Acta Poloniae Pharmaceutica – Drug Research, Vol. 74 No. 3 pp. 881–890, 2017 ISSN 0001-6837
Polish Pharmaceutical Society

NATURAL DRUGS

RESEARCH ON EXTRACTION TECHNOLOGY, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACT FROM LEAVES OF *ABUTILON THEOPHRASTI* MEDIC.

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Abstract: This paper described the extraction procedure and determination method for the total flavonoids in ethanol extract from the leaves of *Abutilon theophrasti* Medic., and evaluated antibacterial and antioxidant activity. Maximum extraction yield was achieved using 60% ethanol, 1 : 30 (g/mL) of a ratio of material to solvent, 20 min of extraction time, 40 kHz of ultrasonic frequency, 100 W of ultrasonic power, 60°C of extraction temperature and two extraction cycles. Total flavonoids content was 16.79 RE mg/g medicinal materials. The extracts had effective antibacterial activity against 24 test strains from *S. aureus* and *E. coli*, MICs ranged from 2.18 to 8.7 mg/mL; it was also revealed that the extracts demonstrated high flavonids content and potent antioxidant activity, achieved by hydroxyl radical, DPPH radical and ABTS radical scavenging. These results indicated that the extract may be a promising plant demonstrating antibacterial and antioxidant activities.

Keywords: *Abutilon theophrasti* Medic. total flavonoids, extraction procedure, antibacterial activities, ABTS, DPPH

Abutilon theophrasti Medic. is a annual herb about 1-2 m high, with upright stems and round heart-shaped alternate leaves. The leaves, with 7-18 cm of the diameter, are apex acuminate, base cordate, crenate and densely pilose on both sides (1). *A. theophrasti*, as one of more common plants in the world, is a traditional Chinese medicine (TCM) widely used in the folk. There are about 150 species in the world, most of which grow in tropics and sub tropics, such as China, India, Japan, Vietnam, Europe and North America (2).

A. theophrasti as a member of Malvaceae, has been utilized for centuries because of the dispelling wind, detoxication and dieresis effects (1, 3). *A. theophrasti* leaves are often used to treat ulcer, swell and venom. Besides, *A. theophrasti* leaves have activities of anti-inflammatory and analgesic (4).

The chemical compounds in *A. theophrasti* leaves have been studied more widely and deeply. The studies on the chemical compounds of *A. theophrasti* leaves were conducted, mainly including flavonoids and phenolic (5, 6), such as protocatechuic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, rutin and naringenin. At present, flavonoids in *A. theophrasti* leaves are attracting more and more chemical, pharmaceutical and nutrition researchers' attention .

Flavonoids and phenolic, widely existing in fruits, vegetables and plants (7-10), were considered to have many pharmacological activities, such as antibacterial action, antioxidant, anti-inflammatory, hepatoprotective activity and anticancer (11-16), so the extraction procedure has been widely researched (17-21).

Antimicrobial agents are widely used in the food, pharmaceuticals and chemical industry as potential inhibitors of decay. However, many synthetic antimicrobial agents used in foods and drugs, such as quinolones and sulfonamides, may produce gastrointestinal reaction, anaphylaxis and other adverse effects (22, 23). On account of this, more attention has been paid to extract natural non-toxic or harmfulless antimicrobial agents in an effort to protect the human or animals body from the damage

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of drug adverse reaction and retard the progress of antibiotic resistance (24, 25). However, there is almost no study focusing on *in vitro* antibacterial activity of the extracts.

Antioxidants were defined as molecules that, at low concentration and specific assay conditions, can delay or prevent oxidation of an oxidizable substrate (26). In recent years, flavonoids compounds have played an important role in food and medical industry because of their strong *in vitro* and *in vivo* antioxidant activities and their ability to scavenge free radicals and scavenging metals (14, 15).

Hence the objective of this research was to extract and determine total flavonoids content from *A. theophrasti* leaves by single factor, orthogonal test and aluminum nitrate-sodium nitrite-sodium hydroxide experimental design methods, as well as to evaluate antibacterial activity and antioxidant activities of the ethanol extracts (EE) by broth micro-dilution method and scavenging hydroxyl radical, DPPH radical and ABTS radical methods were used.

