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Effect of bergamot essential oil and its constituent linalool on myogenic and neuronally-mediated contractions of human and rat isolated colon: potential benefits in complementary treatment of intestinal diseases

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ABSTRACT

Introduction

Bergamot essential oil (BEO) is used in aromatherapy and as an additive to food and drink to promote a citrus flavour. In animal models, BEO can modulate the synaptic functions within the Central Nervous System. However, it is not known if BEO can affect the functions of the gastrointestinal tract, despite being widely used in the food industry. BEO and its components linalool, limonene and linalyl-acetate were therefore examined for their ability to influence neuromuscular contractions of human and rat isolated colon.

Material and Methods

Human colon was obtained at surgery for bowel cancer following informed consent; mucosa-free strips were cut parallel to the circular muscle. Rat colon (Sprague-Dawley) strips were also cut as circular muscle preparations. In most experiments, each strip was suspended between platinum wire electrodes in tissue baths containing Krebs solution (5% CO_2 in O_2; 37°C) under tension (1 or 2g of tension for rat and human muscle strips, respectively) for recording of isometric contractions in response to stimulation of cholinergic nerves using electrical field

stimulation (EFS) or to the application of exogenous stimulants of smooth muscle contraction (acetylcholine (ACh), 5-hydroxytryptamine (5-HT), substance P (SP) or KCl). Cumulative concentration-response curves were obtained for BEO (10^{-6} - 10^{-3} % v/v) and its major components linalool, limonene and linalyl-acetate (10^{-9} - 10^{-4} M). The inhibition of the amplitude of the contractions by each agent was expressed in percentage terms as the mean ± s.e.m of the numbers of patients or animals.

Results

In preliminary experiments, BEO and its components reduced contractions of rat colon caused by ACh, 5-HT or SP. Subsequently concentration-dependent inhibition of both KCl-evoked contractions and neuronally-mediated contractions were demonstrated in response to BEO or its components, with greater potency when tested on the latter. The inhibitory effect of BEO on myogenic and neuronally-mediated contractions was associated largely via the actions of linalool (apparent pIC_{50} 5.6 ± 0.4, n= 4 on KCl-evoked contractions; apparent pIC_{50} 6.7 ± 0.2, n = 4 on neuronally-mediated contractions) in human colon. Similar but less potent activity of linalool was obtained in rat colon (apparent pIC_{50} 5.4 ± 0.3, n = 4 on KCl-evoked contractions; apparent pIC_{50} 5.8 ± 0.1 %, n = 4 on neuronally-mediated contractions).

Conclusion

The results indicated that BEO, largely via the actions of linalool, inhibited both human and rat enteric neurotransmission. Some species differences were found in the ability of these substances to inhibit neuronally-mediated contractions; the rank order in terms of potency (apparent pIC_{50}) in human was: linalool > limonene >> linalyl acetate = BEO, and in rat was: linalyl acetate > limonene = linalool >> BEO. Both BEO and linalool were more potent in human muscle strips, acting at least partly by directly inhibiting muscle contractility. These data provide a potential mechanism for their use as a complementary treatment of gastrointestinal diseases related to increased intestinal motility.

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ABBREVIATIONS

5-HT	5-Hydroxytrptamine, Serotonin
ACh	Acetylcholine
ATP-like	Adenosine triphosphate-like transmitters
ATR	Atropine
BEO	Bergamot essential oil
CNS	Central Nervous System
DA	Dopamine
DMSO	Dimethylsulphoxide
EEG	Electroencephalogram
EFS	Electrical Field Stimulation
EMA	European Medicines Agency

E_{max}	Efficacy (maximal contractile effect obtained)
ENS	Enteric Nervous System
GABA	Gamma-aminobutyric acid neurotransmitter
GI	Gastrointestinal
IBS	Irritable Bowel Syndrome
IBS-C	Irritable Bowel Syndrome with predominant constipation
IBS-D	Irritable Bowel Syndrome with predominant diarrhea
IBS-M	Irritable Bowel Syndrome with constipation mixed with diarrhea
IBS-U	Unsubtyped Irritable Bowel Syndrome
ICC	Interstitial cells of Cajal
I_{max}	Efficacy (maximal inhibitory effect obtained)
IPANs	Intrinsic primary afferent neurons

KCl	Potassium chloride
lim	Limonene
lin	Linalool
NO	Nitric oxide
pEC_{50}	Measurement of potency (negative logarithm of concentration of a substance that produces half of maximal contractile effect)
pIC_{50}	Measurement of potency (negative logarithm of concentration of a substance that produces half of maximal inhibitory effect)
PNS	Peripheral Nervous System
SP	Substance P
TK	Tachykinins
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

PUBLICATIONS

Article

Inhibition of Neuromuscular Contractions of Human and Rat Colon by Bergamot Essential Oil and Linalool: Evidence to Support a Therapeutic Action. Straface, M., Makwana, R., Palmer, A., Rombolà, L., Aleong, J. C., Morrone, L. A., & Sanger, G. J. (2020)**.** *Nutrients*, *12*(5), 1381.

Oral presentation

Bergamot Essential Oil and Its Constituent Linalool, Inhibit Cholinergically-Mediated Contractions of Human and Rat Isolated Colon.

Marilisa Straface, Rajesh Makwana, Laura Rombolà, Joanne ChinAleong, Luigi Morrone, Gareth J Sanger.

Neurogastroenterology & Motility. vol. 31,

4th Biennial Meeting of the European Society of Neurogastroenterology and Motility.

Centro Cultural de Belém, Lisbona, 6 September 2019.

Poster presentation

Effects of Bergamot Essential Oil on Neuronally-Evoked Contractions of Rat Isolated Colon.

Marilisa Straface, Rajesh Makwana, Laura Rombolà, Joanne ChinAleong, Luigi Morrone, Gareth J Sanger.

1st workshop in Translational Medicine, Experimental and Therapeutic Medicine: New Opportunities in Molecular Oncology.

University Club, University of Calabria, 24 June 2019.

CHAPTER 1

1.1 Bergamot essential oil

Bergamot (*Citrus bergamia Risso et Poiteau*) is a citrus fruit plant belonging to the Rutaceae family. The fruit is slightly larger in size than an orange but smaller than a grapefruit, with an ovoid shape, and depending on its ripeness, has a green to yellow coloured rind (Figure 1). It is grown almost exclusively along the southern coast of the Reggio Calabria in Italy (Regional Law n. 41, 14 Oct 2002), primarily for its essential oil, a product that is in great demand by the perfumery and cosmetic industry, but also for pharmaceutical, food and confectionery industries (Calapai and Delbò, 2011).

According to the Farmacopea Ufficiale Italiana (1991) bergamot essential oil (BEO) is obtained by cold pressing of the epicarp and part of the mesocarp of the ripe fruit.

The constituents of BEO can be separated into a volatile and non-volatile fraction using high-performance liquid chromatography (Costa et al., 2010, Donato et al., 2014). The volatile fraction represents 93%–96% of total volume of the oil and consists of two classes of molecules i.e the terpene hydrocarbons (e.g., d-limonene, ß-bisabolene, γ-terpinene, α-pinene, ß-pinene, sabinene, ß-myrcene, terpinolene, and geranyl acetate)

and oxygenated derivatives (e.g. linalool, linalyl acetate, neral, geranial, neryl acetate, and geranyl acetate). The non-volatile fraction (4%–7% of total volume of oil) contains coumarins and psoralens.

Within the volatile fraction, the most abundant compounds are limonene (25.62%–53.19%), linalyl acetate (15.61%–40.37%), and linalool (1.75%–20.26%) (Figure 1) (Melliou et al., 2009).

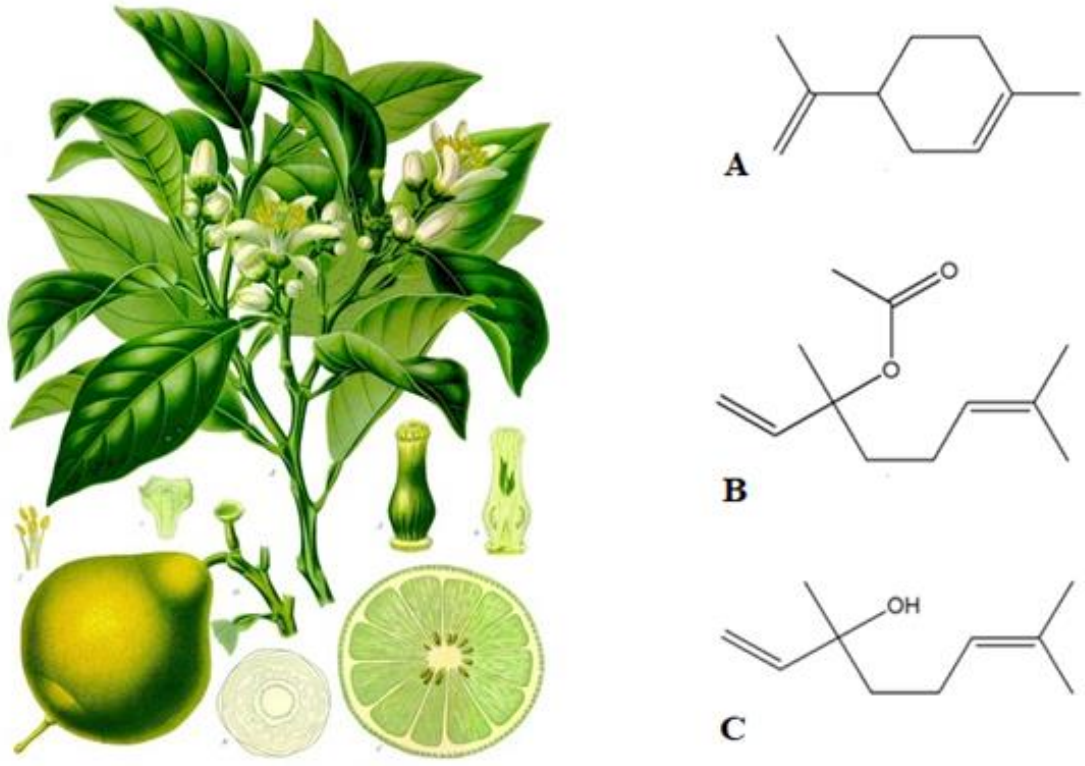


Figure 1: Bergamot fruit.

Image of bergamot fruit and the structure of the main compounds present in volatile fraction of BEO: (A) limonene, (B) linalyl acetate, (C) linalool.

1.2 Traditional uses

Around 80% of the world's population relies on the use of a traditional medicine for their primary health needs (Ekor, 2013). However, such practice is largely based on folklore and schools of traditional medicine rather than evidence-based research.

As reported by the European Medicines Agency (EMA), the use of BEO has long been known in traditional and folk medicine. Historical data shows that BEO has been used since 1725 for a variety of symptoms, such as mouth and skin infections, respiratory and urinary tract infections, gonococcal infections, leucorrhoea, vaginal pruritis and fever (Calapai and Delbò, 2011). An ointment containing a mixture of herbal preparations, including BEO, has been used since 2008 (in Hungry) for mitigation of symptoms (erythema, infiltration, parakeratosis, urticaria) and for nursing of dry, pealing, squamosus skin in mild or moderate psoriasis.

Primarily BEO is used as a topical agent (Calapai and Delbò, 2011) to facilitate wound healing possibly because of its antiseptic, anthelminthic antimicrobial and antifungal properties (Laird et al., 2012, Sanguinetti et al., 2007). These actions have also been attributed to the ability of the BEO phytocomplex to increase oxidative metabolism in human polymorphonuclear leukocytes (Cosentino et al, 2014).

BEO is also widely used as flavouring for drinks and food, however, despite being used in the food and confectionery industry, almost nothing is known about the potential of BEO to influence gastrointestinal functions.

1.3 Preclinical studies

In recent years, animal studies, *in vivo* and *in vitro*, have shown an ability of BEO to influence a range of different effects on neuronal and smooth muscle functions; these studies have been restricted largely to studies with models of central nervous system (CNS) and cardiovascular functions.

In studies involving the CNS, the perfusion of BEO into the hippocampus and the superfusion of BEO over hippocampal synaptosomes, at low concentrations stimulated the release of excitatory amino acids (such as glutamate) by a Ca^{2+}-dependent mechanism, whereas at high concentrations the release was stimulated through a non-Ca^{2+}-dependent carrier mechanism (Morrone et al., 2007). However, the molecular targets on the glutamatergic nerve endings leading to exocytosis, or to the release of mediated glutamate transporter, remain to be discovered (Morrone et al., 2007). In addition, the systemic administration of BEO has been shown to cause a dose-dependent reduction in brain damage induced by focal cerebral ischemia in rats

(Amantea et al., 2009). Under these experimental conditions, BEO did not influence the levels of basal amino acids but reduced the levels of excitatory amino acids in the penumbral region (Amantea et al., 2009). Moreover, in the ischemic penumbra, an improvement of the phosphorylation of protein kinase B (PKB or Akt), levels of the prosurvival gene and of the downstream kinase GSK-3β was observed. Therefore, it was suggested that the protection caused by BEO was the result of its ability to increase the phosphorylation of Akt (Amantea et al., 2009).

In a series of animal behaviour studies Rombolà et al (2017, 2019) showed the anxiolytic-like/relaxant effects of BEO in rats were different to those of benzodiazepines, such as diazepam, because the anxiolytic effects of BEO was associated with the maintenance of alertness in the animals. This behaviour was reflected in the activity of the electroencephalogram (EEG) in which the systemic administration of BEO increased α wave frequencies (related to relaxation) and β waves (associated with the alert and awake state) of the EEG (Rombolà et al., 2009). It was argued that both the behavioural and EEG data supported the hypothesis that neurotransmitters systems (such us serotoninergic neurotransmission), could be involved in the anxiolytic-like/relaxant effects of bergamot oil (Rombolà et al., 2009, 2017, 2019). In separate studies, peripheral nociception (paw-licking/biting behaviour), induced by

local application of capsaicin (activating the transient receptor potential vanilloid type-1 receptors located in C-fibers), was reduced by injection of BEO (Sakurada et al., 2009). Both linalool and linalyl acetate were found to be more potent than BEO in inhibiting the nociceptive response (Sakurada et al., 2009). Further, the antinociceptive effect was prevented by naloxone hydrochloride, suggesting that the phytocomplex or monoterpenes acted through the opioid receptors (Sakurada et al., 2011). In an animal model of chronic pain, characterized by partial ligature of the spinal nerve, it was possible to observe an inhibition of allodynia by BEO (Kuwahata et al., 2013).

Finally, it was found that the survival and proliferation of neuroblastoma cells SH-SY5Y can be inhibit by BEO (Celia et al., 2013), in which the monoterpenes limonene and linalil acetate have been shown to play a central role (Russo et al., 2013).

Away from the nervous system, a study using the carrageenan-induced rat paw oedema test, demonstrated an anti-inflammatory activity of BEO (Karaca et al., 2007). Moreover, in the mouse aorta, BEO induced vasorelaxation by an action on K^+ and Ca^{2+} channels (Kang et al., 2013). Most notably, BEO induced hyperpolarization by activation of the K^+-channels, an effect partially inhibited by tetraethylammonium chloride a K^+-channels blocker (Kang et al., 2013). Furthermore, $CaCl_2$-induced contractions of the aorta were suppressed in tissues pretreated

with BEO, highlighting an ability to block membrane Ca^{2+}-channels (Kang et al., 2013).

Finally, the studies described above also highlight the lack of clarity into the molecular mechanisms by which BEO and its components can exert activity. Probably, this is because of the complexity of the models used to study their actions, most being whole-animal investigations. The use of *in vitro* techniques, such as isolated tissue preparations, could therefore be of great help in identifying one or more mechanisms of action. Interestingly, in one study linalool has been found to inhibit ACh-induced contraction in rat isolated duodenum and ileum (Blanco et al, 2013). In this respect, tissues from the gastrointestinal tract provide useful models with which to investigate the functions of BEO, since the ability of the Enteric Nervous System (ENS) to control muscle contractility allows both neuronal and muscle functions to be studied in the same tissue.

The general aspects of the methodology of isolated tissue preparations includes the use of specific physiological solutions, suitably oxygenated and maintained at a constant optimal temperature (Sanger and Bennett, 1984). Pharmacological studies with isolated preparations (qualitative assays and quantitative dosages of activity) have been of great importance since the last century in the characterization of the activity of compounds endowed with biological properties usable in therapy (Edinburgh staff, 1968). With regards to the use of

gastrointestinal (GI) preparations *in vitro* studies have been performed using isolated segments (e.g. intact lengths of the intestine) or muscle strips (obtained by cutting lengths of muscle approximately parallel to the circular or longitudinal muscle) (Sanger and Bennett, 1984). An important advantage is that such techniques make it possible to use human preparations removed at surgery, thereby avoiding complications arising from the use of animals with unknown species differences in bowel functions and/ or response to BEO (Sanger et al., 2013).

In this study, rat and human isolated colon preparations have been used to evaluate the actions of BEO and its components on muscle contractions evoked by application of substances which directly evoke muscle contraction and also by the use of electrical field stimulation of the intrinsic neurons to cause muscle contraction. Based on the evidence obtained by studies with CNS and cardiovascular models (see above) it was hypothesised that BEO has the potential to interact with both the enteric nervous system and the smooth muscle of the colon and that the components of BEO will act similarly but with different potency and efficacy. Together the data has the potential to give information on the possible therapeutic use of BEO in the treatment of GI disorders, most especially those thought to be associated with increased intestinal motility such as the diarrhoea-predominant form of Irritable Bowel Syndrome.

CHAPTER 2

2.1 Enteric Nervous System

The Enteric Nervous System (ENS) is an extensive intrinsic nervous system in the GI tract, extending from the oesophagus to the anal sphincter. It controls all aspects of the GI function (Furness, 2012) such as the regulation of GI motility, secretion and movement of transmucosal fluid, local blood flow, nutrients management and the interaction with the immune and endocrine system (Furness, 2006). The propagation and mixing of the GI contents is determined by the activity of the muscular layers, whose regulation is determined by the ENS. In particular, the ENS dominates the control of motility of the small and large intestine with the only exception of defecation control, in which the CNS has control through the lumbo-sacral spinal cord centres (Furness, 2006).

A unique property of the ENS, compared to any other section of the Peripheral Nervous System (PNS), is to maintain the functions of the GI tract even in the absence of CNS input (Furness et al., 2014). Overall, the ENS consists of a large number of neurons, from 200 to 600 million in humans (the same number of human spinal cord neurons). These are contained mostly within the myenteric plexus (Auerbach plexus, located between the two smooth muscle layers) and the submucous plexus

(Meissner's plexus, located in the submucosal tunic) (Furness, 2006) (Figure 2). The myenteric plexus forms a continuous network around the bowel and extending from the upper esophagus to the internal anal sphincter, while, in the small and large intestine the submucosal plexus is also present (Furness et al., 2014). The nerves fibres consist of axons and dendrites projecting from the cell bodies of enteric neurons, axons and terminals of extrinsic neurons that project to the gut wall and also the glial cells (Furness et al., 2014).

