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Bronchodilator Phenylpropanoid Glycosides from the Seeds of *Prunus mahaleb* L.

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Abstract: *Prunus mahaleb* seeds were selected for phytochemical study directed by *ex-vivo* bronchodilator effect based on the traditional use for the treatment of respiratory problems. From the active chloroform fraction, five known phenylpropanoid glycosides: *cis*-melilotoside sodium salt (**1**), *cis*-methoxy-melilotoside (**2**), 3-(2-O-*β*-D-glucopyranosyl-4-methoxyphenyl) propanoic acid (**3**), *trans*-methoxy-melilotoside (**4**) and *trans*-melilotoside (**5**) were identified for the first time from genus *Prunus*. Chemical structure of the compounds elucidated by spectroscopic techniques such as 1D, 2D NMR and HR-ESI/MS. Compounds **1**, **2**, **4**, **5** showed promising bronchodilator effects against carbamylcholine (CCh) induced bronchospasm in isolated Guinea-pig trachea while **3** was found completely inactive. The mechanism(s) of action was studied using both CCh, low K^+ (25 mM) and high K^+ (80 mM)-mediated contractions and compound **2** was found distinctly more potent and efficacious against CCh compared to both types of K^+-mediated contractions where partial efficacy was observed, hence showed dual inhibition of cholinergic receptors followed by Ca^{2+} channels. The anticholinergic and Ca^{2+} inhibitory activities of compound **2** were further confirmed when it deflected CCh concentration response curves (CRCs) without suppression, whereas its higher doses shifted Ca^{2+} CRCs similar to verapamil. The bronchodilator effect proved to be mediated via dual anticholinergic and Ca^{2+} channels blocking effects.

Keywords: *Prunus mahaleb* L seeds; bronchodilator; phenylpropanoid glycosides; anticholinergic; calcium channel blocker.

1. Introduction

Many members of the genus *Prunus* are used in traditional medicine. The leaves and blossoms of *P. spinosa* are used as diuretic and for the treatment of peptic ulcers. The fruit of *P. salicina* is given to alleviate joint pain. An infusion of leaves and bark of *P. cerasoides* is used for treatment of

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whooping cough, asthma, dyspepsia, looseness of the bowels and kidney stones [1, 2]. The seed kernel has additionally been utilized to treat loose bowels in youngsters in Sudan, as tranquilizers and vasodilators in Saudi Arabia [3, 4]. *P. mahaleb* seed kernel is used in folk medicine as a tonic for sensory organs and the heart, treatment of respiratory problems, cough, kidney stones and as flavoring agent [5, 6]. A recent study proved that *P. mahaleb* seeds extract effectively prevented the formation of kidney stones in experimental animals [7]. The seed kernels of *P. mahaleb* have strong antimicrobial and antifungal activities and might be significantly important for the pharmaceutical industry [8]. The seeds of *P. mahaleb* reported to contain 27-40% fixed oil composed mainly of linolenic acid and unsaturated acid such as 9,11,13- octadecatrienoic acid. The seeds also have high protein contents reaching 31% in addition to sucrose [9]. The seeds were considered a rich source of α-eleostearic acid and polyunsaturated fatty acids [10]. *β*-sitosterol and *β*-sitosterol glucoside were also reported from the seeds oil [11]. Detailed comparative chemical study between the fruits and seeds of *P. mahaleb* indicated that the fruits have much higher content of total phenols, total flavonoids, condensed tannins, glucose, fructose and sucrose than seeds, as expected. The antioxidant activity of the fruit was reported as ten times more potent as the seeds and also the fruits contain more volatile components (33 volatile components) compared to its seeds where only 11 components were observed [12]. Based on color observations, a study shows that fruits are rich in anthocyanins while the seeds are free from such components [12]. In another study, coumarin, herniarin (7-methoxycoumarin), dihydrocoumarin, and *β*-D-glucoside of 7-methoxycoumaric acid were reported from the *P. mhaleb* seeds [4, 13].