EXPERIMENTAL

Apparatus and reagents

The total flavonoids content (TFC) was determined by DU 730 nucleic acid protein analyzer (Beckman Coulter Inc., USA). Standard of rutin (HPLC purity© 98.0%) was purchased from National Institutes for Food and Drug Control (Shenyang, China). Standards of gentamicin, ofloxacin and ascorbic acid (HPLC purity© 98.0%) were from China Institute of Veterinary Drug and Control (Beijing, China). Nutrient broth and Mueller-Hinton agar were purchased from Beijing Aoboxing Biological Technology Co., Ltd. (Beijing, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All other chemicals and solvents were of analytical grade from China. The antibacterial activity was evaluated by *E. coli* and *S. aureus*. Test strains included standard strains (ATCC 25922 and ATCC 25923) and their clinical isolates. The standard strains were obtained from Culture Collection Center of China supervision of veterinary drug, and clinical isolates were from clinical mastitis cows in Liaoning province in China. These clinical isolates were separated, cultured, identified and preserved by other researcher in our research group.

Sample preparation

The leaves of *A. theophrasti*, Malvaceae family, were collected from Jilin province in China (No.121024). The leaves were cleaned, dried naturally in a shady and dry place during 20 days, and ground to fine powder for extraction.

Extraction of flavonoids compounds

Initially, a 0.5 g powdered leaves sample of *A. theophrasti* was extracted with different concentration ethanol, ratio of material to solvent and exacting time, by ultrasound at 60°C, ultrasonic frequency of 40 kHz, and power of 100 W, in a two-cycle extraction. After extraction, the extract was filtered and combined, then the filtrate was evaporated to dryness. The solid residues were stored in a refrigerator (4°C), dissolved with 10 mL of 60% ethanol until used for analysis.

Method validation

Calibration curves

A reference standard of rutin was dissolved in 60% ethanol. The stock solution of the standard was prepared as 0.25 mg/mL. The calibration standard working solutions were freshly prepared by diluting the stock solution with 60% ethanol in appropriate quantities. The rutin was quantified by comparing its absorbance value to the standard curves. Concentration range for rutin was 3.2-125 μg/mL.

Precision, accuracy, repeatability and stability

The precision of the method was evaluated by six replicate analyses of the same concentration standard solution over consecutive three days. The variations were expressed by relative standard deviation (RSD). The accuracy of the method was determined by analyzing the test samples spiked with three different levels of standards and calculating the percent recoveries of the analytes. Three replicate analyses were carried out at each spiked level. The original amounts of the analytes in the test samples were subtracted from the measured amounts of each spiked sample before calculating recoveries. The repeatability of the method was confirmed by five replicate samples. The repeatability was also calculated by RSD value. The stability of the analytes in the final extracts stored at room temperature was investigated by replicate analysis of the sample solution at 0, 10, 20, 30, 40, 50, 60, 80, 100 and 120 min. Variations were expressed as RSD.

Determination of the total flavonoids content

The TFC in the extracts was determined by classical aluminum nitrate-sodium nitrite-sodium hydroxide colorimetric method (21) with minor modification, and rutin as standard. Five mL of extract solution (0.05 g/mL) was placed in a 10 mL

volumetric flask, and then 0.3 mL of 5% sodium nitrite solution. After 6 min at room temperature, 0.3 mL of 10% aluminum nitrate solution was added. After another 6 min, 4 mL of 4% sodium hydroxide solution was added and volume was made up with 60% ethanol solution. The reaction solution was mixed thoroughly and incubated for 20 min at room temperature. After high-speed centrifugation for 6 min, the absorbance of the supernatant was measured at 510 nm. TFC was defined as follows: extraction yield of total flavonoids (w/w) (%) = (mass of total flavonoids expressed as mg of rutin equivalents/1 g of *A. theophrasti* leaves samples). All tests were performed in triplicate and the means were calculated.

