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Composition and Bronchodilator Activity of the Fruits of *Trachyspermumammi* L. Essential Oil

Mariam K. Alamoudi [a*], Najeeb Ur Rehman [a], Mohammad Ayman A. Salkini [b], Abdulaziz S. Saeedan [a], Omar K. M. Al-Rahimi [c], Maged S. Abdel-Kader [b,d]

[a] *Department of Pharmacology and Toxicology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Saudi Arabia*

[b] *Department of Pharmacognosy, College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Saudi Arabia*

[c] *College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Saudi Arabia*

[d] *Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Alexandria 21215, Egypt*

Abstract

*Trachyspermumammi*is used traditionally to manage respiratory problems. The essential oil of the fruits was analyzed using GC-MS and the bronchodilator potential was explored using isolated guinea-pig trachea in an *ex-vivo* model. The GC-MS analysis revealed that thymol is major compound with 65% yield. *T. ammi* oil exhibited comparable potency in inhibiting contractions caused by carbachol (CCh, 1 µM) and high K^+ (80 mM), with corresponding EC50 = 0.28 mg/mL (0.26-0.31, n=4) and 0.32 mg/mL (0.29-0.35, n=4) in a fashion similar to papaverine known to inhibit both Ca^{+2} channels and the phosphodiesterase enzyme (PDE). In contrast, Verapamil, a selective Ca^{+2} channel blocker, suppressed contractions induced by elevated K^+ levelswith significantly higher potency than those induced by CCh, as indicated by the EC50 = 0.82 µM (0.68–1.02, n=5) and 17.84 µM (15.64–1.86, n=5), respectively. Tissues preincubated with 0.03 and 0.1 mg/mL of the oil were able to attenuate the Ca^{+2} produced contractions and suppressing the maximum response in a manner comparable to verapamil and papaverine further supporting the Ca^{+2} channel inhibitory effect. PDE blockage was settled when tracheal tissues preincubated with *T. ammi* essential oil at 0.1 and 0.3 mg/mL expressed potentiation of isoprenaline relaxant effect against CCh, similar to papaverine. Preincubation with verapamil did not show any potentiation. Therefore, our results demonstrated that *T. ammi*essential oil have bronchodilator activities mediated by dual inhibition of Ca^{+2} channels and PDE, although other mechanisms may also be involved.

Keywords: *Trachyspermumammi* L.; GC-MS; Bronchodilator; Ca^{+2} blockade; Phosphodiesterase inhibition.

1. Introduction

Family Apiaceae composed mainly of aromatic flowering plants. It contains about 440 genera and 3800 species [1]. *Trachyspermum* is a small genus of about 14 species [2]. Plants essential oils expressed several biological activities including antimicrobial, cytotoxic, anticonvulsant, insecticidal and neuroprotective effects [3–6]. *Trachyspermumammi* L. is known as Ajwain while in Arabic known as "Alnaanikhuk, Royal cumen and Ethiopian cumin [7]. Conventionally, the plant is employed in herbal remedies to treat several conditions such as travel sickness, anorexia, abdominal flatulence, nausea and vomiting [8]. Additionally, it is claimed to possess laxative, stomachic and anthelmintic properties [9]. *T. ammi* can alleviate abdominal pain, hemorrhoids and abdominal tumors [10]. Ajwain is also used as a household folk medicine to treat respiratory problems, such as asthma and bronchitis [11,12]. Essential oil of *T. ammi* contains up to 50% thymol along with other terpenes [13–16]. The oil's antifungal effect was demonstrated against broad spectrum of fungus, including *Aspergillusochraceu, A. oryzae, A. flavus, A. niger, Fusarium. graminearum, F. monoliforme, Pencilliumcitrium, P. madriti, P. viridicatum*, and *Curvularialunata* [16].

In a recent study, we explored the bronchodilator properties of several essential oils derived from plants traditionally used in Saudi Arabia [17,18]. Despite of the common used of *T. ammi* in Traditional medicine the bronchodilator effect was not previously explored. The present study comprises GC-MS analysis of the ethereal oil of *T. ammi* and provides a comprehensive investigation of the mechanism of the bronchodilator action of the oil.

