

INHIBITORY EFFECT OF EUGENOL ON RAT ILEAL MOTILITY *IN VITRO*

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Eugenol, an essential oil of clove oil possesses several different pharmacological properties, including antimicrobial, antifungal, insecticidal and antihelmintic. With regard to the digestive tract, eugenol has shown spasmolytic and relaxant effects. To elucidate some of the mechanisms involved, the effects of eugenol on contractions of isolated rat ileum induced by electrical field stimulation (EFS) were investigated. Eugenol (100 μ M) significantly and reversibly reduced EFS induced contractions by approximately 80%. Control contractions were 2.00 ± 0.18 g, while contractions in the presence of eugenol decreased to 0.40 ± 0.02 g ($n=7$; $P=0.0001$), respectively. Moreover, eugenol (100 μ M) reversibly decreased ileal basal tonus from 0.88 ± 0.04 g to 0.65 ± 0.04 g ($n=7$; $P=0.0002$).

After incubation with the nitric oxide synthase inhibitor nitro-L-arginine methyl ester (L-NAME), eugenol (100 μ M) still significantly inhibited ileum contractions (reduction by 75.30%), from 1.70 ± 0.09 g to 0.42 ± 0.08 g ($n=6$; $P=0.0001$), respectively. Likewise, incubation with L-NAME did not alter the eugenol relaxant effect on ileal basal tonus. Mean control basal tonus was 0.84 ± 0.07 g and significantly decreased after the addition of eugenol (100 μ M), to 0.37 ± 0.09 g ($n=6$; $P=0.0003$). After incubation with 300 μ M of L-NAME mean basal tonus was 0.85 ± 0.08 g, while eugenol significantly relaxed ileal preparations in the presence of L-NAME to 0.34 ± 0.11 g ($n=6$; $P=0.0003$).

Our results suggest that eugenol reversibly inhibits contractions caused by EFS and induces relaxation of rat ileum via a mechanism largely independent of NO activity.

Key words: eugenol, ileum, NO, L-NAME

INTRODUCTION

Eugenol, an aromatic substance is an essential oil and major component of clove oil (from *Eugenia caryophyllus*). Eugenol is also found in large quantities in the essential oils of many other plants, most of them widely used in traditional folk medicine (Leal-Cardoso *et al.*, 2002; Magalhaes *et al.*, 2004). The frequent usage of different essential oils in folk medicine consequently increases the interest of

investigators in their pharmacodynamic properties, as well as their mechanism of action.

Experimental studies have shown that eugenol appears to have several different pharmacological properties including antimicrobial, antifungal, insecticidal and antihelminthic actions (Burt, 2004; Pessao *et al.*, 2002; López-Malo *et al.*, 2002; Asha *et al.*, 2001). On the other hand, many authors reported that this herbal medicine shows important relaxant properties in the blood vessels and smooth muscles in the digestive tract. Damiani *et al.* (2003) reported that eugenol causes relaxation of blood vessels smooth muscle through blockade of voltage and ligand dependent ion channels. In addition, according to Lahlou *et al.* (2004) i.v. treatment of both anesthetized and conscious rats with methyleugenol lowers blood pressure probably through active vascular relaxation.

With regard to the digestive tract, it has been shown that methyleugenol relaxes the isolated ileum and inhibits contractions induced by stimulation of voltage-dependent and receptor-operated channels (Lima *et al.*, 2000).

Leal-Cardoso *et al.* (2002) showed that eugenol relaxes the basal tonus of the ileum precontracted with KCl. In the same study authors reported that eugenol reversibly inhibited ileal contractions induced by submaximal concentrations of ACh and K⁺ with IC₅₀ values of approximately 228 and 237 µM. The relaxant effect of eugenol on basal tonus of the ileum precontracted with KCl was unaltered by tetrodotoxin, L-NAME, hexamethonium and indometacin.