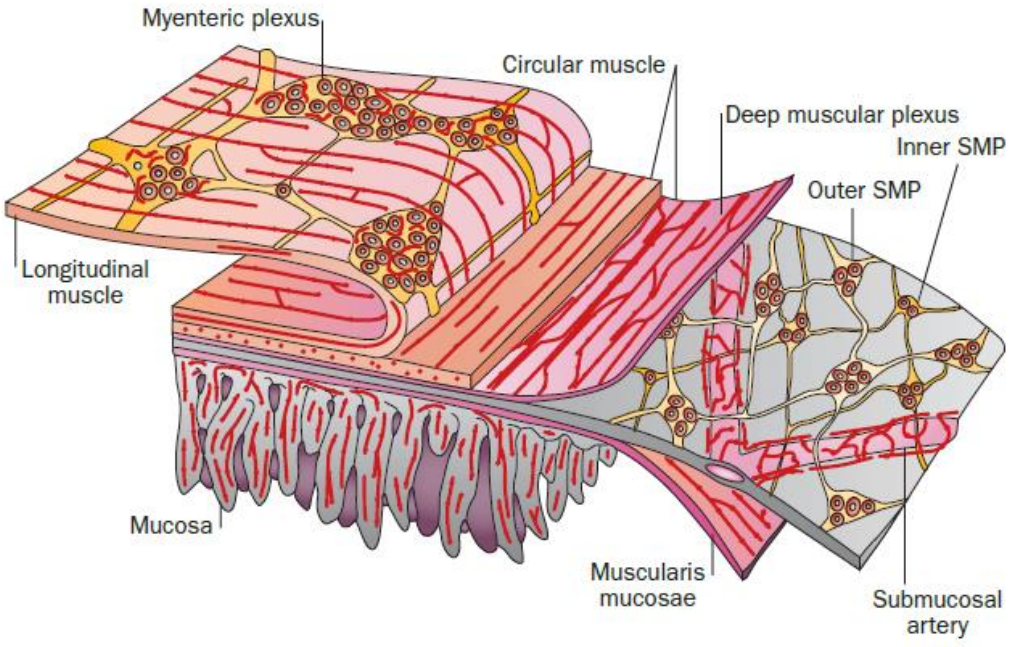


Figure 2: The organization of the ENS of human and medium–large mammals.

The ENS is characterized by ganglionated plexuses, the myenteric plexus and the submucosal plexus (SMP). Ganglia and plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic

arteries and the mucosa are connected by nerve fibre bundles (Adapted from Furness, 2006).

2.2 Types of enteric neurons

From the functional point of view, it is possible to distinguish three main classes of neurons: a) intrinsic primary afferent neurons (IPANs) (also referred to as intrinsic sensory neurons), b) interneurons and c) motoneurones.

IPANs are connected to each other, with the interneurons and directly with the motor neurons, while, the interneurons connect with other interneurons and with the motor neurons (Furness et al., 2014). The IPANs are large multi-axonal neurons (type II morphology) that respond to chemical stimuli, mechanical distortion of mucosa or external musculature. From 10 to 30 % of neurons in the submucosal and myenteric plexus in the small and large intestine, are formed by cell bodies of IPANs, but these neurones are not present in the esophagus (Furness, 2006).

Amongst the motor neurons are muscle motor neurons, secretomotor neurons, secretomotor/vasodilator neurons, motor neurons for enteroendocrine cells and for innervation of lymphoid follicles (Furness et al 2014). Overall, it is possible to distinguish two types of motor neurons, namely the excitatory and inhibitory motor neurons that

innervate the longitudinal and circular smooth muscle and the muscularis mucosae, and which are uni-axonal (Furness et al., 2014). Their chemical mediators are the excitatory transmitters, acetylcholine (ACh) and the tachykinins (TK), and the inhibitory transmitters, nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate-like (ATP-like) (Furness et al., 2014). In all mammals the circular muscle is mainly innervated by motor neurons that have the circular soma in the myenteric plexus, and probably in the human the circular muscle also has an innervation coming from the submucosal plexus (Furness et al., 2014). The cell bodies of motor neurons that innervate the longitudinal muscle are located in the myenteric plexus of small mammals, but in larger mammals some motor neurons of the longitudinal muscles have cell bodies in the external submucous plexus (Timmermans et al., 1992).

The interneurons are distinguished in a type of interneurons with oral (ascending) projections and two types of interneurons with anal projection (descending) (Furness et al., 2014). The ascending interneurons are cholinergic and participate in the local motor reflex; the descending interneurons are of two types: a) descending interneurons which synthesize ACh, NO or serotonin (5-HT); b) descending interneurons that synthesize ACh and somatostatin and participate in the conduction of the migrating motor complex (MMC) along the

intestine. Some types of interneurons also have mechanoceptive properties and contribute to relaxation reflex (Mongardi Fantaguzzi et al., 2009).

Another class of enteric neurons is represented by secretory and secretomotor/vasodilator neurons that regulate the transport of electrolytes and water through the intestinal mucosa (Vanner and Macnaughton, 2004).

An important non-neuronal component of the ENS is represented by the enteric glial cells, which resemble the CNS astrocytes and in analogy to the latter, not only contribute to create a protective microenvironment, but can also play a functional role in the transfer enteric information responding to a variety of neuroligands (Gulbransen and Sharkey, 2014).

2.3 Enteric neurotransmissions

The main excitatory neurotransmitter released from the myenteric and submucosal plexus is acetylcholine, which elicits contractions of the gastrointestinal muscle and secretions through the activation of specific muscarinic receptors (Hirota and McKay, 2006). The M_1 receptor is present on epithelial cells and in the intestinal crypts, the M_2 receptor subtype on smooth muscle cells, the M_2 and M_4 subtypes are colocalized on myenteric neurons expressing choline acetyltransferase (ChAT) and

M_3 subtype at the crypt and smooth muscle cells (Caulfield, 1993; Eglen et al., 1996, Hirota and McKay, 2006: Khan et al., 2013).

In addition to ACh, tachykinins (TKs) such as substance P (SP), neurokinin A and neurokinin B (NK_A, NK_B), are another class of excitatory neurotransmitters released from both enteric plexuses. They act at neurokinin (NK) receptors and can be released from the same neurons that release ACh. In the rat colon NK_1 and NK_2 receptors are mainly located on the circular muscle and on the cell bodies in the lamina propria (Appleyard et al., 2006).

Non-cholinergic non-adrenergic inhibitory neurotransmitters (NANCs) are considered: NO, VIP and ATP are the most common (Nishiyama et al., 2014). The release of NO generally causes muscle relaxation through a reduction of the levels of cytoplasmic Ca^{2+} and the desensitization of the Ca^{2+}-dependent contractile apparatus (Kwon et al., 2000). ATP and VIP, with different pathways, causes relaxation of muscle cells (Waseda et al., 2005; Sandgren et al., 2003).

In mammals, about 95% of 5-HT is present in the intestine and derives mostly from enterochromaffin cells, but also from mast cells (in rodents) and myenteric serotonergic neurons, which are descending interneurons that project to the submucosal plexus. The 5-HT released by enterochromaffin cells activates the IPANs that initiate the motility of the intestine, while the descending serotoninergic interneurons, which

activate the inhibitory neurons, produce a tonic inhibition of the circular smooth muscle and promote secretion (Mawe and Hoffman, 2013). Most 5-HT receptors are present in the gut and all of these are located within the ENS, in motor neurons (5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4), on IPANs that influence intestinal sensitivity (5-HT_3, 5-HT_4 and 5-HT_7) and on smooth muscle (5-HT_1, 5-HT_2, 5-HT_4, 5-HT_7) (Sanger, 2008).

Dopamine (DA) is a neurotransmitter of the CNS, which acts as a negative modulator of intestinal motility through stimulation of the receptor families D_1 and D_2. Activation of D_2-like receptors causes an inhibitory effect on the spontaneous activity of the circular colonic muscle, while the activation of D_1-like receptors reduces the release of ACh, through a pathway involving enteric inhibitory neurotransmitters (Martinucci et al., 2015). Both, D_1 and D_2-like receptors are present within the GI tract (Auteri et al., 2016).

Another important enteric neurotransmitter is glutamate, whose metabotropic and ionotropic receptors are present both on intrinsic and extrinsic neurons. In particular, these receptors are located at the level of the glial cells in the myenteric and submucosal plexus of the colon and in the myenteric plexus of the small intestine (Filpa et al., 2016). Glutamate also has an indirect excitatory action that is associated with the activation of cholinergic neurons (Wiley et al., 1991) mediated by the

activation of N-methyl-D-aspartate (NMDA) receptors (Giaroni et al., 2003).

The gamma-aminobutyric acid neurotransmitter (GABA) is also involved in the regulation of ENS, in fact $GABA_A$ and $GABA_B$ receptors are present at presynaptic level and controlling the release of ACh in intestinal smooth muscle (Auteri et al., 2014). Administration of GABA at low concentrations seems to improve the release of ACh mainly involving the $GABA_A$ receptor by increasing peristalsis; at high concentrations it reduces the release of ACh by activating the decreasing peristalsis of the $GABA_B$ receptor (Auteri et al., 2014).

Finally, the manner by which the Sympathetic Nervous System interacts with the ENS and other systems of the GI tract should be noted. These extrinsic neurons usually cause relaxation of the intestinal smooth muscle through the release of noradrenaline which in turn acts at the α- and ß-adrenergic receptors. The $ß_1$, $ß_2$ and $ß_3$ receptors are present on the interstitial cells of Cajal (ICC) of the small intestine, while the $ß_1$ and $ß_3$ receptors are present on the colon ICC (Nasser et al., 2006). In the myenteric plexus of rat and mouse, $α_2$ receptors are widely distributed on the neurons and enteric glia cells. Furthermore, a wide expression of $ß_1$ and $ß_2$ receptors is observed both in the myenteric plexus and in the submucosal plexus (Nasser et al., 2006).

CHAPTER 3

3.1 Irritable Bowel Syndrome

The correct functioning of the GI tract is essential to sustaining life, however, GI tract disorders are common, affecting the mucosa, musculature and neuronal innervation from the esophagus to the colon. Abnormalities of GI function can lead to life-threatening diseases such as chronic inflammatory bowel disease (IBD) or to conditions that seriously affect quality of life, such as gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) (Enck et al., 2016).

IBS is a functional GI disorder (not associated with structural or biochemical abnormalities and detectable with current routine diagnostic tools), characterised by abdominal pain and a disturbed bowel habit over a period of at least three months, with a prevalence of approximately 11% of the world population (Lovell et al., 2012; Enck et al., 2016). The incidence of IBS decreases with advancing age (> 50 years) (Lovell et al., 2012), is similar in children and adolescents compared to adults, and is not necessarily carried through from childhood to the adult (Goodwin et al., 2013), moreover, also the family aggregation has been reported (Saito et al., 2010).

Patients with IBS, in general, experience greater sensitization of normal intestinal functions, whose symptoms are abdominal pain, cramps, changes in intestinal habits and defecation disorders. All of these symptoms can severely affect the quality of life of patients. Based on the predominant symptoms, patients with IBS can be classified into subgroups, i.e. those with constipation (IBS-C), diarrhea (IBS-D), constipation mixed with diarrhea (IBS-M) and in an unsubtyped (IBS-U) (Enck et al., 2016).

3.2 Aetiology and risk factors

The Aetiology remains largely unknown, however, psychic disorders (Saito and Talley, 2008), genetic predisposition (Fukudo et al., 2011) and environmental factors (Rahimi et al., 2009) may underlie the manifestation of pathology. Numerous studies highlight the involvement of the immune system in IBS, food antigens and bile acids, brain-intestine axis (Enck et al., 2016). Moreover, it seems that increased epithelial permeability is very important in the manifestation of post-infectious IBS (in particular for IBS-D) (Bischoff et al., 2014). Based on clinical observation, infectious gastroenteritis is a strong risk factor for IBS development (Thabane et al., 2007). Moreover, the immunohistochemical data of patients with IBS show greater infiltration of T cells and mast cells in the mucosa of the small and large intestine (Barbara et al., 2011).

The increase in bile acids (mainly present in the IBS-D subgroup) influence the intestinal habit by accelerating the transit of the colon and inducing diarrhea and visceral hypersensitivity (Valentin et al., 2015). The gastrointestinal microbiota, considered as an ecosystem that inhabits the entire GI tract, has a systemic influence on our health (Enck et al., 2016). Evidence of an involvement of the altered composition of the intestinal microbiota in IBS pathophysiology has accumulated (Rajilić-Stojanović et al., 2011). Anxiety and depression can determine abdominal symptoms associated with an altered peripheral regulation of intestinal function, alteration of signaling between the intestine and the brain (Hungin et al., 2015; Enck et al., 2016). In addition, the same pathology can result in mental disorders associated with feelings of shame, fear and embarrassment (North et al., 2007; Drossman et al., 2009).

3.3 Current Treatment

Currently there is no curative treatment for IBS, and although a substantial number of patients will experience spontaneous remission over time, therapy is based only on symptom relief (Enck et al., 2016). Treatment options usually include the combination of dietary and pharmacological interventions aimed at managing the predominant symptom and the main therapeutic classes are represented by compounds belonging to antispasmodic, antidepressant, laxative, prokinetic,

antidiarrheal and probiotic drugs (Enck et al., 2016). These include the most recently introduced medications, namely the intestinal secretagogues (Chloride channel type-2 inhibitors, Guanylate cyclase-C receptor activators) and bile acid transporter inhibitors (Camilleri, 2012). However, none of the available treatments can fully alleviate the complex symptoms of IBS. Furthermore, the chronic use of these drugs may be characterized by the appearance of numerous side effects. Over the years all these problems have led patients with functional gastrointestinal disorders to use of products of vegetable origin, and in particular natural extracts obtained for example from *Mentha piperita, Aloe vera, Curcuma spp., Hypericum perforatum* (Rahimi and Abdollahi, 2012, Chang, 2014, Grundmann and Yoon., 2014). From *Mentha piperita,* a naturally-occurring carminative herb, it is possible obtain peppermint oil which is widely and commonly used in the traditional medicine to improve symptoms related with various gastrointestinal disorders including IBS (Ford et al., 2008; Alammar et al., 2019). This property of peppermint oil has been linked with menthol, a principal component of the essential oil, which blocks Ca^{2+} channels in smooth muscle cells, thus producing antispasmodic effect in the gut (Amato et al., 2014). Some studies have also showed the ability of menthol to act on Transient Receptor Potential (TRP) channels (a family of ion channels, Behrendt et al, 2004; Fothergill et al., 2016; Paschke et al., 2017). This inhibitory effect and the natural

origin makes peppermint oil a safe and effective therapy to improve abdominal pain and global symptoms in IBS patients (Alammar et al., 2019).

AIM OF RESEARCH

BEO is widely used in traditional and complementary medicine for the treatment of various disorders (Calapai and Delbò, 2011; Ni et al., 2013; Section 1). However, a limited number of studies have examined the rational basis for the use of BEO. The majority of published data has indicated that BEO can produce neurobiological effects in animal models, which originated, at least in part, from interference with the basic mechanisms that regulate synaptic plasticity in both physiological and pathological conditions in CNS (Morrone et al., 2007; Amantea et al., 2009; Rombolà et al., 2016; Rombolà et al., 2019). In view of these evidences, it is likely that the phytocomplex may show a potential activity on the neurotransmission of the ENS. Furthermore, the study of Kang et al. (2013), suggests that BEO may also act on intestinal smooth muscle to cause relaxation, as observed in the mouse aorta. If BEO were found to affect intestinal function in a potentially beneficial manner, BEO or a constituent of BEO could therefore be useful as a complementary treatment of intestinal diseases such as IBS, where the chronic use of drugs is characterized by the appearance of numerous side effects, that over the years have led patients to use products of natural origin (Rahimi and Abdollahi, 2012; Grundmann and Yoon, 2014).

In this regard, the purpose for this study was to investigate the ability of BEO and its components to modulate intestinal functions. It was hypothesised that BEO has the potential to interact with both the ENS and the smooth muscle of the colon and that the different components of BEO will act similarly but perhaps with different potency and efficacy.

To test this hypothesis, it must be recognised that although the basic functions of the GI tract are similar among different mammals, major anatomical and functional differences exist between species (Sanger et al., 2011). For example, rodents do not have the ability to vomit, have a large cecum (degenerated in the appendix in humans) and the colon is relatively short without presenting the arrangement of longitudinal muscle in three discrete bands (taenia) (present in human colon) (Sanger et al., 2011). Moreover, it is necessary to consider the selection of positive genes during evolution, that have conferred specific human variations that cannot be reproduced by animal models (Vamathevan et al., 2008). Therefore, it is important that this study investigates the activities of BEO in *ex-vivo* models using both rat and human intestinal preparations, in order to highlight the translation value and / or the presence of differences between species.

The research aims were:

1. To evaluate the effects of BEO on the contractile activity of rat isolated jejunum, ileum and colon induced by different muscle stimulants.
2. Determine if BEO and when tested separately, the constituents of BEO, could modulate neuromuscular contractions evoked in rat colon by electrical field stimulation of the intrinsic neurons, and to compare such activity with the ability to modulate contractions of the colon induced directly by potassium chloride (KCl).
3. Compare the actions of BEO and its individual constituents on the neuromuscular contractions evoked by electrical field stimulation in the human colon and on contractions evoked by KCl.

MATERIALS AND METHODS

Human colon

Following ethical approval (REC 15/LO/2127), informed written consent was obtained for use of macroscopically normal ascending and descending colon (5–10 cm from tumour) from patients undergoing elective surgery for non-obstructing bowel cancer (n= 8 females and n= 7 meals, median age of 61, range of age it was between 43-81). No patient had previous chemoradiotherapy or diagnosis of inflammatory bowel disease. Tissue was immersed into Krebs solution (in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5), pre-gassed with 95% O_2 and 5% CO_2 and within in 60–120 min after surgery was transferred to the laboratory. The mucosa, *muscularis* mucosa and submucosal plexus were removed by blunt dissection and discarded. Muscle strips (~15 mm long and ~5 mm wide) were cut approximately parallel to the circular muscle fibres. These were used immediately or after overnight storage (~15 hours) at 4°C in fresh, pre-oxygenated Krebs solution.

Rat intestine

The animal experiments were carried out in accordance with the Italian directives of the D.L.gs n. 26/2014 and UK Animal Scientific Procedures Act (1986) and approved by UNICAL and QMUL ethics committee. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results. Initial experiments were performed in Italy using tissue from male Wistar rats (250-300 g, Charles River Italia Srl, IT). Subsequent experiments were performed in the UK using Sprague-Dawley of both sexes (150g, Charles River Laboratories, Margate, UK). All animals were kept in a controlled temperature (22 ± 1°C), humidity (55 ± 10%) and 12-h light-dark cycle. Both sexes were segregated in separate cages with food and water provided *ad libitum*, and were used after a minimum of 6 days of acclimatisation to their new environment since their arrival in the animal unit.

The rats were sacrificed by exposure to 4% isoflurane air or to a rising concentration of CO_2 followed by for cervical dislocation. The jejunum, ileum and colon were excised and immersed in Krebs solution. Each tissue was cleared of their intraluminal contents and cut into full thickness strips along the longitudinal (UNICAL) or circular axis (QMUL) (15 mm long and 5 mm wide).

Experimental protocol

Human and rat muscular strips was mounted in 10 or 40 ml tissue baths between platinum wire stimulation electrodes (15 mm in length, 10 mm apart). Changes in muscle tension were recorded in milliNewtons (mN) using an isometric transducer (MLT201/D, AD Instruments, Chalgrove, United Kingdom) connected to an AcqKnowledge data acquisition system version 3.8.1 (BIOPAC Systems Inc., CA, USA) on a personal computer (Dell, UK, www.dell.com/uk). The stimulation electrodes were connected to an STG2008 stimulator (Multi Chanel Systems, Reukingen, Germany).

15 min later, human and rat strips were stretched respectively by 20 mN and 10 mN and allowed to equilibrate for 60 min for fresh tissues or up to 150 min for tissues stored during night, with periodic renewals of Krebs solution (at 15 min intervals).

Pilot studies were first conducted using rat jejunum, ileum and colon to look for any ability of BEO and its components to modulate contractions evoked by exogenously applied ACh (10^{-6} M), 5-HT (10^{-8} M) or SP (10^{-7} M). To obtain reproducible responses to use as control, these substances (ACh, 5-HT and SP), were administrated and left in the tissue baths for 5 min. After obtaining of 3 reproducible contractions, BEO (2.5 x 10^{-5} to 2.5 x 10^{-3} % v/v), linalool (2.5 x 10^{-6} M to 2.5 x 10^{-4} M), limonene (2.5 x 10^{-6} M to 2.5 x 10^{-3} M) and linalyl acetate (2.5 x 10^{-6} M to 2.5 x 10^{-3} M) were administered and left in the tissue baths for 15 min. At the end

of this period ACh, 5-HT or SP were again administered and the height of resulting contraction was compared with the height of contraction obtained without any pre-treatment.