Broth micro-dilution assay

MIC values of all the strains were evaluated using micro-well dilution method as described by Eloff (27) with some modifications. Overnight cultures of *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and 22 clinical isolates in Mueller-Hinton agar, were adjusted to 0.5 McFarland turbidity standard (1×10^8 CFU/mL) and then diluted 1 : 100 with sterile nutrient broth. The solid residue of the total flavonoids extract was dissolved in water to obtain an initial concentration of stock solution of 17.4 mg/mL. Ten serial twofold dilutions of the stock solution were prepared to a final concentration of 17.4 to 0.034 mg/mL. Briefly, each well of 96-well plate was filled with 100 µL of the EE solution and 100 µL bacteria inoculums. The antibiotic gentamicin and ofloxacin (in similar volume with test sample) were respectively included as positive controls in each assay. The plates were covered and incubated at 37°C for 24 h. The MIC values were defined as the lowest concentration of samples showing clear wells or with complete inhibition of bacteria. The experiment was repeated in triplicate.

Antioxidant activity assay

Measurement of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by the Smirnoff method with some modification (28). Adopting fixed reaction time method, different volume (0.1, 0.2, 0.4, 0.8, 1.0 mL) of EE samples with concentration of 870.7 µg/mL were dissolved. The sample solution was mixed with 2 mM/L $FeSO_4$ (3.0 mL), 1 mM/L H_2O_2 (3.0 mL), 6 mM/L salicylic acid-ethanol (3.0 mL) and distilled water to 10 mL for 15 min at 37°C. The hydroxyl radical was detected by monitoring absorbance at 510 nm. The distilled water and ascorbic acid (Vc) were used as the blank control and positive control, respectively. The assay was done in triplicate. The hydroxyl radical scavenging activity was expressed as:

$$\text{Scavening rate } (\%) = \frac{A_{\text{blank control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100$$

where $A_{\text{blank control}}$ was the absorbance of distilled water, and A_{sample} was the absorbance of the flavonoids extract or Vc sample mixed with reaction solution.

Measurement of DPPH radical scavenging activity

The DPPH· radical scavenging activity was measured according to previous studies with a few modifications. Briefly, 2 mL of 0.1 mM ethanolic solution of DPPH· radicals were added to different volume (0.1, 0.2, 0.5, 1.0, 2.0 mL) of the EE samples with concentration of 870.7 µg/mL and distilled water to 4 mL. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at room temperature in the dark. Vc was used as the positive control and distilled water as the blank control. Each sample was measured in triplicate. The scavenging effect was calculated according to the following equation:

$$\text{Scavening rate } (\%) = \frac{A_{\text{blank control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100$$

where $A_{\text{blank control}}$ was the absorbance of distilled water, and A_{sample} was the absorbance of the flavonoids extract or Vc sample mixed with reaction solution.

Measurement of $ABTS^+$ radical scavenging activity

Antioxidant activity (AA) was measured using an improved ABTS method as described by Re et al. (29). ABTS (7 mM/L) was dissolved in distilled water, to which potassium persulfate (2.45 mM/L) was added, generating the $ABTS^+$ radical cation. The solution was incubated overnight at room temperature protected from light exposure. Phosphate buffer saline (PBS) (pH 7.4) was prepared by dissolving 8.5 g NaCl, 5.54 g $Na_2HPO_4 \cdot 12H_2O$ and 0.26 g $NaH_2PO_4 \cdot 2H_2O$/L in distilled water. The pH was adjusted to 7.4 using NaOH 0.1 M/L. The radical cation $ABTS^+$ was diluted in PBS before use, to an absorbance of 0.700 ± 0.02 at 734 nm.

Three and half mL of ABTS radicals were added to 0.5 mL of the different concentration (87.07, 174.1, 348.3, 696.6, 870.7 µg/mL) of the EE sample. The absorbance of the mixture was measured at 734 nm after 15 min of incubation at room temperature in the dark. Vc was used as the positive control and distilled water as the blank control. The

scavenging effect was calculated according to the following equation:

$$\text{Scavening rate (\%)} = \frac{A_{\text{blank control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100$$

where $A_{\text{blank control}}$ was the absorbance of distilled water, and A_{sample} was the absorbance of the flavonoids extract or Vc sample mixed with reaction solution.

Statistical analysis

All experimental results were centred on using three parallel measurements, and expressed in the mean ± standard error of mean (SEM). Statistical analysis was performed using a one-way ANOVA and significant differences among groups were determined by Duncan's multiple range tests at a significance level of p values < 0.01 with the SPSS 19.0 (SPSS19.0 for WINDOWS; IBM Co., USA). In all statistical analyses, a probability of p values < 0.05 was considered significant.