In the present study, we decided to characterize the effects of eugenol in a smooth muscle preparation of rat's isolated ileum. While, it was interesting to study the effect of eugenol on contractions induced by electrical field stimulation in the isolated rat ileum, as well as a potential role of nitric oxide (NO) in the eugenol actions on gastrointestinal motility.

MATERIAL AND METHODS

Drugs

Eugenol (standardized in concentration of 95% in ethyl-alcohol) was a gift from Company ESSENTICO (commercial producer of eugenol in Serbia), and dissolved in ethanol, 10 and 100 µM were tested. The volume added in the bath never exceeded 0.1% ethanol/25 mL and the contact time for both tested concentrations of eugenol before electrical stimulation was 2.5 minutes. Nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma (Italy), dissolved in distilled water and added to the bath (0.1 mL). Incubation time for each ileum preparation with L-NAME was 30 minutes prior to electrical stimulation.

Animals

Male Wistar rats, weighing 150-200 g, were housed under standard conditions for laboratory animals in groups of five with controlled 12-h light/dark cycles and temperatures between 21 and 24°C. The day before the experiments, the animals were fasted overnight, but allowed *ad libitum* access to water. All procedures in the study conformed to EEC Directive 86/609. Rats were euthanized by cervical dislocation [in accordance with *Home Office Code of*

Practice (1997). The Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986. HMSO. ISBN 0-10-265397-6] and the abdomen was immediately opened. The segments of ileum (2 cm long) were removed and placed in Krebs solution (mM): NaCl 139.9, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.04, NaHCO₃ 11.9, NaH₂PO₄ 0.4 and glucose 5.5 at 37°C.

Procedures

A segment of the ileum was placed in a longitudinal direction in a 25 mL muscle bath, filled with bubbled (95% O₂ / 5% CO₂) Krebs solution at 37°C. The upper end of the preparation was tied to an isotonic transducer (EI Unit, Belgrade, load 1.0 g) connected to software PC Biodata-F (EI Unit, Belgrade). After 30 minutes equilibration period, until a stable baseline was attained, preparations were subjected to electrical field stimulation by packages containing 5 stimulations every 60 seconds (EFS, 50 Hz, for 2 sec, 0.5 ms pulse duration), with resting periods of 5 minutes between each package. EFS was delivered by electrodes placed around the preparation using a Narco Bio stimulator, (Systems Inc, Houston, USA). After stable control contractions evoked by EFS had been recorded (contractions were stable and reproducible for a period of at least 30 minutes), the responses were observed in the presence of different concentrations of eugenol and nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) synthase inhibitor.

Statistical analyses

Results are expressed as means \pm SEM in grams (g) of contraction. Data were analyzed by Paired t-test, using GraphPad Prism, Version 4.0.

RESULTS

Eugenol significantly reduced contraction caused by EFS. Control contractions were 1.45 ± 0.23 g, while in the presence of 10 μ M eugenol, contractions decreased to 0.92 ± 0.20 g (~36% reduction) (n=5; P=0.0056), respectively (Figure 1). The inhibitory effect of eugenol (10 μ M) on contractions caused by EFS were completely reversible; after washing contractions were not significantly different compared to initial control values. On the other hand, this concentration of eugenol did not have significant effects on amplitude and tonus of spontaneous ileal motor activity. Figure 2 shows representative recordings of eugenol inhibitory effects on EFS-induced contractions of rat ileum.

Higher concentrations of eugenol (100 μ M) caused a more pronounced inhibition of EFS-induced contractions (~80%); control contractions were 2.00 ± 0.18 g, while contractions in the presence of 100 μ M of eugenol were 0.40 ± 0.02 g (n=7; P=0.0001), respectively (Figure 2 and 3). Whereas, 10 μ M of eugenol had no effect on basal tonus of spontaneous ileum motor activity, higher concentrations (100 μ M) significantly decreased ileal basal tonus from control values of 0.88 ± 0.04 g to 0.65 ± 0.04 g after treatment (n=7; P=0.0002). Likewise, this effect of eugenol was reversible and disappeared after washing, basal tonus increased from 0.56 ± 0.03 g to 0.84 ± 0.05 g (n=7; P=0.002) (Figure 4).

