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Full Length Research Paper

Spasmolytic activity of *p*-menthane esters

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The present study aimed to investigate the relationships between the chemical structure and spasmolytic activity of 10 *p*-menthane monoterpene esters found in aromatic plants. All of the monoterpenes studied had spasmolytic actions in isolated guinea pig ileum. The neo-isopulegyl acetate and the 4-terpinyl acetate were the most potent, while the perillyl acetate was the least potent *p*-menthane ester. This difference in potency may be due to the position of the acetate group linked to C-7 in perillyl acetate, a structural feature different from that of the other esters. Our results revealed that stereochemistry and electronic density highly influenced spasmolytic activity in smooth muscle. Moreover, the presence of an aromatic ring or changes in the position of the ethyl acetate group in the *p*-menthane skeleton had little effect on the spasmolytic activity of monoterpene esters. Therefore, our data suggest that appropriate modifications of monoterpene structure can be associated with improved potency and may lead to the development of new antispasmodic drugs.

Key words: Monoterpene, essential oils, spasmolytic activity, smooth muscle, pharmacological activity.

INTRODUCTION

Evaluation of the therapeutic potential of medicinal plants has been the focus of intense studies and chemical constituents, such as flavonoids, alkaloids, terpenes, tannins and lignans, among others, have been proven to possess pharmacological properties in preclinical testing on animals. In this context, plant-derived essential oils have been widely used for medicinal applications, particularly in the pharmaceutical, cosmetic, natural-product and food industries (Bakkali et al., 2008). Many essential oils exhibit pharmacological activities owing to the presence of various structurally diverse bioactive chemical components, such as monoterpenes. Several compounds have actions on smooth muscle and nerve endings, thereby decreasing gastric and intestinal motility and producing an antispasmodic response (Somlyo et al., 1994). An increasing number of studies carried out in animals have shown several beneficial pharmacological properties of essential oils, such as hypotensive and vasorelaxant (Edris, 2007), spasmolytic (De Sousa et al., 2010), anticonvulsant (De Almeida et al., 2011), analgesic (De Sousa, 2011), and anti-inflammatory activity (Mendes et al., 2010). Functional problems of the gastrointestinal tract, such as irritable bowel syndrome, intestinal disorders and diarrhea are very common in humans and are characterized by a broad spectrum of symptoms mostly related to paralysis of intestinal motor function (Heinle et al., 2006).

Essential oils have been demonstrated to have spasmolytic actions in a variety of smooth muscle types and this effect is attributed to its chemical constituents, including monoterpenes such as α-pinene, (Camara et al., 2003), citral (Sadraei et al., 2003), pulegone (Soares et al., 2005), and linalool (Mazzanti et al., 1998), which are found in *Plectranthus barbatus, Melissa officinalis, Mentha* x *villosa,* and *Hyssopus officinalis* L., respectively. The essential oil of *Thymus leptophyllus* has also been demonstrated to have significant spasmolytic effects against contractions induced by acetylcholine in isolated rat duodenum (Zafra-Polo et al., 1989). The major constituent of this oil is the monoterpene ester linalyl acetate, which makes up 68.5% of the oil. In a recent study, we showed that monoterpene acyclic esters, such as citronelyl acetate and linalyl acetate, exhibit significant spasmolytic actions in isolated guinea pig ileum (De Sousa et al., 2011). The differences in pharmacological effects are probably due to the structural

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Abbreviations: TLC, Thin layer chromatography; **SEM,** standard error of the mean; **NMR**, nuclear magnetic resonance.

diversity of chemical constituents present in essential oils (Perry et al., 2003). It is, however, necessary to investigate new drugs that exert therapeutic actions against disorders caused by intestinal diseases. Therefore, in this study we evaluated the spasmolytic activity of structurally related monoterpene esters.

MATERIALS AND METHODS

Chemicals and solutions

(-)-Menthol, *cis*-carveol, (-)-isopulegol, neo-isopulegol, 4-terpineol, Perillyl alcohol , (+)-*p*-ment-1-en-9-ol, (-)-α-terpineol, carvacrol and thymol were purchased from Aldrich Chemical Company (Jacksonville, FL, USA). All esters were dissolved as emulsions in 10% Tween-80.

