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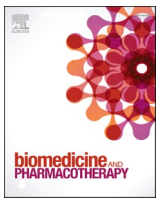

Inhibitory effect and underlying mechanism of essential oil of *Prangos ferulacea Lindl* (L.) on spontaneous and induced uterine contractions in non-pregnant rats

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ABSTRACT

Evidence suggests the use of natural compounds as support in the management of uterine contractility disorders. We recently demonstrated that the essential oil of Apiacea *Prangos ferulacea* (L.) (Prangoil) modulates intestinal smooth muscle contractility. Thus, we aimed to evaluate if Prangoil could also affect the contractility of uterine muscle in non-pregnant rat and to investigate the related action mechanism/s. The effects of the aromatic monoterpenes, β-ocimene and carvacrol, constituents of Prangoil, were also evaluated. Spontaneous contractions and contraction-induced by K^+-depolarization and oxytocin in rat uterus were recorded *in vitro*, using organ bath technique. Prangoil reduced the amplitude of spontaneous contractions as well as responses to KCl and oxytocin. β-ocimene and carvacrol matched oil inhibitory effects. Prangoil effects were not affected by nitrergic and adenylyl cyclase inhibitors or non-specific potassium channel blocker, but they were reduced by nifedipine, L-type calcium channel inhibitor, or 2-aminoethoxydiphenylborate (2-APB), membrane-permeant inositol 1,4,5-triphosphate receptor inhibitor. The response to β-ocimene was reduced by nifedipine and by 2-APB (20 μM), whilst carvacrol inhibitory effect was attenuated only by nifedipine. In conclusion, Prangoil, and its components, β-ocimene and carvacrol, reduced spontaneous and KCl or oxytocin-induced contractions of rat myometrium, mainly modulating extracellular Ca^{2+} influx through L-Type channels and Ca^{2+} release from the intracellular store. Further studies could contribute to evaluate the potential use of Prangoil against disorders characterized by abnormal uterine contractions.

1. Introduction

Uterine contractility regulation is fundamental in many physiological reproductive processes, such as menstruation, sperm and embryo transport, implantation, gestation and parturition. Most of the uterine disorders such as implantation failure, dysmenorrhea, endometriosis, or preterm labor could be caused by an abnormal contractility [1]. Currently, tocolytic agents, such as β_2 adrenergic receptor agonists and calcium channel blockers, such as nifedipine, are used to reduce uterine contractions in spasmodic disorders and in the treatment of preterm labor [2,3]. However, these drugs can also affect other targets, leading to adverse side effects [4,5]. Thus, due the limited number of safe and effective therapies for most uterine disorders, there is an urgent clinical need to improve the treatment of diseases characterized by irregular uterine contractions.

Historically, natural products have played a crucial role in the process of drug discovery and development [6–8]. These products and their derivatives are being used as support to classical treatments. Moreover, they could provide clues for identifying cost-effective therapies with fewer side effects for management of many diseases, including those derived from defective uterine smooth muscle contractility. In this context, Apiaceae as *Prangos ferulacea Lindl.* (L.) have demonstrated to have therapeutic effects and have been used in traditional medicine for treatment of different diseases [9,10]. It is noteworthy that *Prangos ferulacea* has been used as carminative, emollient, tonic for gastrointestinal disorders, and it shows flatulent, sedative, anti-inflammatory, anti-viral and anthelmintic effects. *Prangos ferulacea* extract has been shown also to modulate smooth muscle contractility [11]. Recently,

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chemical composition and biological activities of the essential oil from aerial parts of *Prangos ferulacea* Lindl. (L.) (syn. *Cachrys ferulacea* (L.) Calest) (Prangoil), which grows wild in Sicily, have been reported. The essential oil is characterized by a high amount of monoterpene hydrocarbons, being β-ocimene the major component (59%), and it shows antioxidant, anti-acetylcholinesterase and cytotoxic activities. [12]. Moreover, we have recently reported that both Prangoil and β-ocimene have inhibitory effects on the contractility of the rat small and large intestine[13]. Therefore, in consideration of the absence of information about possible effects of Prangoil on uterine contractility, we aimed, at first, to investigate the potential tocolytic action of Prangoil on non-pregnant rat uterus and the related action mechanism/s. Subsequently, since aromatic monoterpens are reported to be able to modulate smooth muscle contractility [13–18], we investigated the contribution to the overall Prangoil effects of β-ocimene, the main constituent of the oil, and of carvacrol, which is reported to have tocolytic effect on the pregnant rat uterus [15]. The results of this study could provide new insights for the development of new tocolytic drugs for treating pathological conditions, such as uterine spasmodic disorders or dysmenorrhea, characterized by smooth muscle contraction dysregulation.

2. Materials and methods

2.1. Plant Materials

The areal parts (flowers and leaves) of *Prangos ferulacea* (L.) Lindl. were collected from a wild source in Sicily (Piano Zucchi, Palermo, Italy), and stored in the Herbarium of the University of Palermo (Voucher No. PAL 109762). Essential oil was extracted by hydrodistillation (Department STEBICEF, University of Palermo, Italy), analyzed by GC\MS analysis, as reported by Bruno's group [12]. The monoterpene hydrocarbons, β-ocimene (59%), α - pinene (5.6%), carvacrol (3.6%), sabinene (2.8%) and p-cymene (2.0%) constitute the major components of oil.