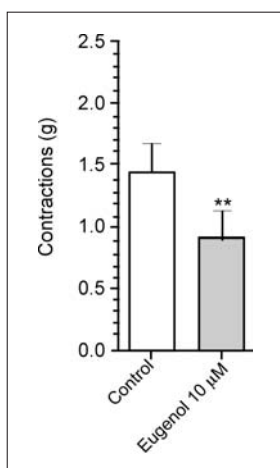


Figure 1. Inhibitory effect of eugenol (10 µM) on contractions (g) of isolated rat ileum caused by EFS (mean ± SEM, n=5, **-P=0.0056)

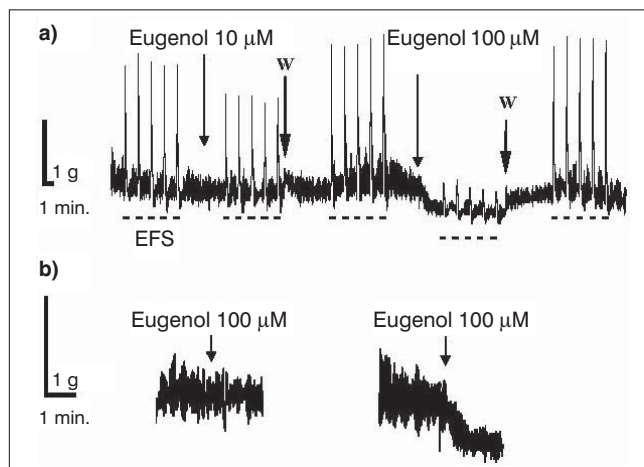


Figure 2. a) Tracing of rat ileal contractions caused by electrical field stimulation (EFS) in the presence of eugenol (10 µM and 100 µM); b) Tracing of ileal basal tonus after application of eugenol (10 and 100 µM)

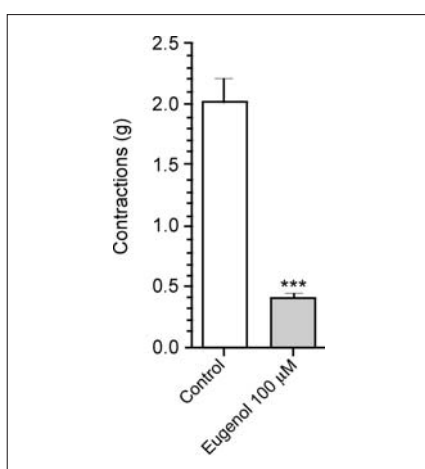


Figure 3. Inhibitory effect of eugenol (100 µM) on contractions (g) of isolated rat ileum caused by EFS (mean ± SEM, n=7, ***-P=0.0001)

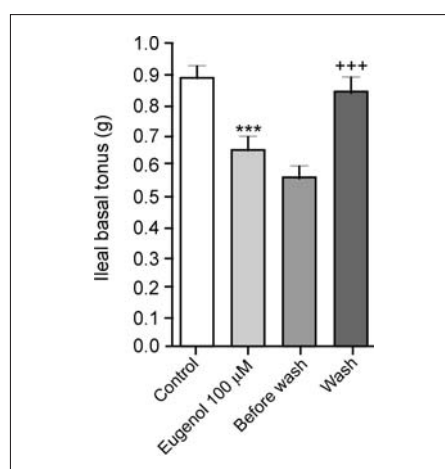


Figure 4. Relaxant effect of eugenol (100 µM) on ileal basal tonus (g) (mean ± SEM, n=7; ***-P=0.0002 Control vs. eugenol; +++-P=0.002 Before washing and after)

