Journal of Applied Pharmaceutical Science Vol. 3 (01), pp. 055-059, January, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.30110
ISSN 2231-3354

Total phenolic and flavonoid content, antioxidant and antimicrobial activity of extracts from *Tordylium maximum*

Jelena S. Matejić[1]*, Ana M. Džamić[2], Tatjana M. Mihajilov-Krstev[3], Vladimir N. Ranđelović[3], Zoran Đ. Krivošej[4], Petar D. Marin[2]

[1]University of Niš, Faculty of Medicine, 18 000 Niš, Serbia.
[2]University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac", 11 000 Belgrade, Serbia.
[3]University of Niš, Faculty of Science and Mathematics, 18 000 Niš, Serbia.
[4]University of Pristina, Department of Biology, Faculty of Natural Sciences, 38 220 Kosovska Mitrovica, Serbia.

ARTICLE INFO

Article history:
Received on: 18/12/2012
Revised on: 07/01/2013
Accepted on: 21/01/2013
Available online: 28/01/2013

Key words:
Tordylium maximum, Apiaceae, antioxidant, antimicrobial, DPPH, ABTS, phenols, flavonoids

ABSTRACT

The present study describes the total phenolic and flavonoid content and *in vitro* antioxidant and antimicrobial activity of methanol and water extracts from *Tordylium maximum* L. wild growing in Serbia. The total phenolic content in the extracts was determined using Folin-Ciocalteu reagent and their amounts ranged between 74.59 to 83.36 mg GA (gallic acid)/g. The concentrations of flavonoids in the extracts varied from 20.48 to 47.71 mg Qu (quercetin)/g. Antioxidant activity was analyzed using DPPH reagent. Antioxidant activity ranged from 4.042 to 7.825 IC_{50} (mg/ml) and from 2.48 to 2.78 mg VitC (vitamin C)/g when tested with the DPPH and ABTS reagents, respectively, using BHA and VitC as controls. The antimicrobial activity of the extracts was investigated using a micro-well dilution assay against the most common human gastrointestinal pathogenic bacterial strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. This finding suggests that *T. maximum* may be considered as a natural source of antioxidants and antimicrobial agents.

INTRODUCTION

Apiaceae (=Umbelliferae) is a well known family of aromatic and economically important plants, of more than 2500-3000 species in the world (Lawrence, 1969; Pimenov and Leonov, 1993). The genus *Tordylium* L. contains 1 species, *Tordylium maximum* L., in the flora of Serbia (Nikolić, 1973). *T. maximum* is biennial or annual plant growing up to a height of 30-130 cm. This species is distributed in the south and central Europe, though doubtfully native in the northern part of its range (Tutin, 1968).

Recently, the isolation of some flavonoids and a series of antifungal and cytotoxic coumarins from *T. apulum* L. used as spice in Greece were published (Kofinas *et al*., 1998). Also, antibacterial activity of the oil from the aerial parts of *T. apulum* was investigated. Tested oil was characterized by α-humulene (28.7%), octyl hexanoate (11.7 %) and farnesyl acetone (9.8 %) were found as the main components in the oil (Kofinas, 1993).

Moreover, the leaves of *T. apulum* exhibited a remarkable activity in lipid peroxidation assay (Pieroni *et al*., 2002). Trillini *et al*. (2006) also investigated the essential oil of *T. apulum* from Italy, and found (E)-β-ocimene (17.3 %), α-humulene (11.4 %) and octyl octanoate (8.8 %) as major constituents. Aerial parts of the some *Tordylium* species growing in Turkey were examined and their major constituents were determined as *β*-caryophyllene (19.5%), caryophyllene oxyde (18.3%), α-bisabolene (13.1 %) in the oil of *T. trachycarpum;* 2-tridecanone (11.3 %), caryophyllene oxyde (10.0 %) in the oil of *T. lanatum;* α-bisabolene (20.6 %), β-caryophyllene (8.1%), caryophyllene oxyde (6.8 %) in the oil of *T. aegyptiacum; α*-bisabolene (13.5 %), calamenene (9.1 %), α-humulene (5.7 %) in the oil of *T. syriacum;* octyl 2-methylbutyrate (19.7 %), octyl hexanoate (16.6 %), 1-octanol (8.8 %) in the oil of *T. pustulosum* (Tosun, 2011). Several methods are available to evaluate antioxidant activities of natural compounds in foods or biological systems. Two methods commonly used in antioxidant activity assays are the DPPH and ABTS.