RESULTS AND DISCUSSION

Optimization of extraction procedure

On the basis of the references (30-34) and practical experience, the effect of three main factors including ethanol concentration, the ratio of material to solvent and extraction time were investigated by the single factor experiment.

The effect of ethanol concentration on the extraction yield of total flavonoids

Ethanol concentration was an important parameter of the total flavonoids extraction. Usually, it was not a constant or linear relationship between ethanol concentration and the extraction yield of total flavonoids. Here, it was, respectively, set at 20%, 40%, 60%, 80% and 100% to examine the influence of different ethanol concentration on the yield of the total flavonoids extraction when other extraction conditions were as follows: the ratio of material to solvent 1 : 20 (g/mL) and extraction time 20 min.

Figure 1 indicated that the yield of total flavonoids rose gradually with the increase of ethanol concentration, and then reached the peak at 60%, and finally dropped from 60% to 100%. Therefore, 50%, 60% and 70% were selected for further optimization in orthogonal test.

The effects of the ratio of material to solvent on the extraction yield of total flavonoids

The choice of the ratio of material to solvent was another important step. If the ratio of material to solvent is too small, the total flavonoids in medicines material could not be extracted fully. If the ratio of material to solvent is too big, this would waste of experimental material. So it is necessary to screen the most suitable the ratio of material to solvent for extraction of targeted flavonoids. In this study, effect of different ratio of material to solvent (1 : 10, 1 : 15, 1 : 20, 1 : 25 and 1 : 30 g/mL) on the extraction yield was investigated when the other extraction conditions were as follows: ethanol concentration 60% and extraction time 20 min.

The results are showed in Figure 2. The yield increased greatly when the ratio increased from 1 : 10 to 1 : 20, and then it maintained a mild slope

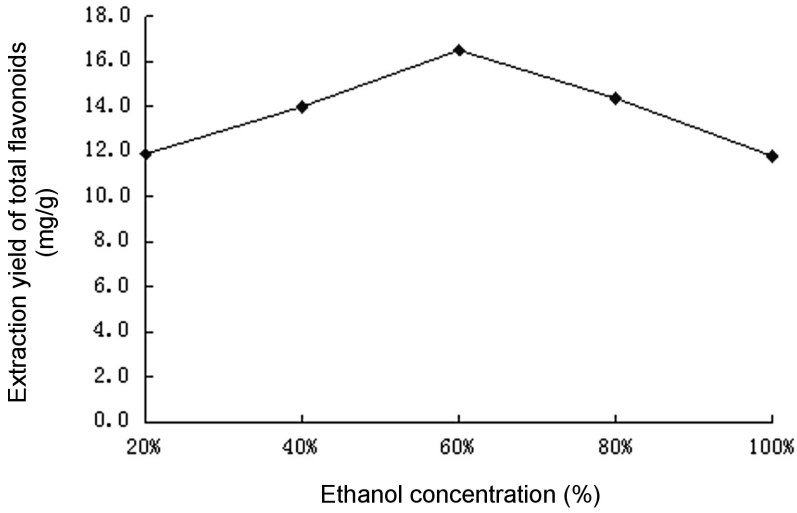

Figure 1. Effect of ethanol concentration on the extraction yield of total flavonoids