Following control EFS and stimulations in the presence of eugenol (100 μ M), the same ileum preparations were incubated for 30 minutes with 300 μ M of L-NAME to examine the potential role of NO in the eugenol relaxant effect. The control contractions were 1.80 ± 0.19 g while, in presence of eugenol (100 μ M) contractions significantly decreased, to 0.43 ± 0.05 g ($n=6$; $P=0.0008$), respectively. Contractions of the same preparations, after 30 minutes incubation with 300 μ M of L-NAME were 1.70 ± 0.09 g, with no significant differences regarding control contractions ($P=0.8465$). In the presence of L-NAME (300 μ M) eugenol (100 μ M) still significantly inhibits contractions (reduction of 75.30%), being 0.42 ± 0.08 g ($n=6$; $P=0.0001$) (Figure 5). The inhibitory effect of eugenol on ileum contractions caused by EFS was not significantly different after incubation with L-NAME. Likewise, incubation with 300 μ M of L-NAME did not influence the eugenol relaxant effect on ileal basal tonus. For these experiments mean control basal tonus was 0.84 ± 0.07 g and significantly decreased after addition of eugenol (100 μ M) to 0.37 ± 0.09 g ($n=6$; $P=0.0003$). After incubation with 300 μ M of L-NAME the mean basal tonus was 0.85 ± 0.08 g, while eugenol significantly relaxed ileal preparations in the presence of L-NAME to 0.34 ± 0.11 g ($n=6$; $P=0.0003$), respectively (Figure 6). Figure 7 shows representative recordings of eugenol inhibitory effects on EFS-induced contractions and basal tonus of rat ileum before and after incubation with L-NAME (300 μ M).

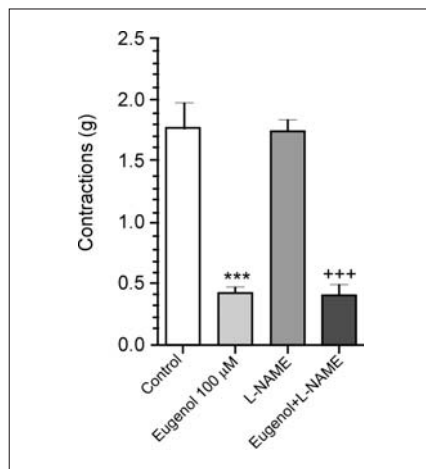


Figure 5. Inhibitory effect of eugenol (100 μ M) on contractions (g) of the isolated rat ileum caused by EFS and the effect of eugenol on the same preparations after incubation with L-NAME (300 μ M) (mean \pm SEM, $n=6$; ***- $P=0.0008$ Control vs. eugenol; +++- $P=0.0001$ L-NAME vs. eugenol + L NAME)

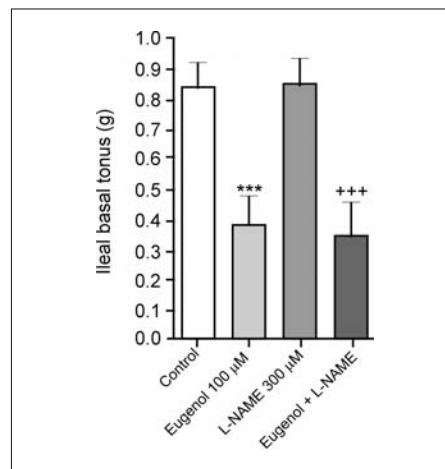


Figure 6. Relaxant effect of eugenol (100 μ M) on ileal basal tonus (g) and the effect of eugenol after incubation with L-NAME (300 μ M) (mean \pm SEM, $n=6$; ***- $P=0.0003$ Control vs. eugenol; +++- $P=0.0003$ L-NAME vs. eugenol + L NAME)

