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Achillea fragrantissima Essential Oil: Composition and Detailed Pharmacodynamics Study of the Bronchodilator Activity

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Abstract: The bronchodilator effect of the *Achillea fragrantissima* essential oil (AFO) was studied in guinea pigs' tracheas and the influence of drying on the quantity and composition of AFO was studied using GC-MS and GC analyses. AFO produced a complete and potent relaxation against carbachol (CCh), while lower potency and partial efficacy were observed against high K^+ (80 mM), thus producing dual inhibitory effects similar to dicyclomine. The anticholinergic-like action was further confirmed when pre-incubation tracheal tissues were used at lower concentrations with AFO displacing the CCh concentration-response curves (CRCs) to the right in a competitive manner similar to atropine. However, non-parallel shifts in CCh CRCs were observed with higher doses, similar to dicyclomine. Further confirmation of the CCB-like effect was obtained from the non-specific deflection of Ca^{++} CRCs toward the right using the pre-incubated tissues with AFO in Ca^{++} free medium, similar to verapamil. When AFO was tested against low K^+-mediated contractions to explore the possible involvement of additional antispasmodic mechanism(s), AFO interestingly showed a complete inhibition with a higher potency. This inhibition was found to be sensitive to tetraethylammonium (TEA) and 4-aminopyridine (4-AP), whereas glibenclamide (Gb) remained inactive. These results show that AFO possesses bronchodilator effects predominantly from its anticholinergic and K^+ channel activation followed by weak Ca^{++} channels inhibition.

Keywords: *Achillea fragrantissima* oil; GC-MS; bronchodilator; anticholinergic; Ca^{++} channel blocker; potassium channel opener; guinea pig trachea

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1. Introduction

Achillea fragrantissima is a flowering plant belonging to the Asteraceae family. *Achillea* is a genus that comprises approximately 100 species. Most family members of this genus are characterized by the biosynthesis of sesquiterpene lactones and flavonoids [1]. Members of the genus *Achillea* are well-known in traditional medicine. They are used for the treatment of many aliments such as stomach pain, menstrual disorders, bleeding, hemorrhoids, gastrointestinal tract inflammation, rheumatism, allergic rhinitis, and pneumonia. They are also useful during breast feeding and possess wound-healing potential [2] *A. fragrantissima* is popular in folk medicine for many Arab countries and is used for the management of some common health problems including diabetes, respiratory disorders, gastrointestinal disturbances, dysmenorrhea, eye infections, smallpox, fever, headaches, and fatigue [3–5]. *A. fragrantissima* is also used in Saudi Arabia as for its anticancer, antibacterial, antifungal,

antimalarial, appetizer, treatment of respiratory problem, aphrodisiac, antispasmodic, and relaxant effects [6]. The plant extract as well as the isolated methoxylated flavonoids exhibit promising antitumor activities attributed to their antioxidant potential [7]. Studies of the essential oil of the plants collected from Jourdan revealed the presence of α-thujone (33.8%) and β-sesquiphellandrene (28.6%) as the principal constituents [8]. The volatile nature of the essential oil extracted from different medicinal plants is the most effective for treating respiratory diseases as their volatile nature can help in the delivery to the site intended to be treated [9]. The importance of essential oil use in bronchoconstriction can be estimated from the official inclusion of more than 25 essential oils in European Pharmacopoeia [10]. Among them, thyme, anise, eucalyptus, fennel, and peppermint are the mostly used in practice for addressing respiratory ailments such as bronchoconstriction [11].

The current study aims to quantitatively and quantitatively evaluate the effect of drying on *A. fragrantissima* oil (AFO). The brochodilator effect of AFO was evaluated with detailed pharmacodynamics explored in the isolated tracheal tissues of guinea pigs using an ex vivo experimental organ bath setup.

2. Materials and Methods

2.1. Plant Material

The plants of *Achillea fragrantissima* (Forssk.) Sch.Bip. were collected in February, 2021 from Faydat Alhayra (29°32′49.7″ N 43°22′47.6″ E), west of Rafha, North of Saudi Arabia and close to the border with Iraq. The plant was authenticated by the taxonomist Dr. Mohammad Atiqur Rahman, MAP-PRC, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. A voucher specimen (#18507) was preserved at the center's herbarium.

2.2. Chemicals

Carbamylcholine, verapamil, dicyclomine, atropine, glibenclamide, tetraethylammonium, and 4-aminopyridine were obtained from Merck (Rahway, NJ, USA) previously, Sigma-Aldric.