Asthma is one of the important airways ailments, characterized by periodic wheezing following cough and chest rigidity mainly because of the obstruction in outflowing air [14]. Current drugs used as bronchodilator for the management of asthma mainly belongs to three categories; Anticholinergic agents, *β*2 adrenergic agonists and xanthine derivatives that have multiple side effects such as dry mouth, blurred vision, nausea, decreased gut mobility, urinary retention, tachycardia, anorexia, headache, sleep disturbance and cardiac arrhythmias [15, 16]. Many patients believe that herbal-derived medications are effective and comparatively safer [17]. Previous studies on the bronchodilator effects of some plants revealed that the effect could be mediated either by inhibition of muscarinic and histamine (H1) receptors, stimulatory effects on the *β*-adrenergic receptors or activation of potassium channels and inhibition of calcium channels [18]. There is interest in developing new classes of bronchodilators to overcome the limits that characterize the existing classes and their long-term use raises safety concerns [19].

Few preliminary studies were conducted to support the traditional uses of *P. mahaleb* seed kernels against cough, asthma, vasodilator, kidney stones and tranquilizers and to explore the corresponding secondary metabolites. Many herbal mixtures containing *P. mahaleb* seed are used in Egypt to manage cough and broncheal asthma [6]. Based on this practice in the absence of scientific evidences the current study was designed to verify the traditional use of *P. mahaleb* seeds as bronchodilator, identify the components responsible for this action and to explore in detail the mechanism(s) of the bronchodilator effect.

2. Materials and Methods

2.1. General

Melting points were uncorrected and recorded on an open capillary tubes Thermosystem FP800 Mettler FP80 central processor supplied with FP81 MBC cell apparatus. Ultraviolet absorptions were measure on a UV–Visible spectrophotometer (Unicum Heyios). ^{1}H-, ^{13}C NMR and 2D-NMR experiments were conducted using UltraShield Plus 500 MHz (Bruker) spectrometer operating at 500 MHz for protons and 125 MHz for carbon atoms located at the NMR Unite at the College of Pharmacy, Prince Sattam Bin Abdulaziz University). Chemical shift values are reported in δ (ppm) and the coupling constants (*J*) are reported in Hertz (Hz). Spectrum were referenced using the residual solvent peak, HRESI/MS were measured utilizing UPLC-Quadrupole Orbitrap MS Model UPLC RS Ultimate 3000- Q UPLC RS Ultimate 3000 - Q (Thermo Scientific). Silica gel 60/230-400 mesh (EM Science) were used for column chromatography. TLC plates silica gel 60 F254 (Merck) were used for

TLC screening. Carbamylcholine, verapamil, atropine and dicyclomine were purchased from Merck (NJ, USA) previously, Sigma-Aldrich.

2.2. Plant Material

The seeds of *Prunus mahaleb* L. were purchased in October 2020 from the local market in Riyadh city and were identified by Prof Saniya Kamal, Prof of Taxonomy, College of Science, Alexandria University. Voucher specimen (ALX 121020) was deposited at the Department of Botany, College of Science, Alexandria University, Alexandria, Egypt.

2.3. Extraction and Isolation

Two kg of the crushed seeds were exhaustedly extracted with 95% ethanol by maceration at room temperature. The extract was concentrated using rotary vacuum evaporator then subjected to liquid-liquid fractionation using *n*-Hexane (31.20 g), Chloroform (5.21 g) and Ethyl Acetate (5.38 g). The bronchodilator activity of the total extract was trapped to the chloroform fraction. Five grams of the active chloroform fraction were chromatographed on 200 g silica gel column eluting with Chloroform followed by Chloroform/Methanol mixtures in a gradient system to yield fractions (**A**-**F**). Fraction **E** eluted with 10% Methanol in Chloroform (412 mg) was subjected to MPLC on RP18 silica gel column eluting with water Methanol mixtures in a gradient system to afford 21 mg of **1**, 136 mg of **2**, 10 mg of **3** and 17 mg of **4**. Fraction **F** (2.44 g) after purification on silica gel column followed by MPLC on RP18 silica gel column eluting with water/ methanol mixtures in a gradient system afforded 14 mg of **1**, 22 mg of **2** and 13 mg of **5**.

2.4. Characterization of the Isolated Compounds

cis-Melilotoside sodium (***1***) : Yellowish matrix; UV λ_{max} (MeOH) 252, 293 nm; ^{1}H and ^{13}C NMR see Table S1. HRESI-MS: *m/z* 325.0925 (Calculated 325.0923) [M^{+}-1], 651.1931 (Calculated 651.1925) [$2M^{+}$-1], 349.0875 (Calculated 349.0899) [M^{+}+Na].