* *Corresponding Author*
Email: jekamatejic@gmail.com

ABTS is soluble in both aqueous and organic solvents, and it reacts relatively rapidly compared to DPPH, which normally takes several hours for the reaction to be completed. Color interference of the DPPH assay with samples that contain anthocyanins leads to under-estimation of antioxidant activity. However, this problem does not occur with the ABTS assay, especially when the absorbance is measured at 734 nm (Arnao, 2000). In this work, the antioxidant and antimicrobial activity of the *T. maximum* methanol and water extracts are reported. As far as we know, this is the first report of the antioxidant and antimicrobial activity of this species.

MATERIALS AND METHODS

Chemicals

Organic solvents were purchased from,, Zorka pharma Šabac, Serbia. Gallic acid, 3-tert-butyl-4-hydroxyanisole (BHA) and 2,2-dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent was purchased from Merck, Darmstadl, Germany. Sodium carbonate anhydrous (Na_2CO_3), potassium acetate ($C_2H_3KO_2$), potassium peroxidisulphate ($K_2O_8S_2$) and L(+)-ascorbic acid (Vitamin C) were purchased from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven Belgium. Aluminium nitrate nonahydrate ($Al(NO_3)_3x9H_2O$) was purchased from Fluka Chemie AG, Buchs, Switzerland. ABTS and quercetin hydrate were obtained from TCI Europe NV, Boerenveldsweg, Belgium. All other solvents and chemicals were of analytical grade.

Plant material

Aerial parts of plant *Tordylium maximum* were collected in July 2003 from Ozren mountain (Soko Banja). A voucher specimens for *T. maximum* (BEOU TM 60324), have been deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

Preparation of plant extract

Plant material was air dried in the dark and grounded to a powder. The aerial plant parts (10 g) were powdered and extracted with 100 ml of methanol. The mixture was exposed to ultrasound for 30 min and after 24 h of standing in the dark was filtered. The methanol solvent was removed by evaporation under the reduced pressure, at maximum temperature of 40ºC. Water extract was frozen and later dried by freeze-drying. After evaporation of the solvent, the crude extract was subjected to subsequent analysis.

Determination of total phenolic content

The total phenolic content of extract was determined spectrophotometrically by Folin-Ciocalteu method according to the procedure reported by Singleton *et al.* (1999) with some modifications. Briefly, 300 µl of methanol and water extracts solution and 1500 µl of 1:10 Folin-Ciocalteu reagent were mixed and after 6 minutes in the dark, 1200 µl of sodium carbonate (7.5%) were added. After 2 h in the dark of incubation at room temperature, the absorbance at 740 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). The total phenolic concentration was calculated from gallic acid (GA) calibration curve (10-100 mg/L). Data were expressed as gallic acid equivalents (GA)/g of extracts averaged from 3 measurements.

Determination of flavonoid content

The total flavonoid content was evaluated using aluminium nitrate nonahydrate according to the procedure reported by Woisky and Salatino (1998) with some modifications. The sample for determination was prepared by mixing 600 µl of methanol and water extract solution and 2580 µl of mixture (80% C_2H_5OH, 10% $Al(NO_3)_3$ x 9 H_2O and 1M $C_2H_3KO_2$). After 40 min of incubation at room temperature, the absorbance at 415 nm was measured using Shimadzu, UV-Visible PC 1650 spectrophotometer. The total flavonoid concentration in methanol extract was calculated from quercetin hydrate (Qu) calibration curve (10-100 mg/L) and expressed as quercetin equivalents (Qu)/g of dry extract. Measurements were done in triplicates.

Evaluation of DPPH scavenging activity

The antioxidant activity of extracts was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method. This assay uses stable radical DPPH as reagent (Blois, 1958). Methanolic and water solution of investigated extract (300 µl) (the extract concentrations between 1000 and 7500 µg/ml) was added to 2700 µl methanolic solution of DPPH radical (concentration of 0.04 mg/ml) and after shaking reaction mixture was left to react in the dark for 30 minutes at room temperature. Absorbance of remaining DPPH radical was measured on 517 nm after that time (A1). Every concentration was done in triplicate and the same was done with Vitamin C and BHA, known antioxidants. Blank probes were done in the same way using methanol instead of investigated solution (A0). The decrease of absorption of DPPH solution is calculated by equation:

% of absorption decreased (on 517 nm) = (A0-A1) x100/ A0

Concentrations which decrease absorption of DPPH solution for 50% (IC_{50}) were obtained from the curve dependence of absorption of DPPH solution on 517 nm from concentration for each compound and standard antioxidant. For calculation of these values, Origin 7.0 software was used. Tests were carried out in triplicate.