2.3. Preparation of AFO

A sample quantity of 250 g comprising the fresh plant and 100 g of shade dried aerial parts (acquired from 250 gm fresh stems after drying) of *A. fragrantissima* were used for AFO extraction for a time period of 5 h using hydrodistillation with Clevenger apparatus (Dolphin Instruments, Mumbai, India). The fresh plants were cut using a knife into small pieces (approximately 2 cm long), while the dried plants in the shade under controlled temperature were ground and used for AFO preparation. After the hydrodistillation process, the AFO layers were collected after separation and the water layers were extracted with diethyl ether; the ethereal layers were combined, dehydrated with sodium sulfate (anhydrous), and the ether layer was evaporated using a rotary evaporator to leave pure AFO. From this oil, 300 mg/mL stock solution in propylene glycol is produced for biological testing. Each experiment was performed in triplicate.

2.4. GC-MS Analysis

AFO samples of 5 ppm concentrations were prepared in MeOH. From each sample, 1µL was injected for GC-MS analysis using an Agilent Model 7890 MSD GC-MS instrument (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an (30 m × 0.25 mm i.d., 0.25 µm coating) HP-5MS capillary column. The injections were performed via the Autosampler in the split-less mode in triplicates. The temperature program started at 60 °C and was unchanged for 10 min, then increased by 4 °C/min until it reached 220 °C, from which it was held for 5 min. Then, the temperature was increased by 10 °C/min to reach a final temperature of 290 °C and kept constant for 5 min with for a total run time of 67 min. Helium (99.999% purity) was the used carrier gas with a flow rate of 1.0 mL/min. Quadrupole mass spectroscopy analysis was recorded using the EI ionization mode set at 70 Ev. The range of mass detection was adjusted between 30 and 600 m/z. AFO compound

identification was performed by comparing the obtained MS spectra with the library of the NIST 2017 (National Institute of Standards and Technology) data base. The results analysis and control were achieved using the MASSHUNTER software (Version B.04.xx, Agilent Technologies Inc., Santa Clara, CA USA).

2.5. GC Analysis

GC chromatograms were obtained using a GC Agilent 7890B equipped with the capillary column HP-5 19091J-413 (30 m × 0.25 mm) along with FID detector applying conditions used for GC-MS analysis. *n*-Alkanes series were used to calculate the relative retention index (RRI) applied for peak identification. The area for each peak was measured automatically to enable the quantification of the peaks (Figure 1).