Subsequently, the effect of BEO and its components was studied for an ability to modulate contractions evoked by electrical field stimulation (EFS) or by KCl. EFS was applied with frequency of 5 Hz, pulse width 0.5 ms, for 10 s every 1 min at a voltage 10% higher than that required to obtain maximal contractions (Broad et al, 2012). Each substance was administered after at least 30 min in which the basic muscle tension and the amplitude of the contractions evoked by EFS had become stable. The muscle strips were used to obtain cumulative concentration-response curves for BEO (from 10^{-6} to 10^{-3} % v/v), linalool (from 10^{-9} to 10^{-4} M), linalyl acetate (from 10^{-9} to 10^{-4} M) and limonene (from 10^{-9} to 10^{-4} M). The intervals between administrations of substances (BEO, linalool, linalyl acetate and limonene) were always of 15 minutes. Moreover, in order to confirm the neurogenic nature of contractions and that these were due to a release of acetylcholine, some tissues have been treated with the neurotoxin and sodium channel blocker tetrodotoxin (10^{-6} M) and with the muscarinic receptor antagonist atropine (10^{-6}M).

A submaximally-effective concentration of KCl (40-60 mM caused ~ 40% of the maximum contraction) was used in both tissues, human and rat colon. After at least 30 min in which the basic muscle tension and the

amplitude of the spontaneous contractions had become stable, the submaximally-effective concentration of KCl was applied. BEO (10^{-5} to 10^{-3} % v/v) and linalool (10^{-7} to 10^{-4} M) were administered cumulatively (at intervals of 15 min) when KCl-evoked contraction became stable.

Substances

Atropine, acetylcholine chloride, carbamylcholine chloride, tetrodotoxin, (-)-linalool and (R)-(+)-limonene were purchased from Sigma-Aldrich, IT and UK; linalyl acetate was purchased from ThermoFisher scientific, Geel-West, UK. The crude bergamot essential oil was used as it represents the form marketed for personal human use and for therapeutic use. BEO was kindly provided by "Capua Company1880 S.r.l.," Campo Calabro, Reggio Calabria (Italy) and chromatographic results on the certificate of analysis confirm that the essential oil contained (R)-(+)-limonene, ~48.6%; linalyl acetate, ~23.6%; (-)-linalool, ~5.5% of the total volume (v/v) (see Appendix 1 for analysis certificate).

Acetylcholine (ACh), atropine (ATR) and tetrodotoxin (TTX) were dissolved in distilled water, whereas the other drugs: BEO, (-)-linalool, linalyl acetate and (R)-(+)-limonene, were dissolved dimethylsulphoxide (DMSO). The total volume of the solvents added to the tissue baths did not exceed 1% of the bath volume.

Data analysis

To study the effects on the contractions evoked by ACh, 5-HT or SP, the height of the contractions in the absence and in the presence of the tested substances was measured. The changes obtained were expressed as a mean percentage compared with control contractions.

To study the effects on neuronally-mediated contractions, the amplitude at last 3 EFS-responses of each interval between doses was measured. The changes obtained were expressed as mean percentage compared with at last 3 contractions of each interval obtained in control-responses tissues.

To study the effects on KCl-evoked contractions, the height of the baseline tension at last 5 min of each interval between doses was measured. The changes obtained were expressed as a mean percentage compared with the height of the baseline tension of the last 5 minutes of each interval obtained in control-responses tissues.

GraphPad PRISM 7.0 for Windows was used (Graph-Pad Software, La Jolla, CA, USA). The cumulative concentration-effect curves in the absence and presence of a tested substance were fitted by non-linear regression to a four-parameter Hill equation (Equation 1). The concentration-response data were plotted as the mean ± standard error of the mean (mean ± s.e.m), n values represent the number of patients or animals from whom tissues were used.

To minimise potential desensitisation, only one muscle strip was used per drug treatment from a given patient or animal.

$p\mathrm{EC}_{50}$ and $p\mathrm{IC}_{50}$ show negative logarithm of concentration of a substance that produces half of maximal contractile or inhibitory effect obtained; E_{max} and I_{max} show the maximal contractile or inhibitory effect obtained. However, it was not possible to obtain a very high concentration in bath volume, for this reason, these values we can be defined as apparent $p\mathrm{IC}_{50}$ and I_{max}.

$$E = Basal + \frac{E_{max} - Basal}{1 + 10^{(LogEC_{50} - Log[A])^{n_H}}} \qquad \text{(Equation 1)}$$

The statistical significance of any difference between unpaired data was determined using Student's test (*t*-test), and $P<0.05$ was considered significant.

Tissue bath

Structurally, the bath is connected to a serpentine containing the oxygenated physiologic solution. The wholes are surrounded by a jacket in which water heated to 37-38°C circulates. The isolated preparation is kept alive through the complete immersion in a heated physiological solution that ensures a continuous supply of nutrients and oxygen.

Furthermore, the isolated preparation is fixed at one end to the bottom of the bath, and with the other end is connected to the transducer. The transducers used were isometric that measured the variations in force. Each transducer is connected to an amplifier whose purpose is to improve the signal, increasing or decreasing the intensity of the signal to guarantee an easy interpretation of the signals (Edinburgh staff, 1968). The intestinal preparations are suspended in the tissue bath under 1-2 g of tension (respectively for rat and human tissue) to facilitate the initiation of the spontaneous muscular activity (Sanger and Bennett, 1984).

Electrical stimulation of autonomic nerves

The electrical field stimulation (EFS) generate a nerve response in muscle strips (Sanger and Bennett, 1984). The electrodes are positioned on the sides of the strips and stimulate only a small part, causing a response capable of propagating (Sanger and Bennett, 1984).

In this research, the EFS was used following parameters: frequency 5 Hz, pulse width 0.5 ms, for 10 s every 1 min (Cellek et al., 2006; Broad et al., 2012). The choice of these parameters was determined on the basis of previous studies, which demonstrated how this type of electrical stimulation is associated with reproducible and long-lasting responses (about 150 min) (Cellek et al., 2006; Broad et al., 2012). Furthermore,

these parameters of EFS are associated with the neuronal release of mediators such as ACh, TKs and NO which can determine the formation of three different shapes of EFS-evoked contractions in human isolated circular muscle of colon (Cellek et al., 2006). These contractions have been classified into monophasic response (release of ACh during EFS), biphasic response (nitrergic relaxation during EFS followed by cholinergic contraction after termination of EFS) and triphasic response (cholinergic contraction and nitrergic relaxation during EFS, and a tachykininergic contraction after EFS) (Cellek et al., 2006).

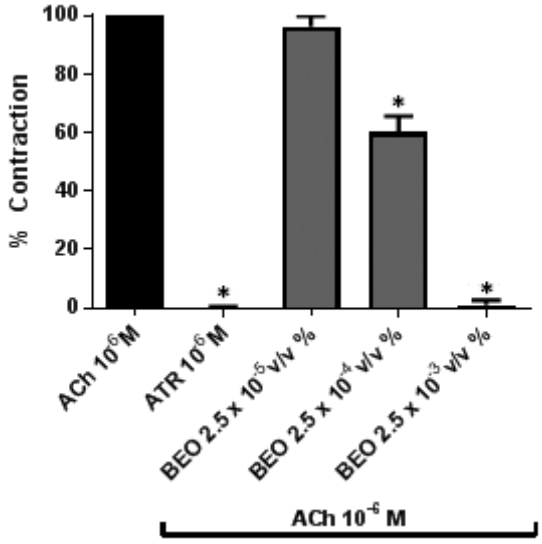
RESULTS I

I.I Effect of BEO on acetylcholine-evoked contractions in jejunum, ileum and colon of rat

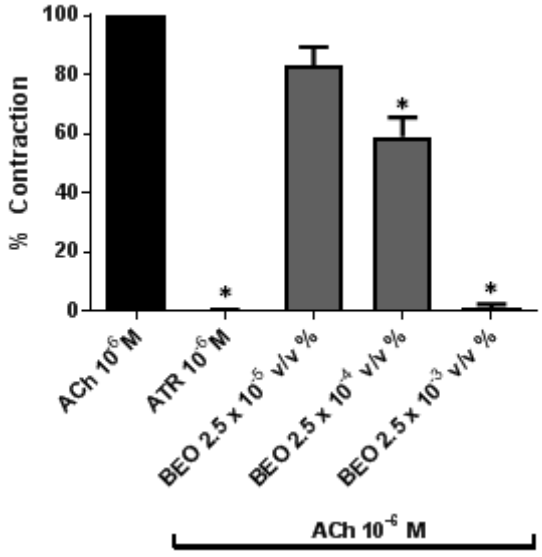
Administration of ACh (10^{-6} M) elicited a contraction in isolated jejunum, ileum and colon of rat. Pre-treatment with ATR (10^{-6} M) for 15 min completely inhibited this response highlighting the involvement of muscarinic receptors (Figure 3). Application of BEO (2.5 x 10^{-5} to 2.5 x 10^{-3} % v/v) resulted in a concentration-dependent decrease in the height of the contraction elicited by ACh (10^{-6} M) (Figure 3). The rank order in terms of I_{max} for the various tissue was: ileum ≥ jejunum ≥ colon. Table 1 summarizes all I_{max} obtained in each tissue.

Figure 3

A)


% Contraction
100
80
60
40
20
0
*
*
*
ACh 10-6M
ATR 10-6M
BEO 2.5 x 10-5 v/v %
BEO 2.5 x 10-4 v/v %
BEO 2.5 x 10-3 v/v %
ACh 10-6 M

B)


% Contraction
100
80
60
40
20
0
*
*
*
ACh 10-6M
ATR 10-6M
BEO 2.5 x 10-5 v/v %
BEO 2.5 x 10-4 v/v %
BEO 2.5 x 10-3 v/v %
ACh 10-6 M

C)

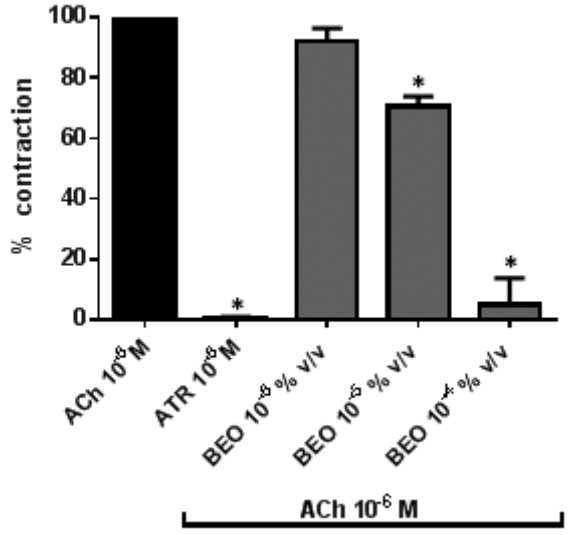


Figure 3: Effect of BEO on ACh-evoked contraction in jejunum, ileum and colon of rat.

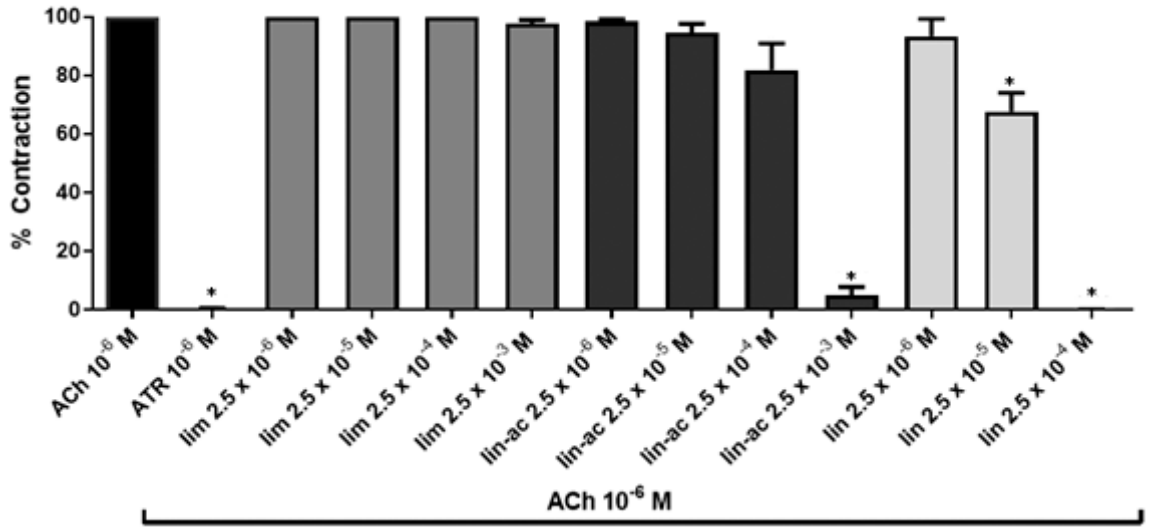
Panels show the effect of BEO on ACh-evoked contraction: (A) jejunum, (B) ileum and (C) colon of rat. Each point represents the mean of 4 rats. Vertical lines show standard error of mean. *=P<0.05 shows the statistical significance between the concentrations of BEO tested on ACh-contraction versus ACh-contraction control (*t*-tests). BEO= bergamot essential oil, ACh= acetylcholine, ATR= atropine.

I.II Effect of linalool, linalyl acetate and limonene on acetylcholine-evoked contractions in jejunum, ileum and colon of rat

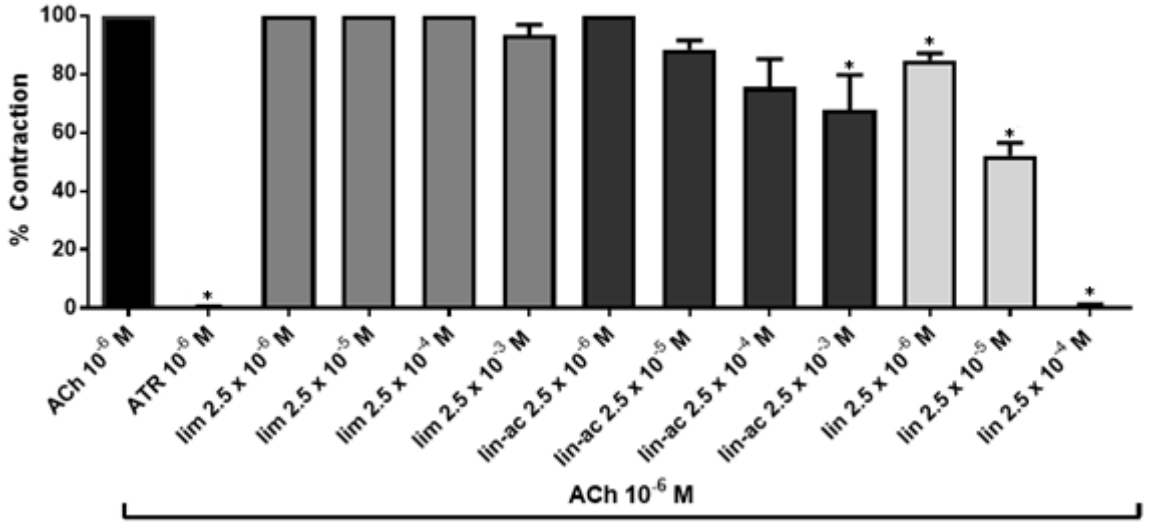
The actions of three constituents of BEO, linalool, linalyl acetate and limonene were evaluated and compared on contractions evoked by ACh. The administration of single constituents (2.5 x 10^{-6} M, 2.5 x 10^{-5} M, 2.5 x 10^{-4} M) resulted in a concentration-dependent decrease in the height of ACh-contractions (10^{-6} M) (Figure 4). Linalool showed the maximum inhibition in all types of tissues, particularly, the rank order in terms of I_{max} for the various tissues was: jejunum ≥ ileum ≥ colon. Linalyl acetate started to show an inhibition of cholinergic contraction from 2.5x10^{-5} M in ileum and jejunum, but did not show effects statistically significant in colon (Figure 4). The rank order in terms of I_{max} for the tissues was: jejunum >> ileum. The last constituent tested, limonene, produced a small inhibition with the highest concentration tested (2.5 x 10^{-3} M), however, this was not statistically significant in any tissues (see Figure 4). Table 1 summarizes all I_{max} obtained in each tissue.

Figure 4

A)


% Contraction
100
80
60
40
20
0
ACh 10-6 M
ATR 10-6 M
lim 2.5 x 10-6 M
lim 2.5 x 10-5 M
lim 2.5 x 10-4 M
lim 2.5 x 10-3 M
lin-ac 2.5 x 10-6 M
lin-ac 2.5 x 10-5 M
lin-ac 2.5 x 10-4 M
lin-ac 2.5 x 10-3 M
lin 2.5 x 10-6 M
lin 2.5 x 10-5 M
lin 2.5 x 10-4 M
ACh 10-6 M

B)


% Contraction
100
80
60
40
20
0
ACh 10-6 M
ATR 10-6 M
lim 2.5 x 10-6 M
lim 2.5 x 10-5 M
lim 2.5 x 10-4 M
lim 2.5 x 10-3 M
lin-ac 2.5 x 10-6 M
lin-ac 2.5 x 10-5 M
lin-ac 2.5 x 10-4 M
lin-ac 2.5 x 10-3 M
lin 2.5 x 10-6 M
lin 2.5 x 10-5 M
lin 2.5 x 10-4 M
ACh 10-6 M

C)

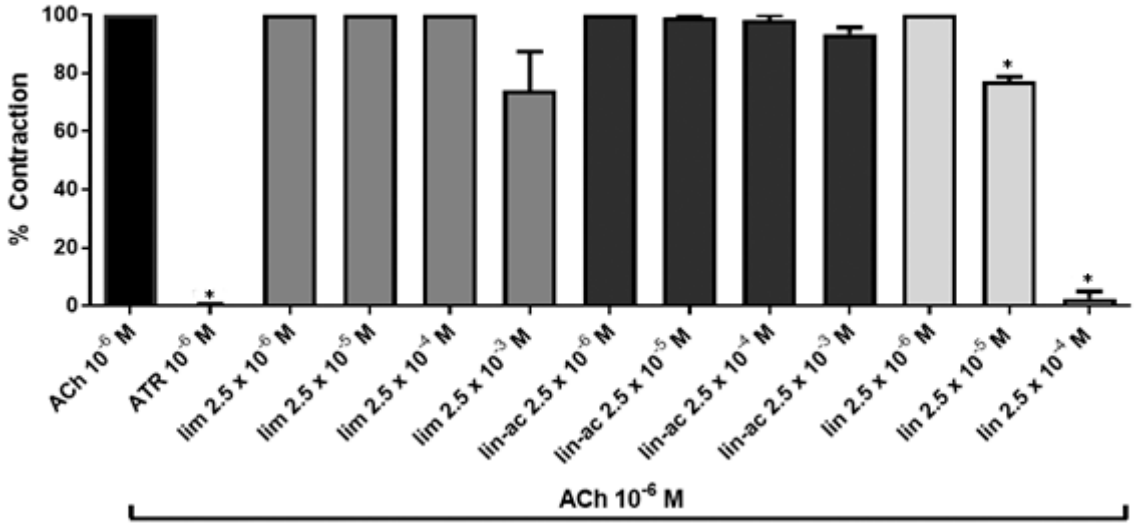


Figure 4: Effect of the components of BEO on ACh-evoked contraction in jejunum, ileum and colon of rat.

