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Spasmolytic Activity of the Ethanolic Extract and Essential Oil of *Aloysia citriodora* Palau

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ABSTRACT The leaves of *Aloysia citriodora* Palau (Jordanian "malliseh") are traditionally used to remediate irritative gastrointestinal symptoms. The aim of this study is to assess the spasmolytic activity of the ethanolic extract and essential oil of "malliseh" on spasmogen-induced isometric tension in small intestine isolated from Wistar rats (*Rattus norvegicus*). Isometric tension of mounted tissue preparations was continuously measured. Tissue preparations were serially pretreated with increasing concentrations of either the ethanolic extract or the essential oil. Following each pretreatment, tissue preparations were treated with a single application of acetylcholine chloride, barium chloride, or potassium chloride. The ethanolic extract of "malliseh" exhibited spasmolytic activity against acetylcholine- and barium-induced isometric tension, but not potassium-induced isometric tension. The essential oil of "malliseh" partially inhibited acetylcholine-, barium- and potassium-induced isometric tension. The traditional use of "malliseh" may be justified. The ethanolic extract and essential oil of "malliseh" induce dose-dependent, reversible inhibition of spasmogen-induced isometric tension in small intestine isolated from rats, albeit by distinct mechanisms.

INTRODUCTION

Aloysia citriodora Palau (family *Verbenaceae*) is an aromatic plant native to South America, where it is commonly known as "cedrón" (Ragone et al. 2007). The plant is widely cultivated in Jordan and goes by the transliterated local name "malliseh" (Afifi and Abu-Irmaileh 2000). Jordanian "malliseh" is grown in domestic herb gardens for its leaves, which are used to scent and flavour black tea. The leaves exude a characteristic lemony aroma, hence their culinary use and the specific epithet of the plant. "Malliseh" is used in the traditional remediation of numerous and diverse symptoms, including a constellation of irritative gastrointestinal symptoms (Abuhamdah et al. 2013).

The laboratory rat is a popular model organism in preclinical studies. For instance, candidate spasmolytic agents are often assessed using smooth muscle tissue isolated from Wistar and Sprague-Dawley rats (*Rattus norvegicus*). Indeed, the spasmolytic activity of *Aloysia citriodora* Palau has been documented in numerous *in vitro* studies on both rat strains (Ragone et al. 2007; Ponce-Monter et al. 2010; Calzada et al. 2010; Lenoir et al. 2012; Consolini et al. 2011). For instance, Ponce-Montor et al. demonstrated the inhibitory effect of the hexane extract of the plant on spasmogen-induced contractile activity in uterine strips isolated from Wistar rats (Ponce-Monter et al. 2010). The spasmolytic activity of the plant was reproduced in two additional *in vitro* studies investigating the effects of methanolic and aqueous extracts of *Aloysia*

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citriodora Palau on small intestine (isolated from Sprague-Dawley rats) and duodenum (isolated from Wistar rats), respectively (Ragone et al. 2007; Calzada et al. 2010). Additionally, an infusion of the plant was shown to be protective against chemical-induced colitis in Sprague-Dawley rats (Lenoir et al. 2012). Aqueous extracts of other plants in the genus, namely *Aloysia polystachya* (Griseb.) Moldenke and *Aloysia gratissima* (Gillies and Hook.) Tronc., also demonstrated spasmolytic activity in duodenum and ileum isolated from Sprague-Dawley rats, albeit as a result of a different set of spasmolytic components (Consolini et al. 2011).

To date, chemical constituents of the aqueous extract of *Aloysia citriodora* Palau have been studied once only (Ragone et al. 2007). The flavonoid vitexin was identified as a spasmolytic component of the aqueous extract. Vitexin accounts for a proportion of the spasmolytic activity, while other components remain to be elucidated. On the other hand, the essential oil profile of *Aloysia citriodora* Palau has been investigated in numerous studies, two of which are based in Jordan (Abuhamdah et al. 2015; Di Leo Lira et al. 2008; Hudaib et al. 2013; Moein et al. 2014). However, spasmolytic components of the oil, if any indeed exist, have not been identified. In fact, the spasmolytic activity of the essential oil of *Aloysia citriodora* Palau has yet to be investigated.