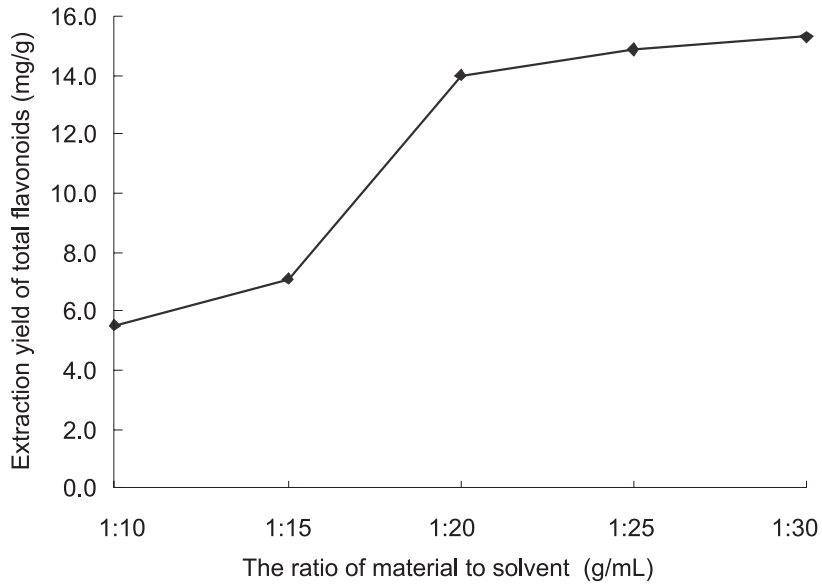

Figure 2. Effect of the ratio of material to solvent on the extraction yield of total flavonoids

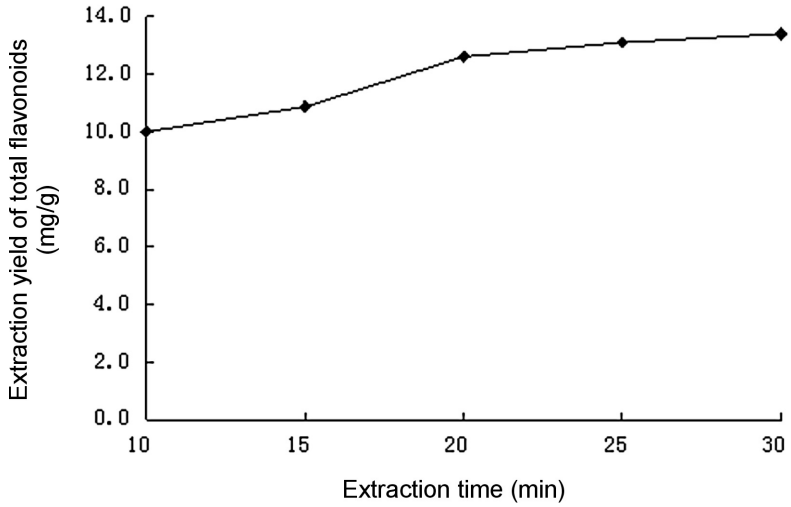

Figure 3. Effect of extracting time on the extraction yield of total flavonoids

when the ratio of material to solvent increased. Taking into account of cost, the ratios of material to solvent range of 1 : 20 to 1 : 30 were further refined.

The effects of extracting time on the extraction yield of total flavonoids

Extraction time was also an important factor for extraction of active constituents from plant materials. It was associated with the final extraction efficiency, yield of total flavonoids and the experimental cost. In this study, different extraction times (10, 15, 20, 25, and 30 min) were tested when the other extraction conditions were as follows: ethanol concentration 60% and the ratio of material to solvent 1 : 20.

The results are displayed in Figure 3. It could be found that the extraction yield improved with the increase of the extraction time from 10 to 25 min, and increased slowly after 20 min. Thus, the extraction time 20, 25 and 30 min were chosen for the following screening.

Design for $L_9(3^4)$ orthogonal test

Considering the above experiment results, ethanol concentration (A), the ratio of material to

solvent (B) and extraction time (C) were selected as the experimental factors and a $L_9(3^4)$ orthogonal test was designed. Taking the TFC as the investigation index, the best extraction procedure was optimized.

The results of variance analysis showed that the importance of three factors to affect the total flavonoids extraction yield was A > B > C, and the A factor with statistical significantly difference ($p < 0.05$). The optimal extraction condition was $A_2B_3C_1$, i.e., ethanol concentration 60%, the ratio of material to solvent 1 : 30 and extraction time 20 min.

The results of method validation

Calibration

Calibration curve corresponding to rutin was A = 0.0051 × C + 0.0043, where A is the absorbance value and C is the rutin concentration. The calibration curve has shown good linearity function response in concentration range from 1.6 to 62.5 μg/mL and the correlation coefficient (*r*) was 0.9995.