2.2. Animals

Animal procedures were carried out in compliance with the European Community directives (1986/609/EEC; 2010/63/EU) regulating the use of animals in research, recognized and adopted by the Italian Government. All efforts were made to reduce animal suffering and the number of animals utilized. The research was conducted on tissues obtained after animal sacrifice and then it did not require ethic committee approval.

Isolated tissue bath technique was used to measure mechanical activity of uterine musculature as previously described [19]. In brief, fifteen virgin Wistar rats in oestrus phase (220–250 g, Envigo, S Pietro al Natisone- Italy) as determined by examination of a vaginal smear, were euthanized using 2% isoflurane anesthesia followed by cervical dislocation.

Muscle strips (2 mm × 10 mm) were dissected from uterine horns and the endometrial layer was gently removed. Then, the strips were placed individually in a 10 mL organ bath containing Krebs solution aerated with a carbogenic mixture (95% O_2, 5% CO_2) at 37 °C and pH= 7.4. Mechanical activity was recorded by means of isometric force transducers (FORT 10, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy) using a data acquisition system (PowerLab/400 system, Ugo Basile, Italy). Preparations, under a passive tension of 1 g, were left to equilibrate for at least 45 min, changing the Krebs solution every 15 min. All strips developed spontaneous mechanical activity. After the equilibration period, preparations were tested with 5 nM oxytocin (OXY) or KCl solution (60 mM) until stable responses were attained.

In order to examine the effect on the spontaneous contraction of rat uterus, Prangoil (6–200 μg/mL) was added cumulatively to the organ bath, leaving each concentration in contact for about 10 min. To study the effect of the essential oil on an agonist-induced contraction, the tissues were precontracted with KCl (60 mM) or OXY (5 nM), then Prangoil (6–200 μg/mL) was cumulatively added during the sustained tonic phase, with an interval of 10 min between each addition.

To investigate the possible mechanism/s related to the observed effects, submaximal dose of Prangoil was tested in the presence of: i) Nω-nitro-L-arginine methyl ester (L-NAME, 100 μM, [20]), a nitric oxide synthase inhibitor, ii) 1 H-[1,2,4]oxadiazolo [4,3,A]quinoxaline-1-one (ODQ, 10 μM), a soluble guanylyl cyclase selective inhibitor [13], iii) 9-(Tetrahydro-2′-furyl)adenine, adenylyl cyclase selective inhibitor (SQ 22,536, 10 μM[21]), iv) tetraethylammonium (TEA, 20 mM [19]), non-specific K^+ channel inhibitor.

Then, to investigate the effect of Prangoil, on the extracellular calcium influx or on calcium release from the intracellular calcium stores, Prangoil responses were also tested in the presence of nifedipine (5 nM [22]), L-type calcium channel blocker, or 2-Aminoethoxydiphenylborate (2-APB, 20 μM [22]), membrane-permeant inositol 1,4,5-triphosphate (IP_3) receptor blocker. Preliminary experiments allowed us to identify the dose of nifedipine (5 nM) able to reduce KCl response without significantly affect the amplitude of the spontaneous contractions.

All the drugs were left in contact with the tissue for 30 min before adding Prangoil. Each preparation was tested with a single blocker.

In a separate set of the experiment, we investigated also the response to β - ocimene or carvacrol alone or in combination on the uterine spontaneous contractions, at a range dose corresponding to their content in Prangoil (59% and 3.6%, respectively). Then β - ocimene or carvacrol, at the doses corresponding to their content in Prangoil submaximal dose, were studied on KCl or OXY precontracted strips or in the presence of nifedipine (5 nM), L-type channel blocker, or 2-APB, (20 μM). Also, in this set of experiments all the inhibitory drugs were left in contact with the tissue for 30 min, before adding β - ocimene or carvacrol.

2.3. Drugs

The composition of Krebs solution was (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. The following drugs used were: 2-Aminoethoxydiphenylborate (2-APB), carvacrol, β - ocimene, nifedipine, Nω-nitro-L-arginine methyl ester (L-NAME), 1 H-[1,2,4] oxadiazolo [4, 3-a]quinoxalin-1-one (ODQ), oxytocin, Prangoil, tetraethylammonium (TEA), 9-(Tetrahydro-2′-furyl) adenine (SQ 22,536) (Sigma- Aldrich, Inc., St. Louis, USA). Carvacrol, ODQ, β-ocimene and Prangoil were dissolved in dimethysulphoxide (DMSO) and further diluted in Krebs. The maximal final concentration of DMSO in the organ bath was 0.5%, which did not affect the contractility of the myometrium.

All the other drugs were dissolved in distilled water. On the day of the experiment, the stock solutions were diluted in Krebs and then added on the bath.

2.4. Statistical analysis

Data are expressed as means ± SEM; *n* indicates the number of animals on which observations were made. In order to determine the inhibitory effects produced by each concentration of the compounds, the amplitude of the phasic contractions was measured 10 min prior to the treatment and compared to that observed 10 min after the treatment. Data are reported in absolute value (mg).

Responses were fitted to sigmoid curves (Prism 5.0, Graph-PAD, San Diego, CA, USA) and IC_{50} values with 95% confidence limits (CLs) were determined. The IC_{50} value was defined as the concentration required to produce 50% of the maximal inhibition. Statistically significant differences were calculated by Student's t-test or by means of analysis of variance, followed by Dunnett's test, when appropriate. A probability value less than 0.05 was regarded as significant.