*Corresponding author e-mail: m.alamoudi@psau.edu.sa.; (Mariam K. Alamoudi).
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2. Experimental

2.1. Plant materials

The fruits of *Trachyspermumammi* L. were purchased from Riyadh city local market, Saudi Arabia. The plant's identity was confirmed by Mona Alwahibi, PhD., Department of Botany and Microbiology, College of Science at KSU, Riyadh. The fruits were preserved under voucher number MSA 10832 at the Department of Pharmacognosy, College of Pharmacy, PSAU.

2.2. Preparation of the essential oil

Samples of 150 g of *T. ammi* fruits were grinded and immediately used for oil preparations. The oil was prepared by the hydrodistillation method and performed for 5 h using a round-bottom flask of 1 L capacity along with Clevenger-type apparatus [17]. At the end of the experiment, the oil layer and the water condensates were separated using 50 mL separating funnel. The process was run in triplicate.

2.3. GC/MS Analysis

Chloroform was used as a solvent to dilute oil samples in order to obtain 1 ppm concentration. Injection of 1 µL of the solutions to Agilent GC/MS instrument Model 7890 MSD were performed with the help of autosampler adjusted to the splitless mode. Helium (99.999% purity) was the carrier gas adjusted to flow rate = 1.2 mL/min. HP5MS 30 m length column with 0.25mm i.d. and 0.25 µm thickness was used in theanalysis. The Injector temperature was set at constant temperature of 280 °C. The instrument was programmed with a start temperature of 70 °C for 5 min, then gradual raising of the temperature at rate = 2 °C/min till 120 °C where it was hold for 2 mins followed by increasing rate of 15 °C/min to 290 °C that was kept for 2 mins. The ionization in the mass spectrometer was performed at 70 Ev and mass range was set between 30-600 Daltons. The ion-source temperature was adjusted to 280 °C. Chromatograms of standard thymol and carvacrol and their mixture were generated under similar conditions.

2.4. GC/FID Analysis

Chromatograms obtained under conditions similar to the GC/MS analysis were generated using FID detector. The instrument Model 7890 was programmed to start temperature at 70 °C hold for 5 min, followed by gradual raising at rate = 2 °C/min till 120 °C where it was hold for 2 mins. Temperature was then increased at rate of 15 °C/min to 290 °C that was kept for 2 mins. Peak quantification was recorded by automatically measuring the area of each peak. The mass range was set between 30-600 Daltons. The ion-source temperature was set at 280 °C.

2.5. Chemicals and Reagents

The following chemicals of analytical grade were obtained from Sigma Company, St. Louis, MO, USA; Chloroform, ether, sodium sulphate, thymol, carvacrol, carbamylcholine (CCh), isoprenaline, verapamil and papaverine. Details of reagents (salts) to prepare physiological buffer solution (Tyrod) are as follows; potassium chloride (Sigma Co), sodium chloride, calcium chloride, glucose, sodium bicarbonate magnesium sulphate, potassium and dihydrogen phosphate (Merck, Germany).

2.6. Animals

Guinea pigs weighing around 0.6 kg of either gender were obtained from the animal care unit of KSU and housed at the Animal Care Unit at the College of Pharmacy, PSAU, KSA. The accommodation of the guinea pigs was regulated to a constant temperature range of 23-25 °C. Free access to drinking water and commercial grade animal nourishment were provided to the animals. All the *ex vivo* experiments performed adhering to the guidelines established by the Institute of Laboratory Animal Resources, Commission on Life Sciences, NRC. The PSAU Bio-Ethical Research Committee (BERC) approved the study protocol under the reference number BERC-001-12-19.