Acetylation of alcohols and phenols: General method

The starting materials (alcohol or phenol) (1 g) were treated with a mixture of Ac_2O/py (6:10, v/v) at reflux and stirred until they completely disappeared. The reaction was monitored by TLC. Reaction times for monoterpene esters were 24 or 48 h. After completion, the reaction mixture was quenched with cold water (30 ml), and product was extracted with $CHCl_3$ (90 ml), washed first with saturated copper sulfate and then with water, dried over anhydrous sodium sulfate and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel and was eluted with hexane–EtOAc (8:2) to obtain the esters.

Animals

Male guinea pigs (weighing 300 to 400 g) were used in these experiments. They were obtained from the Central Animal House of the Federal University of Sergipe, São Cristóvão, Brazil. Two days before experiments, the animals were housed at 23 ± 2°C under a light/dark cycle (6 to 18 h in light and 18 to 6 h in dark) in the Animal Facility of the Department of Physiology. The animals fasted for 16 h before the experiments but were allowed free access to water. The animal experiments were approved by the Ethics Committee on Research Animals of the Federal University of Sergipe, São Cristóvão, Brazil, on 04/24/2009 with protocol number 14/09.

Tissue preparation

The animals were killed by cervical dislocation and exsanguination through the carotid arteries. A 2.0 cm full-thickness segment of the distal portion of the ileum (1 cm proximal to the ileocecal sphincter) was removed and suspended under 1 g of resting tension in a 10 ml organ bath containing Tyrode solution (composition in $mmol \cdot L^{-1}$: NaCl, 137; KCl, 2.7, $MgCl_2 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 1.8; NaH_2PO_4, 0.4; $NaHCO_3$, 12; glucose, 5.5). Tissues were maintained at 37°C and continuously bubbled with atmospheric air. The ileal strips were allowed to equilibrate for 60 min with washing every 15 min in Tyrode solution. The muscle strips were connected to a force transducer coupled to an amplifier-recorder (GOLD, Ohio, USA), and isometric contractions were recorded on a computer.

Experimental protocol

After equilibration, ileal muscle tone was increased by addition of 3 µM bethanechol. When muscle tension had stabilized, test compounds were cumulatively added in separate preparations to obtain concentration-response curves. Relaxation was measured as the reduction of bethanechol-induced tone and responses were normalized by calculating percent relaxation of bethanechol-induced tone. In order to compare spasmolytic potencies of the acetates, the concentration required to obtain a half-maximum response (EC_{50}) was calculated by nonlinear regression from the concentration-response curves of each ester.

Data presentation and statistical analysis

The data are represented as mean and variability as 95% confidence intervals or standard error of the mean (SEM) of the relaxation responses in muscle strips obtained from 6 animals. Statistical analysis was performed using one-way analysis of variance followed by the Turkey's test. A probability level of 0.05 was considered significant.

RESULTS

The present study compared the spasmolytic activity of monoterpene esters using isolated guinea pig ileum, precontracted by bethanechol (3 µM). Acetylation of alcohols and phenols using a mixture of Ac_2O/py afforded purified esters, with a yield of approximately 82.5%. Thin layer chromatography (TLC) analysis of the reaction products indicated that the esters had been synthesized. The isolated compounds were identified on the basis of spectral characterization (IR, ^{13}C, and ^{1}H nuclear magnetic resonance) (NMR), chromatographic TLC behavior and comparison with data from the literature (Figure 1). Each of the 10 esters that were tested exerted spasmolytic activity in guinea pig ileum (Table 1), which was expressed by reduction of tonus induced by bethanechol. This activity was concentration dependent and started at concentration of 10^{-7} molar and maximum response was obtained at concentration range of 10^{-3} - 10^{-2} molar.