3. Results

3.1. Prangoil effects on rat uterus spontaneous contractile activity

Myometrial smooth muscle strips showed spontaneous contractions with amplitude of 1.92 ± 0.16 g and frequency of 0.91 ± 0.04 c.p.m (contractions per minute) (n=14). Cumulative applications of Prangoil (6–200 μg/mL) progressively and significantly inhibited, in a dose-dependent manner, the amplitude of the spontaneous uterine contractions (IC_{50} = 54.50 μg/mL, 95% CL 46.73–67.90 μg/mL) without affecting basal tone, as shown in Fig. 1A. Prangoil inhibitory effects started at the concentration of 12.5 μg/mL and at the dose of 200 μg/mL contractions were abolished (Fig. 1A,B). No evident effect on the frequency of spontaneous contraction was observed.

3.2. Prangoil effects on contractions induced by high-K^+ solution or oxytocin

High K^+ solution (KCl 60 mM) induced a contraction characterized by an initially rapid increase in the force, followed by a decline, reaching a plateau within 2 min, which was maintained until washout. Cumulative addition of Prangoil (6–200 μg/mL) during the plateau phase induced relaxation of the KCl contracted strips in a concentration-dependent manner (IC_{50} 50.25 μg/mL 95%Cl 32.83–76.94 μg/mL) (Fig. 2).

As expected, oxytocin (OXY, 5 nM), increased the muscular tone and the amplitude of contractile activity of the uterine muscular strips. As shown in Fig. 2 Oxytocin-induced contractions were gradually inhibited by increasing Prangoil concentrations (6–200 μg/mL) (IC_{50} 79.22 μg/mL 95%Cl 46.16–135.51 μg/mL). Multiple comparison suggests that Prangoil was more potent in inhibiting KCl-induced contraction than OXY-induced contraction ($p < 0.05$). These results may suggest that the Prangoil could interfere with extracellular calcium influx, targeting the L-type Ca^{2+} channels, and with calcium mobilization from sarcoplasmic reticulum via Phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP_3) pathway.

* $P < 0.05$ compared to control condition.

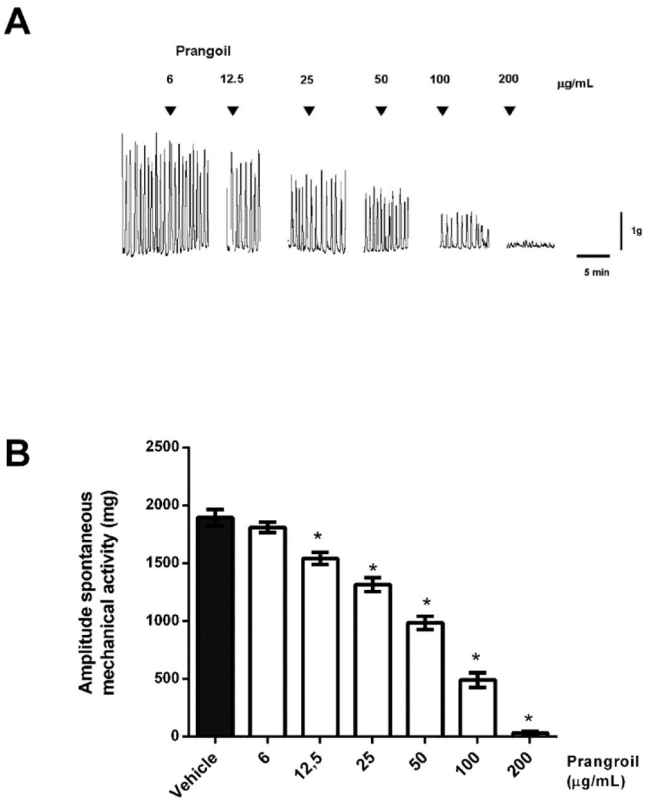

Fig. 1. Inhibition of the amplitude of spontaneous contractions of isolated rat uterine strips. A) Original tracings showing the effects induced by increasing concentrations of Prangoil (6–200 μg/mL) on the amplitude of spontaneous contractions of isolated rat uterine strips. B) Histogram showing the effects on the spontaneous contraction amplitude induced by Prangoil (6–200 μg/mL) in isolated rat uterine strips. All data are means ± SEM (n=14) and expressed in absolute value (mg).

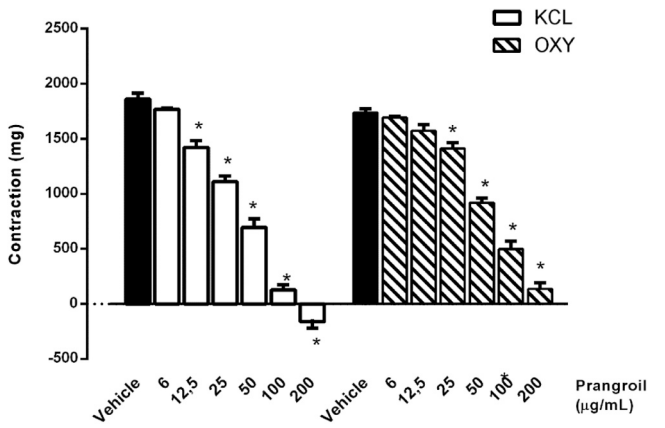

Fig. 2. Inhibition of the contraction induced by high K^+ solution or by OXY in isolated rat uterine strips. Histogram showing the inhibitory actions of the Prangoil (6–200 μg/mL) on the contraction induced by high K^+ (60 mM) solution or by 5 nM OXY in isolated rat uterine strips. Data are means (n = 5 each) ± SEM and are expressed in absolute value (mg).