2.7. Guinea Pig Tracheal muscles

Cervical dislocation was performed to scarify the trachea of guinea pigs following a forceful jerk at the trachea of the animals, which were sacrificed with their heads suspended in an erect position between the middle and index figure. After being dissected, the tracheal tube was maintained in Kreb's solution, an appropriate physiological buffer. Seven to eight distinct tissues were isolated from the tracheal tube, with each tissue ring being exposed through a longitudinal incision in the cartilage that was perpendicular and facing the smooth muscle. The tracheal tissue was then mounted in a 10 mL tissue buffer supplemented with Kreb's solution, kept at 37 °C aeriated with carbogen (95% O_2 and 5% CO_2). The buffer components were (mM): KCl 4.7, KH_2PO_4 1.3, NaCl 118.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.7 with pH 7.4. Each tracheal strip was subjected to a constant tension of 1 g for the duration of the experiment. A 60-minute equilibration period was observed prior to application of the tested materials. During this time the buffer was replaced every 15 minutes. For tissue stabilization, CCh (1 µM) was employed until constant superimposable contractions were accomplished. The relaxation caused by the tested oil and standards was determined by adding increasing concentrations to get concentration-dependent responses.

2.8. Determination of the mechanism of the Bronchodilator's effect

In order to investigate the existence of calcium channel blocking (CCB) activity of the essential oil of *T. ammi*, the standard Kreb's solution in the tissue-organ baths was gradually substituted with a Kreb's solution that is rich in K^+ and lacks Ca^{+2}after the tissues were mounted and stabilized [19]. Following that, concentration response curves (CRCs) for Ca^{+2} were constructed in the presence and absence of different *T. ammi* oil concentrations. Verapamil served as the positive control. Phosphodiesterase inhibitory-like activity of *T. ammi* oil was investigated by constructing inhibitory CRCs of isoprenaline against CCh (1 µM)-induced contractions [20]. To examine the potentiating effect of *T. ammi* oil on the inhibitory activity of

isoprenaline, the experiments were replicated in the presence of the oil. The resulting responses were compared with those obtained using the standard drug papaverine [21]. An isometric transducer was used to achieve the responses on an organ bath (emkaBATH, France) with iox software (2.10.8.6) installed.

2.9. Statistical Analyses

Results are presented as mean ± standard error of the mean (SEM, n represents the number of experiments). EC_{50} with 95% confidence intervals (CI) represents the median effective potency. A *p-value* of less than 0.05 was accounted as significant result. Relaxation concentration response curves (CRCs) were inspected using non-linear regression utilizing the GraphPad program (GraphPAD, San Diego, CA, USA).

3. Results

3.1. Preparation of the essential oil

The essential oils produced from 150 g of the fruits were 7.94 g calculated as 5.30% w/w yield. The oil was kept at 4 °C for analysis and biology.

3.2. GC/MS and GC/FID investigation

The MASSHUNTER software was used for managing the process and analysis of the findings.Identification of the oil's components was accomplished by comparison of the compounds' retention time (RT), MS spectra as well as retention indices (RI) relative to the standard n-alkanes series of C7-C40 (49452-U) (RRI) with the published values of the National Institute of Standards and Technology database, Gaithersburg, MD, USA (NIST 2017). Results of the essential oil analyses as well as thymol and carvacrol are presented in Table 1 and Figure S1, S2.