DISCUSSION

The aim of this study was to evaluate the spasmolytic potency of *p*-menthane monoterpene esters (Figure 1) and to determine structure-activity relationships of these compounds. Knowledge of the pharmacological structure-activity relationships of natural, synthetic, or semi-synthetic molecules is of fundamental importance in the development of new drugs with spasmolytic activity and appropriate pharmacological profiles (Edris, 2007). Among the esters tested, 1 and 2 (Table 1) were equipotent with each other but were more potent than each of the other compounds ($p > 0.05$). In the monoterpene ester 2, the functional group is attached to tertiary carbon, C-4, while in ester 1, the functional group is connected to a stereogenic secondary carbon, C-3, which has less steric hindrance and has also stereogenic centers in C-1 and C-4. These structural differences did

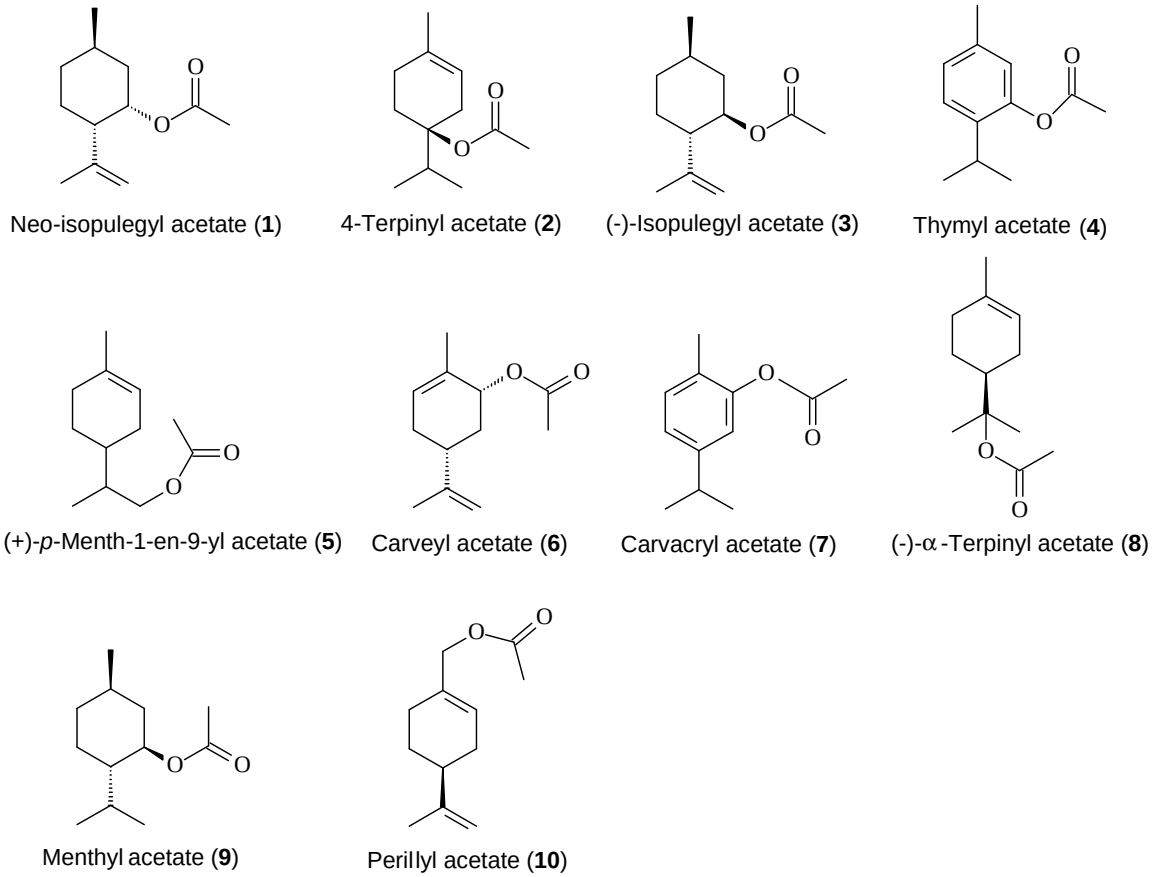

Figure 1. Chemical structures of monoterpene esters used in this study.

not result in differences in the spasmolytic activities of the 2 compounds. Between the diastereoisomers 1 and 3, 3 was found to be less potent ($p < 0.001$), suggesting that the change in configuration of the stereogenic center at C-3 influences spasmolytic potency. Between ester 4 and 9, 4 was a more potent spasmolytic ($p < 0.001$). This result shows that the aromaticity in ester 4 improves pharmacological potency. Factors such as electronic density and planar structure may be involved in this potentiation. On comparing esters 8 and 2, which are positional isomers, we observed that the presence of the acetate group at C-4, the endocyclic carbon, increased spasmolytic potency. Similarly, between esters 4 and 7, which differ in the position of the functional groups linked to C-3 and C-2, ester 4 was slightly more effective ($p < 0.001$).

