1    # Eugenol induces potent vasorelaxation in uterine arteries

2    # from pregnant goats – A promising natural therapeutic

3    # agent for hypertensive disorders of pregnancy

4

5

6    Running title: Vasorelaxation effect of eugenol in uterine arteries

7

8

9    Parija SC[1][¶]*, Jandhyam H[1][¶], Mohanty BP[2,3,&], Parasar P[4,&] and Nayak NR[4,5,&]

10

11   [1]Department of Pharmacology and Toxicology, College of Veterinary Sciences and Animal

12   Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751003, India

13   [2]Fishery Resource & Environmental Management (FREM) Division, ICAR- Central Inland

14   Fisheries Research Institute, Barrackpore, Kolkata 700120, India.

15   [3]Fisheries Science Division, Krishi Anusandhan Bhawan II, Indian Council of Agricultural

16   and Research, New Delhi 110012, India

17   [4]Department of Obstetrics and Gynaecology, Wayne State University, MI-48201, USA

18   [5]Department of Obstetrics and Gynaecology, University of Missouri, Kanas City, MO-64108,

19   USA

20

21   *Corresponding Author:

22   Email address:parijasc1963@gmail.com (SCP)

23

24   [¶]These authors contributed equally to this work.

25   [&]These authors also contributed equally to this work.

26

1

# Abstract

Hypertensive disorders of pregnancy, including preeclampsia, affect about 8-13% of pregnancies and are the leading causes of pregnancy related maternal mortality worldwide. Poorly controlled high blood pressure during pregnancy increases the risk of pregnancy complications and development of future cardiovascular diseases. However, the choice for antihypertensive therapy during pregnancy has been limited due to side effects of many commonly used antihypertensive drugs and lack of other proven safe treatment options. Eugenol is a natural phenolic compound and the main component of clove oil. It is known for its antioxidant, anti-inflammatory and vasorelaxant actions. These beneficial effects of eugenol make it as an excellent therapeutic candidate for treatment of hypertensive disorders of pregnancy. Thus, as a first step, we compared the vasorelaxant effect of eugenol on the middle uterine arterial (MUA) rings from pregnant and nonpregnant goats. Additionally, we examined the potential involvement of the transient receptor potential channel 1 (TRPV1) in mediating the actions of eugenol and compared the effects with a known TRPV1 channel agonist, capsaicin. Isometric tension was measured in MUA rings from endometrial-myometrial junctions of pregnant and nonpregnant goats precontracted with phenylephrine, using a highly sensitive isometric force transducer and an automatic organ bath. The concentration-dependent contractile response curves of eugenol were compared to capsaicin, with and without pre-incubation of the MUA rings with a selective and non-selective TRPV1 antagonists, capsazepine (CAPZ) and Ruthenium Red (RR), respectively. Capsaicin induced concentration-dependent vasorelaxation in nonpregnant PE precontracted MUA rings and the concentration-response curve shifted to the right with significantly reduced $pIC_{50}$ and $R_{max}$ values in the presence of CAPZ and RR. The effects were similar in MUA rings from pregnant animals, except that there was a moderate increase in $pIC_{50}$ values in the presence of RR. Similarly, eugenol induced concentration-dependent vasorelaxation in both nonpregnant and pregnant PE precontracted MUA rings and the effects were markedly antagonized by

53    CAPZ and RR. However, compared to capsaicin, the $R_{max}$ of eugenol was increased 31.25%

54    in nonpregnant and 97.99% in pregnant MUA rings. These results suggest that eugenol has

55    highly potent vasorelaxant effect in MUAs and its effect is partly mediated through activation

56    of the TRPV1 channel. Most importantly, its vasorelaxant effect is about three-fold

57    augmented in pregnancy, suggesting its potential value as a nutraceutical agent and

58    therapeutic candidate for treatment of hypertensive disorders of pregnancy.