Objectives

The aim of this study is to investigate the spasmolytic activity of the ethanolic extract of "malliseh" (EEM) and the essential oil of "malliseh" (EOM) on small intestine isolated from Wistar rats.

METHODOLOGY

Plant Material

Fresh leaves of *Aloysia citriodora* Palau were collected during April 2016 from the School of Agriculture, The University of Jordan. The plant was authenticated by Professor Suleiman Al-Olimat (Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology). A voucher specimen is deposited in the institutional herbarium at the School of Pharmacy, The University of Jordan (AC-V1). The plant name has been checked with www. worldfloraonline. org (23 December 2019).

Preparation of the Ethanolic Extract of "Malliseh"

Fresh leaves (100 grams) were extracted by reflux with one litre of ninety-six percent ethanol for one hour. Following overnight incubation at room temperature (20°C), the extract was filtered using 125 mm Whatman® Grade 1 filter paper. The solvent was evaporated to dryness under reduced pressure using a rotary evaporator (Heidolph Laborota, Germany). The residue was dried further by incubation for eight days at room temperature. The yield was two percent, representing 2.01 grams of extract out of 100 grams of fresh leaves. The crude extract was stored in an airtight glass container and refrigerated until use. For the experiments, a portion of the residue (0.1 grams) was dissolved in 10 mL of Krebs-Henseleit solution. Further dilutions of the stock solution were prepared using the same solution.

Isolation of the Essential Oil of "Malliseh"

Fresh leaves (500 grams) were hydrodistilled with one litre of water for three hours using a Clevenger-type apparatus. The essential oil was dried with anhydrous sodium sulphate and stored at 4°C. The stock solution and further dilutions were prepared using Krebs-Henseleit solution. The composition of the essential oil was analysed using an AutoSystem XL gas chromatograph coupled with a TurboMass quadrupole mass spectrometer (PerkinElmer, Shelton, CT, USA). Chromatography was performed on a 30 m × 0.25 mm ID × 0.25 ìm DB-5 MS column (J&W Scientific, Rancho Cordova, CA, USA) using a temperature program of 40-300°C at a rate of 3°C per min. The results were previously reported (Abuhamdah et al. 2015).

Animals and Tissue Preparation

The study protocol was approved by the Institutional Review Board (The University of

Jordan Deanship of Scientific Research). Male albino Wistar rats (test-naïve; age range, 8-10 weeks; weight range, 250-300 g) were obtained from the animal colony of The University of Jordan School of Medicine. Following a 24-hour water fast, rats were anesthetised by ether inhalation and sacrificed by cervical dislocation. The abdomen was opened by a midline incision and the intestines were removed. A segment of small intestine, measuring approximately 10 cm, was excised. The segment was immediately immersed in Krebs-Henseleit solution. The solution was maintained at 37°C and gassed with a carbogen mixture of ninety-five percent oxygen and five percent carbon dioxide. Intraluminal contents were gently flushed out with Krebs-Henseleit solution. Four 1.5-cm tissue sections were prepared per rat. Preparations were mounted in organ baths filled with aerated Krebs-Henseleit solution and connected to an isometric force transducer. Isometric tension was continuously measured using a data acquisition system. The measure was recorded using LabChart 8 software. Preparations were allowed to equilibrate in the bathing medium for 30 minutes under 2 grams of preload.