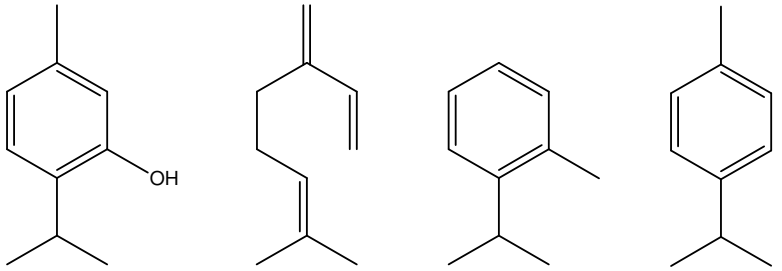
3.3. Bronchodilator Effect on Trachea

The study was performed in an *ex-vivo* model utilized isolated guinea-pig trachea. The oil of *T. ammi* effectively suppressed contractions generated by carbachol (CCh, 1 µM) and high K^+ (80 mM) at comparable potencies. The EC_{50} were 0.28 mg/mL (0.26-0.31, n=4) and 0.32 mg/mL (0.29-0.35, n=4), respectively (Figure 2A). Figure 2B indicated that an analogous inhibitory pattern of the oil was noted compared to papaverine, that acts as a binary blocker of phosphodiesterase enzyme (PDE) and Ca^{+2} channels. EC_{50} values for papaverine were 10.20 µM (10.82-12.46, n=5) and 10.96 µM (0.96-10.42, n=5), respectively. In Figure 2C, it was demonstrated that a selective Ca^{+2} channel blocker, verapamil, exhibited greater potency in suppressing contractions caused by high K^+ compared to contractions caused by CCh. The EC_{50} values for verapamil's effect on high K^+-induced contractions and CCh-induced contractions were 0.82 µM (0.68 – 1.02, n=5) and 17.84 µM (15.64-18.56, n=5), respectively.

Table 1: Essential oil composition of *the fruits of Trachyspermumammi*

	Name	RT	RI	RI*	%
1	β-myrcene	7.9076	992	992 [22]	0.755
2	o-Cymene	9.4924	1030	1029 [23]	15.955
3	γ-Terpinene	10.6697	1060	1060 [24]	17.857
4	Thymol	20.2951	1293	1292 [25]	65.432
Total		**99.999**			

*Reported RI.



Thymol **β-Myrcene** **o-Cymene** **γ-Terpinene**

Figure 1: Chemical structures of *Trachyspermumammi* oil components

3.4. Ca^{+2} Channel Inhibitory Effect

The Ca^{+2} channel inhibitory action was further confirmed by preincubation of the tissue with 0.03 and 0.1 mg/mL of the oil resulted in attenuation in concentration-dependent manner the Ca^{+2} concentration response curves (CRCs) with lowering the maximum response (Figure 3A). Similarly, verapamil (Figure 3B) and papaverine (Figure 3C), also suppressed and shifted the Ca^{+2} CRCs towards right and thus showed non-specific antagonistic effects.

3.5. PDE- Inhibitory-Like Effect

The PDE inhibitory-like action of *T. ammi* essential oil was authenticated by preincubation of the tracheal tissues with 0.1 and 0.3 mg/mL of the oil. This treatment potentiated and shifted the isoprenaline inhibitory CRCs constructed against CCh towards left (Figure 4A) almost identical with papaverine (1 and 3 µM) (Figure 4B) whereasverapamil preincubation at 0.1 and 0.3 µM did not show any potentiation (Figure 4C).

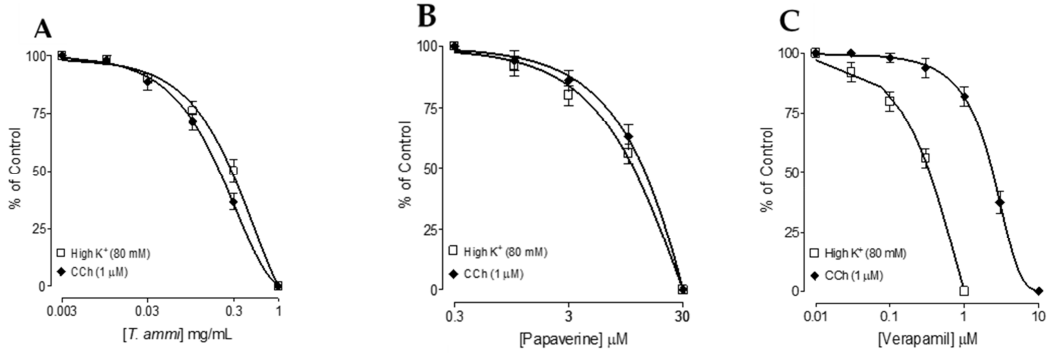


Figure 2: Concentration response inhibitory curves of (A) *T. ammi* oil, (B) papaverine and (C) verapamil on CCh and high K^+- persuaded contractions using guinea-pig trachea (n=4).