59    **Key words**: capsaicin, eugenol, TRPV1, uterine artery, pregnancy, hypertension,

60    vasorelaxation

# Introduction

62    In pregnancy the hypertensive disorders may be chronic (pre-existing) or gestational. When

63    blood pressure is maintained at 140/95mmHg before pregnancy to 20th week of gestation is

64    designated as chronic. If such blood pressure is prolonged beyond 20th week of pregnancy it

65    is defined as gestational hypertension. The gestational hypertension is resolved within 42

66    days after delivery [1,2]. Current treatment for hypertensive pregnancy disorders is to control

67    and manage blood pressure and seizures with antihypertensive and magnesium sulphate [3],

68    however, the ultimate option is to deliver foetus and placenta [4]. Blood pressure levels

69    requiring therapy in pregnancy have been set at higher systolic and diastolic levels compared

70    to the general population. Therapeutic intervention is recommended if blood pressure during

71    pregnancy is maintained at $\leq$ 149/95 mmHg. The first choice of drugs are methyldopa and

72    labetalol followed by nifedipine to treat severe form of hypertension during pregnancy [2, 5].

73    Other drugs like ACE-inhibitors and angiotensin II receptor blockers (ARBs) is

74    contraindicated during pregnancy [2, 5] due to adverse foetal outcomes like intrauterine

75    growth retardation, neonatal hypotension, oligohydramnios, and patent ductus arteriosus [6,

76    7]. Similarly, diuretics can cause placental hyper perfusion and β-blockers except labetolol

77    and atenolol has been reported to be associated with intrauterine growth restriction, preterm

78    delivery, neonatal hypoglycemia and bradycardia [1]. Although, plethora of small therapeutic

79    molecules have been developed in treating hypertensive disorders of pregnancy, they have

80    varying success [9-10]. Based on clinical observational studies, practically none of them are

81    evaluated as per the guidelines of rational pharmacotherapy during pregnancy. In recent

82    years, adverse foetal outcomes have resulted from use of non-recommended antihypertensive

83    drugs pregnant women in different countries [11]. In order to overcome such crisis, use of

84    nutraceutical derived antihypertensive agents would be a better option without any risk for

85    adverse foetal outcomes.

86    Hypertensive disorders of pregnancy include range of conditions which, if not treated, may

87    lead to preeclampsia [12] and eclampsia, a severe form of hypertension and convulsions and

88    may also consequent into coma and death causing an estimated 14% pregnancy-related

89    maternal deaths. Preeclamptic mothers have higher risk of cardiovascular diseases including

90    chronic heart failure, hypertension, and stroke [8]. Preeclampsia may also associate with

91    intrauterine growth restriction (IUGR) which could cause premature births. These pregnancy

92    complications are particularly of importance in developing countries where incidence of

93    preeclampsia is greater and maternal mortality and preterm births are higher compared to that

94    of developed countries [13-15].

95    While there is no definitive mechanism or aetiology of preeclampsia, common risk factors are

96    pre-existing diabetes [16], obesity [17], African American ethnicity, and family history of

97    preeclampsia [18-19]. During embryo implantation, placental trophoblast cells invade the

98    uterine arteries (spiral arteries in humans) and induce its remodelling, while obliterating the

99    tunica media of the myometrial spiral arteries which allows the arteries to accommodate

100   increased blood flow to nourish the developing foetus [20]. This abnormal spiral artery

101   remodelling was seen and described over five decades ago in pregnant women who were

102   hypertensive [21]. It has since been shown to be the central pathogenic factor in pregnancies

103   complicated by intrauterine growth restriction, gestational hypertension, and preeclampsia

104   [20]. Defective trophoblastic invasion with associated uteroplacental hypoperfusion may lead

105   to preeclampsia and has been supported by animal and human studies [22-23]. Therapeutic

106   vasorelaxation of spiral arteries could improve placental blood flow, reducing risks to the

107   fetus, and potentially alleviate maternal hypertension.