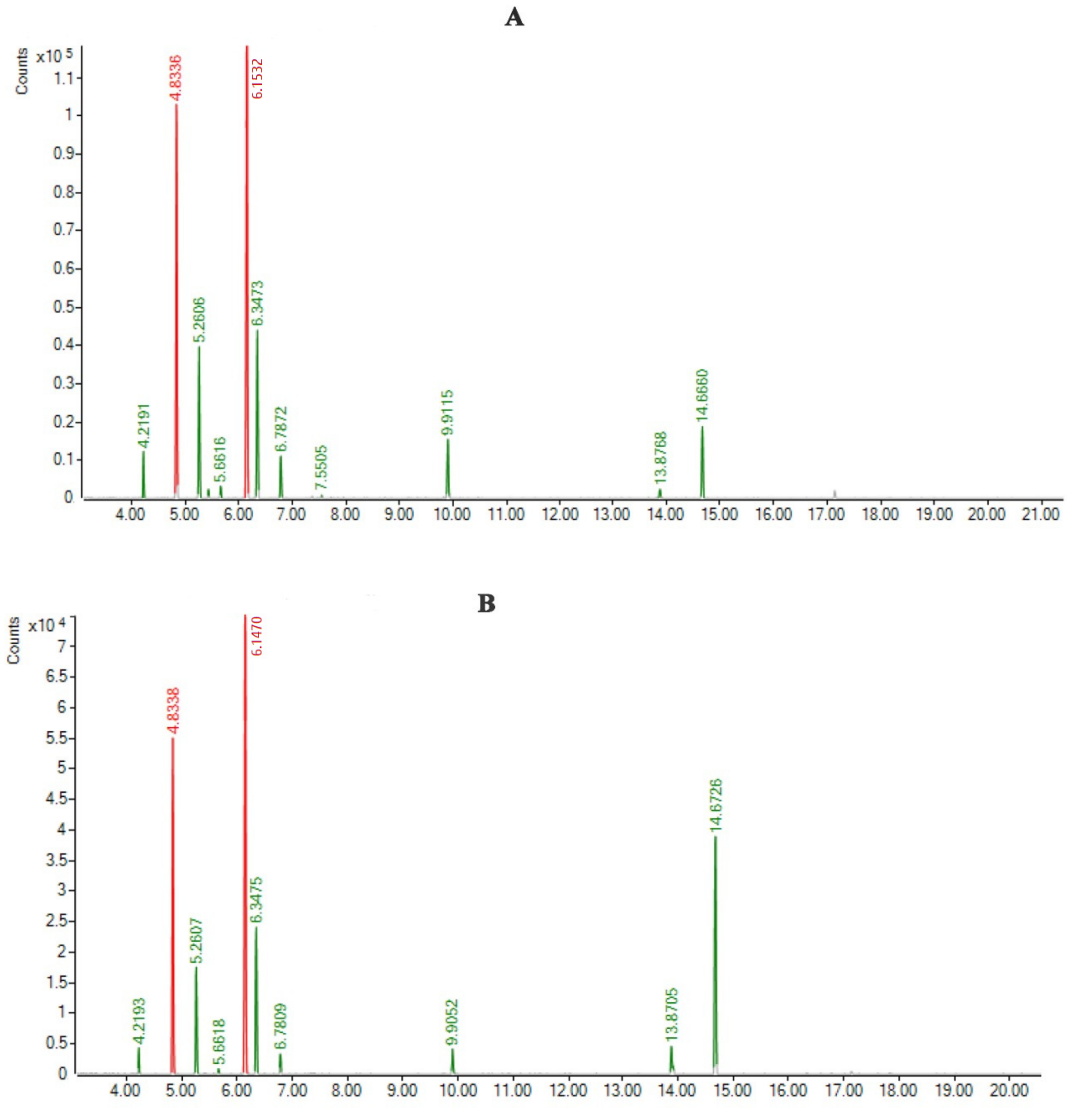

Figure 1. GC chromatogram of AFO obtained from fresh (**A**) and dried (**B**) plant samples.

2.6. Bronchoduilator Study

2.6.1. Animals

Experimental guinea pigs of 500–550 g weight of either gender were obtained from the King Saud University animal laboratory. All the animals received due care at the Animal Care center of the College of Pharmacy, PSAU, KSA where a standard temperature of 23–25 °C was maintained while a standard commercial diet and running water ad libitum was provided to all tested animals. Animal ethical guidelines for laboratory

animals were followed during experimental assays with compliance to the guidance of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [12]. The ex vivo protocol followed in this study has been registered with reference number BERC-001-12-19 by Bio-Ethical Research Committee (BERC) at PSAU.

2.6.2. Isolated Tissue Preparation

Animals were humanely sacrificed by cervical dislocation. The tracheal tubes were removed and preserved in an ice-cold Krebs–Henseleit solution immediately. The composition of the Krebs–Henseleit solution was (mM) as follows: $NaHCO_3$ 25.0, NaCl 118.0, KH_2PO_4 1.2, KCI 4.7, $MgSO_4$ 1.2, glucose 11, and $CaCl_2$ 2.5 [13]. Carbogen (95% O_2: 5% CO_2) was passed through the solution that was maintained at 37 °C.

The accompanied connective tissue and fats were removed with care and the tracheas were cut into rings 2–3 mm wide. The rings were open opposite to the tracheal muscles and fitted together forming a tracheal chain [14]. The preparation under testing was mounted in the 20 mL tissue bath containing Kreb's solution at 37 °C with continuous aeration with carbogen (95% O_2 and 5% CO_2). A tension of 1 g was applied and kept constant throughout the experiment to each tracheal strip.

Soon after an hour stabilization period is provided to the tracheal tissues in an organ bath, the preparations were chemically contracted using a bronchoconstrictor drug; carbamylcholine (CCh, 1 μM) and potassium chloride having final bath concentrations of 80 mM and K^+-25 mM while the tissue tension was recorded using an emkaBATH data acquisition system (France) equipped with isometric force transducer. After getting stable bronchoconstriction, different concentrations of AFO as well as the used standard drugs were tested in a cumulative way to record the bronchial relaxant response [15].

2.6.3. Determination of the Possible Mechanisms of Action

To determine the involvement of antimuscarinic and Ca^{++} channels' inhibitor-like pharmacodynamics in the relaxant effect of AFO, the CCh-mediated CRC were obtained in the absence and presence of the pre-incubated tracheal tissues with two increasing doses of AFO; the results were compared with parallel experiments conducted using pre-incubated tissues with atropine, an anticholinergic [16]; dicyclomine, a dual inhibitor of muscarinic receptors; and Ca^{++} channels [17].