3.3. Action mechanism/s underlying Prangoil inhibitory effects

To investigate the action mechanism/s underlying the Prangoil inhibitory effects, different inhibitors of the main signaling pathways leading to the relaxation of uterine smooth muscle were tested on the response induced by the submaximal dose of Prangoil.

The Prangoil effects were unaffected by pretreatment with L-NAME (100 μM), a nitric oxide (NO) synthase inhibitor, (Fig. 3A, B) or ODQ (10 μM), guanylyl cyclase (GC) inhibitor, implying that NO/GC/cyclic guanosine monophosphates (cGMP) pathway is not involved in the observed effects (Fig. 3A,B).

Another crucial signaling pathway related to smooth muscle relaxation involves increase in the intracellular levels of cyclic adenosine monophosphates (cAMP) and the related downstream events. However, in our preparations the responses to Prangoil were not significantly modified in the presence of SQ 22,536 (10 μM), adenylyl cyclase inhibitor, allowing us to discard the involvement of cAMP in the Prangoil-induced inhibitory effects (Fig. 3A,B). Moreover, Prangoil effects remained unaltered in the presence of TEA (20 mM), non-specific K^+ channel blocker, (Fig. 3A,B), discarding the participation of TEA-sensitive K^+ channels in the Prangoil action mechanism.

TEA, *per se*, induced a transient increase in the amplitude of the spontaneous contractions, while none of the other blockers had any effects on the muscular contractility.

In addition, nifedipine, L-type Ca^{2+} channel blockers, at a concentration of 5 nM, which did not affect the spontaneous activity, significantly reduced the response to Prangoil. 5 nMnifedipine was able to reduce contraction to KCl, used as positive control (Fig. 3A,C,D).

Furthermore, oil responses were tested in the presence of 2-APB, membrane- permeant IP_3 receptor inhibitor. Prangoil effects on the spontaneous activity were significantly attenuated by 2-APB (20 μM), suggesting that oil could also interfere with Ca^{2+} release from the sarcoplasmic reticulum, decreasing cytoplasmic Ca^{2+} concentration (Fig. 3A,C). To exclude non-specific effects, 2-APB was tested against OXY, which increases IP_3 synthesis and release of Ca^{2+} from IP_3-