Precision, accuracy, repeatability and stability

The RSDs corresponding to the intra- and inter-day precision were 0.95% and 1.77% ($n = 6$), respectively. The average recovery of rutin was 91.9%, with the RSD 5.8%. The RSD of the reproducibility assay was 1.78% ($n = 5$). The RSD of the assay results at different time intervals was 0.34% ($n = 5$), which indicated that the sample solution was stable at room temperature for at least 120 min.

The total flavonoid content in ethanol extract

The validated ultraviolet spectrophotometry method was applied to the determination of the TFC in leaves extract of *A. theophrasti*. The content of the total flavonoid extract was (16.79 ± 0.19) mg/g ($n = 3$).

Table 1. MIC for the total flavonoids extract of leaves from *Abutilon theophrasti* Medic. and standards.

Strains	Gentamicin (μg/mL)	Ofloxacin (μg/mL)	Total flavonoids (mg/mL)
E. coli ATCC25922	2.0	0.25	8.71 ± 0.22
E. coli clinical isolate No.1	2.0	0.5	8.51 ± 0.15
E. coli clinical isolate No.2	8.0	< 0.125	8.66 ± 0.06
E. coli clinical isolate No.3	< 0.125	1.0	8.59 ± 0.11
E. coli clinical isolate No.4	16.0	< 0.125	8.71 ± 0.15
E. coli clinical isolate No.5	8.0	< 0.125	8.52 ± 0.06
E. coli clinical isolate No.6	0.5	< 0.125	8.65 ± 0.07
E. coli clinical isolate No.7	< 0.125	0.125	8.67 ± 0.08
E. coli clinical isolate No.8	4.0	< 0.125	8.62 ± 0.14
E. coli clinical isolate No.9	4.0	< 0.125	8.68 ± 0.08
E. coli clinical isolate No.10	< 0.125	< 0.125	8.66 ± 0.09
E. coli clinical isolate No.11	< 0.125	< 0.125	8.68 ± 0.12
S. aureus ATCC25923	2.0	< 0.125	8.63 ± 0.08
S. aureus clinical isolate No.1	0.25	4.0	8.64 ± 0.16
S. aureus clinical isolate No.2	2.0	0.25	8.68 ± 0.07
S. aureus clinical isolate No.3	1.0	0.125	8.75 ± 0.16
S. aureus clinical isolate No.4	0.25	0.25	8.65 ± 0.15
S. aureus clinical isolate No.5	8.0	4.0	4.37 ± 0.17
S. aureus clinical isolate No.6	4.0	0.125	8.59 ± 0.16
S. aureus clinical isolate No.7	> 64	0.25	8.63 ± 0.14
S. aureus clinical isolate No.8	2.0	0.125	4.30 ± 0.13
S. aureus clinical isolate No.9	2.0	< 0.125	4.36 ± 0.20
S. aureus clinical isolate No.10	< 0.125	< 0.125	2.21 ± 0.12
S. aureus clinical isolate No.11	0.5	4.0	4.36 ± 0.18

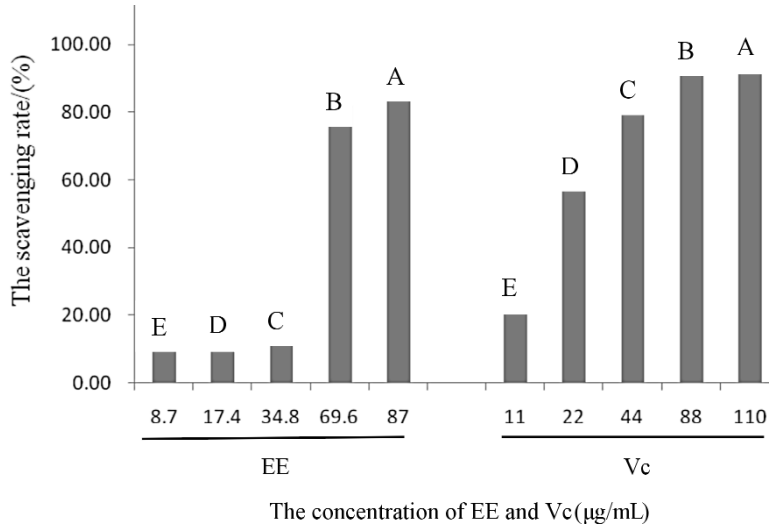

Figure 4. The scavenging effect on OH of EE and Vc. Means with different letters indicate significant differences at $p < 0.01$ according to DMRT