*cis-Methoxy-melilotoside (**2**)*: Yellowish matrix; UV λ_{max} (MeOH) 264, 308 nm; ^{1}H and ^{13}C NMR see Table S2. HRESI-MS: *m/z* 355.1029 (Calculated 325.1029) [M^{+}-1], 711.2137 (Calculated 711.2136) [$2M^{+}$-1], 379.0994 (Cal. 379.1005) [M^{+}+Na].

*3-(2-O-β-D-Glucopyranosyl-4-methoxyphenyl)propanoic acid (**3**)*: White needles, m.p. 194 ^{0}C; UV λ_{max} (MeOH) 250, 279 nm; ^{1}H and ^{13}C NMR see Table S2. HRESI-MS: *m/z* 357.1191 (Cal. 325.1186) [M^{+}-1], 715.2457 (Calculated 715.2450) [$2M^{+}$-1], 381.1146 (Calculated 381.1162) [M^{+}+Na].

*trans-Methoxy-melilotoside (**4**)*: Yellowish matrix; UV λ_{max} (MeOH) 274, 321 nm; ^{1}H and ^{13}C NMR see Table S2. HRESI-MS: *m/z* 355.1038 (Calculated 325.1029) [M^{+}-1], 379.0991 (Calculated 379.1005) [M^{+}+Na].

*trans-Melilotoside (**5**)*: Yellowish matrix, UV λ_{max} (MeOH) 276, 318 nm; ^{1}H and ^{13}C NMR see Table S1. HRESI-MS: *m/z* 325.0927 (Calculated 325.0923) [M^{+}-1], 651.1929 (Calculated 651.1925) [$2M^{+}$-1], 349.0898 (Calculated 349.0899) [M^{+}+Na].

2.5. Bronchodilator Effect

Experiments were conducted following the guide lines of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [20]. The study has been approved by Bio-Ethical Research Committee (BERC) at Prince Sattam Bin Abdulaziz University with reference number BERC-001-12-19.

Guinea-pigs (500-550 g) of either sex and local breed were procured from the lab animal unit of King Saud University. Animals were kept at the Animal Care Unit, College of Pharmacy, PSAU, KSA, maintained at 23-25^{0}C and given commercial standard diet and tap water *ad libitum*. Guinea-pigs were humanely killed by cervical dislocation. The tracheal tubes were immediately removed and placed in ice-cold Krebs solution. Krebs solution was gassed with 95% O_2: 5% CO_2 at 37°C. Krebs solution was prepared from (mM): NaCl 118.0, $NaHCO_3$ 25.0, KCI 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.0 [21]

Prepared guinea-pig tracheal strips were mounted in a 20 mL tissue bath containing Kreb's solution, maintained at 37°C. A tension of 1 g was applied to each of the tracheal strips and was kept constant throughout the experiment. After an equilibration period of at least 60 min, the preparations were tested for contractile responses to carbamylcholine (CCh, 1 µM), repeatedly using isometric force transducer, connected to emkaBATH data acquisition system (France). Once the tonic contraction became stable, the plant materials or pure compounds were added in increasing concentrations (0.01 to 10 mg/mL) to the bath by the cumulative method to assess their bronchial relaxant activity against CCh or K^+-induced sustained spasms [22].

To explore the possible involvement of *β*-adrenoceptor in the bronchodilator effect of the tested compound, the tracheal relaxation response curves against CCh were compared with the tissues pre-incubated with the *β*-adrenoceptor blocker propranolol (Prop. 1 µM). The involvement of additional multiple types of bronchodilatory-like mechanisms(s), relaxation response curves were obtained against CCh (1 µM), low K^+ (25 mM) and high K^+ (80 mM) and the inhibitory effect was expressed as percentage of the control contraction mediated by the added spasmogen. Muscarinic receptor antagonist-like action was explored by cumulative addition of sample different concentration (0.01 to 10 mg/mL) against sustained contraction obtained with CCh (1 µM) and its inhibitory effect was compared with dicyclomine, a dual inhibitor of muscarinic receptors and Ca^{2+} channels [23] and the selective muscarinic antagonist atropine [24]. For further confirmation of the antimuscarinc actions, the CRCs of CCh was constructed in the absence and presence of the pre-incubated tracheal tissues with low and high concentration of samples and the pattern of the inhibitory CRCs were compared with curves obtained in the presence of dicyclomine and atropine. The parallel displacement of the CCh CRCs towards right is indication of competitive-like mechanism of the test material [25] whereas non parallel displacement to the right with suppression of maximum response indicates non-specific antagonistic-like nature of the test substances [23].