108   Vascular tone is regulated in part by transient receptor potential $Ca^{++}$ channels, including

109   TRPV1, a polymodal nociceptor member of the TRPV subfamily. TRPV1 can be activated

110   by capsaicin, noxious heat, extracellular protons, vanilloids, and voltage-dependent

111   depolarization [20]. It is predominately expressed in sensory nerves and non-neuronal tissues

112   and can cause vasodilation through calcitonin gene related peptide (CGRP) and mediate

113   vascular functions via baroreceptor reflex [24]. Its functions in various organs include pain

114   perception, attenuation of inflammatory pain, cell migration, and modulation of receptor

115   activity [25]. TRPV1 in the vascular system plays a vital role in regulation of

116   vasoconstriction and vasodilation [26].

117   Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) is the principal active constituent of hot

118   chilli peppers and has been used as a spice for enhancing flavour and as an analgesic [27].

119   Capsaicin has significant stimulating effects, including stimulation of thermogenesis,

120   catecholamine secretion from the adrenal medulla, adipogenesis via enhancement of energy

121   metabolism, and weight reduction [28]. Capsaicin is also a selective agonist for the TRPV1

122   cation channel [29] and capsaicin-induced vasorelaxation has been reported in human

123   coronary artery, rat aorta [30], guinea pig ileum [31], human and porcine coronary arteries

124   [32], rat mesenteric artery [33], and goat mesenteric artery [34].

125     Another naturally occurring compound with vasorelaxant effects is eugenol (4-allyl-2-

126   methoxyphenol), a chief component of clove oil. Its myorelaxant [35] and vasorelaxant

127   effects in rat [36-37], rabbit thoracic aorta, and rat mesenteric vascular bed [38] suggest it

128   could be used in the treatment of hypertension. Due to the presence of a vanillyl moiety like

129   that in capsaicin, eugenol may also cause vasorelaxation via activation of TRPV1 channels

130   [39]. Eugenol may dilate resistant arteries by increasing the expression of its target genes,

5

131    sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) and big potassium (BK) calcium

132    activated potassium channels (KCa 1.1 or KCNMA1 gene) channel [40-41], thereby relaxing

133    vascular smooth muscle cells and decreasing blood pressure. Information regarding the

134    effects of capsaicin and eugenol on uterine arteries via activation of TRPV1 channels is

135    lacking. Utilizing a novel *Capra hircus* MUA denuded from endo-myometrial junction, a

136    novel arterial model similar to human uterine arteries, and combining functional and

137    electrophysiological studies, we demonstrate that naturally occurring compounds, capsaicin

138    and eugenol, induce vasorelaxation in pregnant and nonpregnant goat MUA and this effect is

139    mediated through endometrial TRPV1. Especially, eugenol due to its higher effectiveness

140    could be used therapeutically to control hypertension and is a potential candidate to treat

141    effects of preeclampsia associated with reduce spiral artery blood flow.

142

## Materials and methods

144    **Ethical guidelines**

145    Protocols were approved by the institutional ethical committee on Animal Research

146    (433/CPCSEA/CVS vide ID.No.1586(6)/CVS/dt.03.05.2016).

147    **Drugs and Chemicals**

148    Commercially available phenylephrine (PE), capsaicin, eugenol, and CAPZ were obtained

149    from Sigma-Aldrich, USA, and RR from MP Biochemicals, India. PE was prepared in triple

150    distilled water whereas Capsaicin and CAPZ, were prepared by dissolving in 70% ethanol.

151    **Preparation of endo-myometrial junction middle uterine artery rings**

152    Non-pregnant and pregnant uteri intact with broad ligaments and uterine arteries were

153    obtained and placed into aerated ice-cold Modified Krebs-Henseleit Saline (MKHS) (118

154    mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 11.9 mM NaHCO3, 1.2 mM

155    KH2PO4, and 11.1 mM dextrose, pH 7.4). The arteries from myometrial and endometrial

156    junction were promptly excised. Secondary branches of uterine arteries supplying the uterine

6

157 horns were carefully cleansed off of fascia and connective tissue in MKHS solution under

158 continuous aeration. The arteries were cut into ~2 mm long segments of circular rings.