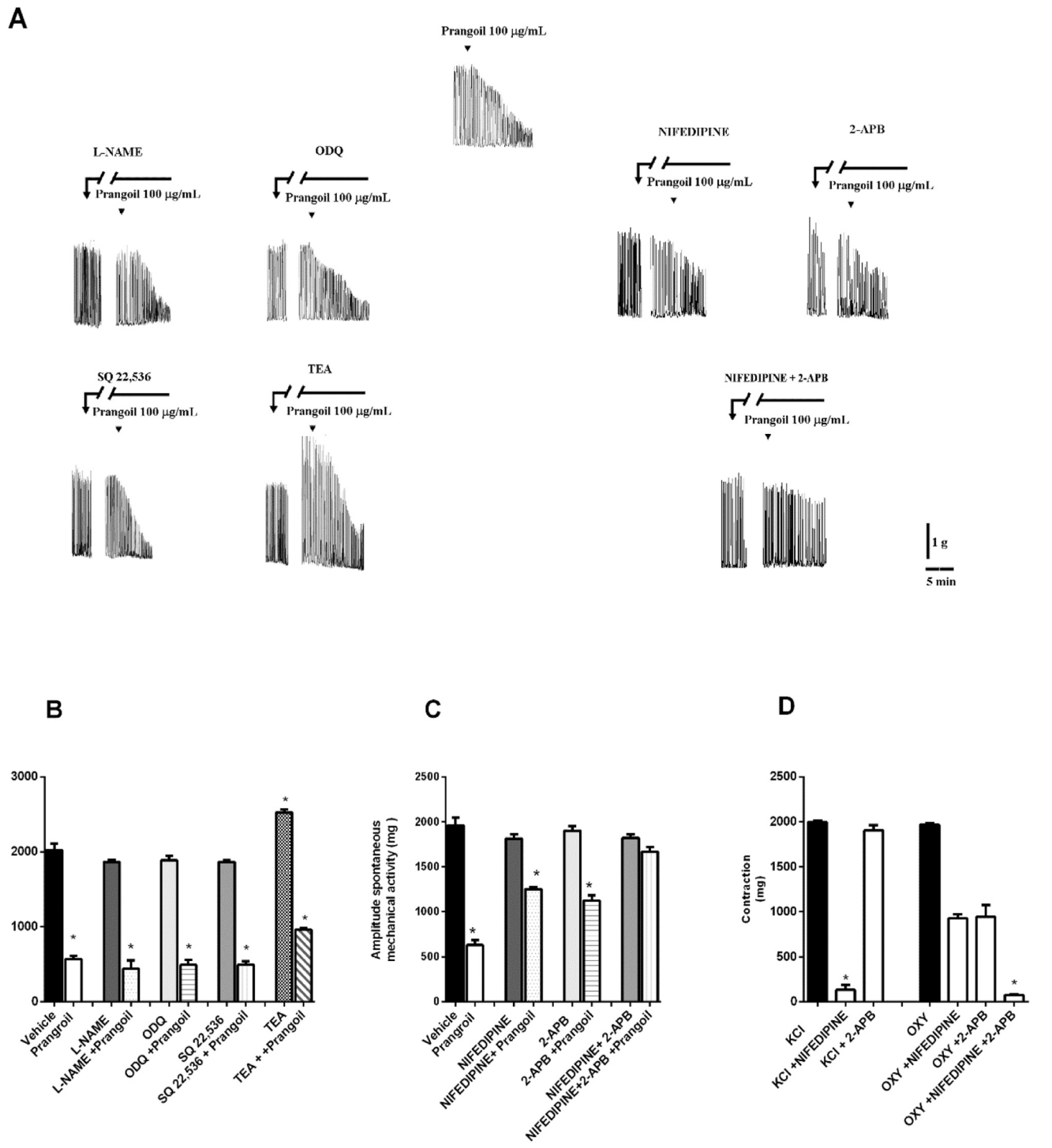

Fig. 3. Mechanism of action of Prangoil. A) Original tracings showing the inhibitory effects induced by the submaximal dose of Prangoil (100 μg/mL) in the presence of L-NAME (100 μM), a blocker of the NO synthase, ODQ (10 μM), soluble guanylyl cyclase inhibitor, SQ 22,536 (10 μM), adenylyl cyclase inhibitor, tetraethylammonium (TEA, 20 mM), non-specific K^+ channel inhibitor, nifedipine (5 nM) non-selective L-type channel blocker, 2-APB (10 μM), blocker of IP_3 receptor or in the combined presence of nifedipine and 2-APB. B) Histogram showing the inhibitory effects induced by the submaximal dose of Prangoil (100 μg/mL) on the amplitude of uterine spontaneous mechanical activity before and after: L-NAME (100 μM), a blocker of the NO synthase, or ODQ (10 μM), soluble guanylyl cyclase inhibitor, or SQ 22,536 (10 μM), adenylyl cyclase inhibitor or tetraethylammonium (TEA, 20 mM), non-specific K^+ channel inhibitor. All data are means ± SEM (n = 4 each) and expressed in absolute value (mg). * $P < 0.05$ compared to control condition. C) Histogram showing the inhibitory effect induced by the submaximal dose of Prangoil (100 μg/mL) on the amplitude of uterine spontaneous mechanical activity before and after: nifedipine (5 nM) non-selective L-Type channel blocker, or 2-APB (10 μM), blocker of IP_3 receptor or by the joint application of nifedipine and 2-APB. All data are means ± SEM (n = 5 each) and expressed in absolute value (mg). * $P < 0.05$ compared to control condition. § $P < 0.05$ compared to Prangoil effect. D). Histogram showing the response to high (60 mM) K^+ solution or OXY (5 nM) before and after nifedipine (5 nM) L-Type channel blocker, or 2-APB (10 μM), blocker of IP_3 receptor, or by the joint application of nifedipine and 2-APB in isolated rat uterine strips. Data are means ± SEM (n = 4) and expressed in absolute value (mg).* $P < 0.05$ compared to control condition.

sensitive Ca^{2+} stores. As shown in Fig. 3D the contractile effect of OXY was significantly attenuated in the presence of 20 μM 2-APB.

The joint application of 5 nM nifedipine and 20 μM 2-APB abolished the oil-induced inhibitory effects (Fig. 3A,C).

3.4. Effects of the monoterpenes β-ocimene and carvacrol

Cumulative applications of β-ocimene (3.6–120.0 μg/mL) or carvacrol (0.2–7.2 μg/mL), the range doses corresponding to their content in Prangoil (59% and 3.6% respectively), significantly decreased, in a dose-dependent manner, uterine spontaneous contractions, (Fig. 4A,B). To abolish the spontaneous contractions, the doses of β-ocimene or carvacrol were increased to 240 μg/mL or 14.5 μg/mL, respectively (Fig. 4B). The inhibitory effect of both compounds was reversible after washout. Joint application of β-ocimene and carvacrol at the submaximal doses of 59 μg /mL and 3.6 μg /mL, respectively, irrespective of the order of application, induced a further significant inhibition of spontaneous activity, comparable with inhibition induced by the corresponding dose of Prangoil (Fig. 4C).

The effects induced by β-ocimene (59 μg/mL) or carvacrol (3.6 μg/mL) were further investigated in the presence of nifedipine or 2-APB. The response to β-ocimene was significantly reduced by nifedipine (5 nM) and by 2-APB (20 μM), suggesting a modulation of Ca^{2+} influx from extracellular medium as well as of calcium release from the sarcoplasmic reticulum (Fig. 5A). On the contrary, carvacrol inhibitory effect was significantly attenuated by nifedipine (5 nM), but not by 2-APB 20 μM, suggesting an action exclusively as Ca^{2+}-channel blocker (Fig. 5A).

β-ocimene and carvacrol alone or in combination were able to reduce the amplitude of the contractile responses produced by high K^+ solution or oxytocin (Fig. 5 B).