Panels show: (A) jejunum, (B) ileum and (C) colon of rat. Each point represents the mean of 4-5 rats. Vertical lines show standard error of mean. *=P<0.05 shows the statistical significance between the concentrations of linalool, linalyl acetate and limonene tested on ACh-contraction versus ACh-contraction control (*t*-tests). ACh= acetylcholine, ATR= atropine, lin= (-)-linalool, lin-ac = linalyl acetate, lim= (R)-(+)-limonene.

Table 1: Maximum inhibition (I_{max}) of ACh-evoked contraction in jejunum, ileum and colon of rat.

	Rat					
	Jejunum		**Ileum**		**Colon**	
	I_{max} (%)	**n**	**I_{max} (%)**	**n**	**I_{max} (%)**	**n**
BEO	98.2±1*****	4	99.3±0.3*****	4	95±12.3*****	4
Linalool	99.8±0.2*****	5	99.4±0.1*****	5	97.5±1*****	5
Linalyl acetate	95±2.5*****	4	32.3±11.5***†**	4	6.4±3.9**†**	4
Limonene	2.3±1.3†	4	2.3±1.3†	4	25.8±13.3†	4

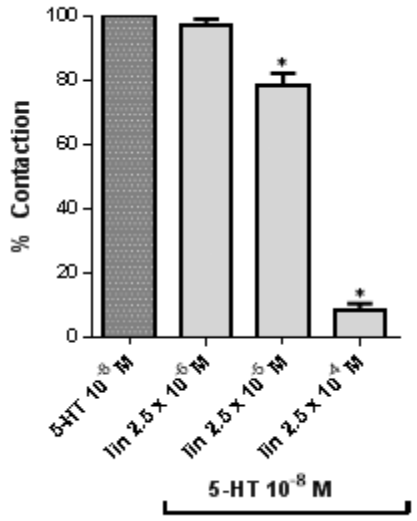
Each point represents the mean and standard error of the mean of n animals. *****=P<0.05 shows the statistical significance between the highest concentration of BEO, linalool, linalyl acetate and limonene tested on ACh-contraction versus ACh-contraction control (*t*-tests). **†** = shows the apparent I_{max}. BEO = bergamot essential oil, ACh= acetylcholine

I.III Effect of linalool on 5-HT and SP-evoked contraction in rat colon

From previous experiments, we started to see that linalool was the most active component of BEO. To observe whether linalool showed specific or non-specific activity, it was tested on contractions evoked by substances (5-HT (10^{-8}M) and SP (10^{-7}M)) other than ACh. However, repeated exposures of the muscle to a given concentration of 5-HT or SP did not produce reproducible responses in ileum and jejunum, so it was not possible to obtain results. In the colon it was possible to obtain responses to repeated exposures to 5-HT and SP. In these experiments, linalool (2.5 x 10^{-6} M and 2.5 x 10^{-5} M, 2.5 x 10^{-4} M) resulted in a concentration-dependent reduction in the height of the 5-HT- and SP-induced contractions (Figure 5). The maximum inhibition of 5-HT- and SP-responses (I_{max}) obtained is showed in Table 2.

Figure 5

A)



B)

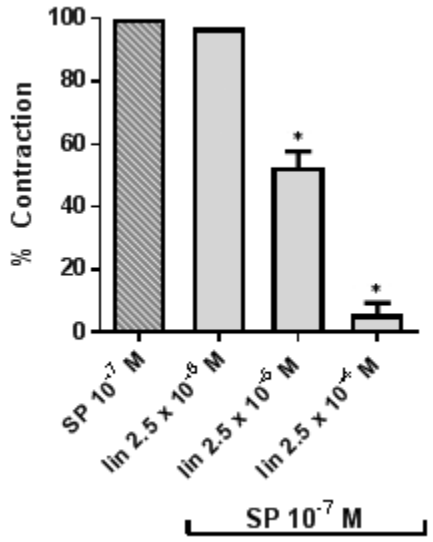


Figure 5: Effect of linalool on 5-HT and SP-evoked contraction in rat colon.

Panels (A) and (B) show respectively the effect of linalool on 5-HT and SP-evoked contraction. Each point represents the mean of 3 or 4 rats. Vertical

lines show standard error of mean. *=P<0.05 shows the statistical significance between the concentrations of linalool tested on 5-HT- and SP-contraction versus 5-HT- and SP-contraction control (*t*-tests). SP= substance P, 5-HT= serotonin, lin= (-)-linalool.

Table 2: Maximum inhibition (I_{max}) of linalool on 5-HT and SP-evoked contraction in rat colon.

	Colon			
	5-HT		SP	
	I_{max}	n	I_{max}	n
linalool	92.3 ± 3.2 %*	4	94.2 ± 2.3 %*	3

Each point represents the mean and standard error of the mean of 3 or 4 animals. *=P<0.05 shows the statistical significance between the highest concentrations of linalool tested on 5-HT- and SP-contraction versus 5-HT- and SP-contraction control (*t*-tests). SP= substance P, 5-HT= serotonin.

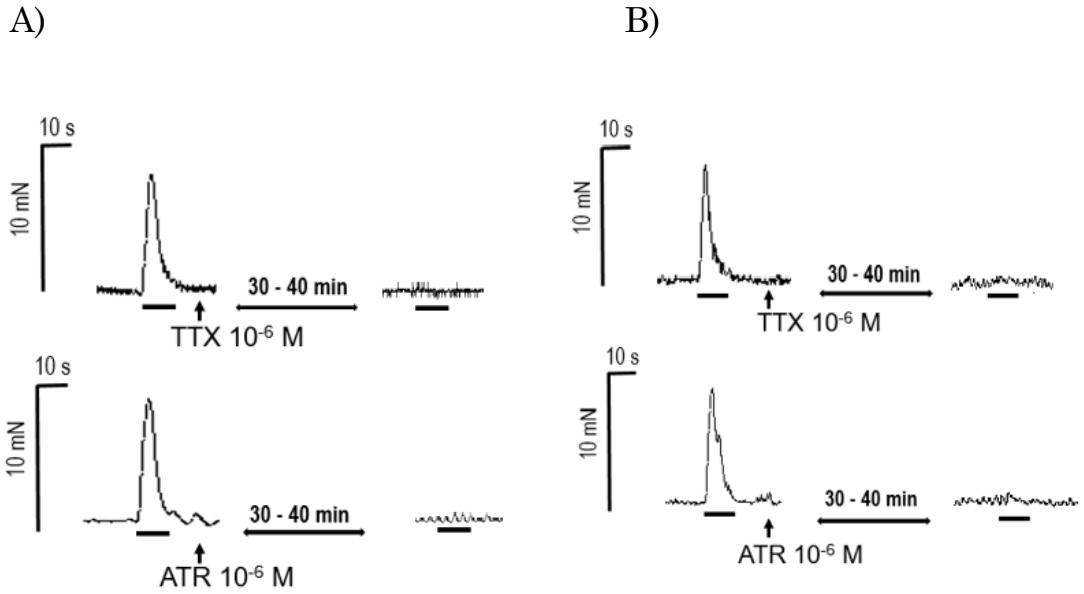
RESULTS II

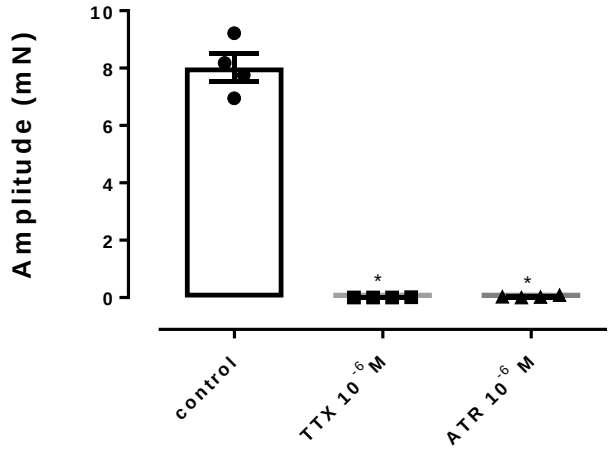
II.I Neuronally-mediated contractions in human and rat colon

After first obtaining consistent responses EFS elicited monophasic contractions of 8.3 ± 0.5 mN (n=4) in human and 6.9 ± 0.7 mN (n=8) in rat colon.

Time-matched vehicle controls were without effect (human: 1 ± 3.7 % inhibition, n= 3; rat: 5.9 ± 2% inhibition, n=4). However, in the presence of TTX (10^{-6} M) the resulting amplitude of EFS-contraction was 0.1 ± 0.3 mN (n=4) in human and was of 0.5 ± 0.3 mN (n=4) in rat, with an inhibition respectively equal to 97.9 ± 0.5 % ($P<0.05$) (n=4), and to 96.6 ± 4.2% ($P<0.05$) (n=4). In presence of ATR (10^{-6} M) the resulting amplitude was 0.4 ± 0.1 mN with an inhibition equal to 98.6 ± 0.3% ($P<0.05$) (n=4) in human, and was 0.1 ± 0.8 mN (amplitude) with an inhibition of 97.2 ± 1.7% ($P<0.05$) (n=4) in rat (Figure 6).

Figure 6


A)
B)
10 s
10 mN
30 - 40 min
TTX 10-6 M
ATR 10-6 M


C)
Amplitude (mN)
10
8
6
4
2
0
*
control
TTX 10-6 M
ATR 10-6 M

D)

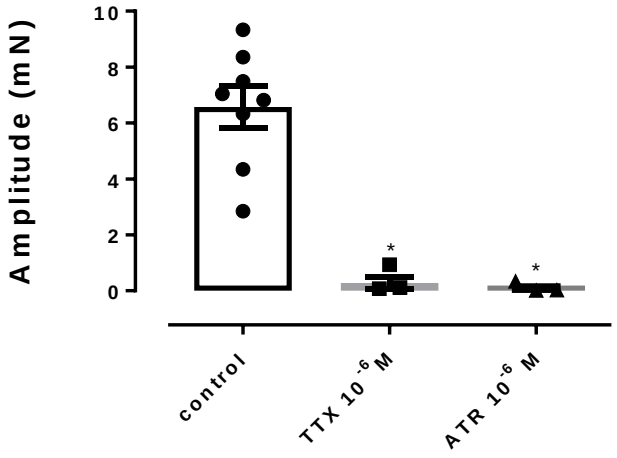


Figure 6: Effect of TTX and ATR on EFS-evoked contraction in human and rat colon.

The representative contraction evoked by EFS before and after administration of TTX or ATR is showed in panel (A) for human and in panel (B) for rat colon. Panels (C) and (D) show the amplitude of EFS-contractions respectively for human and rat colon. Each point represents the mean of n patients and animals: control n= 4, TTX n= 4, ATR= n= 4 in human; control n= 8, TTX n= 4, ATR n= 4 in rat. Vertical lines show standard error of mean. EFS (frequency 5 Hz, pulse width 0.5 ms, for 10 s every 1 min). *=P<0.05 shows the statistical significance between the concentrations of TTX and ATR tested on EFS-contraction control (*t*-tests). Control= distilled water, TTX= tetrodotoxin, ATR= atropine, EFS= Electrical Field Stimulation.

II.II Effect of BEO on neuronally-mediated contractions in human and rat colon

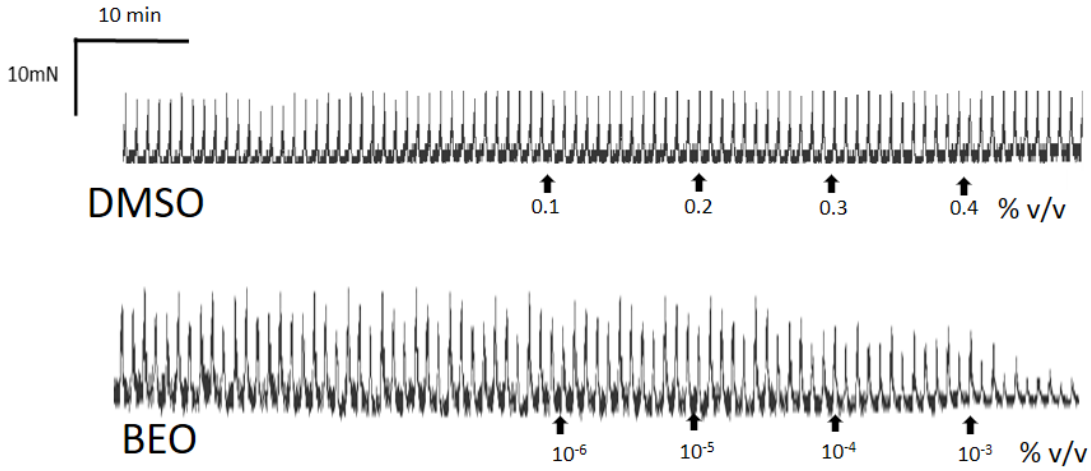
In time-matched, vehicle-controlled studies, comparing the EFS-evoked contractions between the beginning and at the end of the experiment, no statistically significant difference were observed (P>0.05) (Figure 7).

For the human colon, cumulative application of BEO (10^{-6}-10^{-3} % v/v) reduced the amplitude of EFS-evoked contractions in a concentration-dependent manner, the activity being consistently observed between 10^{-5} to 10^{-3} % v/v. In these experiments, the results obtained using ascending and descending colon were combined; similar activity was observed when using either of these tissues (a statistical comparison was not appropriate given the low numbers of tissues used from each region) (see Figure 7 for combined results and Table 3 for the singular results obtained in ascending and descending colon). Overall, the apparent $p\text{IC}_{50}$ was 3.8 ± 0.3 and the highest concentration tested showed an apparent I_{max} of 55.8 ± 4.2%, (P<0.05) (n = 5) (Figure 7 and Table 3).

In proximal and distal colon of the rat, application of the highest tested concentration of BEO inhibited the EFS-evoked contractions with an apparent $p\text{IC}_{50}$= 4 ± 0.3, and an apparent I_{max}= 56.3 ± 2.2, (P<0.05) (n = 4) (Figure 7 and Table 4). No statistical difference was observed between the proximal and distal colon (P>0.05).

Figure 7

A)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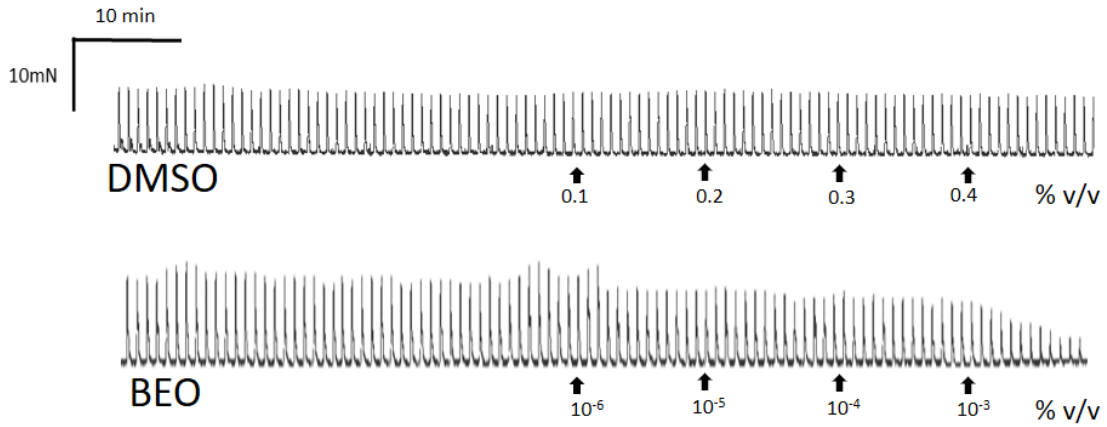
% v/v
BEO
10-6
10-5
10-4
10-3
% v/v

B)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BEO
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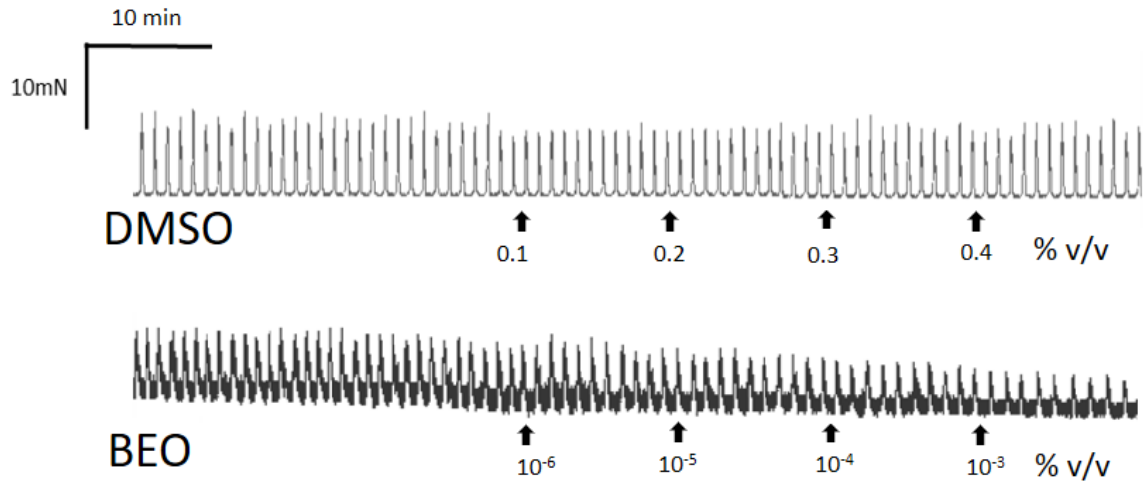
% v/v

C)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BEO
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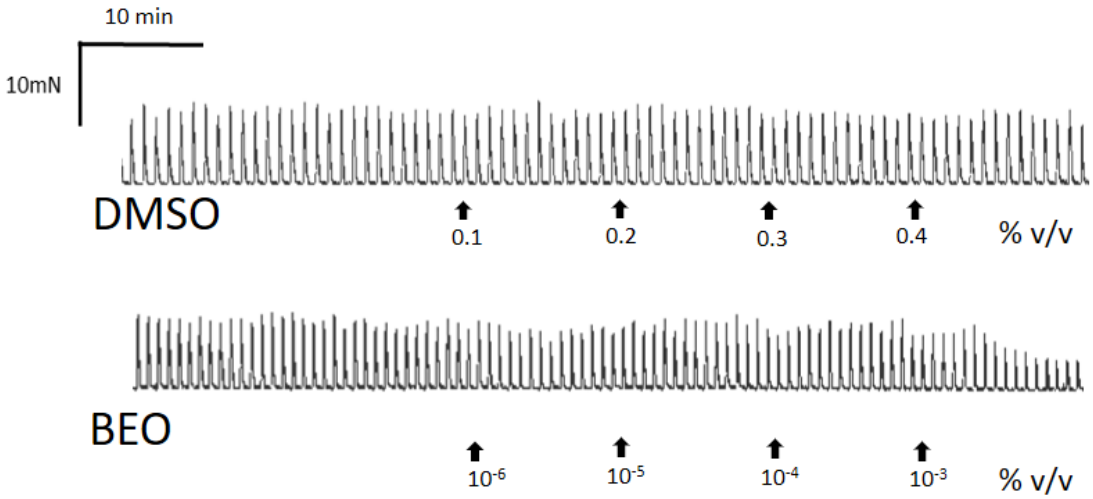
10-3
% v/v

D)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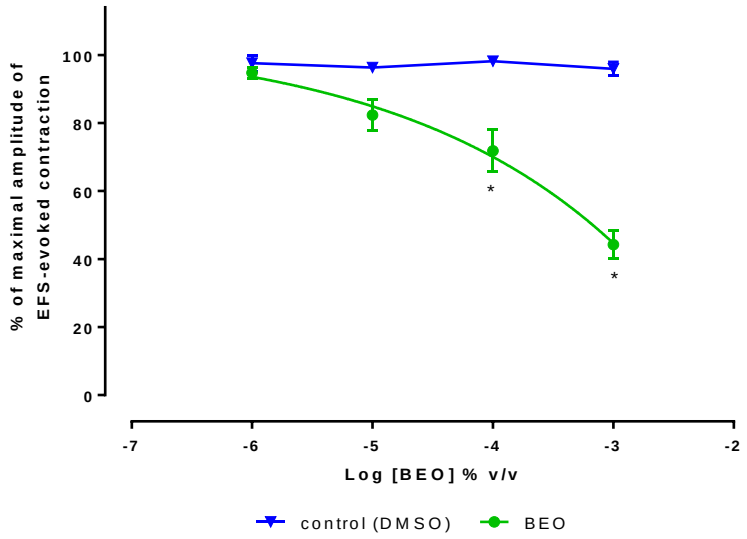
E)



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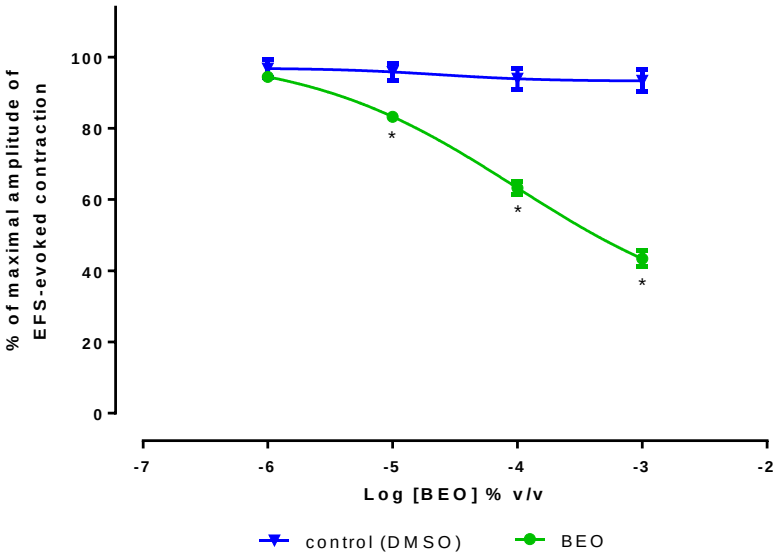


Figure 7: Effect of BEO on EFS-evoked contractions in human and rat colon.