Experimental Protocol

Following a control trial, tissue preparations were pretreated with either EEM or EOM and the bathing medium was allowed to sit. After 20 minutes, the bathing medium was washed out and replaced with a treatment solution consisting of a fixed concentration of one of three spasmogens, namely, acetylcholine chloride (30 μM), potassium chloride (60 mM), or barium chloride (5 mM). The treatment solutions had been prepared using Krebs-Henseleit solution. Spasmogen concentrations were determined based on cumulative dose-response curves from a pilot study. For each type of treatment, up to six concentrations of EEM (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 150 μg/mL and 200 μg/mL) or five concentrations of EOM (1.5 μg/mL, 15 μg/mL, 150 μg/mL, 300 μg/mL and 600 μg/mL) were serially tested. The number of concentrations studied was predetermined according to experimental time constraints. In each experimental cycle, one rat was studied (four tissue sections; serial pretreatment with increasing concentrations of one plant extract; serial treatment with a fixed concentration of one spasmogen). One rat was studied per experimentation day. On experimentation days, the protocol was initiated at 9:00 AM. Rats were serially allocated to pretreatment-treatment cycles in the following order: EEM and acetylcholine chloride, EEM and potassium chloride, EEM and barium chloride, EOM and acetylcholine chloride, EOM and potassium chloride, EOM and barium chloride. In total, six rats were studied (one rat per full pretreatment-treatment cycle).

Drugs and Solutions

Acetylcholine chloride, barium chloride, potassium chloride, sodium chloride, potassium chloride, magnesium sulfate, monopotassium phosphate, calcium chloride, sodium bicarbonate and dextrose were obtained from Sigma-Aldrich (Amman, Jordan). Krebs-Henseleit solution was composed of the following: sodium chloride (128 mM), potassium chloride (4.8 mM), magnesium sulfate (1.1 mM), monopotassium phosphate (1.2 mM), calcium chloride (1.25 mM), sodium bicarbonate (25 mM) and dextrose (12 mM).

Data Analysis

LabChart data were viewed using LabChart Reader. The data analyst (JZA) was blinded to the pretreatment-treatment conditions. Four values were returned per preparation for a given pretreatment–treatment condition: maximum isometric tension during the final 3-minute pretreatment period, maximum isometric tension during the initial 1-minute post-treatment period, mean isometric tension during the final 3-minute pretreatment period, and, mean isometric tension in the time period between the 1-minute post-treatment mark and the 3-minute post-treatment mark. Differences between the two former values were computed and termed “maximum isometric tension change”. Differences between the two latter values were computed and termed “mean isometric tension change”. Data were manually entered into the IBM SPSS Statistics Data Editor and subsequent analyses were performed using this software package. One-way repeated measures analyses of variance were conducted in order to assess differences in isometric tension change between pretreatment conditions for a given type of plant extract, treatment condition and type of measure. Underlying assumptions were tenable, as assessed by the Shapiro-Wilk

test and inspection of boxplots. Greenhouse-Geisser correction was invariably applied. The omnibus test was followed by polynomial contrasts, wherein linear, quadratic and cubic trend components were evaluated. The false discovery rate was controlled using the Benjamini-Hochberg procedure. Numerical data are presented according to the recommendations of Cole (2015). A P value < 0.05 was considered to indicate statistical significance.

RESULTS

Spasmolytic Activity of the Ethanolic Extract of "Malliseh"

Concentration-dependent differences in maximum isometric tension change were statistically significant for one out of three treatments, namely, acetylcholine chloride (P = 0.01), but neither barium chloride (P = 0.07) nor potassium chloride (P = 0.07). Trend analysis for acetylcholine chloride treatment revealed a statistically significant linear component (P = 0.002). In addition, mean isometric tension change was statistically significantly different between pretreatment conditions following treatment with acetylcholine chloride (P = 0.02) and barium chloride (P = 0.03), but not potassium chloride (P = 0.2). Data trends for acetylcholine chloride and barium chloride treatments were statistically significant for both linear (P = 0.006 and P = 0.02, respectively) and quadratic (P = 0.02 and P = 0.048, respectively) components. The dose-response curves are depicted in Figure 1.