Although esters 10 and 5 are both primary esters, interestingly, compound 5 was more potent in promoting the pharmacological response. Spasmolytic activity has been demonstrated for several major constituents of essential oils, such as (-)-menthol and menthyl acetate, which are found in plants of the genus *Mentha*, and carvacryl and carvacryl acetate, found in other plants, such *Satureja montana* L. and *Origanum compactum*, respectively (Grigoleit and Grigoleit, 2005b; Baser, 2008; Rivero-Cruz, et al., 2010). Most of these compounds belong to the class of oxygenated *p*-menthane monoterpenes and a series of *in vitro* experiments have shown antispasmodic actions on smooth muscle fibers isolated from guinea pig tissues, including trachea and ileum (Reiter and Brandt, 1985; Taddei et al., 1988) and sphincter of Oddi (Giachetti et al., 1988). These studies revealed that the high spasmolytic potential can be attributed to monoterpenes present in essential oils. In fact, there are several reports of the antispasmodic potential of monoterpenoids. For example, the effects of thymol on the spontaneous contractile activity of circular smooth-muscle strips from guinea pig stomach and vena portae have been evaluated *in vitro* (Beer et al., 2007). Another example is a comparative study performed with chemical analogues of rotundifolone, the major component of essential oil from *Mentha* x *villosa* leaves. These compounds also had spasmolytic activity in isolated guinea pig ileum. The experimental data also suggested that the functional groups present and their positions, on the ring of rotundifolone influence spasmolytic activity (De Sousa et al., 2008). All monoterpenes evaluated in this study had relaxing effects

Table 1. EC_{50} and maximum response values obtained from concentration-response curves in isolated guinea pig ileum.

Compound	Mean EC_{50} (95% CI)	$E_{máx}$ (%)
Neo-isopulegyl acetate	6.5×10^{-6} (3.0×10^{-6} - 1.4×10^{-5})	113.2 ± 2.2
4-Terpinyl acetate	1.0×10^{-5} (4.7×10^{-6} - 2.2×10^{-5})	103.7 ± 5.8
(-)-Isopulegyl acetate	1.6×10^{-5} (1.3×10^{-5} - 2.1×10^{-5})	114.0 ± 3.4
Thymyl acetate	1.7×10^{-5} (8.9×10^{-6} - 3.2×10^{-5})	116.0 ± 4.3
(+)-*p*-Menth-1-en-9-yl acetate	2.7×10^{-5} (1.2×10^{-5} - 6.2×10^{-5})	107.4 ± 1.6
Carveyl acetate	3.0×10^{-5} (2.2×10^{-5} - 4.3×10^{-5})	109.0 ± 1.6
Carvacryl acetate	3.7×10^{-5} (2.8×10^{-5} - 5.0×10^{-5})	120.6 ± 4.3
(-)-α-Terpinyl acetate	5.3×10^{-5} (2.7×10^{-5} - 1.0×10^{-4})	109.4 ± 5.3
Menthyl acetate	7.3×10^{-5} (4.0×10^{-5} - 1.3×10^{-4})	107.0 ± 1.9
Perillyl acetate	2.2×10^{-4} (1.2×10^{-4} - 4.0×10^{-4})	131.0 ± 4.9

The EC_{50} values were expressed in mol/L and the data were analyzed with one way ANOVA followed by Tukey's test (n = 6).

on intestinal smooth muscle. The main finding of this study was that monoterpene esters have spasmolytic effect in smooth muscle and we also established the structure-activity relationships between 10 *p*-menthane monoterpene esters from several aromatic plants.

Conclusion

The study showed that ethyl acetate groups and their position on the *p*-menthane structure, as well as differences in stereogenic centers, influence the spasmolytic potency of monoterpenes.

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