Panels (A-D) show the experimental records illustrating the pharmacology of responses to BEO in human and rat isolated colon: (A)

human descending colon, (B) human ascending colon, (C) rat proximal colon and (D) rat distal colon. Panels (E) and (F) show concentration–response curves for BEO, respectively, from combined data obtained in human ascending and descending and in rat proximal and distal colon. Each point represents the mean of n patients or animals: DMSO (n= 4 in human and n= 5 in rat); BEO in human, n=5 (of which n= 2 ascending and n= 3 descending colon); BEO in rat, n=8 (of which n= 4 proximal, n= 4 distal colon). The cumulative concentration-effect were fitted by non-linear regression to a four-parameter Hill equation. Vertical lines show standard error of mean. EFS (5 Hz, pulse width 0.5 ms, for 10s every 1 min). BEO= bergamot essential oil, DMSO= dimethylsulphoxide, EFS= Electrical Field Stimulation. * $P \leq 0.05$ versus control (*t*-tests).

II.III Effect of linalool, linalyl acetate and limonene on neuronally-mediated contractions in human and rat colon

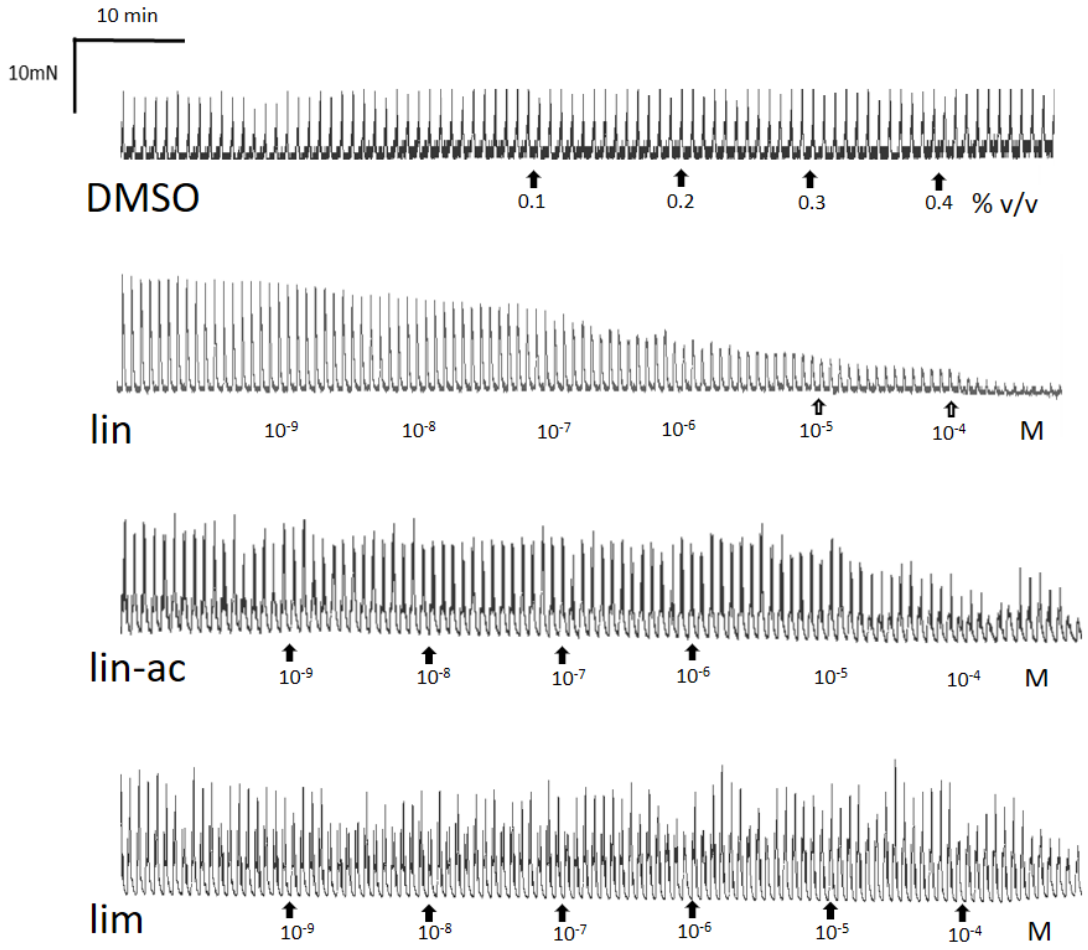
In human colon, administration of linalool, linalyl acetate or limonene (10^{-9}-10^{-4} M), resulted in a concentration-dependent inhibition of EFS-evoked contractions (Figure 8 to see combined results and Table 3 to see all singular results obtained in ascending and descending colon). However, this effect was greater for linalool showing an apparent pIC_{50}=

6.7 ± 0.2 and an apparent I_{max}= 76.8 ± 6.9%, (P<0.05) (n= 4) (Figure 8 and Table 9). The inhibitory effect of linalyl acetate had an apparent pIC_{50}= 4.4 ± 8 and an apparent I_{max}= 53.3 ± 2.9, (P<0.05) (n= 4) (Figure 8 and Table 3). The lowest activity was observed for limonene with an apparent pIC_{50}= 5.5 ± 0.2, apparent I_{max}= 27.5 ± 4.3%, (P<0.05) (n= 3) (Figure 8 and Table 3). In general, similar activity was observed in the ascending and descending colon, although the n-values for each region were too small for a meaningful statistical comparison (Figure 8 and Table 3).

Inhibition of EFS-meditated contraction was also observed in the rat isolated colon, where again, linalool was the most effective of the compounds (Figure 9 to see combined results, and Table 4 to see all singular results obtained in proximal and distal colon). In particular, linalool showed the following apparent pIC_{50}= 5.8 ± 0.1 and apparent I_{max}= 75.3 ± 1.9, (P<0.05) (n= 4) (Figure 9 and Table 4). Linalyl acetate gave an inhibitory effect equal to apparent pIC_{50}= 7 ± 0.2 and an apparent I_{max}= 49.5 ± 1.7%, (P<0.05) (n=4). Limonene, also in this case, showed the lowest inhibitory effect with an apparent pIC_{50}= 6.1 ± 0.3, and apparent I_{max}= 24.7 ± 1.5%, (P<0.05) (n=4) (Figure 9 and Table 4). Similar activity, without any statistical significance (P>0.05) was observed in proximal and distal colon (Figure 9 and Table 4).

Figure 8

A)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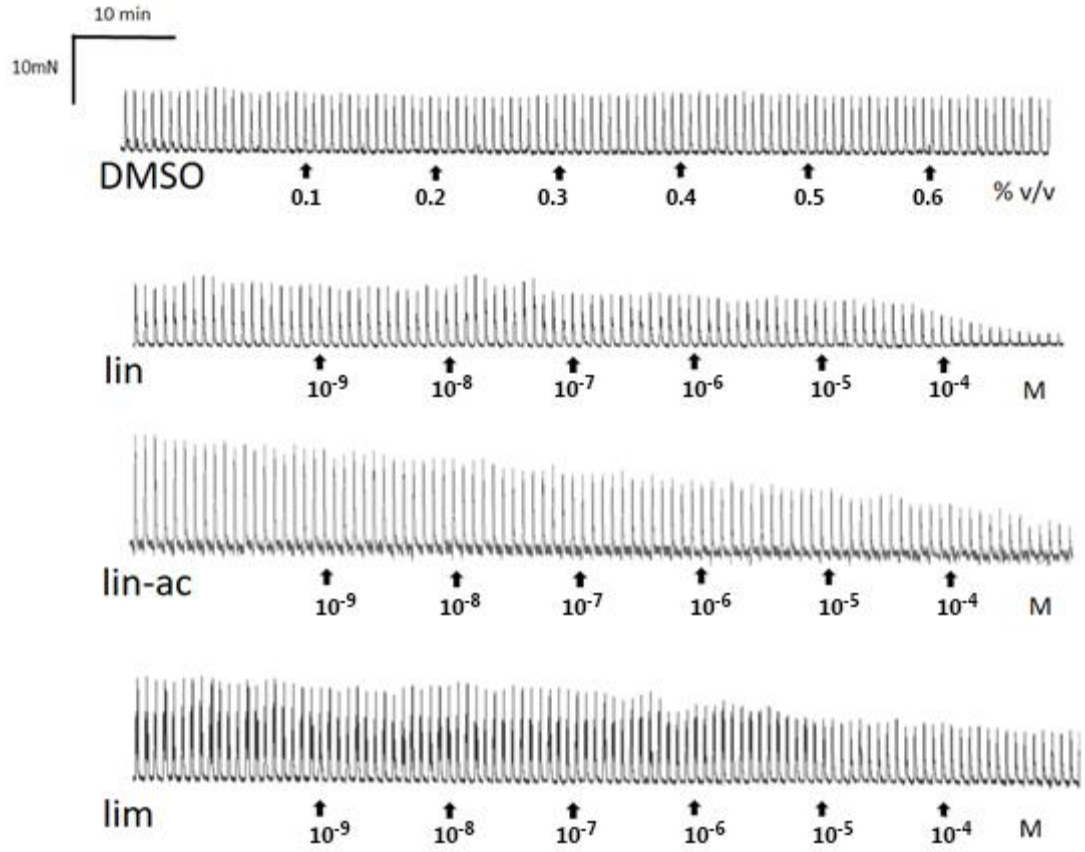
B)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10-4
M

C)

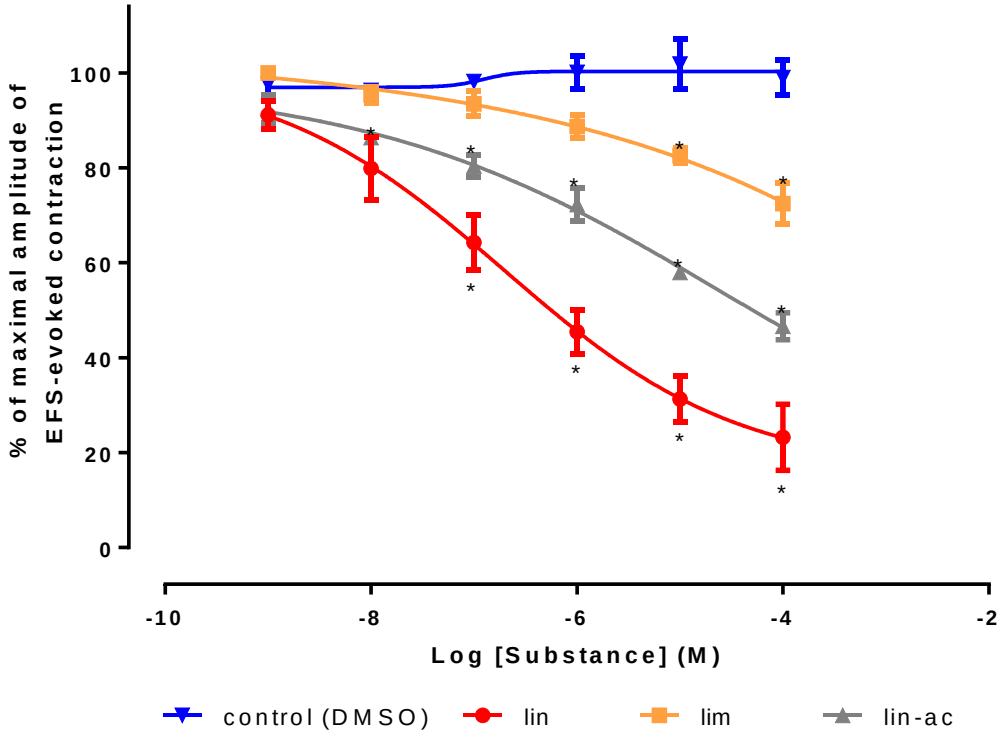


Figure 8: Effect of components of BEO on EFS-evoked contractions in human colon.

Panels (A) and (B) show experimental records illustrating the pharmacology of responses to linalool, linalyl acetate and limonene in: (A) human ascending colon (B) human descending colon.

Panel (C) shows the concentration–response curves determined by linalool, linalyl acetate and limonene. Each point represents the mean

and standard error of the mean of n patients: DMSO n= 4 (n= 2 ascending and n= 2 descending colon), BEO n= 5 (n= 2 ascending and n= 3 descending colon); linalool n= 4 (n= 2 ascending and n= 2 descending colon); linalyl acetate n= 4 (n= 2 ascending and n=3 descending colon); limonene n= 3 (n= 2 ascending and n= 3 descending colon). The cumulative concentration-effect were fitted by non-linear regression to a four-parameter Hill equation. Vertical lines show standard error of mean. EFS (5 Hz, pulse width 0.5 ms, for 10 s every 1 min). lin= (-)-linalool, lin-ac= linalyl acetate, lim = (R)-(+)-limonene, DMSO= dimethylsulphoxide, EFS= electrical field stimulation. * $P \leq 0.05$ versus control (*t*-tests).

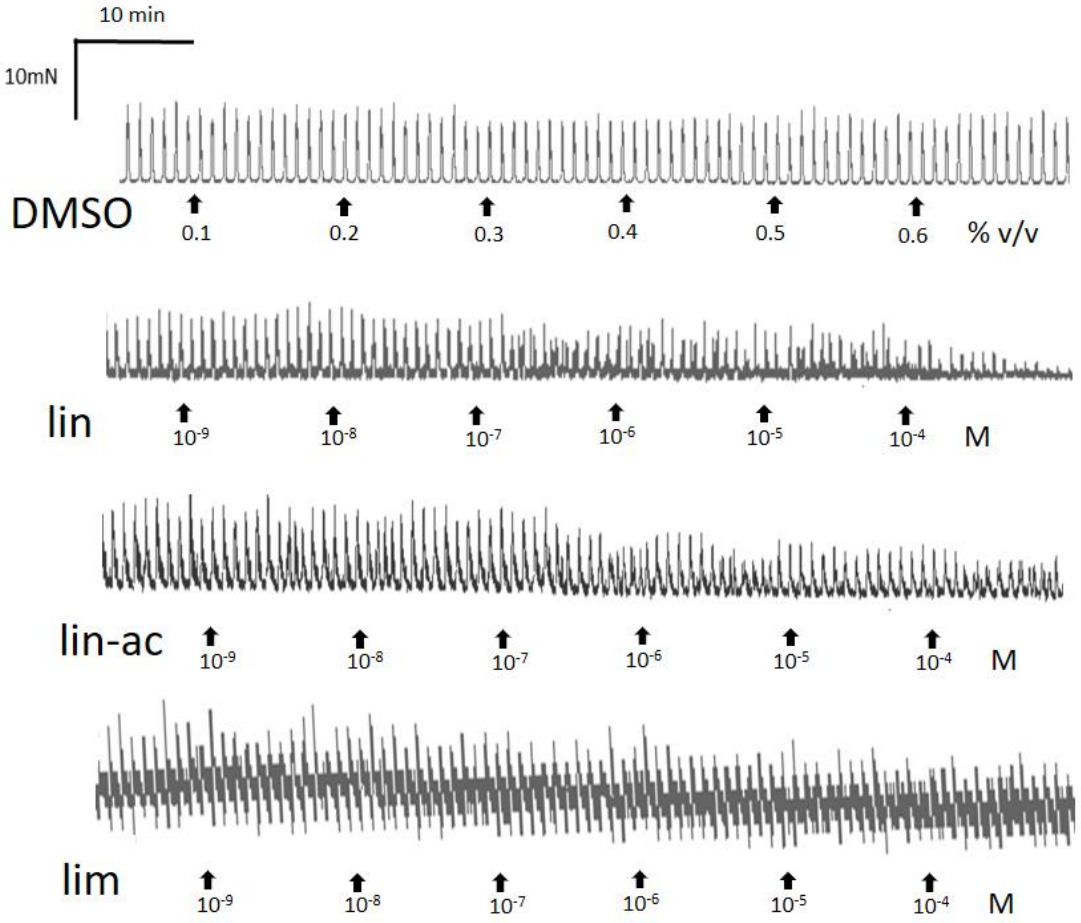
Table 3: Apparent *p*IC$_{50}$ and apparent I$_{max}$ for the inhibition of EFS-evoked contractions in human colon.

	Human								
	Ascending			**Descending**			**Ascending+Descending**		
	apparent *p*IC$_{50}$	**apparent I$_{max}$ (%)**	**n**	**apparent *p*IC$_{50}$**	**apparent I$_{max}$ (%)**	**n**	**apparent *p*IC$_{50}$**	**apparent I$_{max}$ (%)**	**n**
Control (DMSO)	-	4.6 ± 1.4	2	-	3.2 ± 3.2	2	-	3.9 ± 2.7	4
BEO	4.7 ± 0.3	59.1 ± 6.6	2	3.1 ± 0.6	53.1 ± 11.3	3	3.8 ± 0.3	55.8± 4.2*****	5
Linalool	6.3 ± 0.3	88.8 ± 5.4	2	6.8 ± 0.2	64.8 ± 3.2	2	6.7 ± 0.2	76.8± 6.9*****	4
Linalyl acetate	4.7 ± 0.6	58.4 ± 0.4	2	6.3 ± 0.3	48.4 ± 0.2	2	4.4 ± 8	53.3 ± 2.9*****	4
Limonene	5.2 ± 0.3	30.6 ± 5.3	2	5.9	21.4	1	5.5 ± 0.2	27.5 ± 4.3*****	3

Each point represents the mean and standard error of the mean of n patients, *=P<0.05 shows the statistical significance between the highest concentrations of BEO, linalool, linalyl acetate and limonene tested on EFS-contraction versus EFS-contraction control (*t*-test). BEO = bergamot essential oil; DMSO= dimethylsulphoxide.