Spasmolytic Activity of the Essential Oil of "Malliseh"

Pretreatment of rat small intestine with increasing concentrations of EOM resulted in sta-

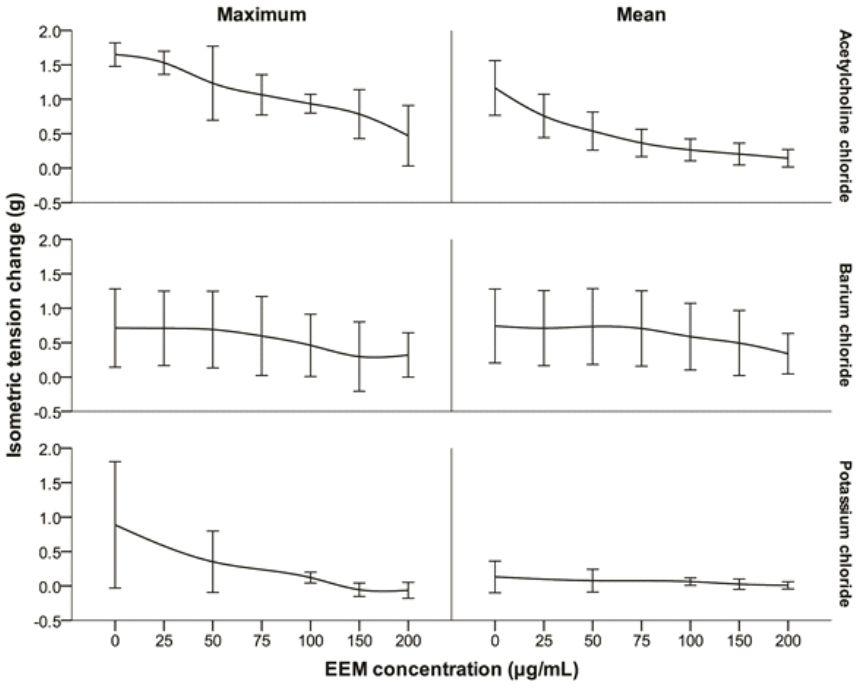

Fig. 1. Maximum and mean changes in isometric tension (g) in the post-treatment period relative to the pretreatment period for each treatment condition given pretreatment with varying concentrations (in ig/mL) of the ethanolic extract of "malliseh" (EEM). Results are the mean of *n*=4 repetitions. Error bars represent the 95 percent confidence interval
Source: Author

tistically significant differences in maximum isometric tension change following treatment with barium chloride ($P = 0.02$) and potassium chloride ($P = 0.04$), but not acetylcholine chloride ($P = 0.1$). The data trend for barium chloride treatment was statistically significant for both linear ($P = 0.03$) and cubic ($P = 0.002$) components. The data trend for potassium chloride treatment was statistically significant solely for a linear component ($P = 0.01$). Concentration-dependent differences in mean isometric tension change were statistically significant for all three treatments, namely, acetylcholine chloride ($P = 0.03$), barium chloride ($P = 0.0495$) and potassium chloride ($P = 0.03$). There was a statistically significant linear component ($P = 0.005$) in the trend analysis for acetylcholine treatment. On the other hand, the trend analysis for barium chloride treatment was statistically significant for both quadratic ($P = 0.002$) and cubic ($P = 0.02$) components. Finally, the trend analysis for potassium chloride treatment was statistically significant solely for a quadratic component ($P = 0.004$). The dose-response curves are depicted in Figure 2.

DISCUSSION

The researchers assessed the dose-response effect of EEM and EOM on spasmogen-induced isometric tension in small intestine isolated from Wistar rats. In normal physiological conditions, endogenous spasmogens effect contraction in smooth muscles by either one, or both, of two means, that is, increasing cytosolic calcium ion concentration, and increasing calcium sensitivity of myosin regulatory light chain phosphorylation. Increase in the cytosolic calcium ion concentration is primarily mediated by the influx of extracellular calcium ions through plasma membrane L-type calcium ion channels (Karaki et al. 1997). The treatments used in the present study, namely, acetylcholine chloride, barium chloride and potassium chloride, exert their spasmogenic effect by overlapping, yet distinct mechanisms.

