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Original article

The Effect of Extraction Method on the Major Constituents and Biological Effects of *Trachyspermum ammi* L. Fruits

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Abstract

Background and objectives: Variety of extraction methods coupled with definite solvents could increase the removal rate of major constituents from plants. This research has been conducted to evaluate the effect of extraction methods on the main group of compounds, cytotoxicity, anti acetylcholinesterase (AChE) and antioxidant activity of *Trachyspermum ammi* fruits. **Methods**: To compare the quality of extracts earned from maceration and reflux techniques, the amounts of total phenolics, flavonoids and antioxidant property of *T. ammi'*s fruits extracts were determined; moreover, the cytotoxic activity against Human acute lymphoblastic leukemia (ALL) cell lines (NALM-6) was conducted using MTT assay. Anti-acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity of both extracts were also examined by Ellman's method. **Results**: The extraction yield of the plant was significantly higher for maceration compared to reflux extraction. Also, both antioxidant activity and total flavonoid contents (IC_{50}=132.95 µg/mL and 140.15 mg catechin/g dry extract, respectively) showed higher amounts considerably in the maceration extraction. In reverse, the content of phenolic compounds (147.28 mg gallic acid/g dry extract and 16.6 mg thymol/g dry extract) was elevated in the refluxed extract. The result exerted moderate inhibition on butyrylcholinesterase activity ($IC_{50=}$ 394.161 µg/mL) and cytotoxicity (IC_{50} =166.92±1.76 µg/mL for NALM-6 cell line) of the extract using maceration. **Conclusion**: The maceration method could provide additional amounts of major constituents and greater biological properties compared to the reflux technique.

Keywords: biological effect; extraction; maceration; reflux; *Trachyspermum ammi*

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Introduction

A wide range of medicinal plants are applied in natural remedies and carry many efficient constituents which can prevent, cure or treat minor or major diseases. The leading step to take the biologically effective ingredients from herbal resources is extraction method [1]. Diversity in plant extraction methods could improve the quality and quantity of bioactive compounds of the extract proficiently.

Ajowan (*Trachyspermum ammi* L.) belonging to Apiaceae (Umbalifireae) family is an annual herb with grayish brown fruits. The plant may be up to 90 cm tall with an erect, glabrous or branched stem, which grows originally in India and is cultivated widespread throughout Asia [2-4].

Phytochemical studies on the extract of *T. ammi's* seeds have revealed the presence of alkaloids, phenolics, steroids, glycosides, tannins, and flavonoids [5]. Javed et al. have reported the allelopathic effects of the plant alkaloids [6]. Also, phytosterols of the plant extract could be considered as sex hormones in herbal remedies [7]. Moreover, phenolic compounds of *T. ammi* extract have shown antimicrobial, cytotoxic activity, anti-inflammatory, and antiviral effects [8]. According to some other studies, flavonoids of Ajwan have exerted multiple biological effects, including free radical scavenging abilities, antispasmodic, germicide, and antifungal activities [9].

Biological studies on the extract or essential oil of *T. ammi* have illustrated various activities. The plant essential oil or extract has been used for the treatment of colitis, flatulence, indigestion, dyspepsia, and diarrhea and for helminthic therapy [10,11]. Some of the therapeutic properties of the plant is due to the essential oil components, in which, thymol as the major phenolic compound, has been reported to be an antispasmodic, germicide and antifungal agent [6]. According to Moein *et al.* research, *T. ammi* fruits' essential oil was known to be effective against respiratory and gastrointestinal diseases [12] . A research was managed by Chauhan *et al*., has demonstrated the cytotoxicity and analgesic activity of *T. ammi* fruits ethanol extract which was meaningfully relevant to its antioxidant power and total phenolic content [13].

Ajowan's fruits are used in Iranian traditional medicine for treatment of abdominal pains, relieving flatulence, nausea, vomiting, burp, spasmodic disorders and infectious-worm diseases [14].

Based on many reports, choosing a suitable extraction method can significantly affect the quality of the extract, biological activity and the quantity of extracted major compounds. In the present study, two different extraction methods were applied on *T. ammi* fruits by using cold maceration and reflux extraction. The aim of the research was to compare the effect of extraction technics and heating on the amount of total flavonoids ad phenolics content in the extract of *T. ammi* fruits. Also, the antioxidant activity, anti acetylcholinesterase and anti butylcholinesterase effect and cytotoxicity of both extract were evaluated.