4. Discussion

Vegetables and medicinal plants provide various nutrients and nutraceuticals and are becoming increasingly important as essential foods in human diets and health care. Aromatic plants, abundant in essential oils, are regarded as valuable and easily available natural resource for producing novel compounds, suitable as therapeutic candidates, since essential oils may easily cross cellular membranes and influence a range of molecular targets, as ion channels and enzymes [23]. While essential oils have garnered attention for the anti-oxidant, antifungal, antimicrobial, antiparasitic, antinociceptive, anti-inflammatory or antitumoral properties [24], their antispasmodic

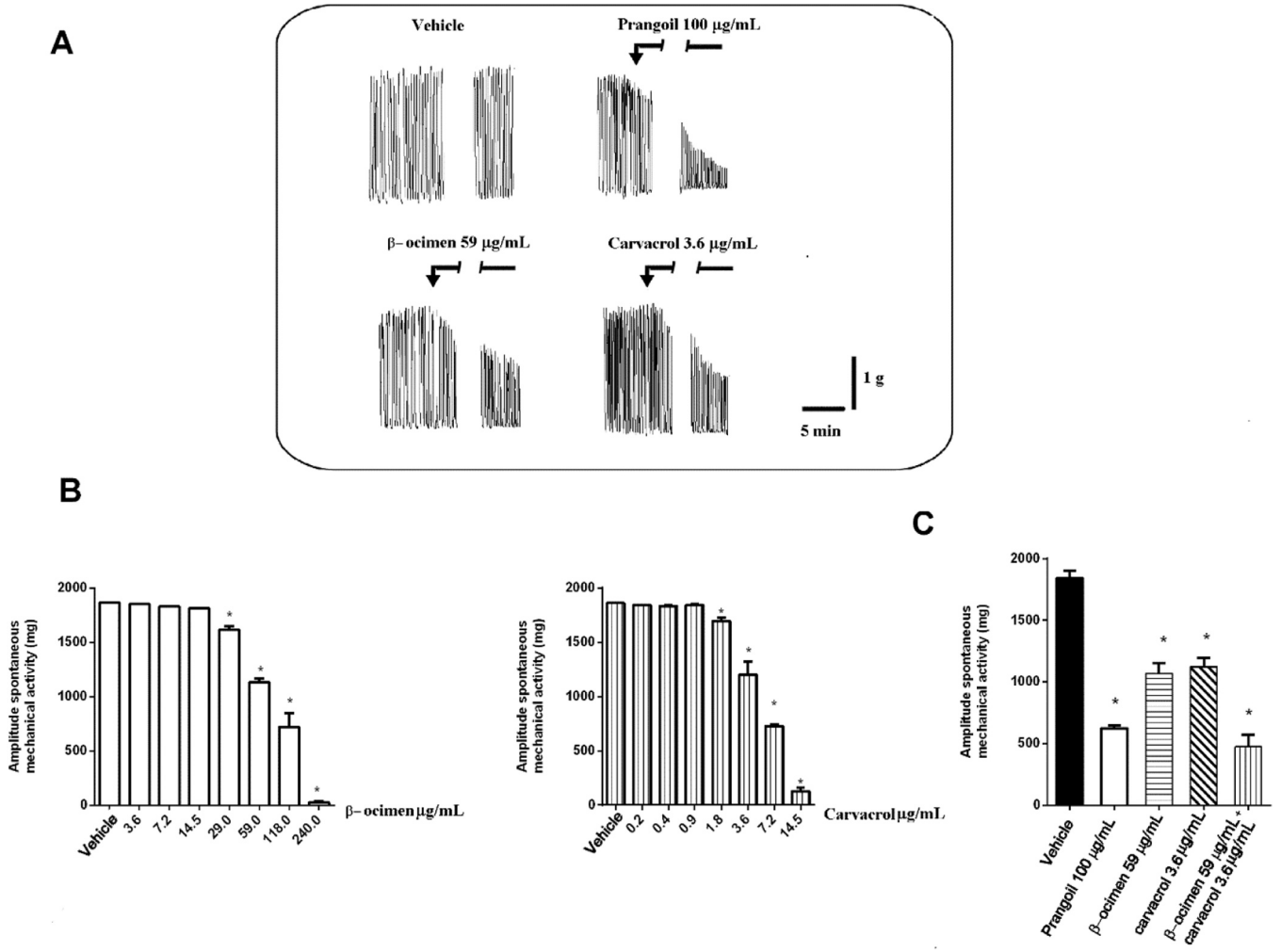

Fig. 4. Inhibitory effects of β − ocimene or carvacrol in isolated rat uterine strips. A) Original tracing showing the inhibitory effect of submaximal dose of Prangoil (100 μg/mL) or β - ocimene (59 μg/mL) or carvacrol (3.6 μg/mL) on the amplitude of spontaneous mechanical activity of isolated rat uterine strips. B) Histograms showing the effects of β − ocimene (3.6–240 μg/mL) or carvacrol (0.2–14.5 μg/mL) on the amplitude of the spontaneous contraction in isolated rat uterine strips. C) Histogram showing the effects on the amplitude of the spontaneous contraction induced by submaximal dose of Prangoil (100 μg/mL), β - ocimene (59 μg/mL) or carvacrol (3.6 μg/mL) or by the join application of β - ocimene (59 μg/mL) and carvacrol (3.6 μg/mL) in isolated rat uterine strips. All data are means ± SEM (n = 5 each) and expressed in absolute value (mg). $P < 0.05$ compared to control condition.

