RELAXANT EFFECTS OF ESSENTIAL OILS OF EUCALYPTUS CAMALDULENSIS ON AORTIC RINGS IN MALE ALBINO RATS

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(Accepted for publication: June 9, 2013)

Abstract
The essential oil (EO) produced a potent dilation in aortic rings with percentage of relaxation 95.659%. The significant relaxant effect on thoracic aortic rings was inhibited by GLIB (10⁻⁵) and 4-AP (1mM) with percentages of relaxation, 74.577% and 51.494%, respectively. On the other hand, vasorelaxation in aortic rings did not influence by pretreatment with TEA and BaCl₂. Pretreatment of aortic rings with nifedipine, significantly blocked the relaxation to 38.7%. On the other hand, the dose-response shifted to the right in endothelium denuded preparation. The percentage of relaxation in endothelium-denuded was 82.28%. Preincubation of aortic rings with L-NAME and Indomethacin altered the dilation from control significantly, and the percentage of relaxation found to be 60.18% and 77.077%, respectively. The present study suggest that the EO of E. camaldulensis depresses the aortic force development, probably acting as a Ca²⁺ channel antagonism and partially via NO and cyclooxygenase pathways, the involvement of K_{ATP} and Kᵥ channels in the vasorelaxation induced by EO.

Keywords: Eucalyptus camaldulensis, essential oils, vasorelaxation

Introduction
Eucalyptus genus is native to Australia (Silva et al., 2003) and Tasmania (Gruenwald et al., 2000), which were cultivated in Europe, Africa, Asia and America. Eucalyptus is one of the world’s most important and most widely planted genera. It includes more than 700 species and belongs to the family of Mirtaceae (Cheng et al., 2009). Eucalyptus trees can grow up to 45 meter in height with a single stem. The trees are towering and fully leafed hanged downwards. The leaves are commonly lanceolate, broad at the base and tapering to the tip (Gruenwald et al., 2000). Eucalyptus leaves are covered with oil glands, and represent the most important part which are reach in oils, and useful to extract the essential oils (Braun and Cohn, 2007). Medicinal properties of Eucalyptus species have been reported to be due to the presence of EO (Bhatti et al., 2007). Eucalyptus camaldulensis is a well-known aromatic and medicinal plant. It is known as a river red gum or Murray red gum tree (Akin et al., 2010). The use of aromatic plants in phytotherapy is mostly due to biological activities of their EO (Gruenwald et al., 2000). The EO of E. tereticornis showed myorelaxant effects on guinea-pig isolated trachea. The effect seems to be result from a complex interaction between its monoterpinoid constituents (Coelho-de-Souza et al., 2005). Lima et al. (2010) concluded that EO of E. tereticornis produced myorelaxant effects on rat isolated tracheal rings, but potentiates ACh-induced contraction. Monoterpenes α- and β-pinene are involved in its potentiating action, but are not responsible for its myorelaxant effects. Juca et al. (2011) found that the EO of E. tereticornis and its constituents decreased the retention. In anesthetized rats, α- and β-pinene induced contraction in rat gastric strips, while enhanced the meal progression in the duodenum. On the other hand, the EO relaxed the gastric strips in vitro but relaxed the duodenum. They conclude that EO oils accelerate the gastric emptying of liquid, and its effect is partially attributed to its active constituent's α- and β-pinene.

The main monoterpane constituent 1,8-cineole was tested its effect in several studies. Lahlou et al. (2002) for the first time showed physiological evidence that treatment with 1,8-cineole in either anesthetized or conscious rats elicits hypotension. They suggested that the vasorelaxant effect induced by 1, 8-cineole, probably because of reduction in peripheral vascular resistance caused by direct relaxation of vascular smooth muscles. Another study showed that the relaxation effect of 1,8-cineole on papillary muscle preparation from rat ventricle, probably by inhibition of calcium ion (Ca²⁺) influx through the membrane (Sorares et al., 2005). Nascimento et al. (2009) found that 1,8-cineole decreased rat bronchial resistance with similar efficacy as phenoterol. On the other hand, 1,8-cineole caused a concentration-dependent relaxation in guinea-pig tracheal rings precontracted by carbachol or potassium ion.
(K⁺) (80mM). They found that 1,8-cineole relaxed rat and guinea-pig airway smooth muscle by a nonspecific mechanism. Tracheal myorelaxant effect of 1,8-cineole acts preferentially on contractile responses elicited electromechanically in guinea-pig airway (Bastos et al., 2009).