Material and Methods

Plant material

Fruits of *T. ammi* were purchased from traditional herbal market, Tehran, Iran on June, 2015. A voucher specimen has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (PMP-657).

Chemicals and apparatus

Most of chemicals and reagents were of analytical grade or purest quality and purchased from Sigma- Aldrich, Merck (USA). N-hydroxyethylpiperazone-n-2-ethanesulfonic acid, fetal bovine serum and RPMI 1640 medium were purchased from Biosera, England. The microplate reader ELX800 model, was supplied from BioTek, USA. The UV visible spectrophotometer Optozem (South Korea), 2120 UV Plus, double-beam was used and equipped with 8 of 1.0 cm path length glass cells.

Extraction

The extraction of dried and finely powdered aerial parts of *T. ammi* was carried out using two different technics. In maceration method 257.5 g of the plant was extracted with ethanol 70%

continuously (1860 mL×7 days).The filtered and completely dried extract was applied to measure all tests.
In the reflux method, ethanol 70% (600 mL) was added to 200g of dry powdered fruits and heated for 1 hour. The filtered liquid solution was evaporated with a rotary evaporator and the dry extract was stored at 4º C until usage.

Assay of the total phenolics content as gallic acid
Total phenolics content was estimated by the Folin-Ciocalteu method, based on the procedure suggested by Pourmorad *et al*. [15] with some modifications. Briefly, 0.5 mL of the plant extract (500 µg/mL) or gallic acid (25-150 µg/mL standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 mL) and aqueous Na_2CO_3 (4 mL, 1M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. Gallic acid was used as the standard for calibration curve. Total phenolics content values were expressed in terms of mg equal gallic acid in 1 g dry extract.

Assay of total phenolics content as thymol
The total amount of phenolics as thymol was determined by the amino-antipyrine method with some modifications [16]. Five mL of the extract (40 µg/mL) was diluted to 100 mL with 40 mL of ethanol 40% and distilled water. Then, 5 mL of this solution was transferred to a 100-mL volumetric flask and dilute with 45 mL distilled water and 0.5 mL NH_3 (0.5%). In the next step, one mL of 4-aminoantipyrine was also added to the solution and mixed well. After that, 4 mL of the potassium ferricyanide (III) 2% solution was transferred to the mixture and was shaken again. Finally, after 5 min and exposing the Emerson reaction [17], the solution was washed with chloroform (25 mL×3 times) and the total volume of the solution was adjusted with chloroform to 100 mL in a flask. The absorbance level of the sample was measured versus prepared reagent blank at 455 nm. Total phenolics percentage was calculated in the following equation.

Total phenolics as thymol (g/100g) = 0.62×E

In which, E is the absorbance level of the sample. The total phenolics assay was measured three times for each extract. Total phenolics amount was reported based on mg of thymol per gram of dry extract.

Antioxidant activity test (DPPH)
The ability of the extract for inhibition of free radicals was assessed by the method of Arabshahi and Urooj [18]. The aliquots of plant extract (20 to 100 µL) were mixed with a methanol solution of DPPH (1 mM, 600 µL) and brought to 6 mL with solvent. After incubating in dark and room temperature, the absorbance was measured at 517 nm. A DPPH-blank sample (containing 5.4 mL of methanol and 600 µL of DPPH-solution) was prepared. The percent decrease in absorbance was recorded for each concentration and percentage inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = [(A_{DPPH} - A_{Extract}) / A_{DPPH}] \times 100$$

A_{DPPH} was the absorbance value of the DPPH/blank sample and $A_{Extract}$ was the absorbance value of the test solution. The concentration of the extract (µg) which caused 50% inhibition of free radicals (IC_{50}, µg/mL) was reported based on the plot of the inhibition percentage against sample concentrations [19].