**Isometric tension assay**

160 Endothelium intact middle uterine arterial rings were mounted between two fine stainless-

161 steel L-shaped hooks [42] and kept under a resting tension of 1.5 gm at 37.0±0.5 ºC in a

162 thermostatically controlled automatic organ bath (Pan Lab) of 20 mL capacity aerated with

163 carbogen (95% $O_2$, 5% $CO_2$). Change in isometric tension was measured by a highly sensitive

164 isometric force transducer (Model: MLT0201, AD instrument, Australia) and analysed using

165 Chart 7.1.3 software.

**Experimental protocols**

167 The arterial rings were precontracted by exposure to PE ($10^{-5}$M) to produce a maximal,

168 sustained contraction, then challenged with either cumulative concentrations of Capsaicin

169 ($10^{-9}$ to 10 x $10^{-5}$ M) or Eugenol ($10^{-9}$ to $10^{-5}$ M). To investigate the involvement of TRP

170 channels in Eugenol and Capsaicin responses, arterial rings were pre-treated with CAPZ ($10^{-6}$

171 M), specific blocker of TRPV1, or RR ($10^{-6}$ M), a non-selective TRP channels blocker, for a

172 period of 30 minutes. The concentration-response curves (CRCs) for capsaicin- and eugenol-

173 treated arterial rings were compared with those of untreated controls. The CRCs for capsaicin

174 and eugenol in the presence and absence of RR and CAPZ were also determined. CRCs were

175 plotted and the $pIC_{50}$ calculated for each condition. The $pIC_{50}$ and $R_{max}/R_{Bmax}$ of the CRCs

176 were compared for each set of experiments.

**Data analysis and statistics**

178 Data are presented as the mean ± S.E.M. Concentration-response curves are based on the

179 percent relaxation from the agonist-induced contraction (control), and the curves were fitted

180 using interactive non-linear regression in Graph Pad Prism (Graph Pad Prism Software, San

181 Diego, CA, USA). The $R_{max}/R_{Bmax}$, mean threshold concentration, and $-logIC_{50}/pIC_{50}$ were

182    calculated in Graph Pad Prism. Unpaired Student's t-test was used to compare groups. A

183    value of $p < 0.05$ were considered statistically significant.

# Results

### Capsaicin-induced vasorelaxation of middle uterine arteries

186    Middle uterine arterial rings from endometrial and myometrial junction were mounted and a

187    contraction was induced with PE. Bath application of capsaicin caused concentration-

188    dependent relaxation of this contraction. Capsaicin caused concentration-related

189    vasorelaxation in PE pre-contracted MUA rings of nonpregnant animals ($R_{max}$ =

190    28.06±0.67%, $pIC_{50}$ = 7.28±0.06; n = 6) (Table 1). When MUA were pre-incubated for 30

191    min with CAPZ ($10^{-6}$ M), it inhibited Capsaicin-induced relaxation as demonstrated by the

192    rightward shift in the CRC and statistically significant decrease in $R_{Bmax}$ and $pIC_{50}$

193    (7.52±0.2% and 5.68±0.04, respectively; P<0.001) (Fig 1A). Similarly, pre-incubation with

194    RR shifted the CRC to the right with significant decreases in $R_{Bmax}$ (4.71±0.11%; P<0.001)

195    and $pIC_{50}$ (6.88±0.12; P<0.05) (Fig 1A).