Pinto et al. (2009) found that the potent vasorelaxant effect induced by EO of Alpinia zeraumbet, could not be fully attributed to the activity of the main constituent 1,8-cineol, and appears totally dependent of the integrity of the functional vascular endothelium. Furthermore, the monoterpenesthymol and carvacrol induced an endothelium independent relaxation in rat aortic rings, probably involving inhibition of Ca²⁺ release from sarcoplasmic reticulum (SR), reducing the sensitivity of contractile elements to Ca²⁺ across the membrane (Peixoto-Neves et al., 2010). In rat cerebral artery, carvacrol also caused a potent endothelium-dependent vasodilatation. Carvacrol caused Ca²⁺ influx leading to activate Ca²⁺-dependent K⁺ channels (KCa) channels. Opening KCa channels produced hyperpolarization of plasma membrane of endothelial cells and vascular smooth muscles, thereby resulting vasorelaxation (Earley et al., 2010). The whole cell patch clamp showed that carvacrol and thymol were able to inhibit the currents for L-type Ca²⁺ channels in cardiomyocytes (Magyar et al., 2004). Moreover, the cardiovascular hypotensive effect of monoterpe α-terpineol was first reported by Saito et al. (1996). Santos et al., 2011 suggest involvement of nitric oxide in vasorelaxation induced by α-terpineol in rat mesenteric vascular bed. Furthermore, studied showed α-terpineol induced vasorelaxation was abolished in pretreatment with L-NAME, due to involvement of nitric oxide in the vasorelaxation of rat mesenteric vascular bed (Magalhaes et al., 2008). Recently, Ribeiro et al. (2010) explained the vasorelaxation induced by α-terpineol was partially endothelium-dependent via nitric oxide release and activation of the NOcGMP-pathway. Other constituent, α-terpinen-4-ol effect was studied by Lahlou et al. (2003) on isolated aortic rings precontracted with depolarizing solution of K⁺, was induced vasorelaxation in concentration-dependent manner. On the other hand, a study by Perez-Hernandez et al. (2009) showed the effect of sesquiterpense. They showed that the hydroxylate darmacadendrene compounds, spathulenol and globule could completely relaxed uterus rings.

Materials and Methods

The extraction and analysis of EO of leaves was performed in the central lab of Organic Chemistry Department, University of Pavia, Italy. The protocol of the study include, hydrodistillation method which was used to extract and detect the EO composition from Eucalyptus camaldulensis. The volatile EO of E. camaldulensis leaves were extracted by steam distillation using Clevenger type apparatus, with circulating mode, 250g of dried powdered leaves. The distillation collected in the condensate collector, and water recirculated automatically into distillation round flask, and the oil accumulated in the condensate collector. After 2 hours of distillation, the oil layer was separated gently and carefully. The obtained oil (2.5ml) was labeled EO₄ stored at -20°C in a sealed vial.

Gas Chromatography

The analysis of EO was performed using Perkin Elmer Auto system GC with capillary column HP5 (length 25m, inner diameter and film thickness 0.32 mm and 0.52 μm, respectively). Nitrogen gas used as a carrier gas at a flow rate 1.5 ml/min. The injector operated in split mode with ratio of 20:1 and temperature 220°C. The GC oven temperature was kept at 60°C for 1 min, then thermal gradient 3°C/min until 100°C for 30 min, then second thermal gradient 10°C/min until 250°C for 5 min. The analysis duration lasted for 64.3 min.

Gas Chromatography-Mass Spectrometry

The analysis of the EO was performed using GC (Model 6890 N) coupled to a bench top MS (Aigilent 5973) Network. Column DB-5, 30m X 0.25 mm; 0.25 μm film thickness. Helium was used as a carrier gas with a flow rate of 1ml/min. The temperature of the injector was 220°C; temperature program, isothermal at 60°C for 1min., then gradually increased to 269°C/5min. The sample split ratio with 1:20 and 1μ of sample was injected. The mass range was 41-350 amu. The identities of compounds were assigned by comparison of their retention indices (RI) that calculated according to Van Den Dool and Kratz (1963) relative to a homologous series of C₈-C₉ n-alkanes under the same conditions.
Further identification was performed by comparing the mass spectrum of compounds with those stored in NIST 98 and Wiley 5 MS Libraries and by comparison of the experimental mass spectrum with literature data (Adams, 2007). Identity was confirmed by the comparison of Kovats retention indices.

