC 35797), , the microrganisms were maintained on Trypticase-soy agar slants and recovered for testing by growth in Trypticase-soy broth for 24 hr.

2. 5. Isolation of the Flavonidal Compounds

Eleven grams of the DCM and ethyl acetate extract (they were pooled together due to similarity in TLC profile) was subjected to a column chromatography Sephadex LH-20 (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and Methanol: Dichloromethane (9: 1) as solvent system. The collected fractions (10 fractions) were subjected to thin layer chromatography using Chloroform: Methanol: Water (9: 1: 0.1) as a mobile phase. Fraction number (5) was showed a strong antioxidant activity as well as activity against Gram-positive bacteria; hence it was subjected for more fractionation, using sephadex-LH 200 packed columns. Seventy grams of F5 was fractinonated using column chromatography (Sephadex LH-20 Pharmacia-LKB Biotechnology, Uppsala, Sweden), Methanol: Dichloromethane (1: 4), and Flow rate:10 ml/h).Fractionation was afforded (8) fractions, which examined by reversed TLC using mobile phase Methanol: Water (7: 3).

Two pure compounds were obtained from this fraction; F-1 (**Hispidulin)** as a yellowish needles (60 mg) and F-2 (**Nepetin)** (23 mg) as a yellowish residue from the last fraction by using preparative HPLC (HPLC- Shimadzu, Japan- CBM-20A, Shim-pack-prep- ODS (H), 65% Methanol: 35% H_2O, 0.8 ml/min , Photo Diode Array Detector, UV-Absorption at 366 nm).

2. 6. Structure Determination of the Flavonidal Compounds

Ultraviolet absorption spectra were obtained in spectroscopic methanol on a Thermo Scientific- Evolution 160 UV -Visible Spectrophotometer. Mass spectroscopic analysis (MS) were obtained using Agilent technologies- 6320- Ion trap LC/MS. Nuclear Magnetic Resonance (NMR); ^{1}H- and ^{13}C-NMR spectra were obtained on the Bruker AM 500 spectrometer (Germany) operating at 500 MHz ($^{1}HNMR$) and 125 MHz ($^{13}CNMR$) in spectroscopic grade $CDCl_3$, CD_3OD and DMSO-d_6. The chemical shifts values are expressed

in δ (ppm) units using (TMS) as an internal standard and the coupling constants (*J*) are expressed in Hertz (Hz). Standard pulse sequences were used for generating COSY, HMQC and HMBC spectra (2D experiments).

2. RESULTS AND DISCUSSION

3.1. Structure Elucidation

Compound F-1 was obtained as a yellowish needles. ^{1}H-NMR spectrum indicated the presence of two singlet aromatic protons each proton integrated for one proton at δ 6.61 and δ 6.79, and were assigned to H-8 and H-3 respectively. Other aromatic protons area were assigned for ring-B protons, two doublet signals at δ 7.92 and δ 6.93 each signal integrating for two equivalent protons were assigned to (H-2', H-6') and (H-3', H-5') respectively. ^{1}H-NMR also indicated the presence of singlet signal integrated for three protons at δ 3.76, was attributed to the methoxy group at C-6. Furthermore, a sharp singlet integrated for one proton at δ 13.09 showed the presence of hydroxyl group at the C-5 position , a broad signal at δ 10-11 assigned to hydroxyl groups at the C-7 and C-4' positions and this was confirmed with HSQC experiment because these protons not showed correlation with any carbon.

^{13}C-NMR spectrum and DEPT experiment indicated the presence of nine quaternary carbons including one carbonyl group at δ 182.1 assigned to C-4, the downfield shift of C-4 indicated the presence of hydroxyl group at C-5, five oxygenated aromatic carbons at δ 163.7, 161.2, 157.4, 152.7 and 152.4 assigned to C-2, C-4', C-7, C-9 and C-5 respectively, other three remaining quaternary carbons at δ 131.3 , 121.1 and 103.9 assigned to C-6, C-1' and C-10 respectively. ^{13}C-NMR spectrum and DEPT experiment indicated also the presence of six methins, two equivalent methin signals at δ 128.4 assigned to C-6' and C-2', other two equivalent methins at δ 115.9 assigned for C-5' and C-3' as well as two methin signals at δ 94.2 and δ 102.3 assigned to C-8 and C-3 respectively. ^{13}C-NMR spectrum also indicated downfield methyl group at δ 59.7 assigned to methoxy group attached to C-6. The correlations between H-2' and H-3', H-6' and H-5' signals were confirmed by COSY experiments. The correlations between each proton signal and the directly attached carbon was achieved by HSQC experiment. The HMBC experiment was the important technique in finding the structure of the compound where two and three bond correlations were observed. H-8 (δ 6.61) correlate with C-10 (δ 103.9) and C-6 (δ 131.3) by three-bond correlation, and correlate with C-7 (δ 157.4), C-9 (δ 152.4) by two-bond correlation, furthermore H-8 also correlate with C-4 (δ 182.1) by four-bond correlation. H-3 (δ 6.79) correlate with C-10 (δ

103.9) and C-1' (δ 121.1) by three-bond correlation, and correlate with C-2 (δ 163.9) and with C-4 (δ 182.0) by two-bond correlation. H-2' and H-6' (δ 7.92) correlate with C-6' and C-2' (δ 128.4), and correlate with C-2 (δ 163.7) and C-4' (δ 161.2) by three- bond correlation. H-3' and H-5' (δ 6.93) correlate with C-5' and C-3' (δ 115.9), and correlate with C-1' (δ 121.1) by three bond correlation and with C-4'(δ 161.2) by two- bond correlation.