To confirm the inhibitory effect of AFO on Ca^{++} channels, the Ca^{++} CRCs were recorded in the absence and presence of pre-incubated tissues with the AFO in tracheal tissues previously made Ca^{++} free. The tracheal tissues were made Ca^{++} free by incubating tissues for an hour in a Ca^{++}-Krebs solution containing EDTA to chelate the intracellular stores of Ca^{++} [18]. The results were compared with parallel experiments conducted with standard drugs, dicyclomine and verapamil, known CCBs [19].

To assess the involvement of K^+ channels' opening-like effects, the spasmolytic effects of the test samples were explored on low K^+ (25 mM) [20]. After getting sustained contraction from low K^+, the test materials were added in a cumulative fashion to obtain the concentration-dependent relaxant responses. The relaxation of the tracheal tissue was demonstrated as the percentage of control contraction mediated by low K^+.

To characterize the specific type of K^+ channels' activation involved in the bronchodilator effect, the bronchodilator effects of the AFO was reproduced against low K^+-mediated contractions in the absence and presence of different K^+ channel antagonists such as tetraethylammonium (TEA, 1 mM), a nonselective K^+ channel blocker [21]; 4-aminopyridine (4-AP, 100 μM), a selective blocker of voltage sensitive K^+ channels [22]; and glibenclamide (Gb, 10 μM), a selective blocker of ATP-dependent K^+ channels [23].

2.7. Statistical Analysis

The recorded findings are presented as mean ± standard error of the mean while "n" represents the number of individual experiments repeated with EC_{50} (the median effective concentrations) with 95% confidence intervals (CI). A one-way analysis of variance and a

Dunnett's test were utilized to determine the bronchodilatation. $p < 0.05$ is considered to be statistically significant. Non-linear regression was applied to statistically analyze the CRCs using a GraphPad program (GraphPad, San Diego, CA, USA).

3. Results

3.1. Preparation of AFO and GC-MS Study

The fresh plants provided 0.694 g AFO from 250 g plant material, while the drying of the same weight resulted in 100 g dry plants that provided 0.545 g (Table 1). GC-MS analysis of the fresh and dry AFO enable the identification of the same nine components representing 98.219 and 99.732% of the AFO prepared from the fresh and dry plants, respectively (Table 2, Figure 2).

Table 1. Yield of AFO from the fresh and dried plant samples of *A. fragrantissima* *.

Condition	Weight (g)	Weight of Oil (g)	% *w*/*w*
Fresh	250	0.694 ± 0.021	0.28
Dried	100	0.545 ± 0.018	0.218 **

* Expressed values are the mean of triplicate determination (n = 3) ± standard deviations. ** The weights of the fresh plant samples were used to calculate the oil percentage.

Table 2. Components of fresh and dried plant samples AFO.

Name of Components	RT	RRI	Area%	
			Fresh	Dry
Yomogi alcohol	4.2191	1000	2.458	1.228
(+)-Santolina alcohol	4.8336	1038	25.643	21.711
artemisia ketone	5.2606	1061	9.949	7.209
α-Thujone	6.1470	1102	34.471	33.397
β-Thujone	6.3475	1110	11.939	10.704
Sabinol	6.7872	1143	3.052	1.268
Sabinyl acetate	9.9115	1291	4.582	1.585
Bicyclosesquiphellandrene	13.8768	1498	0.614	2.562
β-sesquiphellandrene	14.666	1525	5.511	20.068
Monoterpenes%			**92.094**	**77.102**
Sesquiterpenes			**6.125**	**22.63**
Total			98.219	99.732

The bold Monoterpenes and sesquiterpenes represent main groups of compounds.

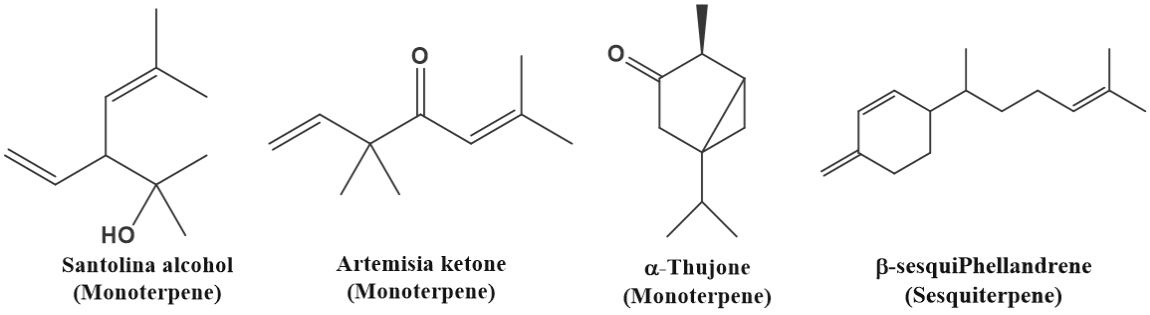

Figure 2. Chemical structure of some components of AFO.