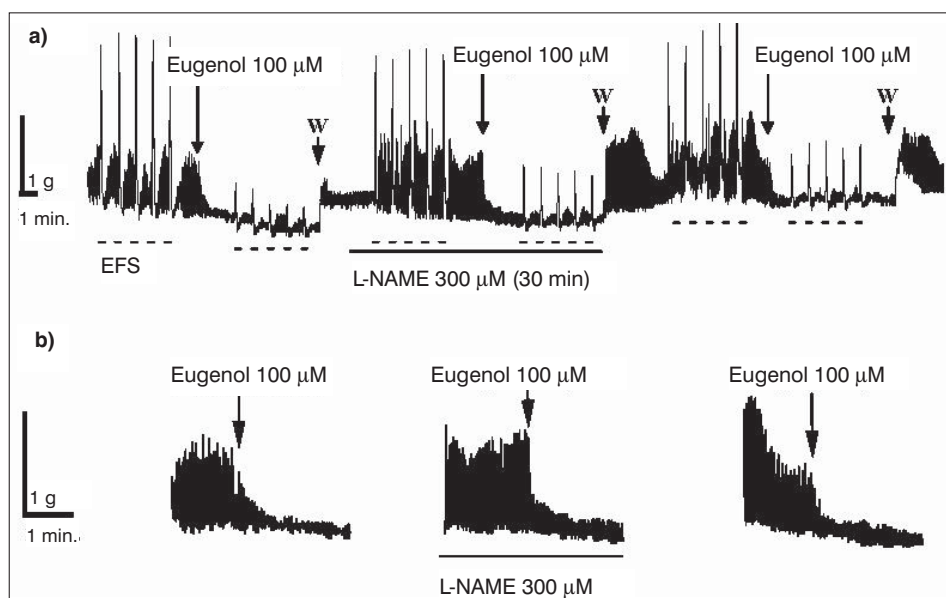


Figure 7. a) Tracing of rat ileal contractions caused by electrical field stimulation (EFS) in the presence of eugenol (100 μM), before and after 30 minutes incubation with L-NAME (300 μM);
b) Tracing of ileal basal tonus after application of eugenol (100 μM) before and after 30 minutes incubation with L-NAME (300 μM)

DISCUSSION

Our results show that eugenol is able to reduce the EFS-induced contractions in the rat isolated ileum. The highest tested concentration of eugenol (100 μM), also significantly relaxes the ileal basal tonus. All noted effects of eugenol were completely reversible and disappeared after washing. Others have shown similar effects of eugenol and methyleugenol. Derived data in our investigation are in accordance with previously published results by Magalhaes *et al.* (2004) that essential oil from *C. Nepetaefolius* (which contains about 14.9% of methyl-eugenol) inhibits ileum contractions induced by ACh, histamine and KCl. In this context Leal Cardoso *et al.* (2002) have reported that pure eugenol reversibly inhibited contractions induced by submaximal concentrations of acetylcholine (ACh) and K^+ with IC_{50} values of approximately 228 and 237 μM , respectively. NO is an established messenger in the gastrointestinal tract (Konturek and Konturek, 1995; Makhlof and Grider, 1993; Stark and Szurszewski, 1992). NO either alone or with different cotransmitters is thought to mediate the inhibitory component of the gut peristaltic wave. There is much evidence that different substances exert their relaxant effects in the gastrointestinal tract through interactions with NO in inhibitory enteric neurons (Ekblad and Sundel, 1997; Tanovic *et al.*, 2001). Therefore, we tested the effect of eugenol on EFS-induced contractions of the

ileum after incubation with L-NAME, an inhibitor of the nitric oxide synthase (NOS) enzyme. L-NAME is widely used for cardiovascular, gastrointestinal and other types of tissue. Its good water solubility makes it a popular experimental tool. In fact, L-NAME has been used in our investigation for studying the possible "nitregic" involvement in the inhibitory and relaxant effects of eugenol on small intestine motility.