**Screening of Biological Activities of Essential Oils**

The animals were injected intraperitoneally with heparin (1500 units/Kg body weight), left for 30 min., to avoid blood clotting and possible damage of endothelium of the aorta. Then the animals were anaesthetized with ketamin (40mg/kg) and xyalzine (10mg/kg) intraperitoneally. The chest cavity was opened, and excess tissues and fat were removed. The aorta was isolated and transferred to a beaker containing aerated Kreb's solution with 95% O₂ and 5% CO₂. Then the aorta was segmented into rings 3-5 mm in length. Isolated thoracic aorta was used in preparations with intact endothelium as well as in aorta where the endothelium had been removed by gentle rubbing of the intimate with syringe needle covered with a piece of cotton. The aortic rings were mounted between two stainless steel hooks, connected by a thread to a force transducer coupled to the transbridge amplifier and Power Lab Data Acquisition system (ML 870, Power Lab, AD Instrument, Sydney, Australia), connected to a computer running chart software (Version 7). The isometric force produced was monitored and recorded. The experiments were performed in 10ml organ baths filled with physiological kreb’s solution at 37°C using thermo regulating system with continuous water circulating throughout the double walled water jacket system, and gassed with a 95% O₂ and 5% CO₂ continuously (pH=7.4). The tension was set at 2g weight for 60 min., and the solution was changed every 15 min. until the resting tone became constant, the experiment was started.

**Statistical Analysis**

The vasorelaxation response was calculated as a percentage of contraction produced by PE was expressed as the mean ± standard error of the mean (SEM). The base line tension was expressed as 100% relaxation, and the tension induced by PE defined as 0% relaxation. All data analysis were fitted with a Hill equation, which the mean effective concentration (Logs of IC50) values were given as geometric mean with 95% confidence intervals (95% CI), using statistics program GraphPadPrism. Two-way analysis of variance (ANOVA) was performed, supported with Bonferroni test when carrying out pair wise comparison between the same doses of different groups using GraphPad program. P-values less than 0.05 (p<0.05) were considered significant.

**Results**

**Vasorelaxant Effects of Eucalyptus camaldulensis Essential Oils and 1,8-Cineol**

The cumulative addition of EO (1X10⁻²-1mg/ml) caused a strong dose-dependent relaxation effect, and reached to complete relaxation. Dose-response curve for EO effect against PE-induced contractions are shown in Figure (1). The EO at concentration from 1X10⁻² – 1mg/ml caused vasorelaxation in rat aortic rings precontracted with PE. The EO produced a potent dilation with percentage of relaxation 95.66%, and the Log IC50’s of 0.3384 mg/ml (with a Log IC50 of CI 95% between0.2974 to 0.3795).

**The Role of Potassium Channels in the Vasorelaxation Induced by EO of E. camaldulensis**

The role of K⁺ channels in vasorelaxation induced by EO was investigated by using TEA (1mM), GLIB (10⁻⁵), BaCl₂ (1mM) and 4-AP (1mM), individually, 20 minutes prior to PE-induced contraction. Dose-response curves for EO effect against PE-induced contractions preincubated with K⁺ channel blockers are shown in Figures (1, 2, 3 and4).The curves were significantly shifted to the right in preincubated aortic rings with either GLIB or 4-AP, and remained unchanged in preincubated aortic rings with either TEA or BaCl₂. There is significant relaxant effect in aortic rings preincubated with either GLIB or 4-AP, with Log IC50’s of 0.4193 mg/ml (with a Log IC50 of CI 95% between 0.2540 to 0.5845) and 0.5467 mg/ml (0.4462 to 0.6473), respectively. Also the percentage of relaxation was reduced in the both, GLIB and 4-AP preparations to 74.577% and 51.494%, respectively. On the other hand, vasorelaxation response, in aortic rings precontracted by PE, was not influenced by TEA and BaCl₂.
The Role of Calcium Channel in the Vasorelaxation Induced by EO of *E. camaldulensis*