The mass spectrum displayed $[M]^+$ at m/z 300.5 corresponding to the molecular ion $C_{16}H_{12}O_6$, which confirmed our structure and other fragments appears at 287, 257and 243.
The UV spectrum in methanol exhibited λmax at 334 (band I) and 275 (band II) nm, indicating its flavonoidal nature. Bathochromic shift was observed with NaOMe, in band I, (+ 60 nm) indicated the presence of a free 4'- hydroxy group. A bathochromic shift with NaOAc, in band II, (+ 39) indicated the presence of a free 7-hydroxyl group. The bathochromic shift in band I exhibited by $AlCl_3$ (+ 27 nm) not significant decreases upon addition of HCl indicated the absence of 3', 4'- dihydroxy groups. These results confirmed the obtained structure (Table 1).

Compound F-2 was obtained as a yellowish residue. ^{1}H-NMR spectrum indicated the presence of two singlet aromatic protons each proton integrated for one proton at δ 6.57 and δ 6.62 proton, and were assigned to H-8 and H-3 respectively. Other aromatic protons were assigned for ring-B protons. The singlet at δ 7.38 integrating for two protons was assigned to H-2' and H-6' and another *ortho*-coupled proton at δ 6.89 (*d*, 8) integrating for one proton and assigned to H-5'. Moreover ^{1}H-NMR spectrum indicated the presence of downfield singlet signal at δ 3.75 and integrated for three protons, assigned for a methoxy protons attached to ring-A in carbon number 6. The correlations between H-2', H-5' and H-6' signals were confirmed by COSY experiments. Four signals appeared as singlets and each integrated for one proton including C_5-OH at δ 13.01, δ10.76, 9.97 and 9.46 assigned to other OH groups.
^{13}C-NMR spectrum and DEPT experiment indicated the presence of ten quaternary carbons including one carbonyl group at δ 182.0 assigned to C-4, six aromatic oxygenated aromatic carbons at δ 163.9, 157.2, 152.8 , 152.3, 149.6 and145.7 assigned to C-2, C-7, C-5, C-9, C-4', C-3' respectively. Other three remaining quaternary carbons at δ 131.3 , 121.5 and 104.0 were assigned to C-6, C-1' and C-10 respectively. Five methine signals at δ 118.9, 116.0, 113.3, 102.3 and 94.0 assigned to C-6', C-5', C-2', C-3, C-8 respectively, also indicated downfield methyl group at δ 59.9 assigned to methoxy group attached to C-6. The correlations between each proton signal and the directly attached carbon was achieved by HSQC experiment. The

HMBC experiment was the important technique in finding the structure of the compound where two and three bond correlations were observed. H-8 (δ 6.57) correlate with C-10 (δ 104.0), C-6 (δ 131.3), C-7 (δ 157.2), C-9 (δ 152.2) by three-bond correlation and with C-4 (δ 182.0) by four-bond correlation. H-3 (δ 6.62) correlate with C-10 (δ 104.0) and C-1' (δ 121.5) by three-bond correlation, and with C-2 (δ 163.9) and C-4 (δ 182.0) by two-bond correlation. H-2' and H-6' (δ 7.41) correlate with C-1', C-4'(δ 149.6), C-3' (δ 145.7) and with C-2 (δ 163.9).

The mass spectrum displayed $[M]^+$ at m/z 316.4 corresponding to the molecular ion $C_{16}H_{12}O_7$, which confirmed our structure and other fragments appears at 270, 257, 241, 229 and 213. The UV spectrum in methanol exhibited λmax at 349 (band I) and 275 (band II) nm, indicating its flavonoidal nature. Bathochromic shift was observed with NaOMe, in band I, (+ 54 nm) indicated the presence of a free 4'- hydroxy group. A bathochromic shift with NaOAc, in band II, (+ 11) indicated the presence of a free 7-hydroxyl group. The bathochromic shift in band I (+ 49 nm) with $AlCl_3$, which decreased to (+34) on the addition of HCl indicated the presence of 3', 4'- dihydroxy groups. The above results confirmed our structure (Table 2).

Table 1. UV spectral data of compound F5-1 (Hispidulin).

λ_{max}	Band I (ring B)	Band II (ring A)
MeOH	334 nm	275 nm
NaOMe	394 nm 327 nm (sh)	276 nm
$AlCl_3$	361 nm	301 nm
$AlCl_3$/HCl	355 nm	299 nm
NaOAc	391 nm	314 nm

* sh = shoulder.