Antibacterial activity

The MIC (Table 1) determined by micro-well dilution method showed that the total flavonoids extract from the leaves of *A. theophrasti* had antibacterial activity towards tested *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and their clinical isolates. According to the MIC, the EE had higher antibacterial activity to the *S. aureus* clinical isolate No.5, 8, 9, 10 and 11 with MIC range 2.21-4.37 mg RE/g than other tested strains with MIC range 8.51-8.75 mg RE/g. It implied that the extract could inhibit the growth of the 24 test strains. Gentamicin and ofloxacin showed greatest antibacterial activity among all the test strains. The water negative control demonstrated good growth. To check any contaminating organisms nutrient broth and Mueller-Hinton agar plates were incubated at 37°C for 24 h. There was no growth in these conditions.

To the best of our knowledge no data are reported concerning antibacterial activity of the EE from the leaves of *A. theophrasti* to isolated strains. The results in Table 1 demonstrated that the extract has almost the same antibacterial activity to standard and isolated strains of *E. coli* and *S. aureus*. This is an indication that the extract is likely to be the potential of antibiotic alternatives, which will provide reference and research basis for solutions of the problem of antibiotic resistance.

Antioxidant activity

Antioxidant activity by the hydroxyl radical method

As shown in Figure 4, both EE and Vc exhibited obvious scavenging activity on hydroxyl radicals in a concentration-dependent manner. The scavenging rates were improved with the increasing of the total flavonoids concentration. At the concentration of 87.07 μg/mL, the inhibition effects of EE was 88.83%. And at the concentration of 88 μg/mL, the inhibition effects of Vc was 97.31%. The results indicated that EE can obviously inhibit ·OH produced by the $FeSO_4$-H_2O_2-salicylic acid system, the antioxidant activity of EE was close to Vc.

In Rollet-Labelle's (35) research report, comparing to other reactive oxygen species, the hydroxyl radical is the most reactive and could induce severe damage to adjacent biomolecules functioning in living cells, which can be prevented and/or inhibited by antioxidants. Therefore, scavenging the hydroxyl radical is important for antioxidant defense in cell or food systems (36).

Antioxidant activity by the DPPH method

As evident from Figure 5, EE displays concentration dependent radical scavenging effects although weaker than that of Vc. This increased dramatically from 42.64% to 97.69% at the concentration range from 43.53 to 217.7 μg/mL for EE as well as the Vc reached 96.76% at 110 μg/mL. When the concentration of EE was over 217.7 μg/mL, the scavenging effect on DPPH· was not of very significant difference.

Antioxidants can protect against the damage caused by free radicals that have been implicated in the etiology of large number of major diseases (37). As a consequence, it was very important for looking for a natural green antioxidants. The results proved

that the antioxidant activity of EE was better evaluated by scavenging of DPPH· radicals method, which was a classic antioxidant assay, and laid the foundation for the research and development of antioxidant.

Antioxidant activity by the ABTS method

Figure 6 showed the scavenging effect of EE and Vc on ABTS radical. As shown, the ABTS radical-scavenging capacity of two samples was dose-dependent. Comparing to the positive control Vc, the scavenging activities of EE significantly increased with the increasing concentrations. At the concentration about 110 μg/mL, the scavenging effects of EE and Vc on the ABTS radical were 87.61% and 99.57%, respectively.

These results suggest that the EE might have a better antioxidant activity estimated by ABTS assay.