3.2. Bronchodilator Activity

AFO caused concentration-dependent relaxation responses (Figure 3A) with complete relaxation against CCh with resultant EC_{50} values of 0.42 mg/mL (0.38–0.46, 95% CI, n = 4), while high K^+ was partially inhibited with a amaximum relaxation of 78% at the highest concentration of 5 mg/mL (Figure 3A). Similarly, dicyclomine also showed a higher potency against CCh compared to high K^+-mediated spasms with resultant EC_{50} values

of 0.36 µM (0.32–0.45, 95% CI, n = 4) and 9.62 µM (8.68–10.24, 95% CI, n = 4), respectively (Figure 3B). Verapamil, on the other hand showed a significantly higher potency to inhibit high K^+ compared to CCh-evoked contractions with respective EC_{50} values of 0.35 µM (0.28–0.43, 95% CI, n = 4) and 5.14 µM (4.26–6.42, 95% CI, n = 4) as shown in Figure 3C. The confirmation of the anticholinergic effect of AFO was processed by the CCh-mediated CRCs constructed in the absence and presence of AFO at a concentration of 0.1 and 0.3 mg/mL where the CRCs of CCh were shifted toward the right without an in-parallel manner or suppression at 0.1 mg/mL; the non-parallel shift was observed at 0.3 mg/mL with a suppression of the maximum response (Figure 4A). Similarly, dicyclomine (Figure 4B) also showed parallel and non-parallel shifts in CCh-mediated CRCs at the respective doses of pre-incubated tissues with 0.1 and 0.3 µM, whereas atropine, an anticholinergic drug, showed a parallel shift toward the right in CCh-CRCs at both doses of 0.01 and 0.03 µM pre-incubation (Figure 4C).

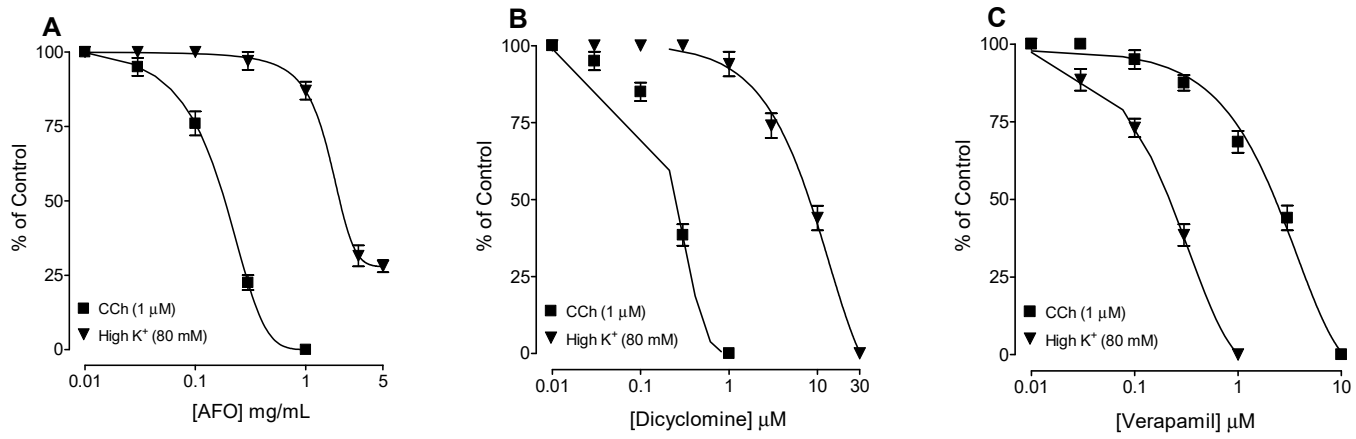

Figure 3. Concentration-dependent inhibitory effects of the (**A**) essential oil (AFO), (**B**) dicyclomine and (**C**) verapamil against carbachol (CCh; 1 µM) and high K^+ (80 mM)-induced contraction in isolated guinea pig tracheal preparations. Symbols represent mean ± SEM; n = 4.