The cumulative addition of EO concentrations (10⁻²-1mg/ml) caused a slight concentration-dependent relaxation in aortic rings preincubated with nifedipine. Dose-response curve for EO effect against PE-induced contractions preincubated with Ca²⁺ channel blocker is shown in (Figure 5). As the results indicates there was highly significance decreased vasorelaxation and dose-response curve was shifted to the right. The percentage of relaxation, Log IC50 and (Log IC50’s of CI 95%) for inhibitory effect of EO on pretreatment aortic rings with Nifedipine, significantly (p< 0.001) blocked the dose-dependent relaxation with percentage of relaxation 38.701% compared to control, with the Log IC50’s of 0.4754mg/ml (with a Log IC50 of CI 95% between0.3415 to 0.6093).
The Role of Endothelium in the Vasorelaxation Induced by EO of *E. camaldulensis*

Dose-response curve for EO effect against PE-induced contractions are shown in Figure (6). In the isolated aortic rings, the EO extract produced a highly significance inhibition and shifted the dose-response curve to the right in endothelium denuded preparation. The percentages of relaxation in both, endothelium-denuded and endothelium-intact preparation were 82.28% and 95.659%, with the Log IC50’s of 0.3975 mg/ml (with a Log IC50 of CI 95% between0.3448 to 0.4501) and0.4553 mg/ml (0.4113 to 0.4994), respectively.

![Figure 6](image_url)

Figure 6. Cumulative dose-response curve for the vasorelaxant effects of EO on control and endothelium-denuded aortic, precontracted with PE (10⁻⁶M).

The Role of Endogenous NO and PGI₂ in Vasorelaxation Induced by EO of *E. camaldulensis*

Dose-response curve for EO effect against PE-induced contractions are shown in Figures (7 and 8). The curves were shifted to the right significantly in both treatments. Treatment of aortic rings with L-NAME and indomethacin altered the dilation significantly on compare with control, and found to be 60.18% and 77.077%, with Log IC50’s of 2.745 mg/ml (with a Log IC50 of CI 95% between0.3402 to 5.149) and 2.352 mg/ml (1.144 to 3.560), respectively.

![Figure 7](image_url)

Figure 7. Cumulative dose-response curve for the vasorelaxant effects of EO on control and preincubated rat aortic rings with L-NAME (3X10⁻⁴M), precontracted with PE (10⁻⁶M).

![Figure 8](image_url)

Figure 8. Cumulative dose-response curve for the vasorelaxant effects of EO on control and preincubated rat aortic rings with Indomethacin (3X10⁻⁵M), precontracted with PE (10⁻⁶M).

Discussion

The effect of EO sample that obtained by hydrodistillation methods, was studied on PE precontracted aortic rings. The vasorelaxation response to EO remained unchanged in PE precontracted aortic rings. On the other hand, the dose-response curve of EO-induced relaxation significantly shifted to the right as compared to EO-induced relaxation. The result of this study, indicate that vasorelaxation in aortic rings induced by EO may be partially attributed to the presence of active constituent, 1,8-cineole which represent the major constituent compound with percentages of 62.7% and 59.09%, respectively, beside other monoterpenes constituents, such as α-terpinol(17.3%), p-cymene (13.5%) and crypton (13.9%) which may mostly be responsible for the vasorelaxation effect. This is partially in agreement with Coelho-de-Souza *et al.*
al. (2005) result who suggested that the vasorelaxation induced by EO of *E. tereticornis*, produced from a complex interaction between its monoterpenes. Recently, Ribeiro et al. (2010) demonstrated that α-terpinol induced vasorelaxation is mediated partially by endothelium via NO release and activation of the NO-cGMP pathway. On the other hand, as compared to our work (Kheder and Al-Habib, 2013), the EO obtained with different methods contained only 2.98% of 1,8-cineole with higher contents of sesquiterpene; valencene (12.55%), spathulenol (10.52%) and globulol (10.25%) which may be responsible mainly for the relaxation induced by EO. Recently, it has been reported that the sesquiterpene; spathulenol and globulol have a strong spasmyloytic activity and they are able to relax the contracted tissue at a concentration of 30µg/ml (Perez-Hernandez et al., 2009). Further study showed that α- and β-pinene are partially involved in the relaxation of both rat gastric and duodenal strips, induced by essential oils of *E.tereticornis* (Jucar et al., 2011). Also these monoterpens are involved in potentiating actions of rat tracheal in vitro induced by EO of *E. tereticornis* (Lima et al., 2010).