Figure 9

A)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lin-ac
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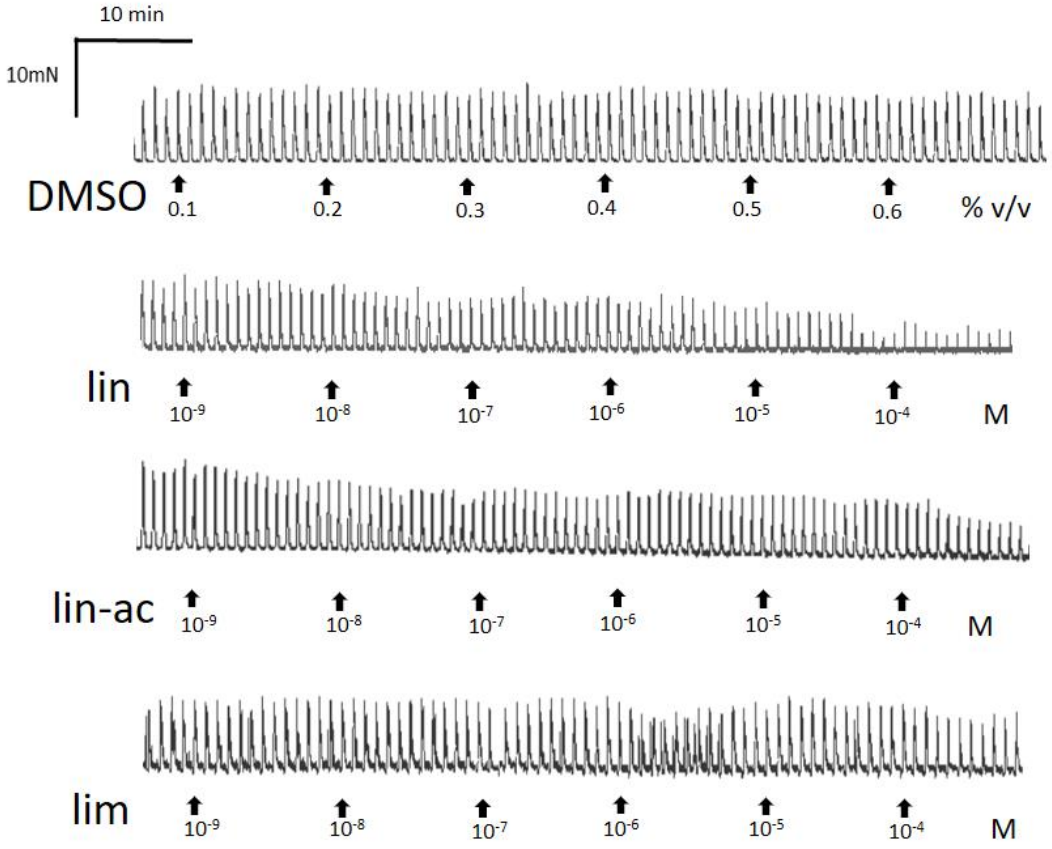
M

B)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C)

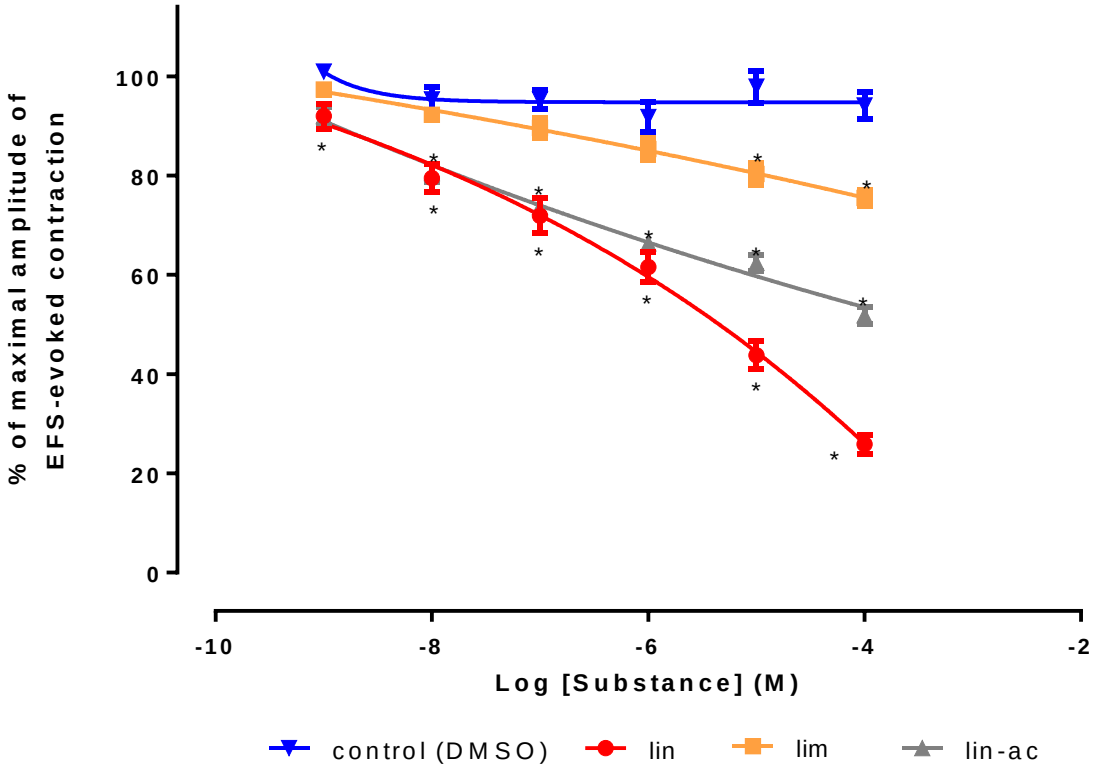


Figure 9: Effect of BEO and its main components on EFS-evoked contractions in rat colon.

Panels (A) and (B) show experimental records illustrating the pharmacology of responses to linalool, linalyl acetate and limonene in: (A) proximal colon (B) distal colon. Panel (C) shows the concentration–response curves determined by linalool, linalyl acetate and limonene. Each point represents the mean of n animals: DMSO n= 4 (of which n= 2 proximal and n= 2 distal colon), BEO, linalool, linalyl acetate and

limonene n= 8 (of which n= 4 proximal and n= 4 distal colon). The cumulative concentration-effect were fitted by non-linear regression to a four-parameter Hill equation. Vertical lines show standard error of mean. EFS (5 Hz, pulse width 0.5 ms, for 10 s every 1 min). lin= (-)-linalool, lin-ac= linalyl acetate, lim= (R)-(+)-limonene, DMSO= dimethylsulphoxide, EFS= Electrical Field Stimulation. * $P \leq 0.05$ versus control (*t*-tests).

Table 4: Apparent *p*IC$_{50}$ and apparent I$_{max}$ for the inhibition of EFS-evoked contractions in rat colon.

	Rat								
	Proximal			Distal			Proximal+Distal		
	apparent *p*IC$_{50}$	apparent I$_{max}$ (%)	n	apparent *p*IC$_{50}$	apparent I$_{max}$ (%)	n	apparent *p*IC$_{50}$	apparent I$_{max}$ (%)	n
Control (DMSO)	-	6.8 ± 4.7	3	-	4.9 ± 3.5	2	-	5.9 ± 2.7	5 (3 rats)
BEO	4.2 ± 0.2	60.8 ± 3.49*	4	4.1 ± 0.1	53.7 ± 2.4*	4	4 ± 0.3	56.3 ± 2.2*	8 (4 rats)
Linalool	5.8 ± 0.2	77.8 ± 1.3*	4	5.8 ± 0.2	69.4 ± 3*	4	5.8 ± 0.1	75.3 ± 1.9*	8 (4 rats)
Linalyl acetate	6.8 ± 0.3	44.5 ± 2.2*	4	7 ± 0.3	49.7 ± 3*	4	7 ± 0.2	49.5 ± 1.7*	8 (4 rats)
Limonene	5.9 ± 0.3	25.2 ± 2.1*	4	6.4 ± 0.4	23.1 ± 2.2*	4	6.1 ± 0.3	24.7 ± 1.5*	8 (4 rats)

Each point represents the mean and standard error of the mean of n animals, *=P<0.05 shows the statistical significance between the highest concentrations of BEO, linalool, linalyl acetate and limonene tested on EFS-contraction versus EFS-contraction control (*t*-tests). BEO= bergamot essential oil; DMSO= dimethylsulphoxide.

II.IV Myogenic KCl-evoked contractions in human and rat colon

Preliminary cumulative concentration-effect curves for KCl (from 20 mM to 120 mM) showed that 40-60 mM KCl caused ~ 40% of the maximum contraction (Figure 10 and 11). These submaximally-effective concentrations were then used for all subsequent experiments to evaluate the actions of BEO and linalool.

Application of KCl (40-60 mM) caused a contraction, the amplitude of which declined slowly but in an approximately constant manner (Figure 10 and 11). In human colon the pEC_{50} = 1.9 ± 1.1 (n= 4) (Table 5), and in rat colon the pEC_{50} = 1.2 ± 0.9 (n= 4) (Table 7).

II.V Effect of BEO and linalool on submaximally-effective concentration of KCl in human and rat colon

In time-matched vehicle control experiments for both the human and rat colon, the contraction induced by KCl (40-60 mM) at the beginning and at the end of the experiment was not statistically significantly different (P>0.05) (Figure 10 and 11). The I_{max}= 3.2 ± 3.3 %: n= 4 in human; I_{max} = 0.9 ± 5.1 %; n=4 in rat.

The effect of BEO (10^{-5}-10^{-3} % v/v) and its constituent linalool (10^{-7}-10^{-4} M) was examined for their ability to relax the muscle strips pre-

contracted with KCl (40-60 mM). Overall, the results obtained from human and rat experiments showed that cumulative application of BEO and linalool caused a concentration-dependent inhibition of the KCl-induced contraction, with a greater sensitivity in human tissues than in rat tissues.

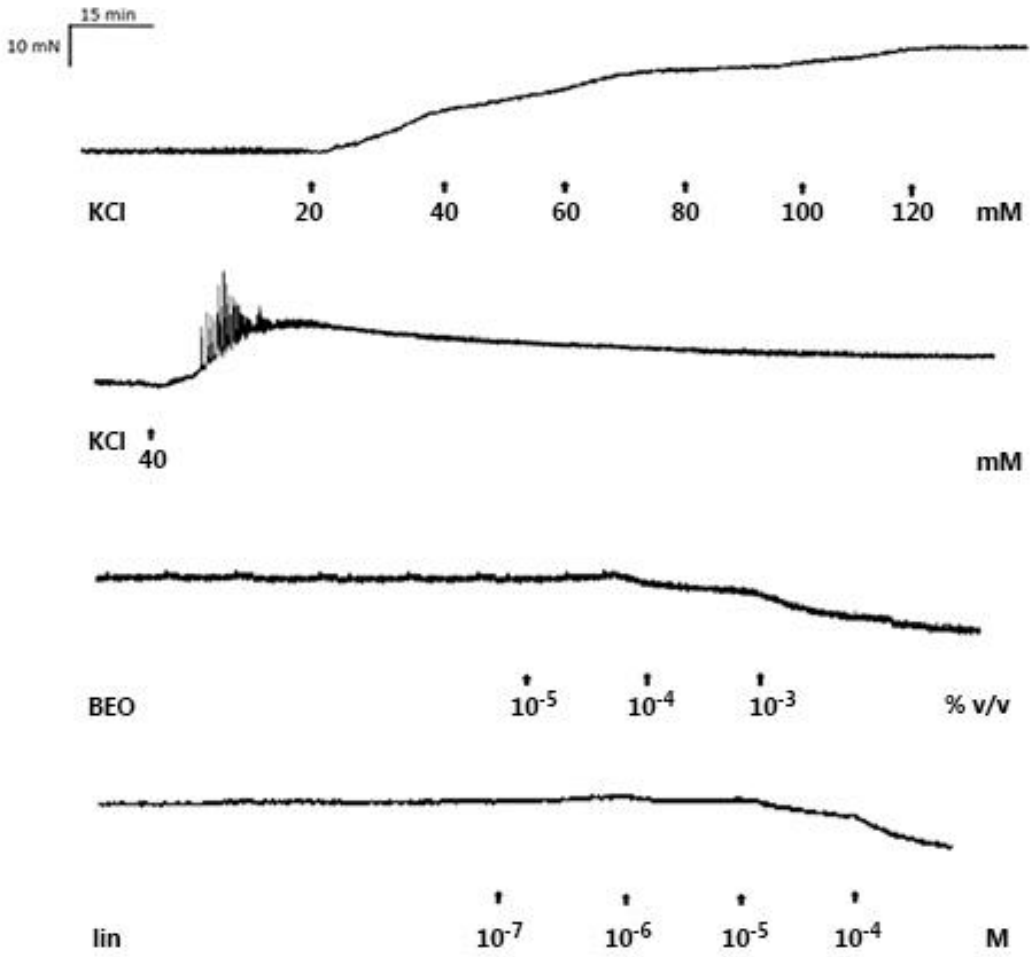
In human colon, the apparent I_{max} = 37.5 ± 4.2 % ($P<0.05$) for BEO, with an apparent pIC_{50}= 4.4 ± 0.3 (n= 4) (Figure 10 to see the combined results and Table 6 to see all singular results obtained in ascending and descending colon). Linalool was more potent than BEO showing an apparent I_{max}= 53.8 ± 4.6%, ($P<0.05$), and an apparent pIC_{50}= 5.6 ± 0.4, (n=4) (Figure 10 and Table 6). Similar activity was found in ascending and descending human colon.

In rat colon application of BEO determined an apparent I_{max}= 26.3 ± 3.8%, ($P<0.05$), and an apparent pIC_{50}= 4.1 ± 0.5 (n= 4) (Figure 11 to see combined results and Table 8 to see all singular results obtained in proximal and distal colon). The results obtained using linalool indicate a better efficacy and potency compared with BEO, in particular the apparent I_{max} was 36.1 ± 4.8%, ($P<0.05$), and the apparent pIC_{50} was 5.4 ± 0.3 (n=4) (Figure 11 and Table 8). Statistically significant difference ($P<0.05$) was found in the effect produced by linalool between proximal and distal colon. In proximal colon the apparent I_{max} was 47.6± 2.3% and the apparent pIC_{50} was 5.6 ± 0.2 (n=4), while, in distal colon the apparent

I_{max} was 21.7 ± 1% and apparent pIC_{50} was 4.8 ± 0.2 (n=4). No statistical difference was observed in the apparent I_{max} and pIC_{50} of BEO between proximal and distal colon (P>0.05) (Table 8).

Figure 10

A)



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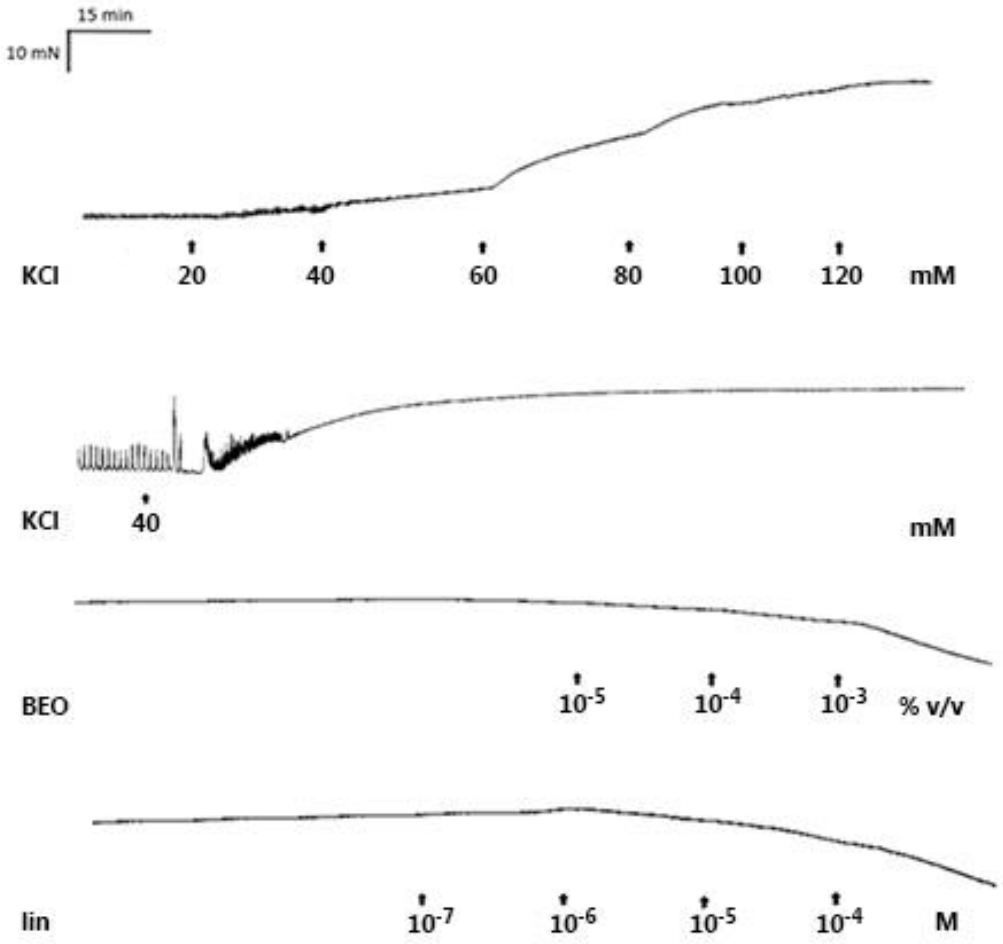
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KCl
20
40
60
80
100
120
mM
KCl
40
mM
BEO
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lin
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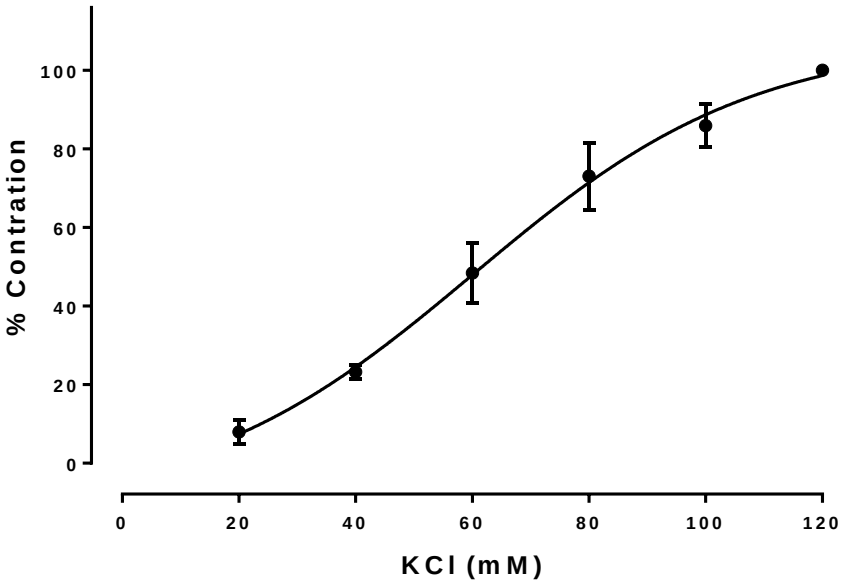
10-6
10-5
10-4
M

C)