Assay of total flavonoids
Total flavonoid content was measured by the aluminum chloride colorimetric assay [20]. An aliquot (250 µg/mL) of extracts and 50-1000 µg/mL of standard solution of catechin were added to 10 mL volumetric flask containing 4 mL of double distilled water. Then 0.3 mL 5% $NaNO_2$ was added to the flask and after 5 min, 0.3 mL $AlCl_3$ (10%) was also added. At 6^{th} min, 2 mL NaOH (1 M) was added and the total volume was made up to 10 mL with double distilled water. The solution was mixed completely and the absorbance level was measured versus prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg catechin equivalents per one gram of dry extract. The total flavonoid assay was measured three times for each Ajowan's plant extracts.

Determination of anticholinesterase activity

The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) by the extracts were measured by the Ellman spectrophotometric method with slight modification [21,22].

Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as Ellman's reagent was used for the measurement of the cholinesterase activity. First, 50 µL of a 100 mM sodium phosphate buffer (pH 8.0), 25 µL of sample solutions and 25 µL of AChE or BChE solution (0.22 U/mL) were mixed and incubated for 15 min at room temperature. Then, 125 µL of 3 mM DTNB was added. Finally, the substrates were added and the absorbance was measured at 412 nm using a microplate reader after 15 min. The solution of all ingredients except the substrates was used as the negative control, and tacrine was applied as the positive control. Both of the extracts were prepared in different concentrations and measured three times. The IC_{50} value (concentration of sample which inhibited 50% of AChE and BuChE), was calculated by a liner regression analysis.

Cytotoxic activity by MTT assay

Antiproliferative effect of *T. ammi* extract on human acute lymphoblastic leukemia (ALL) cancer cell line (Nalm-6) was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. Viable cells could reduce yellow water soluble MTT to water insoluble purple colored formazan crystals by mitochondrial dehydrogenase. The cells in the log-phase of growth were seeded at a concentration of 5×10^4 cells/well in 96-well plate (Nunc, Denmark) in a humidified air atmosphere at 37 °C with 5% CO2. After overnight incubation, the cells were treated in tripicate with 5 µL of extracts at final concentrations of 31, 62.5, 125, 250, 500 µg/mL for 48 h. Etoposide and DMSO or ethanol were used as the positive and negative controls, respectively. After centrifugation, the medium was removed and 200 µL phenol red-free medium containing MTT (Sigma-Aldrich, USA), final concentration of 1 mg/mL, was added to the wells, followed by 4 h incubation. After removing the medium, 100 µL DMSO was added to each well to dissolve purple formasan crystals. The absorbance of each well was measured by using a microplate reader (Gen5, Powerwave xs2, BioTek, America) at 492 nm wavelengths after 30 min shaking. For each extract, IC_{50} value was calculated from concentration response curves by regression analysis [23].

Statistical analysis

All Data have been reported as the mean±SD of triplicate tests and statistical analysis was conducted using Microsoft Excel 2010.

Results and Discussion

The extraction yield was 21.62% w/w for maceration and 13.00% w/w for reflux extraction which were mentioned in the table 1. Also, the results of total phenolics assays of both extracts were shown in the same table. According to the antioxidant assay, the inhibitions of DPPH free radicals against sample concentration were exhibited based on the IC_{50} values for both extracts in the table 1.

Moreover, the total flavonoids content was calculated on mg catechin/g dry extract for both extracted solutions respectively (table 1).

Acetylcholinesterase inhibition activity of both *T. ammi* extracts were investigated for the first time. According to results shown in the table 2, the extract of maceration method showed moderate anti-BuChE activity (IC_{50}=394.161 µg/mL) while its AChE activity was >500 µg/mL. Total refluxed extract of *T. ammi* did not exhibit AChE and BChE inhibitory effects at concentrations up to 500 µg/mL (37.14 % and 42.23%, respectively) (table 2). The effects of both extracts of human acute lymphoblastic leukemia (ALL) were assessed by treating the cells with different concentrations of the samples (table 3). The IC_{50} was found to be 166±1.76 and 198.52 µg/mL for maceration and reflux extracts, respectively. Etoposide as the positive control showed IC_{50} 10.24±0.02 µg/mL. To find a particular extract with the highest amounts of *T. ammi* phytochemical constituents, two extraction methods were compared.