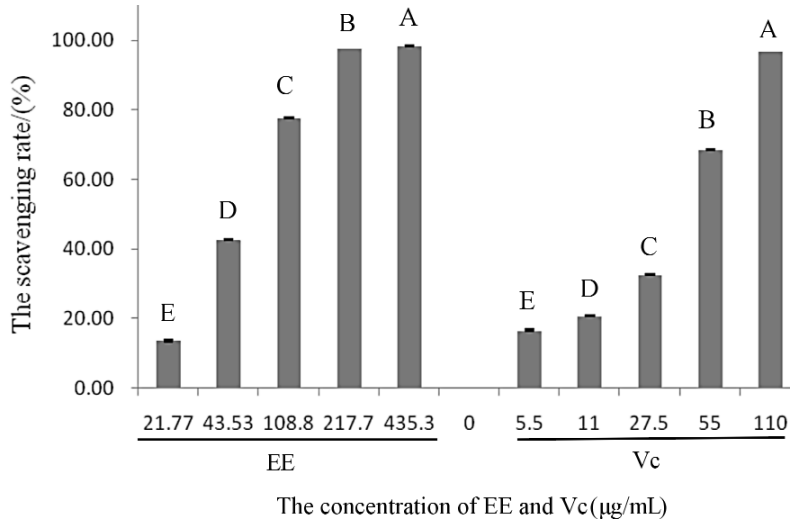

Figure 5. The scavenging effect on DPPH·of EE and Vc. Means with different letters indicate significant differences at $p < 0.01$ according to DMRT

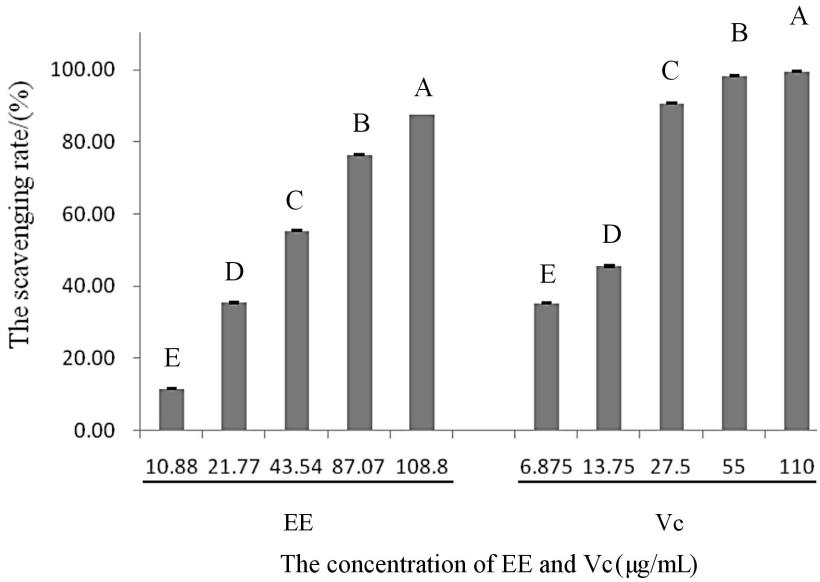

Figure 6. The scavenging effect on ABTS·of EE and Vc. Means with different letters indicate significant differences at $p < 0.01$ according to DMRT

Thus, in comparison with other synthetic antioxidants, the EE was a rich source of natural antioxidant compounds that can potentially be used in food and nutraceutical formulations.

CONCLUSION

The previous studies focused very much on extraction, isolation, purification and identity research of flavonoids constituents from the *A. theophrasti*, nevertheless, few studies focused on extraction procedure, antibacterial activity and antioxidant activity for the total flavonoids in the *A. theophrasti*. In the present study, the extraction procedure, content determination, antibacterial activity and antioxidant activity of the total flavonoids from the leaves of *A. theophrasti* were evaluated. The ultrasonic-assistant extraction of the total flavonoids was investigated with single factor test and three-variable, three-level orthogonal test design for enhancing the total flavonoids extraction yield. The optimum extraction procedure was $A_2B_3C_1$ (60% ethanol of extraction solvent, 1 : 30 of the ratio of material and solvent and 20 min of extracting time). The antibacterial activity of the total flavonoids *in vitro* was determined successfully by broth microdilution method. Based on all the results, it is revealed that the ultrasonic-assistant extraction is an effective method for extraction of the total flavonoids from the leaves of *A. theophrasti*, and the total flavonoids extract exhibits mild antibacterial activity. The present study revealed that EE demonstrated high flavonids content and potent antioxidant activity, achieved by hydroxyl radical, DPPH radical and ABTS radical scavenging. The results clearly revealed that EE from the leaves of *A. theophrasti* could be considered as a potential candidate for developing safe antibacterial and antioxidant agent. The knowledge gained from this study should be useful for further exploitation and application of the resource in clinical research.

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Received: 21. 03. 2016