Evaluation of ABTS radical scavenging activity

For ABTS radical-scavenging activity, the procedure followed the method of Miller and Rice-Evans (1997) with some modifications. The $ABTS^{\cdot+}$ solution was prepared by mixing 19.2 mg of ABTS with 5 ml of potassium persulfate (2.46 mM). The solution was held at room temperature in the dark for 12-16 h before use. The $ABTS^{\cdot+}$ solution (1ml) was diluted with 100-110 ml water, in order to obtain an absorbance 0.7±0.02 at 734 nm. Fresh $ABTS^{\cdot+}$ solution was prepared for each analysis. Antioxidant or standard solutions, 75 µl, were mixed with 3 ml of diluted

$ABTS^{\cdot+}$ solution and incubated at 30ºC for 30'. The absorbance at 734 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). Water was used as a blank. ABTS radical scavenging activity in methanol and water extracts were calculated from Vitamin C (VitC) calibration curve (0-2 mg/L) and expressed as Vitamin C (VitC)/g of dry extract. All experimental measurements were carried out in triplicate and were expressed as average of three analyses ± standard deviation.

Antimicrobial activity

Microbial cultures

The antimicrobial activity of all tested samples was evaluated using laboratory control strains obtained from the American Type Culture Collection: Gram (-) bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076; Gram (+) bacteria: *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC 25923 and yeast *Candida albicans* ATCC 10231.

Micro-well dilution assay

The inocula of the microbial strains were prepared from the overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7-10^8 CFU/ml, depending on genera - consensus standard by the NCCLS) (NCCLS 2003). A serial doubling dilutions of the tested samples (methanol exstract from *Tordylium maximum* species - 100 mg/ml in 30 % ethanol and water extracts-100 mg/ml in 5 % DMSO) were prepared in a 96/well microtiter plate over the range of 50.0 – 0.1 mg/ml in inoculated Mueller-Hinton broth. The final volume was 100 µl and the final microbial concentration was 10^6 CFU/ml in each well. The plates were incubated for 24 h at 37ºC. All experiments were performed in triplicate. Two controls were included - medium with 30% ethanol (negative control) and medium with Streptomycin, Chloramphenicol and Nystatin (positive control). Microbial growth was determined by adding 20 µl of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution (Sartoratto *et al*. 2004). Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the samples inhibiting visible growth (red colored pellet on the bottom of the wells after the addition of TTC). To determine minimal bactericidal/ fungicidal concentrations (MBC/MFC), the broth was taken from each well without visible growth and inoculated in Mueller-Hinton agar (MHA) for 24 h at 37ºC. MBC/MFC was defined as the lowest samples concentration killing 99.9% of bacterial/fungal cells.

RESULTS AND DISCUSSION

Total phenolic content

Total phenolic and flavonoid content, and antioxidant activity (DPPH and ABTS assay) *in vitro* were determined for methanol and water extracst of aerial parts *Tordylium maximum*. The results of the total phenolic content determination of the examined plant extract are presented in **Table 1**. The content of total phenols in different extracts, expressed as gallic acid equivalents (GA) per gram of dry extract, ranged between 74.59 to 83.36 mg GA/g. The highest phenolic content was found in methanol extracts (with 5 mg/ml extract concentration).

Flavonoid content

The summary of quantity of flavonoids detected in the tested extract is shown in **Table 1**. The concentration of flavonoids in methanolic and water extract of aerial parts *T. maximum* was determined using spectrophotometric method with aluminium nitrate nonahydrate. The concentrations of flavonoids in plant extracts ranged from 20.48 to 47.71 mg Qu/g. The highest flavonoid content was identified in methanol extracts (containing 5 mg/ml of extract) Because of their common presence in plants, flavonoids are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Stobiecki and Kachlicki, 2006).

Table. 1: The comparison of the total phenolic content and the total flavonoid, antioxidant capacities by ABTS and DPPH assays content of extracts in *T. maximum* species.