The relaxant effect against high K^+ (80 mM)-induced contractions and low K^+ (25 mM)-induced contractions is used to explore the involvement of Ca^{2+} inhibitory like mechanism or K^+ channel opening-like (KCO) effects respectively. For confirmation of the Ca^{2+} inhibitory like mechanism, tissues were made Ca^{2+} free by incubating in Ca^{2+} free Krebs solution containing a chelating agent (EDTA) for an hour and the $CaCl_2$ CRCs were constructed in Ca^{2+} free medium in the absence and presence of different increasing concentrations of the test material in comparison with the standard Ca^{2+} channel blocker, verapamil [26]. Sustained contraction in response to low K^+ is challenged by the test materials in a cumulative fashion to obtain the concentration-dependent inhibitory responses of the test materials [27]. The inhibitory effect of the tested materials against low K^+ if observed at distinctly lower doses than that observed against high K^+, indicates involvement of KCO whereas a substance causing inhibition of both high and low K^+ at closer dose levels indicates the presence of Ca^{2+} antagonist-like antispasmodic mechanism [28].

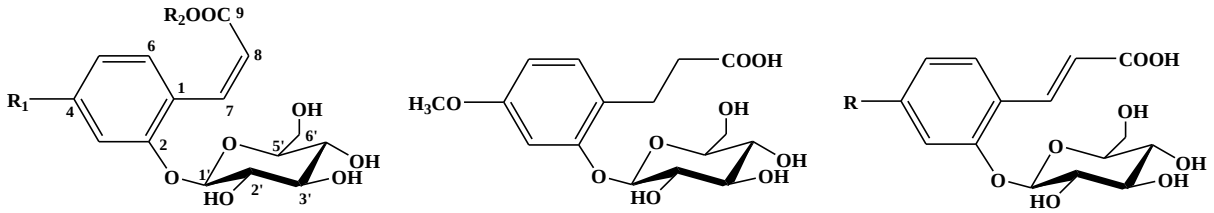
2.6. Statistical Analysis

Data are expressed as mean ± standard error of the mean (n= number of experiment) and the median effective concentrations (EC50) with 95% confidence intervals (CI). One-way analysis of variance followed by Dunnett's test was used to assess the tracheal relaxation CRCs. $P < 0.05$ is considered to be statistically significant. Concentration response curves were analyzed by non-linear regression using GraphPad program (GraphPad, San Diego, CA, USA).

3. Results and Discussion

3.1. Structure Elucidation

The *upfield* part of the spectra of **1** showed 6 oxygenated carbons signals including 5X CH and 1XCH_2. The signals at δ_H 4.85 (d, *J*=6.6), δ_C 101.09 were assigned to H-1′ of glucose. The ^{1}H NMR data of **1** in CD_3OD (Table S1, Figures S1) showed signals for four aromatic protons at δ_H 7.15 (d, *J*=8.25), 7.23 (t, *J*= 7.5), 6.97 (bt, *J*= 6.6), and 7.60 (bs) ppm with their corresponding carbons appeared in the ^{13}C NMR spectrum (Figure S2) at δ_C 116.33, 130.17, 123.14, and 130.96 ppm as indicated from the HSQC experiment (Figure S5) were assigned to positions 3-6, respectively, in a di-substituted aromatic ring. The two quaternary carbons at δ_C 126.86 and 155.90 ppm indicated the presence of alkyl and oxygen substituents at positions 1 and 2 of the aromatic ring. The two methine groups at δ_H 6.84 (d, *J*=12.3 Hz, δ_C 128.75 and δ_H 6.12 (bs), δ_C 128.21 as well as carboxylic carbonyl signal at δ_C 170.42 ppm representing the alkyl substituent. The data of **1** pointed out to *cis*-melilotoside taking in account the *J* value of 12.3 Hz observed for H-7. However, the broad singlet of H-8 was odd. *cis*-Melilotoside literature data assigned the C-7 methine at δ_H 7.31-7.35, δ_C 138.40; C-8 methine at δ_H 5.95-5.99 and δ_C 120.02 [29- 31]. Another report for *cis*-melilotoside assigned the C-7 methine at δ_H 6.83 (d, *J*=12.5 Hz), δ_C 128.30; C-8 methine at δ_H 6.03 (d, *J*=12.5 Hz) and δ_C 128.30 ppm [32]. Although the data in the last reference were different from the previously reported values the authors did not give explanation or cite any references for the compound. Our data for **1** are closer to those of literature [32] except for the splitting of H-8. ^{1}H NMR and ^{13}C NMR in DMSO (Figures S6, S7) showed the two methines at δ_H 6.60 (d, *J*=12.5 Hz), δ_C 123.50 and δ_H 5.92 (d, *J*=11.5 Hz), δ_C 130.29 as well as carboxylic carbonyl signal at δ_C 171.76 ppm representing the alkyl substituent. The *J* value = 12.6 and 11.6 Hz between the two methine protons indicated their *cis*-orientation. The impact of different carboxylates cations on the chemical shifts of protons and carbons in cinnamates and coumarates were studied in comparison with the original free acids [33, 34]. Sodium salts expressed considerable changes in the chemical shifts of the alkyl substituents. Although the studies were conducted on *trans*-isomers, similar effect is expected on the *cis*-compounds. The decrease in the chemical shift value of C-7 in **1** to δ_C 128.75 and the increase of C-8 to δ_C 128.21 ppm in the CD_3OD spectra were in full support of **1** to be *cis*-melilotoside sodium salt. The effect of DMSO was more dramatic on the vinyl moiety as a result of complex formation between the solute molecules and the solvent involving the sodium cation and sulfinyl group of DMSO [35, 36]. HRESI-MS data (Figure S11) showed M^+-Na at *m/z* 325.0925 (Cal. 325.0923), $2M^+$+1-2Na at *m/z* 651.1931 (Cal. 651.1925), and M^+ at *m/z* 349.0875 (Cal. 349.0899) all supporting the molecular formula $C_{15}H_{17}O_8Na$. The structure reported for the phenylpropanoid glycoside *cis*-melilotoside [32] should be revised to *cis*-melilotoside sodium salt.