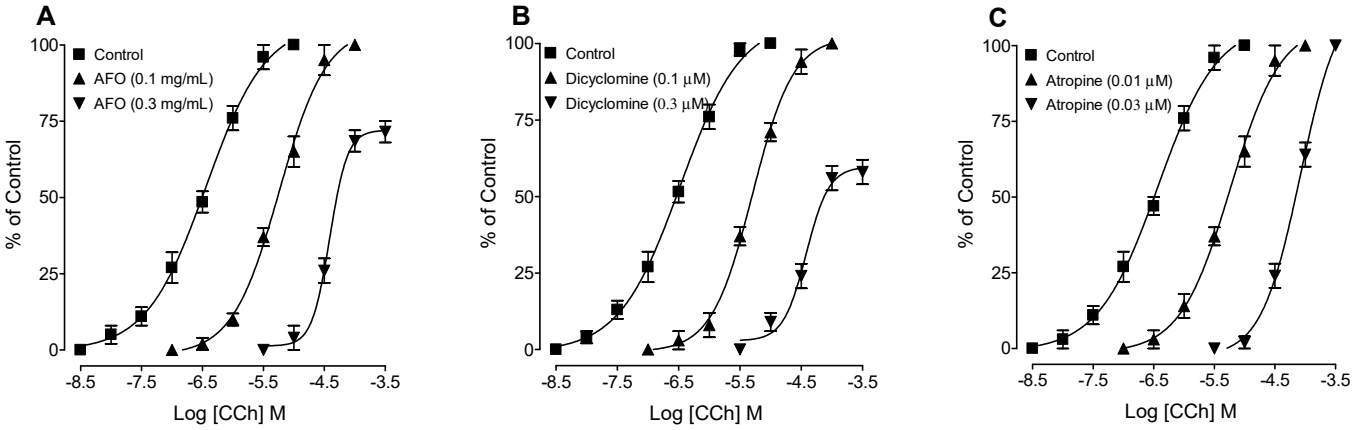

Figure 4. Concentration-response curves of carbachol (CCh) in the absence and presence of the increasing concentrations of the (**A**) essential oil (AFO), (**B**) dicyclomine and (**C**) atropine in isolated guinea pig tracheal preparations. Symbols represent mean ± SEM; n = 5.

The effect of AFO to inhibit Ca^{++} channels was further authenticated when the tracheal tissues pre-incubated with AFO (0.3 and 1 mg/mL) suppressed the Ca^{++}-mediated CRCs with a rightward shift constructed in a Ca^{++}-free medium (Figure 5A). Similarly, the standard drugs, dicyclomine (Figure 5B) and verapamil (Figure 5C), also showed suppression of the Ca^{++} CRCs with a rightward shift, as expected.

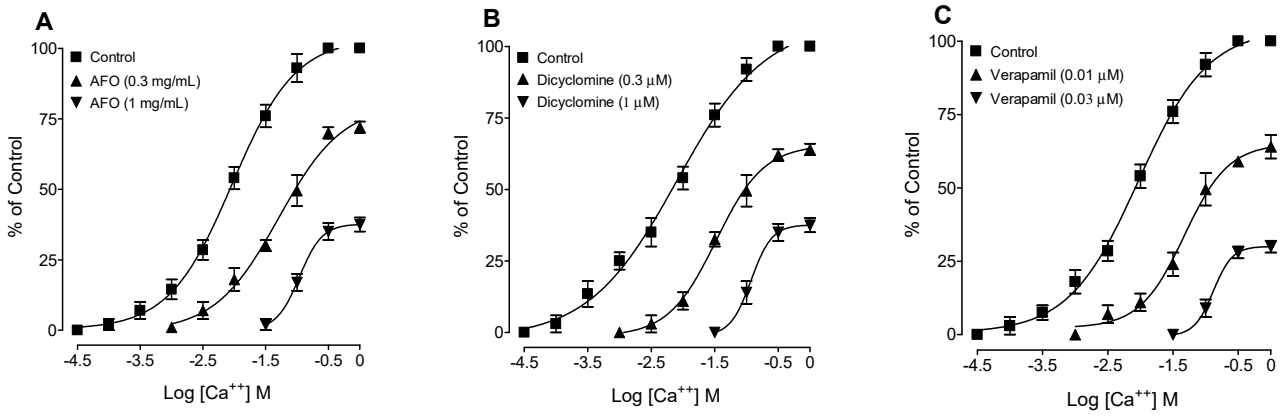

Figure 5. Concentration-response curves of Ca^{++} in the absence and presence of the increasing concentrations of the (**A**) essential oil (AFO), (**B**) dicyclomine and (**C**) verapamil in isolated guinea pig tracheal preparations. Symbols represent mean ± SEM; n = 5.