Muscarinic acetylcholine receptors, expressed on the surface of smooth muscle cells, are activated by acetylcholine. Acetylcholine-induced contractile activity appears to be me-

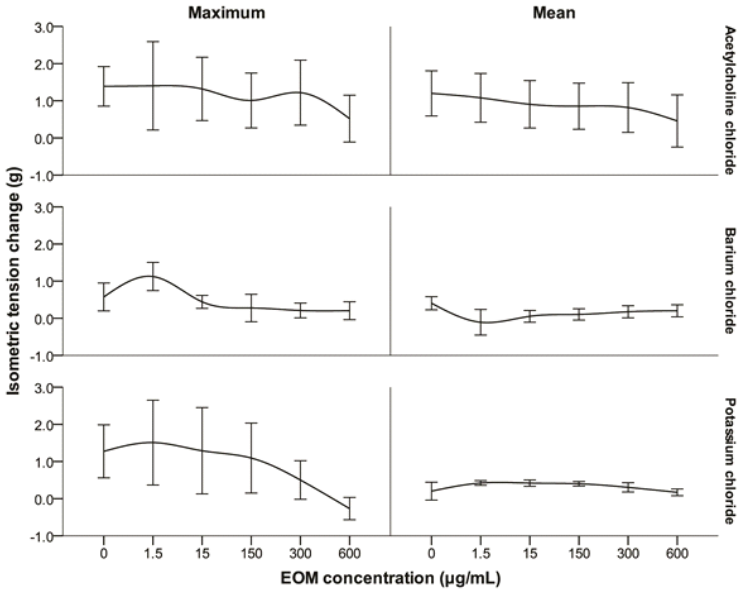

Fig. 2. Maximum and mean changes in isometric tension (g) in the post-treatment period relative to the pretreatment period for each treatment condition given pretreatment with varying concentrations (in ìg/mL) of the essential oil of "malliseh" (EOM). Results are the mean of *n*=4 repetitions. Error bars represent the 95 percent confidence interval.
Source: Author

diated by mobilisation of sarcoplasmic calcium ion stores, direct activation of non-selective cation channels, indirect activation of L-type calcium ion channels, and sensitisation of intracellular contractile elements to calcium ions (Karaki et al. 1997; Unno et al. 2005). In the present study, single-dose application of acetylcholine chloride elicited a characteristic biphasic response under all pretreatment conditions, and maximum increase in isometric tension was achieved during the first minute post-treatment, followed by a stable submaximal plateau throughout the subsequent two minutes. Pretreatment of rat small intestine with increasing concentrations of EEM linearly attenuated the maximum increase in isometric tension following treatment. On the other hand, pretreatment of the rat's small intestine with increasing concentrations of EOM did not significantly alter the maximum increase in isometric tension following treatment. The plateau phase was initially attenuated in a linear fashion with increasing concentrations of both EEM and EOM. However, the effect levelled off at higher concentrations of the former, but not the latter. Overall, both EEM and EOM exhibited inhibitory effects on acetylcholine-induced isometric tension. In agreement with the results, aqueous and hexane extracts of *Aloysia citriodora* Palau have been shown to inhibit acetylcholine- and carbachol-induced contractile activity in smooth muscle (Ragone et al. 2007; Ponce-Monter et al. 2010).