1: R_1= H R_2= Na
2: R_1= OCH_3 R_2= H

3

4: R= OCH_3
5: R= H

Figure 1. Structure of compounds **1- 5**

The NMR and HRESI-MS data of **5** (Figures S33- S39) supported the molecular formula $C_{15}H_{18}O_8$ and isomeric structure compared to **1**. The major difference between **5** and **1** was in the signals of the alky substituent. The two methine groups appeared at δ_H 8.10, δ_C 139.70 and δ_H 6.52, δ_C

118.45 ppm with *J*=16 Hz indicating their *trans*-orientation (Figure S31). These data enable the identification of **5** as the phenylpropanoid glycoside *trans*-melilotoside [29- 32].

The NMR data of **2- 4** indicated the presence of glucosyl moiety (Table S2). The ^{1}HNMR data in the three compounds showed an ABX spin system (Table S2, Figures S12, S19, S26) diagnostic for 1, 2, 4-trisubstituted aromatic ring. Substituents at C-1 and C-2 were similar to those of **1** and **5** as indicated from the C-1 and C-2 chemical shifts (Table S2, Figures S13, S20, S27). The substituents at C-4 were assigned to OCH_3. The chemical shifts of C-4 were δ_C 162.56, 160.77 and 159.35 ppm in **2- 4**, respectively. The methoxyl signals appeared at δ_H 3.73(s), δ_C 55.98; δ_H 3.75(s), δ_C 55.89 and δ_H 3.70(s), δ_C 55.20 ppm in **2- 4**, respectively. Each compound showed additional three carbon resonances for the C-1 alkyl substituents including Carboxylic carbons at δ_C 173.83, 179.20 and 169.96 ppm.

The ^{1}H NMR spectrum of **2** showed two vinyl proton signals at δ_H 6.93 (d, *J*=12.7 Hz), δ_C 132.56 and δ_H 5.85 (d, *J*=12.7 Hz), δ_C 123.34 ppm, while **4** showed two vinyl proton signals at δ_H 8.76 (d, *J*=16 Hz), δ_C 139.20 and δ_H 7.04 (d, *J*=16 Hz), δ_C 117.45 ppm. These data indicated that **2** has *cis*-oriented vinyl protons while **4** is the *trans* isomer. HRESI-MS data of **2** (M^+-1 at *m/z* 355.1029, $2M^+$-1 at *m/z* 711.2137, M^++Na at *m/z* 379.0994, Figure S18) and **4** (M^+-1 at *m/z* 355.1038, M^++Na at *m/z* 379.0991, Figure S30) supported the molecular formula $C_{15}H_{18}O_8$ for the two compounds. The above discussion enables the identification of **2** as *cis*-methoxy-melilotoside previously reported from *Lavandula officinalis* [37] and *Artemisia splendens* [31, 32], while **4** was identified as *trans*-methoxy-melilotoside first reported from *Lavandula officinalis* [37].