In our study the inhibitory effect of eugenol on contractions caused by EFS, as well as the relaxant effect on basal tonus of the ileum, were unaffected by L-NAME, at doses used by other researchers to inhibit NOS-enzyme activity (Bartho *et al.*, 1999; Teague *et al.*, 2002). These results are in accordance with data published by Leal Cardoso *et al.* (2002), that the NO-synthase inhibitor L-NAME did not modify the relaxant effect of eugenol on ileum sections precontracted by KCl. Eugenol relaxant effects on the ileal basal tonus has also been described.

Results from the present study indicate that eugenol has a significant inhibitory action on ileal basal tonus and contractions caused by EFS. Our results, together with previously mentioned data suggest that NO is not involved in the relaxant effect of eugenol on rat ileum. L-NAME does not influence eugenol inhibition of ileal contractions caused by EFS, nor eugenol induced relaxation of the ileal basal tonus. Moreover, all observed effects of eugenol regarding ileal motility are completely reversible. Others have shown that the eugenol inhibitory effect on ileal motility are independent of alterations of transmembrane potential and extracellular Ca^{2+} influx, as well as unaffected by sodium channel blocking agents (tetrodotoxin), ganglion blocking drugs (i.e. hexamethonium) and nonselective COX inhibitor (indomethacin). Our results together with previously published data suggest that eugenol induces reversible inhibition of EFS-caused contractions and causes relaxation of rat ileum probably by direct action on smooth muscle via a mechanism largely independent of NO activity.

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INHIBITORNİ EFEKT EUGENOLA NA MOTILITET ILEUMA PACOVA *IN VITRO*

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SADRŽAJ

Eugenol, aktivni princip ulja karanfilića, poseduje različita farmakološka svojstva, kao što su antimikrobno, antimikotičko, insekticidno i antihelminičko. U digestivnom traktu deluje spazmolitički i relaksantno. U cilju bližeg upoznavanja mehanizma dejstva eugenola, ispitan je njegov efekt na kontrakcije izolovanog ileuma pacova izazvane spoljnom stimulacijom (electrical field stimulation-EFS). Eugenol (100 μ M) signifikantno, reverzibilno redukuje kontrakcije ileuma izazvane EFS za oko 80%. Kontrolne kontrakcije iznosile su $2,00 \pm 0,18$ g, dok su kontrak-

cije u prisutvu eugenola bile $0,40 \pm 0,02$ g ($n=7$; $P=0,0001$). Eugenol ($100 \mu\text{M}$), takođe signifikantno smanjuje i bazalni tonus izolovanog ileuma sa $0,88 \pm 0,04$ g na $0,65 \pm 0,04$ g ($n=7$; $P=0,0002$).

Posle inkubacije sa inhibitorom sintaze azotnog oksida L-NAME, eugenol i dalje signifikantno redukuje kontrakcije ileuma (za 75,30%) sa kontrolnih $1,70 \pm 0,09$ g na $0,42 \pm 0,08$ g ($n=6$; $P=0,0001$). Isto tako, inkubacija sa L-NAME ne utiče na relaksantni efekt eugenola na bazalni tonus ileuma. Kontrolna srednja vrednost bazalnog tonusa bila je $0,84 \pm 0,07$ g i signifikantno je smanjena posle dodavanja eugenola ($100 \mu\text{M}$) na $0,37 \pm 0,09$ g ($n=6$; $P=0,0003$). Posle inkubacije sa $300 \mu\text{M}$ L-NAME, srednji bazalni tonus bio je $0,85 \pm 0,08$ g, dok je eugenol i u prisustvu L-NAME relaksirao izolovani ileum na $0,34 \pm 0,11$ g ($n=6$; $P=0,0003$).

Dobijeni rezultati ukazuju da eugenol reverzibilno inhibiše kontrakcije ileuma izazvane spoljnom stimulacijom i dovodi do relaksacije izolovanog ileuma pacova mehanizmom nezavisnim od aktivnosti azotnog oksida.