196

197    In pregnant goats, capsaicin induced concentration-dependent relaxation in the arterial rings

198    ($R_{max}$ = 23.86± 0.97%, $pIC_{50}$ = 7.01±0.12) (Table 1). When preincubated with CPZ,

199    capsaicin-induced relaxation reduced with a rightward shift in the curve and significant

200    decrease in $R_{Bmax}$ and $pIC_{50}$ (12.42±0.59% and 6.68±0.12, respectively, <0.001) (Fig 1B). In

201    the presence of RR, capsaicin-induced relaxation decreased with  a shift of the curve to the

202    right and a significant decrease in $R_{Bmax}$ (14.33±0.77%; P<0.001) and a non-significant

203    increase in $pIC_{50}$ (7.30±0.10) in the presence of RR (Fig 1B).

### Vasorelaxation induced by Eugenol involves TRPV1 channel activation

205    MUA rings of nonpregnant uteri when incubated with eugenol significantly relaxed the

206    arterial rings precontracted with PE ($R_{max}$ = 36.83±0.66%, pIC50 = 6.93±0.09) (Table 1). To

207    investigate the endothelial-cell dependent mechanism of Eugenol-induced vasorelaxation, the

8

208 experiments were performed in the presence of TRPV1 inhibitors CAPZ and RR. The

209 eugenol CRC shifted to the right with a significant decrease in $R_{Bmax}$ and $pIC_{50}$ in the

210 presence of CAPZ ($R_{Bmax}$ = 3.89±0.35%, $pIC_{50}$ = 5.86±0.03; P<0.001; n=6) (Fig 2A). On the

211 other hand, incubation with RR shifted curve rightward with significant decrease in $R_{Bmax}$

212 (6.57±0.39%; P<0.001) and a nonsignificant decrease in $pIC_{50}$ (6.70±0.13) (Fig2A).

213 In the MUA of pregnant uteri, eugenol induced vasorelaxation ($R_{max}$ 47.24±1.61%, $pIC_{50}$

214 6.84±0.06) (Figure 2B). When incubated with CAPZ, a competitive TRPV1 and TRPM8

215 receptor antagonist, the curve shifted to the right and caused a significant decrease in $R_{Bmax}$

216 (15.67±0.98%; P<0.001) and a nonsignificant increase in $pIC_{50}$ (6.9±0.09) (Figure 2B).

217 However, eugenol-induced relaxation significantly decreased ($R_{Bmax}$ 9.81±0.30%,

218 $pIC_{50}$5.89±0.13; P<0.001; n=6) in the presence of RR (Fig 2B). The mean maximal effects of

219 different ligands relative to capsaicin vasorelaxation (100%) are presented in Fig 3.

220

221 Table-1 Capsaicin- and eugenol-induced vasorelaxation in phenylephrine ($10^{-5}$M)-

222 precontracted MUA rings from nonpregnant (NP) and pregnant (P) *Capra hircus* in the

223 absence (control) or presence of capsazepine (CAPZ) ($10^{-6}$M) or ruthenium red (RR) ($10^{-6}$M).

| | | CAPSAICIN | | | EUGENOL | | |
|---|---|---|---|---|---|---|---|
| | | CON | CAPZ | RR | CON | CAPZ | RR |
| NP | Rmax/RBmax (%) | 28.06±0.67 | 7.52±0.20[a] | 4.71±0.11[a] | 36.83±0.66 | 3.89±0.35[a] | 6.57±0.39[a] |
| | -LOG IC$_{50}$ | 7.28±0.06 | 5.68±0.04[a] | 6.88±0.12[b] | 6.93±0.09 | 5.86±0.03[a] | 6.70±0.13 |
| P | Rmax/RBmax (%) | 23.86±0.97[d] | 12.42±0.59[ac] | 14.33±0.77[ac] | 47.24±1.61[c] | 15.67±098[ac] | 9.81±030[ac] |
| | -LOG IC$_{50}$ | 7.01±0.12 | 6.68±0.12[bc] | 7.30±0.10[d] | 6.84±0.06 | 6.90±0.09[c] | 5.89±0.08[ac] |

[a](p<0.001), [b](p<0.05) represent level of significance between data of each row (treated) compared with the data of control, (CON). [c](p<0.001), [d](p<0.05) represent level of significance between the NP and P. Values are represented as mean±SEM, (n=6). SEM = standard error of mean.