The most important NMR difference between **3** and both **2** and **4** is the disappearance of the vinyl protons and the presence of two methylene signals at δ_H 2.90 (q, *J*= 7.2 Hz), δ_C 25.44, and δ_H 2.56 (bt, *J*= 7.2 Hz), δ_C 35.44 ppm assigned for C-7 and C-8, respectively. Due to the loss of conjugation C-9 carbocyclic carbonyl signal was downfield shifted to δ_C 179.20 ppm. The HRESI-MS data of **3** showed M^+-1 at *m/z* 357.1191, $2M^+$-1 at *m/z* 715.2457, and M^++Na at *m/z* 381.1146 (Figure S25) all supporting the molecular formula $C_{16}H_{22}O_9$ two protons more than **2** and **4**. The data of **3** were similar to those reported for 3-(2-O-*β*-D-glucopyranosyl-4-methoxyphenyl)propanoic acid [38].

3.1. Bronchodilator Effect

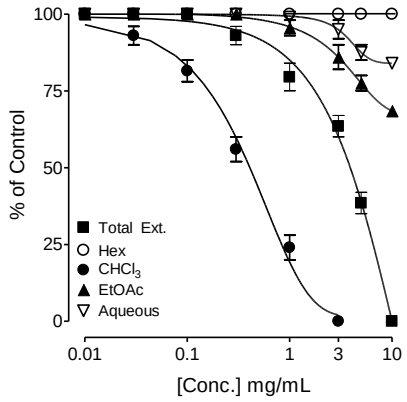


Figure 2. Concentration-dependent inhibitory effects of the total extract of *P. mahaleb* Total Ext., Hex, $CHCl_3$, EtOAc and Aqueous fractions against carbachol (CCh; 1 µM)-induced contractions in isolated guinea-pig tracheal preparations. Symbols represent mean ± SEM; n= 4-5.

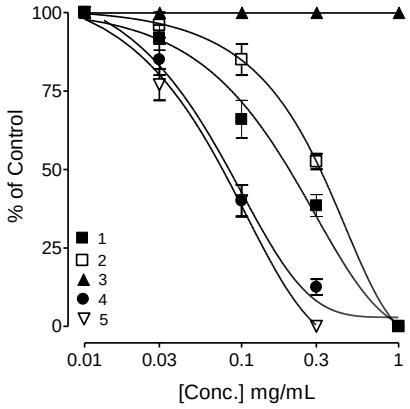


Figure 3. Concentration-dependent inhibitory effects of **1-5.** isolated from chloroform fraction of the *P. mahaleb* against carbachol (CCh; 1 µM)-induced contractions in isolated guinea-pig tracheal preparations. Symbols represent mean ± SEM; n= 4-5.

The total extract of *P. mahaleb* and its fractions were tested for their possible relaxant effects against contractions provoked by carbamylcholine (CCh, 1 µM) in isolated guinea-pig tracheal

preparations. The total extract (0.1 to 10 mg/mL) and the $CHCl_3$ fraction (0.01 to 3 mg/mL), produced concentration-dependent relaxation of the CCh-induced contractions while EtOAc and aqueous fractions produced partial but significant inhibitory activities whereas hexane fraction did not show any relaxation at maximum tested concentration of 10 mg/mL (Figure 2). Bioassay-guided chromatographic purification of the $CHCl_3$ fraction resulted in the isolation of **1- 5** in enough yield to explore their detailed mechanistic activity. When tested against CCh, **1**, **2**, **4** and **5** showed tracheal relaxation (0.01 to 1 mg/mL) with varying potencies while **3** was totally inactive. Compounds **4** and **5** exhibited highest potency with EC50 values of 0.07 mg/mL (0.06-0.09, n=5) and 0.06 mg/mL (0.05-0.07, n=5) respectively, whereas **1** and **2** showed comparable potencies with resultant EC50 values of 0.24 mg/mL (0.21 to 0.27, n=5) and 0.38 mg/mL (0.32 to 0.42, n=4), respectively (Figure 3).