T. maximum	Extract conc. (mg/ml)	Total phenolic content (mg GA/g)	Total flavonoid content (mg Qu/g)	ABTS assay (mg VitC/g)	IC_{50} (mg/ml)
Methanol extracts	5	83.36±0.003	47.71±0.006	2.48±0.010	7.825
Water extracts	3	74.59±0.004	20.48±0.002	2.78±0.010	4.042
BHA	0.1	63.31±0.001	_	2.66±0.005	0.093
Vitamin C	0.1	40.91±0.002	_	_	0.054

Each value in the table was obtained by calculating the average of three analysis ± standard deviation.

DPPH scavenging activity

The effect of an antioxidants on DPPH radical scavenging is due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a, substance that can donate a hydrogen atom, then this gives rise to the reduced form diphenylpicrylhydrazine with the loss of its violet color (Molyneux, 2004). Free radical scavenging capacities of the tested extract was measured by DPPH assay and results are shown in Table 1. According to the results obtained, methanol extract of *T. maximum* was found active with IC_{50} value 7.825 mg/ml of solution and 4.042 mg/ml of solution for water extract. IC_{50} values of the synthetic antioxidant BHA was 0.093 mg/ml and Vitamin C 0.054 mg/ml were determined in parallel experiments. A lower IC_{50} value indicates higher antioxidant activity. Water extract of *T. maximum* aerial parts possessed the strongest antioxidant activities compared to methanol extract.

ABTS scavenging activity

The results from the ABTS assay are shown in **Table 1.** The amount ranged from 2.48 to 2.78 mg VitC/g of *T. maximum* species. The highest content was identified in water extracts from

T. maximum (with 3 mg/ml extract concentration) and the lowest in methanol extracts (with 5 mg/ml extract concentration). Different solvents as water and methanol, (ranged from higher polarity to lower polarity) extracts was used for the study of antioxidant activity. Various solvents were used to achieve extraction of active substances with diversity in their polarity. Water extract of *T. maximum* showed relatively high antioxidant activity that is in accordance with their high concentration of total phenols and flavonoids. Based on results of this study, the extract with the highest antioxidant activity had the highest concentration of phenols. Phenolic compounds are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolics in plants contributes directly to their antioxidant action.

Antimicrobial activity

Methanol extracts of *T. maximum* showed inhibitory antimicrobial activity against all tested strains at concentrations from 0.78-25.0 mg/ml, (Table 2). Water extracts of *T. maximum* did not show inhibitory antimicrobial activity against tested strains. As a reference antibiotics Streptomycin and Chloramphenicol and antimycotic Nystatin were used. Methanol extract was dissolved in 30% aqueous ethanol. This solvent did not show antimicrobial activity (negative control).

Table . 2: Antimicrobial activity of *Tordylium maximum* methanol and water extracts against pathogenic microbial strains using Micro-well dilution assay.

T. maximum	Extracts (MIC/MBC(MFC) in mg/ml)		Referent antibiotics
	methanol extract	water extract	MIC/MBC (MFC) in µg/ml
Gram (-) bacteria			**Streptomycin**
E. coli ATCC 25922	25.0/50.0	50/>50	16,0/16,0
P. aeruginosa ATCC 9027	0.78/>50.0	50/>50	8,0/8,0
S. enteritidis ATCC 13076	1.56/>50.0	50/>50	4,0/4,0
Gram (+) bacteria			**Chloramphenicol**
Bacillus cereus ATCC 10876	1.56/25.0	50/>50	4,0/16,0
L. monocytogenes ATCC15313	6.25/>50.0	50/>50	8,0/16,0
S. aureus ATCC 25923	3.13/25.0	50/>50	1,0/8,0
Fungal strain			**Nystatin**
C. albicans ATCC 10231	12.5/>50.0	50/>50	16,0/16,0

* Streptomycin for Gram (-) bacteria; Chloramphenicol for Gram (+) bacteria and **Abtimycotic Nystatin for *C. albicans*

MIC - minimal inhibitory concentration; MMC – minimal microbicidal concentration

Tested extract showed the best activity against *Bacillus cereus* strains with inhibitory concentration of 1.56 mg/ml and bactericidal concentration of 25.0 mg/ml, This is very important because *Tordylium maximum* is a spice plant while the *B. cereus* is bacteria that often causes food spoilage. Except for *E. coli*, *B. cereus* and *S. aureus* strains, all other strains did not seem bactericide/fungicide activity even at the highest tested concentration of 50.0 mg/ml. Coumarins and flavonol glycosides were found to possess antimicrobial activity against *Bacillus cereus, Candida albicans* and *Cladosporium cucumerinum* strains in previous studies, which were present in large percentage in extracts of plant species of the genus *Tordylium* (Kofinas *et al*., 1993).