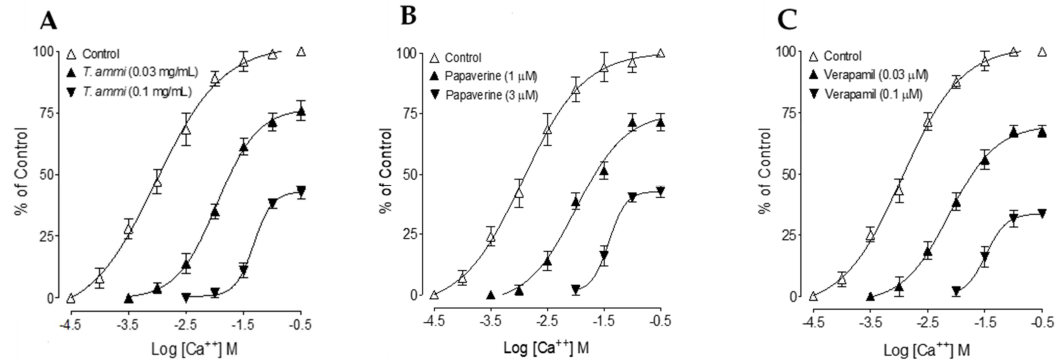


Figure 3: Concentration response curves of Ca+2 plotted in the presence of difference concentrations of (A) *T. ammi* oil, (B) papaverine and (C) verapamil using guinea-pig trachea (n=5).

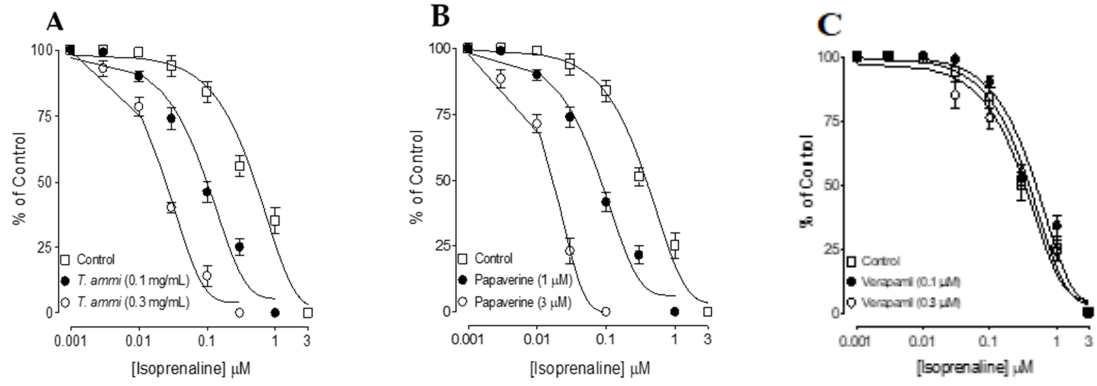


Figure 4: Effect of various concentrations of (A) *T. ammi* oil, (B) papaverine and (C) verapail on the inhibitory effect of isoprenaline against CCh (µM)- incited contraction. The symbols indicated mean ± SEM of 5 experiments.