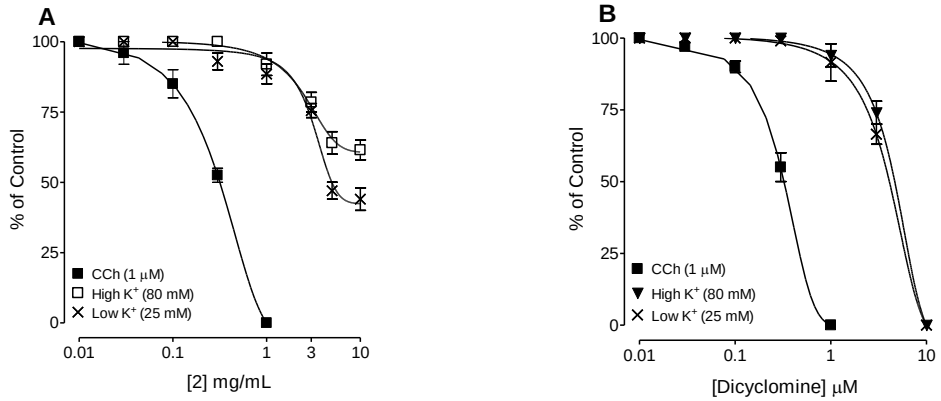


Figure 4: Concentration-dependent inhibitory effects of the **(A) 2** and **(B)** dicyclomine against carbachol (CCh; 1 µM), low K^+ (25 mM) and high K^+ (80 mM)-induced contraction in isolated guinea-pig tracheal preparations. Symbols represent mean ± SEM; n= 4-5.

Due to its high yield, compound **2** was selected for the detailed mechanistic study. The bronchodilation induced by **2** was not significantly attenuated by propranolol (data not shown), suggesting that the relaxant effects of the compound is unrelated to the activation of *β*-adrenoceptors [39]. The tracheal relaxant effect of **2** was studied against the contractions induced by CCh (1 µM), low K^+ (25 mM) and high K^+ (80 mM). Compound **2**, showed significantly higher potency and efficacy against CCh compared to high (80 mM) and low K^+ (25 mM)-mediated contractions (Figure 4A). Similarly, dicyclomine, a dual inhibitor of muscarinic receptors and Ca^{2+} channels also showed selectively high potency against CCh compared to high and low K^+-mediated contractions (Figure 4B).

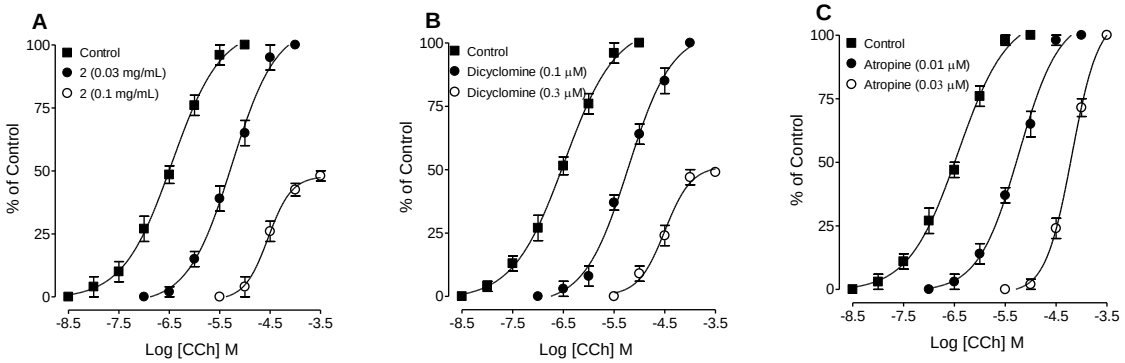


Figure 5. Concentration-response curves of carbachol (CCh) in the absence and presence of the increasing concentrations of the **(A) 2**, (**B)** dicyclomine and **(C)** atropine in isolated guinea-pig tracheal preparations. Symbols represent mean ± SEM; n= 4-5.