Previous studies established the fact that the spasmolytic effect of many medicinal plants may be mediated via K^+ channel opener activity, hence AFO was tested against evoked contractions in the isolated trachea with a low K^+ (25 mM). Interestingly, AFO led to significant relaxation in a concentration-dependent manner with resultant EC_{50} values of 0.26 mg/mL (0.22–0.29, 95% CI; n = 4) as shown in Figure 6A, whereas it failed to antagonize high K^+ (80 mM)-provoked contractions completely (Figures 3A and 6A). This inhibitory effect of AFO against low K^+ was not effected when the tissues were pre-incubated with glibenclamide (10 µM) (Figure 6B); TEA (1 mM) shifted the inhibitory response of AFO toward the right in a significant way (Figure 6C), whereas maximum inhibition was seen with 4-AP (100 µM) as shown in Figure 6D.

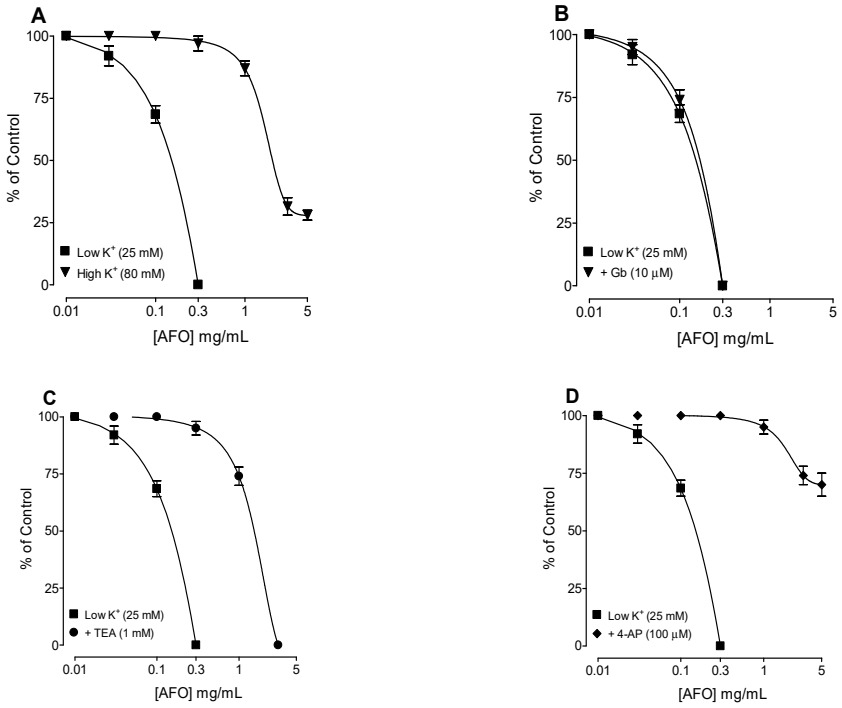

Figure 6. Concentration-dependent inhibitory effects of the essential oil (AFO) against (**A**) low K^+ and high K^+, and low K^+ in the presence of (**B**) glibenclamide (Gb; 10 µM), (**C**) tetraethylammonium (TEA; 1 mM) and (**D**) 4-aminopyridine (4-AP; 100 µM) in isolated guinea pig tracheal tissues. Symbols represent mean ± SEM; n = 4.

4. Discussion

The drying process resulted in a 21.5% loss of the AFO when compared with a fresh plant's yield (Table 1). There was no qualitative difference in the components present in the two AFO samples. The AFO is characterized by the absence of monoterpene hydrocarbons. All the seven identified monoterpenes were oxygenated (Table 2). Only two sesquiphellandrene derivatives could be detected in AFO prepared from the fresh and dry plants. In the AFO prepared from the fresh plants, the percentage of sesquiterpenes was 6.125, which increased to 22.63 in the AFO obtained from dry plant materials. This indicates that the loss in the lighter monoterpenes was greater than in the sesquiterpenes with a higher molecular weight (Figure 2) [24,25].

Among several airway diseases, asthma is the most common and is a major disabling syndrome that accounts for considerable deaths worldwide [26]. Asthma is one of the most important and common airways ailments, expressed as periodic wheezing following cough and chest rigidity mainly because of obstruction of the air passage [27]. The existing pharmacotherapy for the management of asthma has been classified into different classes, including bronchorelaxants, such as cholinergic-antagonists, β-receptor agonists, PDE-inhibitors, and anti-inflammatory drugs such as corticosteroids, mast cells stabilizers, leukotriene inhibitors, K^+ channel openers, and Ca^{++} channel inhibitors [28,29]. The drugs available are expensive (inhaler), beyond the access of laymen in the developing world, and have side-effects, particularly, if employed orally. Hence, less satisfaction is felt by many patients with the conventional medicines as patients need permanent therapy and believe that herbal medications are natural and can sometimes be taken without notifying their physician [30].