Barium-induced contraction of smooth muscle appears to be mediated by extracellular calcium influx through L-type calcium ion channels following membrane depolarisation. Intracellularly, barium ions may directly activate contractile elements (Karaki et al. 1986; Satoh et al. 1987). In the present study, contractile activity in unpretreated tissue preparations featured a consistently reproducible qualitative response following treatment with barium chloride, isometric tension increased to a maximal point during the first minute post-treatment and the selfsame value roughly represented the level of the plateau throughout the subsequent duration of time. This feature was reproduced in tissue preparations pretreated with EEM, albeit with a qualitative difference, that is, the level of the plateau was attenuated with increasing concentrations of the pretreatment. The contractile response of tissue preparations pretreated with EOM, followed by barium chloride treatment, was characterised by a marked "rise-and-collapse" phenomenon. The maximum isometric tension change was greater at the lowest concentration of EOM pretreatment relative to the control trial, accounting for the "rise". At the self-same pretreatment condition, the subsequent plateau contractile activity was surprisingly re-established at a level lower than that of the preceding pretreatment baseline value (hence the "collapse"). This "rise-and-collapse" phenomenon was progressively obliterated with increasing concentrations of EOM pretreatment, such that the maximum and mean isometric tension changes converged in a manner akin to the typical response of unpretreated tissue preparations to barium chloride treatment, albeit at a lower plateau level. All in all, both EEM and EOM induced partial spasmolysis of barium-induced isometric tension.

Potassium-induced smooth muscle contraction occurs as a result of membrane depolarisation and subsequent calcium ion influx through L-type calcium ion channels (Karaki et al. 1997). In the present study, under all pretreatment conditions, single application of potassium chloride produced a biphasic contractile response similar to that previously described for acetylcholine chloride treatment. Neither phase of the contractile response was significantly altered by EEM pretreatment, that is, EEM exhibited neither spasmolytic nor spasmogenic activity on potassium-induced isometric tension. The results are discordant with those of a previous study, which demonstrated a concentration-dependent inhibitory effect of the hexane extract of *Aloysia citriodora* Palau on potassium-induced contractile activity (Ponce-Monter et al. 2010). On the contrary, EOM pretreatment significantly altered both phases of the post-treatment contractile response. Maximum isometric tension change was linearly attenuated with progressive concentrations of EOM, ultimately resulting in an atypical maximum isometric tension decrease (post-treatment relative to pretreatment) at the highest concentration of EOM pretreatment. The post-treatment plateau phase was initially accentuated given EOM pretreatment. However, this effect was obliterated at higher concentrations of the pretreatment. All in all, in

regard to potassium-induced isometric tension, EOM pretreatment exhibited a mild spasmogenic effect at low concentrations and a marked spasmolytic effect at high concentrations.

The ethanolic extract of "malliseh" exhibited spasmolytic activity against acetylcholine- and barium-induced contraction, but not potassium-induced contraction. Based on these results, the inhibitory activity of EEM does not appear to be mediated by antagonism of extracellular calcium influx; rather, an intracellular mechanism of action is more likely. The putative inhibitory mechanism of EEM is supported by the findings of a previous study on the spasmolytic effect of the aqueous extract of *Aloysia citriodora* Palau (Ragone et al. 2007). On the other hand, the essential oil of "malliseh" inhibited acetylcholine, barium- and potassium-induced contraction. Therefore, the mechanistic delineation of EOM in the context of smooth muscle contraction cannot be ascertained from the data in the present study. However, it appears that, at the very least, EEM and EOM exert their spasmolytic action by different mechanisms.

The strengths of the present study include the novel analytical approach. The researchers evaluated trends in contractile activity changes from a longitudinal temporal standpoint with adjustments for spontaneous activity. By using four tissue sections per rat and trend analyses thereafter, the researchers were able to minimise the total number of rats studied. The main limitation is the paucity of mechanistic insight afforded by the study design. While the differential response of contractile tissue to specific spasmogens in the presence of the plant extracts offers a degree of insight, the precise mechanism of action of the extracts cannot be elucidated without further study. In addition, the findings are preliminary and cannot be extrapolated to humans without further investigation.

CONCLUSION

This is the first report of the spasmolytic activity of EEM and EOM on spasmogen-induced isometric tension in the small intestine isolated from Wistar rats. Both the ethanolic extract and the essential oil appear to exert their spasmolytic action by dose-dependent, reversible inhibition.

RECOMMENDATIONS

The mechanisms of action of EEM and EOM appear to be distinct. Therefore, future studies should investigate the additive inhibitory potential of EEM and EOM and their specific mechanisms of action.

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