Table 2. UV spectral data of compound F5-2(Neptin).

λ_{max}	Band I (ring B)	Band II (ring A)
MeOH	349 nm	275 nm
NaOMe	403 nm	266 nm
$AlCl_3$	398 nm	275 nm
$AlCl_3$/HCl	364 nm	284 nm
NaOAc	382	286 nm

3. 2 Antioxidant Test

The isolated compounds were able to reduce the free radical DPPH to the yellow-coloured DPPH. The (DCM/ETOAC) along with the flavonoidal compounds showe strong antioxidant

activity, almost similar in effect to Ascorbic acid. In the β-carotene-linoleic acid model system, flavonoidal compounds were also able to inhibit the discoloration of β-carotene at a oncentration of 1mg/ml as shown in Table.3and Table.4. The total antioxidant value of Hispidulin and Neptin similar in effect to that of standard antioxidant flavonoid Rutin.

Table 3. Free radical activity (DPPH-assay) and antioxidant activity (β-carotene-linoleic acid-assay) of hexane and (DCM/ETOAC) extracts at 1000 µg/ml.

Treatment	Radical scavenging activity (%)					Total antioxidant activity (%)
	10	50	100	500	1000	
Hexane extract	15.4	55.9	74.7	77.0	89	61
(DCM/ETOAC) extract	2.3	10.4	93.4	94.4	95.0	84.6
Ascorbic acid	84.3	90.4	95.6	95.7	98	-
Rutin	-	-	-	-	-	92.3

Table 4. Free radical activity (DPPH-assay) and antioxidant activity (β-carotene-linoleic acid-assay) of hispidulin and nepetin extracts at 1000 µg/ml.

Treatment	Radical scavenging activity (%)				Total antioxidant activity (%)
	10	50	100	500	
Hispidulin	23.8	32.5	68.5	94.1	84.6
Nepetin	31.3	93.5	95.5	95.7	92.3
Ascorbic acid	84.3	90.4	95.6	95.7	-
Rutin	-	-	-	-	92.3

3. 3. Antibacterial Test

The results of the antimicrobial activity are shown in Table .5. It was shown that the two isolated flavonoids showed antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* with MIC values between 50 and 100 µg/ml (Table 5).

Table 5. Antibacterial activity of hispidulin and nepetin.

Organism tested	Hispidulin	Nepetin
Bacillus subtilis	MIC = 50 µg/ml	MIC = 100 µg/ml
Staphylococcus aureus	MIC = 100 µg/ml	MIC = 100 µg/ml
Escherichia coli	-ve at 1mg/ml	-ve at 1mg/ml
Psuedomonas aeruginosa	-ve at 1mg/ml	-ve at 1mg/ml
Mycobacterium smegmatis	-ve at 1mg/ml	-ve at 1mg/ml

*Samples were tested first at 1mg/ml if it is positive, MIC will be determined.

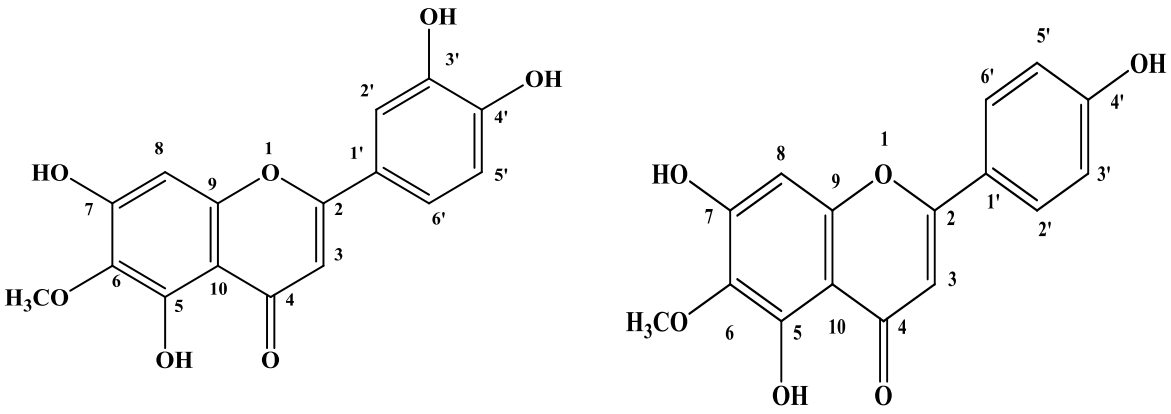

Figure 1: Structures of compounds isolated from *Tarconanthus camphoratus L.*

3. CONCLUSION

Tarconanthus camphoratus may become a potential lead for antioxidants and antimicrobial compounds, specially the results of this study clearly revealed that the crude extracts and isolated compounds showed a potent antioxidant and antimicrobial activities**.**

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Conflicts of Interset

The authors declare no conflict of interest.

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