A. fragrantissima is a medicinal plant with a famous traditional history for its use in the treatment of bronchitis and bronchial asthma [3–6]. To date, no literature has been found to support these claims to the best of our knowledge. The current study aims to verify these traditional uses using isolated guinea pig tracheal tissues, a well-known ex vivo model in airways research [13,31]. The extracted essential oil from *A. fragrantissima* was tested for its possible tracheal smooth-muscle-relaxant effects against contractions provoked by carbamylcholine (CCh, 1 μM) and high K^+ (K^+-80 mM) in isolated tracheal preparations. Interestingly, AFO inhibited both types of evoked contractions with a selectively higher potency and efficacy against CCh, thus suggesting anti-muscarinic and Ca^{++} channel inhibition [32,33]. The anti-muscarinic and CCB-like actions of AFO were further confirmed, respectively, through CCh and Ca^{++} CRCs in the pre-incubated tracheal tissues with different concentrations of the AFO and the relevant standard drugs. A parallel shift of CCh curves without affecting the contractions' efficacy observed at the lower concentration of AFO is a known characteristic of the standard drug atropine, a competitive or specific muscarinic-receptor blocker [32], whereas the next higher dose of AFO exhibited a nonparallel shift in the CCh CRCs with a suppression of the efficacy, thus clearly pointing toward the involvement of non-specific inhibitory components similar to verapamil, a voltage-gated L-Type Ca^{++} antagonist [33]. The double inhibitory actions exhibited by AFO are strengthened by comparing them with the shift in the CCh CRCs obtained with dicyclomine [32], while verapamil, on the other hand, caused deflection of CCh CRCs rightward but also caused a non-parallel shift with a decrease in the efficacy at both concentrations. Atropine, as expected from competitive antagonists, deflected CCh CRCs in a parallel way toward a higher potency but caused no change in the efficacy [32]. The pretreatment of tissues with AFO shifted the Ca^{++} curves to the right accompanied by the suppression of maximum responses, similar to that caused by verapamil, confirming the Ca^{++} antagonistic effect. Interestingly, Ca^{++} antagonists are also useful in bronchoconstriction [34], besides the well-established role of anticholinergics in asthma [35].

According to previously conducted studies, it is experienced that plants with medicinal importance in bronchoconstriction also have an activation effect on K^+ channels [36], hence AFO was tested on low K^+-mediated contractions where it showed the highest potency compared to its anticholinergic and CCB-like effects. The inhibitory effects of AFO

on contractions provoked by low K^+ (25 mM) at a higher potency and efficacy compared to its inhibitory effect on high K^+ indicate their predominant effect on K^+ channel activation, as substances that selectively inhibit the contractions provoked by K^+ (25 mM) at a higher potency and are denoted as potassium channel openers [37]. On the other hand, substances that inhibit the contractions resulting from low (25 mM) and high (80 mM) K^+ concentrations at a comparable potency are termed as Ca^{++} channel blockers [38,39]. These experiments undoubtedly differentiate between the potassium channel openers and calcium channel blocker classes from a mechanistic viewpoint. To explore the contributions of different types of potassium channels, AFO was applied to low K^+ (25 mM)-provoked contractions in tissues pretreated with different K^+ channel antagonists, namely glibenclamide, an ATP-dependent K^+ blocker [23]; TEA, a non-selective K^+ channel blocker [21]; and 4-AP, a voltage-sensitive K^+ channel antagonist [40]. The classes of potassium channel openers could have a potential clinical interest as they have the ability to induce vascular and nonvascular smooth muscle relaxation, including in the respiratory tract muscles [41]. These compounds can open the K^+ channels and consequently cause membrane hyperpolarization by increasing in K^+ efflux, thus causing a decrease in the intracellular free Ca^{++} leading to smooth muscle relaxation [42,43].

5. Conclusions

The process of drying led to some changes in AFO quantity and the relative percentages of the AFO components obtained from fresh and dried stems of *A. fragrantissima*. The drying process resulted in an increase of the sesquiterpenes and decrease of the monoterpenes' relative percentages. The AFO prepared from fresh and dried plants were free from monoterpene hydrocarbons.

Our study also shows that AFO possesses bronchodilator effects mediated predominantly through a muscarinic receptor blockade and triggers the activation of the voltage sensitive and non-specific types of K^+ channels followed by the partial involvement of Ca^{++} channel inhibition. This study supports the traditional use of AFO for the treatment of respiratory disorders with the potential of the essential oil to be developed as a remedy for the management of bronchial asthma.

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