The dual inhibitory-like nature of **2** was further confirmed when the tissue incubated with its lower concentration (0.03 mg/mL) deflected CCh-mediated concentration-response curves (CRCs) to the right without suppression while its next higher concentration (0.1 mg/mL) incubation deflected further CCh curves towards right and also suppressed the maximum response of CCh (Figure 5A), similar to dicyclomine (Figure 5B) whereas atropine, a pure competitive antagonist at muscarinic receptors [24] shifted CCh CRCs towards right without suppression at both concentration (Figure 5C). As compound **2** was also found partially active against K^+-mediated contractions at higher concentrations (Figure 3), which is an indication of its ability to inhibit Ca^{2+} channels, hence further experiments were conducted to confirm the CCB-like effect of compound **2**. For this purpose, the tissues were made Ca^{2+} by one-hour incubation in Ca^{2+} free Krebs solution containing EDTA, a chelating agent [40]. Once the tissue was made Ca^{2+} free, $CaCl_2$ CRCs were constructed in the absence (control) and presence of pre-incubation of tissues with either **2** (0.1 and 0.3 mg/mL) or the voltage gated Ca^{2+} channel blocker (CCB) verapamil [41]. The resultant shift of the Ca^{2+} CRCs construction in Ca^{2+} free medium towards right with suppression of the maximum response (Figure 6A), similar to dicylcomine (Figure 6B) and verapamil (Figure 6C) authenticate non-specific inhibition and confirms the inhibitory effect of compound 2 on voltage-gated Ca^{2+} channels.

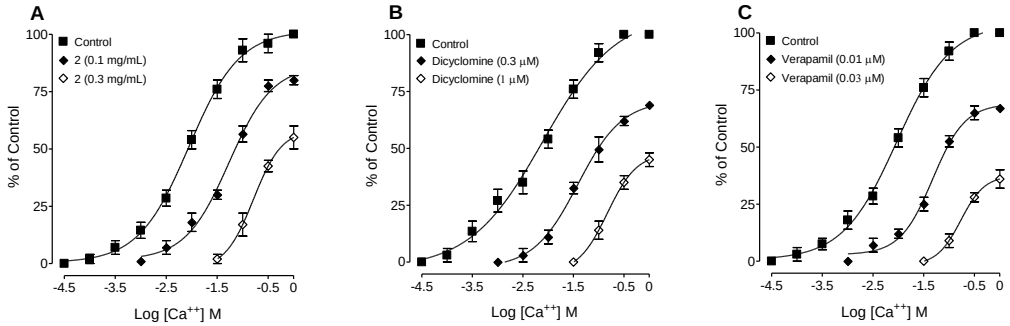


Figure 6. Concentration-response curves of Ca^{2+} in the absence and presence of the increasing concentrations of the **(A) 2**, **(B)** dicyclomine and **(C)** verapamil in isolated guinea-pig tracheal preparations. Symbols represent mean ± SEM; n= 4-5.

The involvement of K^+ channel activation as one of the bronchodilator mechanisms of **2** was excluded based on the findings that its inhibitory effect against low and high K^+-mediated contraction is mediated at comparable concentrations with maximum relaxant effects of 56.0 ± 4.0, (Figure 3A). A substance, that selectively inhibit the contractions provoked by K^+ (25 mM) are denoted as potassium channel openers [42]. On the other hand, substances that inhibit the contractions induced by both concentrations of K^+ (25 and 80 mM) at comparable concentrations are termed as Ca^{2+} channel blockers. The results of these experiments can clearly differentiate between Ca^{2+} channel blockers and K^+ channel openers from a mechanistic viewpoint [28].

The five compounds belonging to the glycosilated phenylpropanoids class. The four active compounds **1**, **2**, **4**, **5** share the presence of vinyl double bond between C-7 and C-8 that seems to be essential for activity as **3** which is lacking this double bond was found totally inactive at the tested concentrations. Compounds **1** and **5**; **2** and **4** are isomeric pairs at the vinyl bond. The two *trans* isomers **4** and **5** are more active than the corresponding *cis* isomers **2** and **1**, respectively. The **1** and **5** pair is lacking the methoxy substitution at C-4. Comparison between **1** and its *cis* analogue with C-4 methoxyl **2** revealed that the methoxylate derivative **2** is less active. Same situation was observed upon comparison of the activity of **5** and the methoxylated analogue **4**. In conclusion, the double bond between C-7 and C-8 seems essential for the bronchodilator effect of these compounds. The trans-orientation of the double bond increases the activity but C-4 methoxylation decreases the activity. Literature survey indicated that this is the first report for the isolation of phenylpropanoid glycosides from the Genus *Prunus*. The bronchodilator effect of this class of secondary metabolites was not previously reported.

In conclusion, *P. mahaleb* possess the bronchodilator effect possibly mediated by dual inhibition of muscarinic receptor blocking and Ca^{2+} ion inhibitory-like actions and thus provide sound basis for its traditional use in hyperactive airways related disorders. However, further molecular studies are recommended to know in-depth precise mechanism(s).

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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