Table 1. The effect of two different extraction methods on phytochemical constituents of *Trachyspermum ammi* L. fruits' extract

Extraction Method	[a] Total phenolics as gallic acid	[b] Total phenolics as thymol	[c] Total flavonoids	[d] Antioxidant activity (IC_{50})
Maceration	101.7	1.19	140.15	132.95
Reflux	147.28	1.66	119.15	159.4

[a] mg gallic acid/g dry extract; [b] mg thymol/g dry extract; [c] mg catechin /g dry extract; [d] µg dry extract/mL solvent

Table 2. Acetylcholinesterase and butyrylcholinesterase inhibory effect of two different extracts of *Trachyspermum ammi* L. fruits

Sample	Acetylcholinesterase IC_{50} (µg/mL)	Acetylcholinesterase Inhibition % (500 µg/mL)	Butyrylcholinesterase IC_{50} (µg/mL)	Butyrylcholinesterase Inhibition % (500µg/mL)
Maceration	>500*	45.22%	394.161	53.38%
Reflux	>500*	37.14%	>500*	42.23%
Tacrine	0.0095± 0.0022	-	0.0020±0.0008	-

* more concentrations were not possible to test because of turbidity in sample tests.

The results demonstrated that the higher amount of extraction yield (%) was revealed in the maceration technique, whereas the total phenolics noticeably increased in the extract attained by reflux method. On the other hand, the radical scavenging activity and total flavonoids contents in the comparing extracts significantly improved in the maceration method.

A research was applied by Ahmad *et al*. on two ethanol extracts of *Eleutherine palmifolia*, from reflux and maceration methods, showed that the ethanol extract of the plant could carry higher amount of major compounds comparing to the extracts using any other solvent. According to the mentioned study, some main spots had disappeared in the TLC of the refluxed extract, because of the direct heating effect and evaporating of volatile components or decomposition of unstable compounds in the higher temperatures [24].

According to the results of our study, direct heating could be the cause of the lower amount of total flavonoids in the reflux technique compared with maceration. The research was applied by Yu *et al*., revealed that after heating treatment of plants, the content of flavanone glycosides decreased significantly. It seems that flavonoids might be destroyed in higher temperature [25]. Moreover, according to the same study, heating could increase the content of phenolic compounds in the herbal extracts considerably. The same pattern was observed in our research in the content of total phenols percentage in the extraction applied by reflux technique.

On the other hand, through the maceration method, the extraction rate was significantly higher than the reflux, hence the total amount of major compounds extracted from the same amount of the crude plant elevated meaningfully in maceration extraction.

The comparison between extraction methods on phytochemicals of *T. ammi* in different studies has been shown in table 3.

In the present study, for the first time, the anti-cholinesterase and cytotoxic activities of two different extracts of *T. ammi* were examined respectively and the results exhibited that the maceration method could improve both mentioned properties of the extract slightly. It seems that the extraction method couldn't be considered as a major factor for improving the biological potency of the *T. ammi*'s extract meaningfully. In conclusion, it seems that the maceration technique with higher extraction yield, more phenolics and flavonoids contents of the extract and greater biological effects would be a more suitable method for extraction of *T. ammi* fruits.

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Table 3. Comparison of compounds and antioxidant property of *Trachyspermum ammi* fruits using different extraction methods and solvents

Extraction method	Extraction solvent	[a]Total phenolics	Antioxidant activity (IC_{50})	[b]Total flavonoids	Ref.
Maceration	Ethanol	101.7	132.95	30.32	Present study
Reflux	Ethanol	147.28	159.4	15.49	Present study
Soxhlet	Methanol	115.1	-	15.32	[26]
Soxhlet	Acetone	73.7	-	6.47	[26]
Soxhlet	Chloroform	92.4	-	12.38	[26]
Soxhlet	Hexane	75.9	-	7.25	[26]
Reflux	Methanol		-	-	[27]
[c]ASE	Methanol	88.00	66.96	-	[28]
[c]ASE	Hexane	67.00	64.88	-	[28]
[c]ASE	Methanol	64.00	68.08	-	[28]
[c]ASE	Hexane	64.00	56.91	-	[28]
Maceration	Methanol	169.56	33.97	-	[29]

[a] mg gallic acid / g dry plant; [b] mg catechin / g dry plant; [c] accelerated solvent extraction

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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