CONCLUSIONS

Methanol and water extracts evaluated from *T. maximum* could be used as protective agents against oxidative stress provoked by DPPH and hydroxyl radicals. They expressed relatively good antioxidant activity in comparison with synthetic antioxidants BHA and Vitamin C. Methanol extract showed the inhibitory effect of low concentrations against selected group of microorganisms. These microorganisms cause food spoilage and indicating their potential use, as natural preservatives, in the future production of healthy food which may prevent gastrointestinal disorders.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministry of Education and Science of the Republic of Serbia for financial support (Grant No. 173029).

REFERENCES

Arnao MB. Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. Trends Food Sci. Tech. 2000; 11: 419–421.

Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181: 1199-1200.

Kofinas C., Chinou I., Loukis A., Harvala C., Maillard M. and Hostettmann K. Flavonoids and bioactive coumarins of *Tordylium apulum*. Phytochem. 1998; 48: 637-641.

Kofinas C., Chinou I., Loukis A., Harvala C., Roussakis C., Maillard M. and Hostettmann K. Cytotoxic coumarins from *Tordylium apulum* and their effects on a non-small-cell bronchial carcinoma line. Planta Med. 1998; 64: 174-176.

Kofinas C., Chinou J., Harvala A. and Gally A. Composition and antibacterial activity of the essential oil of *Tordylium apulum* L. J. Essent. Oil Res. 1993; 5: 33-36.

Lawrence GHM. Taxonomy of Vascular Plants. The Macmillan Company, New York (1969) 642-646.

Miller N., Rice-Evans C. Factors influencing the antioxidant activity determined by the ABTS radical cation assay. Free Radic Res. 1997; 26: 195–199.

Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 2004; 26(2): 211-219.

NCCLS – National Committee for Clinical Laboratory Standards. Performance standards for anti-microbial susceptibility testing: eleventh informational supplement. Document M100-S11, National Committee for Clinical Laboratory Standard, Wayne, PA, USA (2003).

Nikolić, V. (1973). Genus *Tordylium* L. In: M. Josifivić (Ed.), Flora of SR Serbia 5 (pp. 304-306). Belgrade, Serbia: SANU.

Pieroni A., Janiak V., Dürr CM., Lüdeke S., Trachsel E. and Heinrich M. *In vitro* antioxidant activity of non-cultivated vegetables of ethnic Albanians in southern Italy. Phytother. Res. 2002; 16: 467–473.

Pimenov MG. and Leonov MV. The Genera of the Umbellifera. Whitstable Litho: Whitstable, Kent (1993).

Sartoratto A., Machado ALM., Delarmelina C., Figueira GM., Duarte MCT. and Rehder VLG. Composition and antimicrobial activity of

essential oils from aromatic plants used in Brazil. Braz. J. Microbiol. 2004 35: 275–280.

Singleton VL., Orthofer R. and Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Meth Enzymol. 1999; 299: 152–178.

Stobiecki, M., & Kachlicki, P. (2006). Isolation and identification of flavonoids. In: E. Grotewold (Ed.) The Science of Flavonoids (pp 47-69). Springer Science and Business Media, New York. (Second edition: 2008).

Tosun A. Comparative analyses of the essential oils from *Tordylium* L. species growing in Turkey**.** Turk J. Pharm. Sci. 2011; 8(3): 239-246.

Trillini B., Pintore G., Chessa M. and Menghini L. Essential oil composition of *Tordylium apulum* L. from Italy. J. Essent. Oil Res. 2006; 18(1): 51-52.

Tutin, T.G. (1968). Genus *Tordylium* L. In: T.G.Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters and D.A. Webb (Eds.) Flora Europaea 2. (p. 367). Cambridge University Press, London, UK.

Woisky R. and Salatino A. Analysis of propolis: some parameters and procedures for chemical quality control. J. Apic. Res. 1998; 37: 99-105.

How to cite this article:

Jelena S. Matejić, Ana M. Džamić, Tatjana M. Mihajilov-Krstev, Vladimir N. Ranđelović, Zoran Đ. Krivošej, Petar D. Marin., Total phenolic and flavonoid content, antioxidant and antimicrobial activity of extracts from *Tordylium maximum.* J App Pharm Sci. 2013; 3 (01): 055-059.