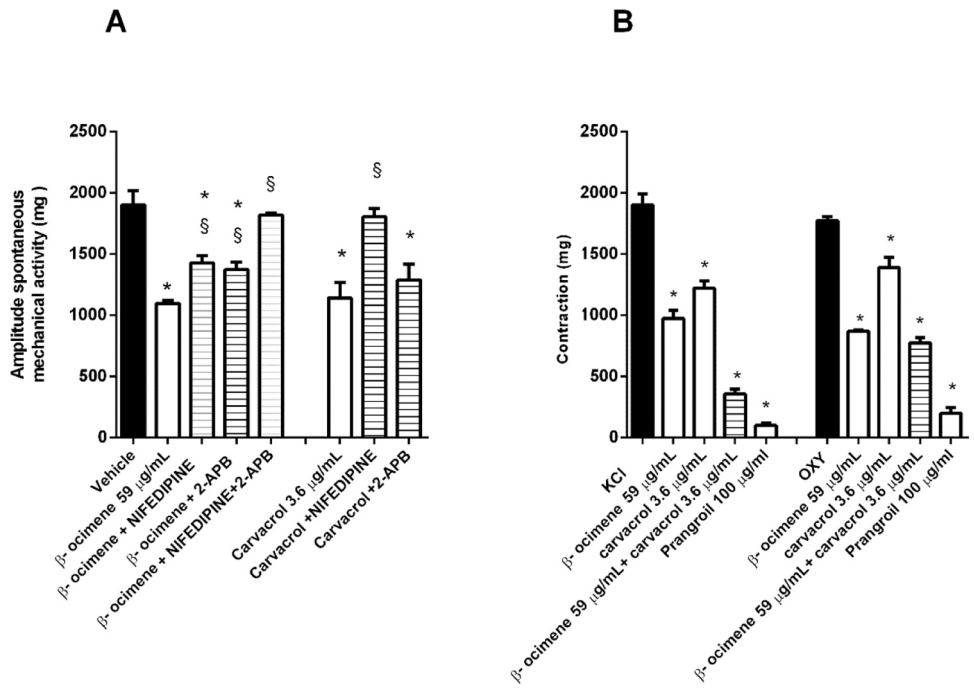

Fig. 5. Mechanism of action of monoterpenes A) Histograms showing the inhibitory effect on the amplitude of spontaneous mechanical activity induced by β - ocimene (59 μg/mL) or carvacrol (3.6 μg/mL) before and after nifedipine (5 nM), L-Type channel blocker, 2-APB (10 μM), blocker of IP_3 receptor or by the joint application of nifedipine and 2-APB, in isolated rat uterine strips. All data are means ± SEM (n = 5 each) and expressed in absolute value (mg). * $P < 0.05$ compared to control condition. § $P < 0.05$ compared to monoterpene response. B) Histograms showing the inhibitory effect on the response to high K^+ (60 mM KCl) solution or OXY (5 nM) induced by β-ocimene (59 μg/mL), carvacrol (3.6 μg/mL), join application of β-ocimene (59 μg/mL) and carvacrol (3.6 μg/mL) or by Prangoil (100 μg/mL). Data are means ± SEM (n=4 each) and expressed in absolute value (mg). *$P < 0.05$ compared to control condition. § $P < 0.05$ compared to monoterpene effects.

effects have been comparatively underexplored, despite being mentioned in traditional medicine. However, some clinical investigations shed some light on the antispasmodic proprieties of essential oils in diverse contexts, as functional dyspepsia, intestinal bowel diseases, infantile colic, or dysmenorrhea, [25].

The Apiaceae family includes numerous members, which have been studied in terms of bioactive compounds and medicinal applications. Researches have revealed the presence of significant substances and the related action mechanisms within Apiaceae plants [9,26] and further studies can ultimately promote their application for medical or industrial purposes. Among the Apiaceae family, we focused our attention on the essential oil (Prangoil) isolated from the aerial part of a Sicilian accession of *Prangos Ferulacea,* previously demonstrate to have antioxidant, anti-acetylcholinesterase (AChE) and cytotoxic activities [12], as well antispasmodic effects on intestinal tissue [13]. Presently, our study unveils a tocolytic effect of Prangoil in non-pregnant rat uterus. The monoterpenes, β- ocimene and carvacrol, matched Prangoil inhibitory effects. The mechanisms driving this tocolytic effect likely entail by inhibition of Ca^{2+} entry through L-type Ca^{2+} channels and Ca^{2+} release from the sarcoplasmic internal stores.

We demonstrated that the essential oil promotes significant inhibitory effects on spontaneous and chemical (high K^+ solution or OXY)-evoked contractions in rat uterus, confirming the ability of Prangoil to modulate negatively smooth muscle contractility, as recently observed in rat intestine [13]. Bruno et al. [12]. showed that the major component in the essential oil from Sicilian *Prangos ferulacea*, Prangoil is represented by aromatic monoterpenes, as β - ocimene and carvacrol, which could exert an impact on the contractility of smooth muscles. As already observed in rat intestine [13], even in rat uterus β- ocimene is able to induce inhibitory effects decreasing, in a concentration-dependent manner, the spontaneous mechanical activity. Moreover, in line with the finding of Munoz-Perez and coll. in pregnant rat uterus [15], our studies reveal that carvacrol induces as well tocolytic effects, being able to reduce significantly the amplitude of the uterine contractions, also in non-pregnant rats. Interestingly, the joint application of both monoterpenes further inhibited the amplitude of the spontaneous contractions, suggesting synergistic inhibitory effects. Therefore, it is possible to speculate that both monoterpenes may be responsible for the Prangoil inhibitory effects, underscoring the significance that also components at low concentrations may be important in determining the oil pharmacological activity.