% Contration
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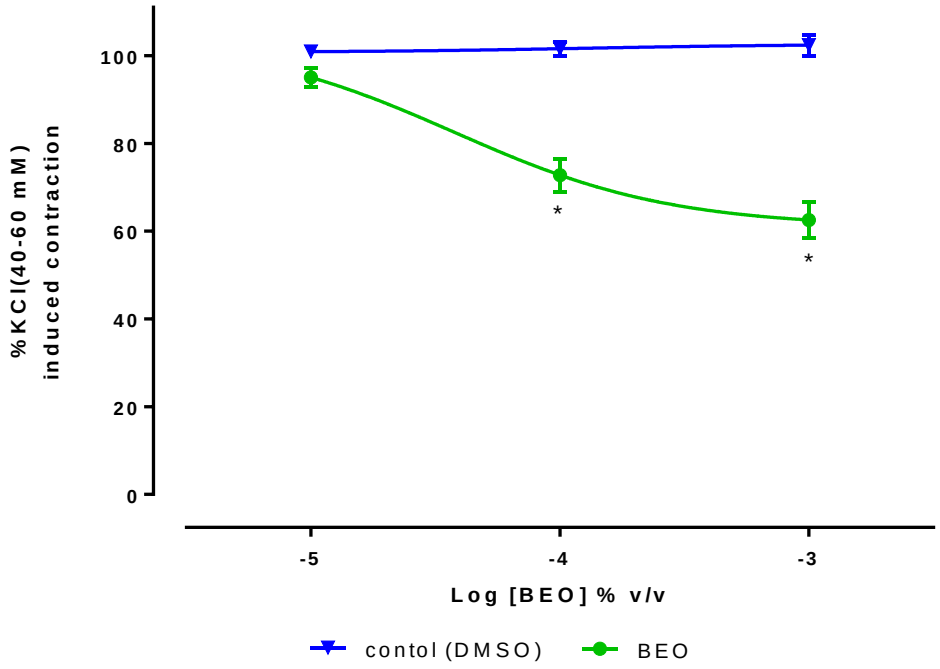
80
60
40
20
0
0
20
40
60
80
100
120
KCl (mM)

D)


%KCl(40-60 mM)
induced contraction
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80
60
40
20
0
*
*
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-4
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Log [BEO] % v/v
contol (DMSO)
BEO

E)

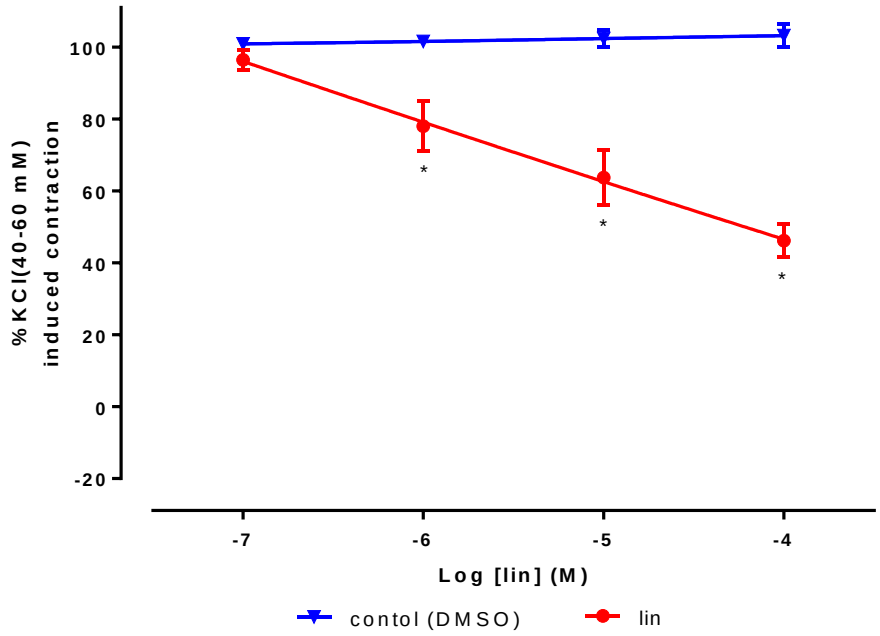


Figure 10: Effect of BEO and linalool on contraction evoked by submaximally-effective concentration of KCl in human colon.

Panels (A) and (B) show experimental records illustrating the pharmacology of responses to BEO and linalool in (A) ascending and (B) descending human colon. Panel (C) show the concentration–response curve for KCl (20mM to 120mM; n= 4 of which n= 2 ascending and n= 2 descending colon). Using submaximally-effective concentrations of KCl (40-60 Mm), panel (D) shows the concentration–response curve for BEO (n= 4 of which n= 2 ascending and n= 2 descending), and panel (E) shows the concentration–response curve for linalool (n= 4 of which n= 2

ascending and n= 2 descending). Each point represents the mean of n patients. Vertical lines show standard error of mean of results combined from ascending and descending colon. BEO= bergamot essential oil; lin= (-)-linalool, DMSO= dimethylsulphoxide. * P≤0.05 versus control (*t*-tests).

Table 5: Comparison of *p*EC$_{50}$ for KCl in human colon.

	Human					
	Ascending		**Descending**		**Ascending+Descending**	
	***p*EC$_{50}$**	**n**	***p*EC$_{50}$**	**n**	***p*EC$_{50}$**	**n**
KCl	2±1.4	2	1.8±0.7	2	1.9±1.1	4

Each value represents the mean and standard error of the mean of n patients.

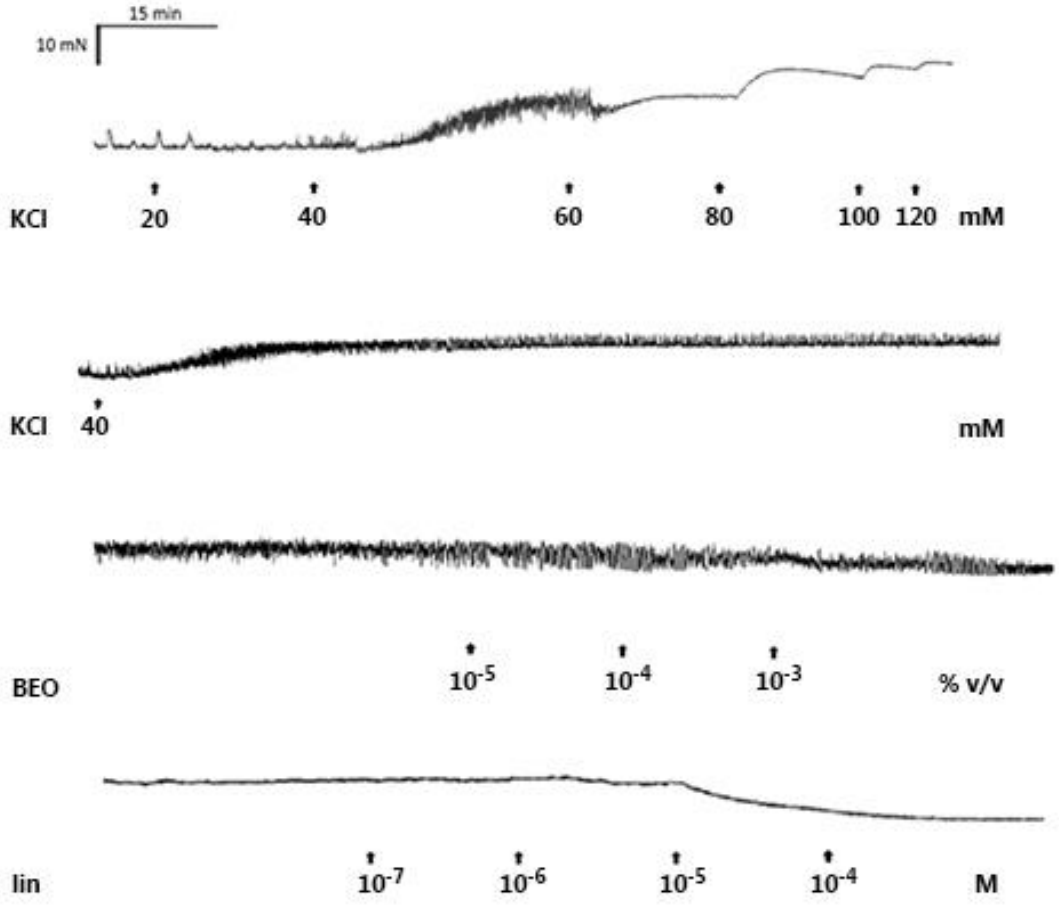
Table 6: Apparent pIC_{50} and apparent I_{max} for the inhibition of contraction evoked by submaximally-effective concentration of KCl in human colon.

	Human								
	Ascending			**Descending**			**Ascending+Descending**		
	apparent pIC_{50}	**apparent I_{max} (%)**	**n**	**apparent pIC_{50}**	**apparent I_{max} (%)**	**n**	**apparent pIC_{50}**	**apparent I_{max} (%)**	**n**
Control (40-60mM KCl)	-	6.41 ± 6.3	2	-	0.2 ± 0.1	2	-	3.2 ± 3.3	4
BEO	4.4 ± 0.8	38.8 ±19.6	2	4.5 ±0.2	36.1±2.9	2	4.4 ± 0.3	37.5 ± 4.2*	4
linalool	5.3 ± 0.2	58.9 ± 4.3	2	5.9 ± 0.5	52.3±4.2	2	5.6 ± 0.4	53.8 ± 4.6*	4

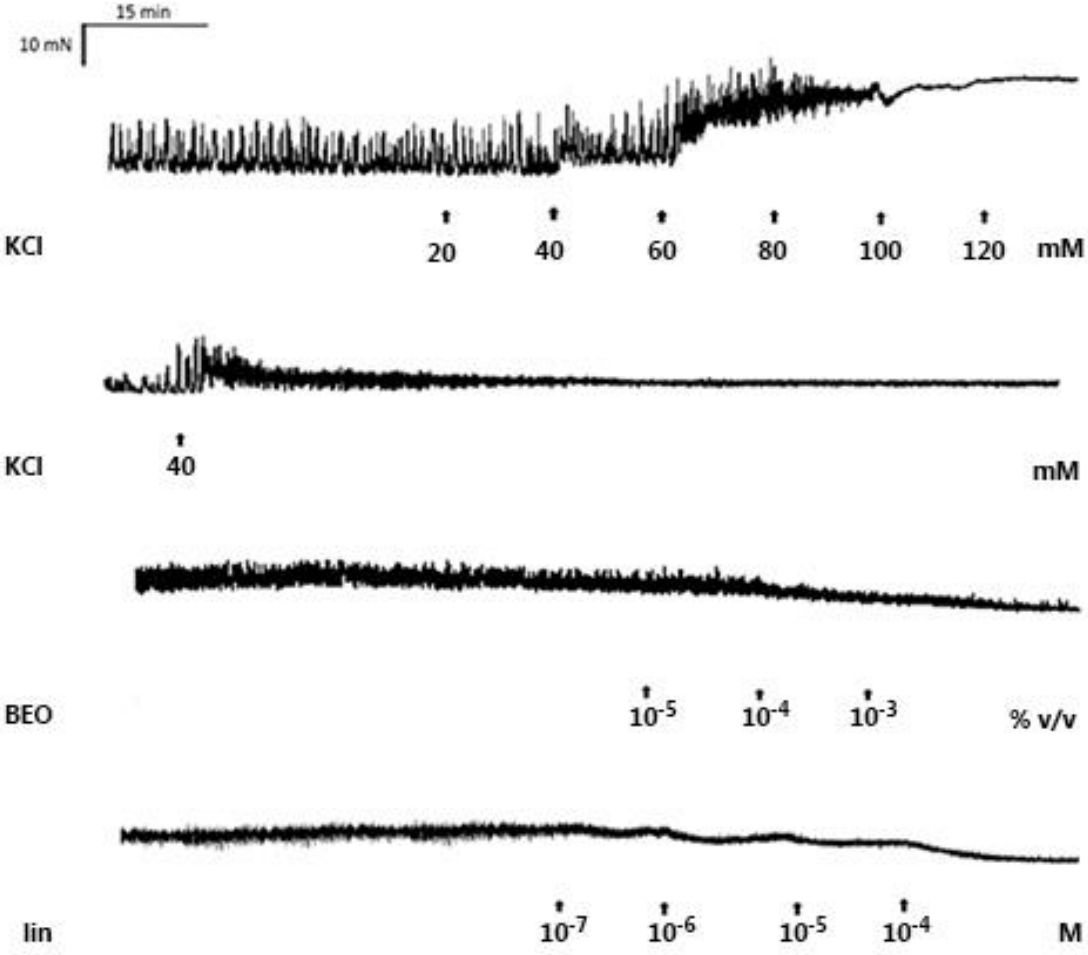
Each value represents the mean and standard error of the mean of n patients, *=P<0.05 shows the statistical significance between the highest concentrations of BEO or linalool tested on KCl-contraction versus KCl-contraction control (*t*-tests). BEO= bergamot essential oil; control= KCl 40-60mM.

Figure 11

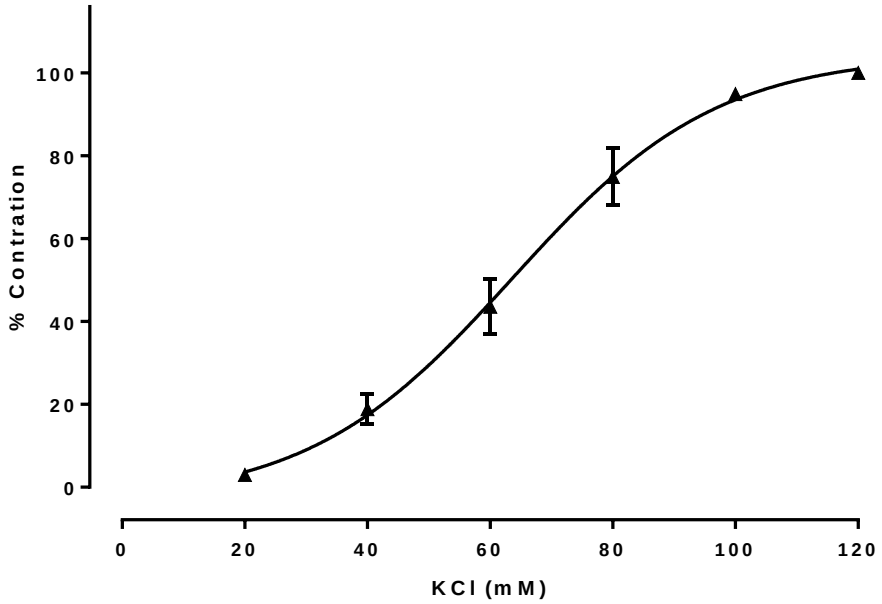
A)


15 min
10 mN
KCl
20
40
60
80
100
120
mM
KCl
40
mM
BEO
10-5
10-4
10-3
% v/v
lin
10-7
10-6
10-5
10-4
M

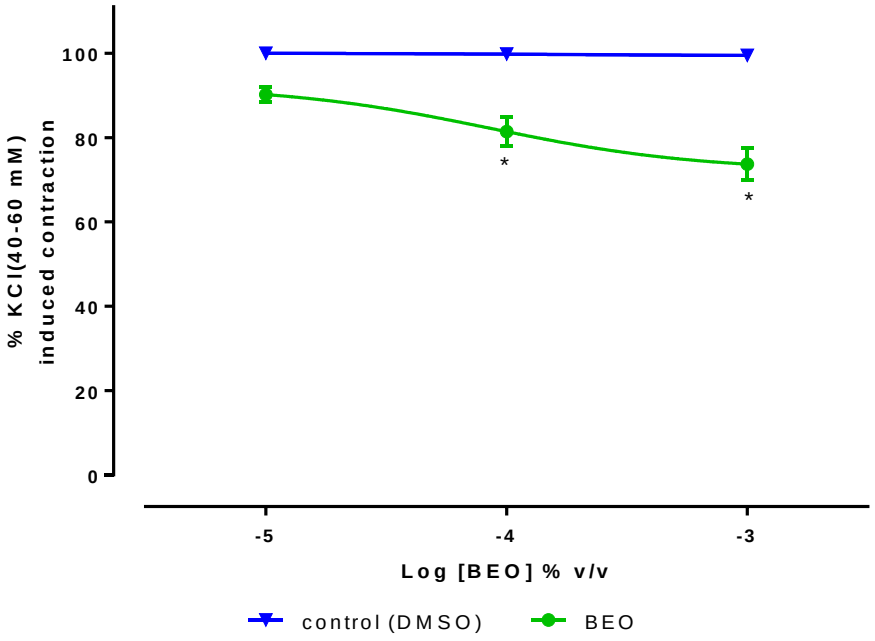
B)


15 min
10 mN
KCl
20
40
60
80
100
120
mM
KCl
40
mM
BEO
10-5
10-4
10-3
% v/v
lin
10-7
10-6
10-5
10-4
M

C)


% Contration
100
80
60
40
20
0
0
20
40
60
80
100
120
KCl (mM)

D)


% KCl(40-60 mM) induced contraction
100
80
60
40
20
0
*
*
-5
-4
-3
Log [BEO] % v/v
control (DMSO)
BEO

E)

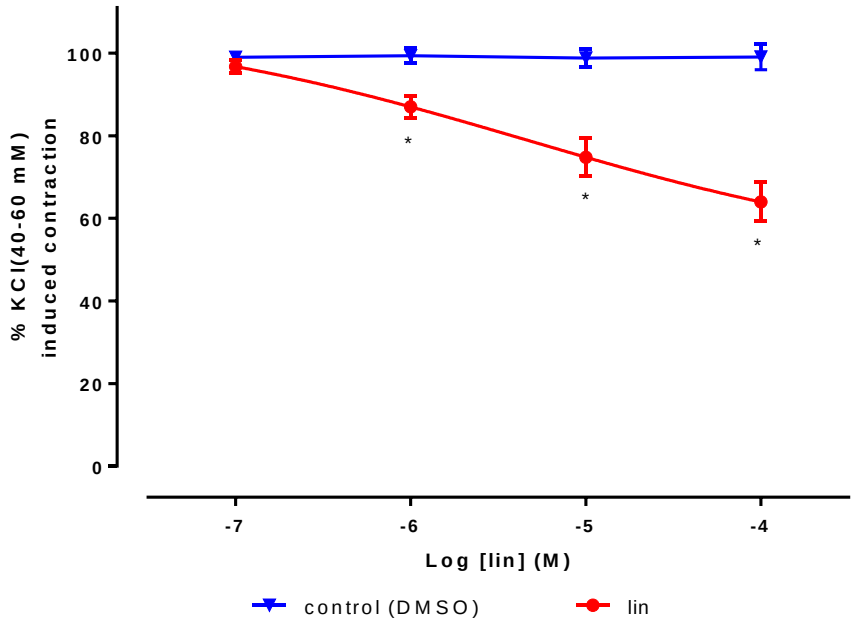


Figure 11: Effect of BEO and linalool on contraction evoked by submaximally-effective concentration of KCl in rat colon.

Panels (A) and (B) show experimental records illustrating the pharmacology of responses to BEO and linalool in (A) proximal and (B) distal rat colon. Panel (C) shows the concentration–response curve for KCl (20mM to 120mM; n=8 of which n=4 proximal and n=4 distal). Panel (D) shows the concentration–response curve for BEO (n=8 of which n=4 proximal and n=4 distal) and panel (E) shows the concentration–response curve for linalool (n=8 of which n=4 proximal and n= 4 distal). Each point represents the mean and standard error of the mean of n animals. Vertical lines show standard error of mean. BEO= bergamot essential oil;

lin= (-)-linalool, DMSO= dimethylsulphoxide. * P≤0.05 versus control (*t*-tests).

Table 7: Comparison of pEC_{50} for KCl in rat colon.

	Rat					
	Proximal		**Distal**		**Proximal + Distal**	
	pEC_{50}	**n**	**PEC_{50}**	**n**	**pEC_{50}**	**n**
KCl	1.3±0.5	2	1.1±0.6	2	1.2±0.9	4

Each value represents the mean and standard error of the mean of n animals.

Table 8: Apparent pIC_{50} and apparent I_{max} for the inhibition of contraction evoked by submaximally-effective concentration of KCl in rat colon.