4. Discussion

The fruits of *T. ammi* yielded 5.30 w/w essential oil prepared by hydrodistillation method. The components of the oil were determined using GC-MS analysis as well as comparison of the retention indices with the values of the National Institute of Standards and Technology database. Thymol was the major component in the oil representing 65.432% (Table 1, Figure1). Carvacrol is a structural isomer of thymol with very close values in all the used parameters. Confirmation of their identification in GC analysis is relatively tough task. For undoubtful identification a solution of thymol and carvacrol with the ratio 2:1 in chloroform was injected under the same conditions of the oil's analysis. The RT of thymol was 20.3143 while that of carvacrol was 20.7736 mins (Figure S1). The RT of the major peak in the essential oil spectrum was identicalwith thymol in full agreement with all the reported data for *T. ammi* fruits essential oil [13–16,26].Due to the recognized therapeutic applications of *T. ammi* in respiratory diseases [11,12], an evaluation wasconducted on the essential oilagainst bronchoconstriction induced by elevated K^+ and CCh ions using the established isolated guinea pig trachealmodel [17]. The bronchoconstriction caused by CChis facilitated by the activation of muscarinic (M3) receptors [27], whereas K+ concentration exceeds 25 mM is supposed to open voltage-gated L-Type Ca^{+2} channels and thus causes depolarization and results in tracheal contractions [28]. Interestingly, *T. ammi*oil demonstrated a concentration-dependent non-selective inhibition of the trachea's contraction induced by CCh and high K^+, which is comparable to papaverine, that is known for its dual inhibition of Ca^{+2} channels and PDE enzyme (Figure 2) [29]. In contrast, the conventional Ca^{+2} channel blocker verapamil [30], exhibited selectively greater potency against K^+, as anticipated for a pure Ca^{+2} antagonist (Figure 2C) [31]. This implies that, similar to papaverine, the airways relaxing action of *T. ammi* essential oil could potentially be driven by a combination of CCB and PDE inhibitory mechanisms. Confirmation of *T. ammi* 's oil CCB activity was obtained from the observed attenuation of Ca^{+2} CRCs, along with reduction in the maximal response, equivalent to the effects of verapamil and papaverine (Figure 3). Similar to papaverine, *T. ammi*'sphosphodiesterase inhibitory like action was confirmed when *T. ammi* boosted isoprenaline's relaxant effect against CCh-incited contraction (Figure 4). Isoprenaline, a nonselective β-adrenoceptor agonist, induces relaxation of the airways by increasing the concentration of cAMP within the cells. Both the $\beta 2$-agonistic action and suppressing PDEs increase cAMP concentration in the respiratory tract and potentiate each other [32]. Enhancement of the isoprenaline inhibitory action by *T. ammi* oil indicates that its airways relaxant mechanism includes PDE inhibition, as PDE inhibitors have been shown to improve the isoprenaline relaxant effect [21,33], nevertheless, the possibility of $\beta 2$-agonistic activity cannot be disregarded. PDE inhibitors are well recognized to be beneficial for the management of asthma [34,35], though stimulation of the heart as a side effect is the main limitation [36]. Interestingly, Ca^{+2} antagonists have demonstrated efficacy in the treatment of bronchoconstriction [37,38] as well as being recognized for cardio suppression effect [39]. Perhaps that Nature intended for *T. ammi* to contain both PDE inhibitors and Ca^{+2} channel blocker components so that the tachycardia that can occur when PDE inhibitors are taken alone can be mitigated. This discovery reinforces the idea that herbal remedies, which are known to have the ability to work synergistically and/or counteract adverse effects, as well as being cost-effective, have merit in evidence-based research [40]. Hence, the simultaneous inhibition of PDE and Ca^{+2} channels might explicate the therapeutic application of *T. ammi* in treating airways hyperactivity and asthma. Thyme oil rich in thymol was reported to alleviates ovalbumin-induced bronchial asthma via modulating Th2 cytokines, IgE, TSLP and ROS [41]. Another study indicated that both Thyme and thymol were able to relief ovalbumin-induced bronchial asthma in mice [42].

These results support our finding for the benefits of T. *ammi* in the management of bronchial asthma. The relaxant effect can be correlated to the major component thymol. Thymol was reported to have smooth muscle relaxant effect [43]. Moreover, many reports indicated that monoterpenes are responsible for the antispasmodic effects of many essential oils [44]. Many natural terpenes are reported in the essential oils with vascular smooth muscles relaxant effect leading to decrease in systemic blood pressure [45]. The intestine, heart and tracheal muscles are all kinds of smooth muscles. Consequently, monoterpene components of *T. ammi* are expected to exert similar relaxant effect on tracheal smooth muscles.

5. Conclusions

Thymol, comprising 65% of the *T. ammi* essential oil constituents and was the principal constituent. The results of the bronchodilator study strongly indicated that the essential oil of *T. ammi* may exert a combination of CCB-like and PDE inhibitory effects. This may explain why this plant is used medicinally as a bronchodilator. While additional research is necessary to determine its safety profile, these findings are likely to support the notion that the plant could be an effective bronchodilator alternative.

6. Conflicts of interest

There are no conflicts to declare.

7. Formatting of funding sources

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