NO pathway activation is able to induce inhibition of uterine contractions [27]. Briefly, the nitric oxide synthase (NOS), converts L-arginine to L-citrulline and NO. NO, derived from the endometrium, diffusing into smooth muscle cells, activates smooth muscle soluble guanylyl cyclase (sGC), leading to the cyclic guanosine monophosphate (cGMP) production and activation of downstream pathway promoting uterus relaxation. Our results show that inhibition of NOS or sGC activity, by L-NAME or ODQ, respectively, failed to interfere with the oil inhibitory effects, indicating that activation of NOS/sGC pathway is not imperative for inducing tocolytic effects. This observation is consistent with previous evidence in rat intestine, where Prangoil was able to induce inhibitory effects without involvement of the nitrergic pathway [13].

Moreover, our results suggest also that the increase in intracellular levels of cAMP, and the activation of the downstream multiple pathways, are not involved in the Prangoil inhibitory effects, since blockade of AC activity did not alter the effects of oil on the rat isolated uterus.

Moreover, K^+ channels play a significant role in regulating the membrane potential. When potassium channels are activated, the membrane undergoes to hyperpolarization, causing a shift in the membrane potential away from the threshold required for the activation of voltage-gated calcium channels. As a result, this leads to the inhibition of muscle contractility. In our experiments, TEA, a non-specific K^+ channel blocker, was unable to affect oil-inducing inhibition of uterine muscle contractility, excluding an involvement of TEA-sensitive K^+ channels in the observed effects. Further studies are required to unravel any possible involvement of TEA-resistant channels in the oil inhibitory

effects.

It is well known that the increase in intracellular calcium concentration, due to the extracellular calcium influx, mainly *via* voltage-operated channels, or to the release from intracellular stores, is essential in the maintenance of the spontaneous active uterine contractions.

Inhibition of voltage-dependent calcium channels is often reported as the mechanism of action of essential oils to promote antispasmodic effects, [25]. In our preparation, the inhibitory effects triggered by Prangoil, β-ocimene, and carvacrol are sensitive to the L-type blocker, nifedipine, indicating that these compounds likely suppress uterine contractile activity by interfering with the opening of voltage-dependent L-type Ca^{2+} channels, thereby shifting the membrane potential of the uterine smooth muscle cells at a negative level. Furthermore, the observation that Prangoil, β -ocimene and carvacrol inhibit KCl-induced contraction, a process mediated by the influx of calcium ions from the extracellular space, through voltage-dependent L-type channels [28], provides support to our hypothesis.

Calcium release from IP_3 sensitive intracellular calcium stores increases the intracellular calcium concentration, and triggers uterine contractions. Interestingly, only Prangoil and β-ocimene effects were significantly reduced in the presence of 2-APB, an IP_3 receptor blocker, and abolished by joint application of 2-APB and nifedipine. These observations strongly imply that both the oil and β-ocimene could not only interfere with calcium entry through voltage-dependent channels, but also affect the calcium release from intracellular stores. In contrast, carvacrol response exhibited no noteworthy difference in the presence of 2-APB, as observed also in vascular [29] and pregnant uterine muscle [15], suggesting that carvacrol may act exclusively as a calcium antagonist on voltage-dependent channels. Lastly, all the tested compounds were able to inhibit the contractile response to oxytocin, the main hormone involved, not only in the physiological mechanism underlying labor, but also in the onset of preterm labor. This hormone induces contraction in uterine muscle by G protein-coupled receptor activation, leading to increased intracellular Ca^{2+} concentration, due to the Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} release from sarcoplasmic reticulum [30,31]. Our results in uterine muscle are overlapped to the results found in the rat intestine[13], indicating a common effect on smooth muscle contractility. However, the most effectiveness of Prangoil against KCl-induced contraction indicates that, in uterine muscle, the Prangoil main mechanism of action is as a blocker of calcium influx through membrane channels.

5. Conclusions

Our results suggest that Prangoil is able to reduce spontaneous and KCl or oxytocin-induced contractions of rat myometrium. This reduction is achieved by interfering with the influx of extracellular Ca^{2+} through L-Type voltage dependent channels and additionally, by reducing Ca^{2+} release from IP_3-sensitive intracellular stores. The components β-ocimene and carvacrol matched Prangoil-induced tocolytic effects.

Since disorders, as dysmenorrhea, are related to abnormal uterine smooth muscle contraction, [32], it is obvious that factors affecting uterine contractions can be useful in treating or managing these issues. The outcomes of our study, showing tocolytic effects of essential oil derived from a Sicilian *Prangos ferulacea* (L.) Lindl., encourage further studies aimed at determining its therapeutic potential.

CRediT authorship contribution statement

Maria Grazia Zizzo: Conceptualization, Investigation, Data analysis and interpretation, Preparation figures, Writing – original draft and editing. **Adele Cicio:** Investigation, Data analysis, Writing – review & editing. **Maurizio Bruno:** Writing – review & editing. **Rosa Serio:** Supervision, Conceptualization, Data interpretation, Writing – review & editing. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors have no competing interests.

Data Availability

Data will be made available from the corresponding author on reasonable request.

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Declarations

Ethics approval not applicable.

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