	Rat								
	Proximal			**Distal**			**Proximal + Distal**		
	apparent pIC_{50}	**apparent I_{max} (%)**	**n**	**apparent pIC_{50}**	**apparent I_{max} (%)**	**n**	**apparent pIC_{50}**	**apparent I_{max} (%)**	**n**
control (40-60mM KCl)	-	1.4 ± 0.73	4	-	0.6 ± 5.6	4	-	0.9 ± 5.1	8 (4 rats)
BEO	4.4 ± 0.4	32.8 ± 3.2*	4	3.7 ± 0.4	19.9 ± 5.5*	4	4.1 ± 0.5	26.3 ± 3.8*	8 (4 rats)
linalool	5.6 ± 0.2	47.6 ± 2.3*	4	4.8 ± 0.2	21.7 ± 1*	4	5.4 ± 0.3	36.1 ± 4.8*	8 (4 rats)

Each value represented the mean and standard error of the mean of n animals, *=P<0.05 shows the statistical significance between the highest concentrations of BEO or linalool tested on KCl-contraction versus KCl-contraction control (*t*-tests). BEO= bergamot essential oil; lin= (-)-linalool, control = KCl 40-60mM.

DISCUSSION

Preliminary experiments: The inhibitory effect of BEO and its constituents in jejunum, ileum and colon of rat

Initially BEO and its major constituents were examined on the various regions of the rat intestinal tract (jejunum, ileum and colon) to identify the presence of any pharmacological activity. In these experiments the contractions induced by ACh, were inhibited by BEO in a concentration-dependent manner and abolished by the highest concentration tested in all three regions of the rat intestine examined. A comparison of the inhibitory effects of the individual constituents showed that linalool was equieffective in all type of tissues tested with an ability to also inhibit SP and 5-HT contractions in rat colon. However, linalyl acetate was only effective in the jejunum and ileum, and limonene was inactive. Overall, these data supported the hypothesis that BEO has an inhibitory effect in the intestinal tract, but it was not possible to highlight a potential action on enteric neurotransmission.

Inhibition by BEO and its constituents of neuronally-mediated contractions in human and rat colon

Preliminary experiments showed that the protocol of EFS evoked predominantly cholinergically-mediated contractions in both human and

rat colon (prevented by tetrodotoxin or by atropine). The application of BEO or its constituents inhibited these contractions in a approximately concentration-dependent manner in both regions of human and rat colon. Some species differences were noted. Thus, in the human colon, linalool was the most potent (apparent $p\text{IC}_{50}$ 6.7) and effective (maximum of 76.8% inhibition) inhibitor of neuromuscular function, although whilst linalool remained the most efficacious inhibitor of neuromuscular function in the rat colon (maximum of 75.3% inhibition), the potency of linalyl acetate ($p\text{IC}_{50}$ 7; maximum inhibition of 49.5%) was greater (linalool $p\text{IC}_{50}$ 5.8).

Since it was not possible to know if BEO or its components inhibited the EFS-evoked contractions by an action on the enteric neurons themselves or by an ability to directly inhibit muscle contraction, additional experiments were performed to evaluate the effects of BEO and linalool on muscle contractions evoked by KCl. Thus, the application of KCl produced depolarization of the muscle with subsequent contraction caused by an increase of intracellular [Ca^{2+}] (Karaki et al., 1997). Also in this case linalool was the most potent and efficacious inhibitor of KCl-induced contractions, compared to BEO with greater sensitivity in human ($p\text{IC}_{50}$=5.6, I_{max}= 53.8 human colon; $p\text{IC}_{50}$=5.4, I_{max}= 36.1 rat colon). A statistically significant difference was found only in the effect induced by linalool on proximal and distal rat colon. Together, these data suggested

that although there is a potential ability of BEO and its constituents to inhibit enteric nerve function, it is difficult to distinguish such activity from a direct ability of these substances to act directly on smooth muscle cells.

Comparisons between the actions of BEO and those of its constituents.

In the first phase of the research, BEO caused a concentration-related maximal inhibition of the ACh-induced contraction of all three regions of the rat intestine examined i.e. the jejunum, ileum and colon. The results obtained by testing its constituents, showed that linalool was the most effective at inhibiting the ACh-evoked contractions.

In human and rat colon application of BEO or its constituents inhibited neuronally mediated contractions in a concentration-dependent manner. Observing the inhibition produced by its constituents, linalool was the most effective at inhibiting the EFS-evoked contractions, which were predominantly cholinergically-mediated.

From the results obtained, it was very likely that the inhibitory effect of BEO was mediated by linalool, despite this constituent accounting for ~5.5 % volume of BEO (see Appendix 1). Furthermore, observing the inhibition produced by linalyl acetate and limonene, and

considering their percentage volumes of ~23.6 % (linalyl acetate) and ~48.6 % (limonene) contained in the total volume of BEO, it is possible to suggest that both linalyl acetate and limonene are not the main pharmacologically active components of the phytocomplex (Appendix 1). It is, however, difficult to obtain a more precise analysis of the contributions made by the individual constituents contained within BEO without knowledge of the actual weights / volume of the constituents within the BEO extract, from which molar concentrations might be calculated. Whereby, it was not possible to directly relate the effective molar concentration of the constitutes obtained from the experiments with those present in the BEO.

Mechanisms of action of BEO and its constituents

Returning to the first part of the experiments, linalool was able to inhibit the contractions evoked by ACh, 5-HT and SP. ACh acted on the M_3 receptor, SP acted on the NK_1 and NK_2 receptors and 5-HT acted on the $5HT_2$ receptor (Prins et al., 1997; Tsukamoto et al., 1997; Mule et al., 2000; Anderson et al., 2014). These receptors are G protein-coupled receptors ($G_{q/11}$) which, following the binding with the ligand, determine the activation the second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Barrett et al., 2019; Birdsall et al., 2019; Andrade et al., 2019). IP_3 activate its receptor on endoplasmic reticulum and

mitochondria determining the release of Ca^{2+} in the cytosol and consequently the muscle contraction (McFadzean and Gibson, 2002; Sarna, 2010). DAG active the protein kinase C (PKC) that phosphorylates other proteins involved in the cellular function (McFadzean and Gibson, 2002; Sarna, 2010). Therefore, these results suggest that linalool could act on the receptor or on the pathway of $G_{q/11}$ protein. However, the contraction evoked by KCl is initiated by depolarization of the muscle fibres with consequent opening of the L-type Ca^{2+} channels. The resulting increase of $[Ca^{2+}]$ intracellular causes a contraction (Karaki et al., 1997). BEO and linalool showed an inhibitory effect on the contraction induced by KCl, suggesting an ability to act directly on smooth muscle cells. In a study conducted with tissue bath technique (Kang et al., 2013), BEO (10^{-2} to 2 $x10^{-1}$ v/v %) gave vasorelaxation of mouse aorta. Kang et al (2013) suggested that the vasorelaxation might be due to an ability of BEO to induce hyperpolarization by activation of K^+-channels, an effect partially inhibited by tetraethylammonium chloride a K^+-channel blocker. Furthermore, the $CaCl_2$ induced contraction suppressed in the tissues pretreated with BEO, highlighting a block of membrane Ca^{2+}-channels (Kang et al., 2013).

Although it seems likely that in their ability to inhibit neuromuscular contractions a major activity of BEO and its constituents was to act directly at the smooth muscle to cause muscle relaxation, it

remains a possibility that BEO or its constituents might also directly inhibit enteric nerve function. In this activity the potency of BEO and its constituents appeared to be greater than that measured when tested against the KCl-evoked contractions. It is difficult to know with any certainty if such differences reflect the differences in types of assay or if this indicates at least some additional ability to directly inhibit neuronal functions.

Vatanparast et al. (2017) suggested that the inhibitory action of linalool (10^{-4} M) in central neurons of *Caucotachea atrolabiata* was exerted mainly on voltage-gated Na^+-channels. The involvement of Na^+ ion was also supported by another study, which investigated the pharmacological effects of linalool on various voltage-gated current in olfactory receptor cells of rat, where linalool (3×10^{-3} M, IC_{50} 5.6×10^{-4} M) showed a better inhibition of Na^+ current compared with other ions currents (Narusuye et al., 2005). It is interesting to note that the concentrations of BEO and linalool in the previous studies, although slightly higher in some cases (Narusuye et al., 2005; Kang et al., 2013) and probably due to the different experimental models used, are very similar to the concentrations used in this study. Together, these data raise the possibility that in the rat and human colon, in addition to a direct inhibitory action on smooth muscle function, BEO and its constituents may inhibit the voltage-gated Na^+-channels within the ENS.

Thus, in summary, it is possible understand that BEO mainly through linalool could be able of acting on different targets, highlighting an ability to influence Na^+, Ca^{2+} and K^+ intracellular levels (Narusuye et al., 2005; Kang et al., 2013; Vatanparast et al., 2017) (see below Figure 12). In nerve cells, it is known that the increase in the intracellular levels of the Na^+ ion is fundamental for initiating the genesis of an action potential, which in turn determines an intracellular increase in the Ca^{2+} ion to guarantee the fusion of synaptic vesicles on the presynaptic membrane. It follows that a reduction in the intracellular levels of Na^+ (due to the action of linalool on the voltage-dependent Na^+ channels) (Narusuye et al., 2005; Vatanparast et al., 2017), reduces the possibility of generating action potential and consequently release of neurotransmitters, including acetylcholine (Cellek et al., 2006).

In muscle cells, linalool may act on K^+-channels and block the membrane voltage-gated Ca^{2+} channels, producing relaxation given by hyperpolarization and by reduction of intracellular [Ca^{2+}] fundamental in the interaction between actin and myosin filaments (Okamura et al., 1993; Kang et al., 2013). Moreover, even if with limited evidence, it is also possible that linalool may inhibit the G protein-coupled receptors ($G_{q/11}$), blocking the contractile response that would ensue. Further studies are needed to confirm the action on the Na^+, K^+, Ca^{2+} channels and on the receptor or on the $G_{q/11}$ protein pathway (Figure 12).

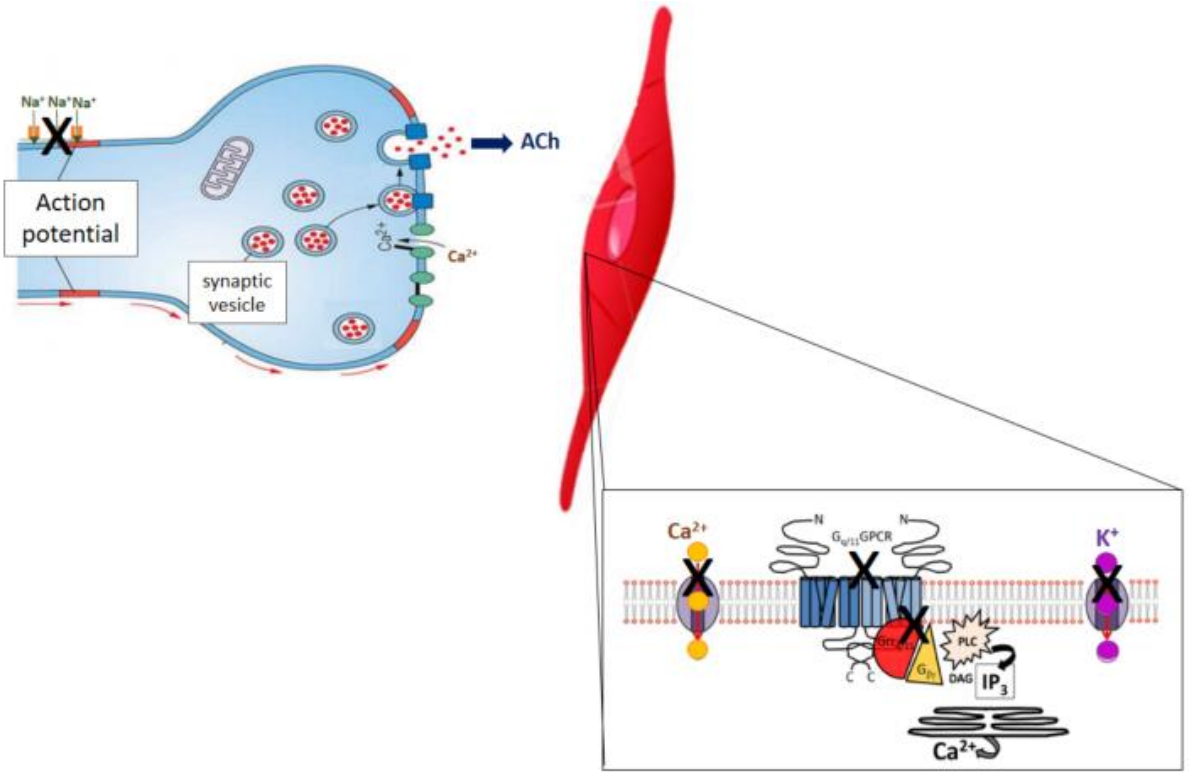


Figure 12: Summary diagram of sites of action.

The picture shows the junction between an enteric neuron (top right) and a smooth muscle cell (top left). Magnification (bottom right) shows membrane targets of smooth muscle cells. The black crosses show the potential targets (Na^+, K^+, Ca^{2+} channels and the receptor or the $G_{q/11}$ protein pathway) inhibited by BEO mainly through the linalool.

Species differences and similarities

The present study demonstrates the ability of BEO and particularly linalool, one of major constituents of BEO, to inhibit human colon neuromuscular contractile activity evoked by EFS and mediated predominantly via activation of cholinergic neurons. Broadly, similar activity was also observed when using EFS to evoke predominantly cholinergically-mediated contractions of rat isolated colon. Nevertheless, small differences suggested species differences in mechanisms of action, where the rank order in terms of potency (apparent $p\mathrm{IC}_{50}$) in human was: linalool > limonene >> linalyl acetate = BEO, and in rat was: linalyl acetate > limonene = linalool >> BEO. However, in both species, in terms of efficacy (apparent I_{max}) linalool was the most efficacious (rank order in human and rat colon was: linalool >> BEO = linalyl acetate >> limonene).

Together, these data suggest that the ability of BEO and its constituents to inhibit neuronally-mediated contractions of the rat colon are similar to those for the human colon. However, the marked differences in potency, compared with the human colon, suggest less dominance of linalool in the overall inhibitory activity of BEO and greater contributions by other constituents of this oil in rat colon.

Limitations

The present research has helped to increase knowledge about BEO, exploring its effect in a new area, the GI tract; however, still many questions remain unanswered. The experimental models made it possible to demonstrate an ability of BEO and its constituents to inhibit human and rat colon motility, but while the action on the smooth muscle was identified with certainty, it was not possible to do so with the action at the level of the ENS. The main limitation of this research is therefore associated with not being able to isolate the effects observed in the ENS from those on smooth muscle.

A further limitation is that for the human colon the technique uses only the muscle tissue, with mucosa removed. This avoids the presence of endogenous interfering substances, released from mucosal or submucosal surfaces (Sanger and Bennett, 1984). The likelihood that an action of BEO or linalool on mucosal functions interfering with the present findings is, however, minimal. Thus, broadly similar data were obtained using rat colon, in which the mucosa was left intact. Nevertheless, further experiments are needed to ascertain if BEO could directly affect epithelial absorptive and secretory functions (using Ussing chambers) or substance release from endocrine cells within the epithelial layer.

The use of human intestinal tissue represents an important advantage of this technique. Thus, demonstrating the effectiveness of

BEO and linalool in human tissue, increases the possibility in obtaining beneficial actions with BEO in patients suffering from intestinal disorders characterized by a high contractile activity. Further research is needed.

Clinical implications

Smooth muscle relaxants can be used to relieve symptoms in patients with intestinal disease such us IBS. IBS is a chronic, functional gastrointestinal syndrome characterized by relapsing abdominal pain and altered bowel habits, with either predominant symptoms of diarrhea and constipation (Schmulson and Drossman, 2017). Currently, a natural remedy, peppermint oil, is widely used in complementary treatment to improve symptoms in patients with IBS (Ford et al., 2008: Alammar et al., 2019). Peppermint oil gives an antispasmodic action associated with its main component menthol, which blocks the Ca^{2+} influx by actions on the L-type Ca^{2+} channels (Amato et al., 2014). In this study, it was observed that linalool is the main antispasmodic component of BEO. It is interesting to observe that both menthol and linalool are monoterpenes. Some studies have compared the effects on Transient Receptor Potential (TRP, a family of ion channels) channels caused by menthol with those obtained from structurally similar substances, including linalool

(Behrendt et al., 2004; Fothergill et al., 2016, Paschke et al., 2017). It appears that only relatively high concentrations of linalool are needed to activate human (10^{-4} M or mouse ($6.7\pm2.0 \times 10^{-3}$M) TRPM8 (Behrendt et al., 2004; Paschke et al., 2017) and to induce a transient increase in short circuit current through TRPA1 in mouse isolated duodenum but not colon mucosa (10^{-4} and 3×10^{-4} M)(Fothergill et al., 2016). From the observations made above, we cannot know if the concentration of linalool contained in the concentrated bergamot oil is such as to guarantee an action on the TRPM8 and TRPA1 receptors. Furthermore, in the hypothesis of an administration of BEO in the GI tract, an important factor to consider would be the dilution that would reduce the concentration of linalool and could make it difficult to have a strong action on the TRP channels.

Therefore, when compared with linalool, menthol has greater potency at TRPM8 and TRPA1, which on the whole could help improve the inhibitory effect. Nevertheless, it is still likely that BEO or linalool, even if without potent ability to interact with TRP channels, may be new candidates among the natural complementary remedies useful for improving symptoms of IBS, notably where there is an increase in intestinal motility like IBS-D, but also in mixed and unsubtyped conditions (IBS-M, IBS-U).

CONCLUSIONS

In conclusion, the results obtained confirm the hypothesis that BEO, largely through the actions of linalool, can inhibit cholinergically-mediated contractions of the human and rat colon, at least partly by acting directly to relax smooth muscle contractility. Consequently, this research supports the use of BEO to promote 'gastrointestinal health', demonstrating potentials benefits in the complementary treatment of intestinal diseases related to increased muscle movement. Further studies are needed to clarify the still poor known mechanism of action and in particular, the possibility that BEO or linalool may act at sites other than calcium or potassium channels.

APPENDIX 1

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CERTIFICATO ANALISI CONSEGNA

Fornitore:CAPU - CAPUA 1880 S.R.L.	Prodotto: 1BGMT-SG - Olio Essenziale di Bergamotto SG
Ordine n°:	Rif.C.A.C.n°:
Quantità cons. 187,4	Lotto: 18/00398 -
Data arrivo merce: 000084 - 11/02/2018	Call n°:

Rev.02

1. Analisi /chimico fisiche

	Analisi richieste		Strumento	Risultato	
✓	Peso specifico	a 20 °C	Anton Paar	0.8708	
✓	Rotazione ottica °C	a 20	Anton Paar	40.9°	
✓	Indice di rifrazione °C	a 20	Anton Paar	1.4696	
✓	Linea CD		LAMBDA 25	0.85	
			LAMBDA EZ 201		
	Perossidi		Foodlab		

2. Analisi Cromatografiche

	GLC	Clarus 500 n°1		KEY PEAKS	%
		Clarus 500 n°1		Limonene	48.61
✓		Autosystem XL	13/00398	linalolo	5.52
		Autosystem		Ac linalile	23.60
				Bisabolene	0.47

Ftalati DEHP assente
CHIRALITA':
(-) linalolo 99.6% (+)linalolo 0.4%
(-) ac linalile 99.8% (+) ac linalile 0.2%
FUROCUMARINE: Bergaptene 2345 ppm, bergamottina 27020ppm

CCP:	alterazione chimica

Data analisi:11/02/2018 Firma operatore : ___G.A.___

3. Valutazione olfattiva. Note

Giudizio olfattivo:	Ok	

CCP:	alterazione organolettica

4. Valutazione all'utilizzo. Note

Prodotto conforme.	X	
Utilizzabile dopo il seguente trattamento		
Prodotto non conforme		Soluzione proposta:

CCP:	torbidità (es. cere)

Data valutazione globale: 11/01/2018 Firma DIG:____M.M ____

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