Our results suggested that the vasorelaxation of EO of *E. camaldulensis* could not fully be responsible to the action of 1,8-cineole, because there are other active constituents of EO which possess a vasorelaxant activity. This effect may be due to the interaction between EO monoterpens and/or sesquiterpenoids constituents.

The role of K' channels in vasorelaxation induced by EO were studied using K' channels blockers TEA, BaCl2, GLIB and 4-AP. The vasorelaxation did not affected in aortic rings preincubated with TEA and BaCl2, these indicate that KCa and Kir channels were not involved in vasorelaxation induced by EO. The role of 1,8-cineole-induced vasorelaxation in aortic rings preincubated with TEA indicates that the vasorelaxation effect remained unchanged. This result coincided with that reported by Pinto et al. (2009) in rat isolated aorta in the presence of TEA for vasorelaxant effect evoked by 1,8-cineole. On the other hand, our results indicated the involvement of KATP and Kv channels in the vasorelaxation induced by EO. Since no data are available on the role of *E camaldulensis* oil constituents in K' channels activity, thus it is difficult to compare our results.

The aortic rings preparation preincubated with nifedipine abolished the relaxation induced by EO. This indicates that the active constituents of *E. camaldulensis* oils mostly act as Ca++ channel antagonist, via either preventing Ca++ influx through voltage dependent Ca++channels (VDCs) of plasma membrane or on the release from SR (Peixoto-Neves et al. 2010). Also the vasorelaxation of aortic rings preincubated with nifedipine abolished in 1,8-cineole-induced relaxation. It can be conclude that the monoterpen 1,8-cineole is one of the most active constituent with carvacrol, which are responsible for EO-induced relaxation. These monoterpenes could cause vasorelaxation via inhibition of Ca++ influx through the plasma membrane (Peixoto-Neves et al. 2010 and Soares et al. 2005). On the other hand, terpineol is another active constituent present in *E camaldulensis* EO and able to induce a concentration-dependent vasorelaxation (Lahlou et al. 2003) at least partially by the endothelium via NO release and activation of NO-cGMP pathway

In denuded aortic rings preincubated either with L-NAME or indomethacin, the vasorelaxation induced by EO was significantly reduced. On the other hand, the vasorelaxation at the final concentration of 1mg/ml in denuded and preincubated aortic rings with indomethacin remained unchanged. These effects may be due to the activity of terpineol which could activate NO-cGMP pathway (Ribeiro et al. 2010). The present study suggest that the EO of *E camaldulensis* depresses the aortic force development, probably acting as a Ca++ channel antagonism and partially via NO and cyclooxygenase pathways(Peixoto-Neves et al. 2010; Ribeiro et al. 2010), the involvement of KATP and Kv channels in the vasorelaxation induced by EO.

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*Eucalyptus camaldulensis* Dehn

تأثير زيوت اليوكاليبتوس المستخلصة من *Eucalyptus camaldulensis* لحلقات الأبر

تمت ملاحظة تأثير استرخائي للزيوت اليوكاليبتوس المستخلصة من *Eucalyptus camaldulensis* في حلقات الأبر، حيث تم تأثیر استرخائي بنسبة 95.69%، وعند معالجة ENC والبطانيات الطلائية، تم تثبيط تأثير استرخائي من قبل 4-AP GLIB. ووصلت نسبة الاسترخاء إلى 77.65 % و59.67 %. في حين أن معالجة ENC بكامل الجلود، تم تثبيط تأثير استرخائي عن طريق 4-AP GLIB. ووصلت نسبة الاسترخاء إلى 77.65 % و59.67 %، اما في حلقات الأبر المعالجة بنيفوسين، فتمت تثبيط الاستجابة الاسترخائية بنسبة 106.79 %، وتم تثبيط تأثير الاسترخائي عن طريق 4-AP GLIB. ووصلت نسبة الاسترخاء إلى 86.90 % و77.68 %

تاثير زيوت اليوكاليبتوس المستخلصة من *Eucalyptus camaldulensis* في حلقات الأبر

المختصر

لا يمكن القول أن تأثير الاسترخائي للزيوت اليوكاليبتوس المستخلصة من *Eucalyptus camaldulensis* قد يكون من خلال عمله عن طريق قنوات الكالسيوم، وجزئيا عن طريق NO وcyclooxygenase، وكهرباء كمانال Ca++ وKATP و Kv.