224

225

# Discussion

Hypertensive disorders of pregnancy are most common medical disorders of pregnancy which complicate 1 in 10 pregnancies. Notably, preeclampsia, a hypertensive disorder of pregnancy, affects 2–8% of pregnancies worldwide. Whereas the rates of occurrence of preeclampsia overall have remained unchanged, the cases of severe preeclampsia have begun to rise over the last several decades [43-44]. Treatment recommendations for hypertension in pregnancy is set at varying levels than in general population. Blood pressure levels requiring therapy in pregnancy have been set at higher systolic and diastolic levels compared to the general population.

Treatment of severe hypertension requires pharmacological intervention using methyldopa, labetalol, and nifedipine; however, current clinical management is limited, and more innovative medical therapies are needed. Labetalol may be associated with foetal growth restriction and nifedipine may inhibit labour. Hydralazine, other antihypertensive drug, has been tested few controlled trials, and may cause neonatal thrombocytopenia. Beta-receptor blockers may reduce uteroplacental blood flow and have the risk of growth restriction when started in the first and second trimester. It also may cause neonatal hypoglycaemia at higher dose. Hydrocholorothiazide may cause volume contraction and electrolyte disorders, however, may be helpful when combined with methyldopa to mitigate compensatory fluid retention. During pregnancy, the challenge is in deciding when to use antihypertensive medications and what level of BP to target. Despite advancements in therapeutic discoveries, it is crucial to consider the safety of the novel agents as therapeutics in pregnant women and to the foetus. Small molecules, despite their role in clinical management, possess varying success rate. Sildenafil, a phosphodiesterase inhibitor, for instance, was originally thought to be a promising uterine vasodilator. However, it did not improve pregnancy outcomes compared to placebo treatment in the women with intrauterine growth restriction [45]. No antihypertensive drug has been proven safe for use during the first trimester [46].

252    Other small molecules such as statins (e.g., Pravastatin) because of their stimulatory effects

253    on hemeoxygenase (HO) and improved vascular functions [47] show clinical promise in

254    treating early onset preeclampsia; however, randomized clinical trials are still in progress

255    [45]. Ouabain, a cardiac glycoside, has been shown to inhibit sFLT-1 mRNA and protein

256    expression through the HIF-1α/heat shock protein 27 pathway in human cytotrophoblasts and

257    explant cultures; however, further investigation of ouabain as a therapeutic is yet to be

258    characterized [45-46]. Novel agents acting upon immediate mediators of vasorelaxation such

259    as TRP ion channels are potential therapeutic candidates for preeclampsia and other

260    hypertensive pregnancy disorders.

261    Therefore, novel natural or endogenous vasorelaxants are needed to treat hypertension

262    during pregnancy with lower risks and better safety during pregnancy. Novel agents acting

263    upon immediate mediators of vasorelaxation such as TRP ion channels are potential

264    therapeutic candidates for preeclampsia and other hypertensive pregnancy disorders. The

265    present study examines the role of natural compounds, capsaicin and eugenol, and assesses

266    the sensitivity of TRPV1 ion channel in our novel model of spiral arteries, the caprine middle

267    uterine arteries. TRP channels, a superfamily of about 28 nonselective cation channels

268    playing key role in vasomotor tone, are divided into 7 subfamilies including TRP Vanilloid

269    (TRPV) [48]. TRPV1 plays key regulatory role in thermal nociception [49] and is important

270    in thermal hyperalgesia after inflammation or tissue injury [50]. TRPV1 channels are found

271    on nociceptive axons in rat vagina [51] and human cervix [52]. In addition, estrogen enhances

272    pain evoked by uterine distension via a TRPV1 receptor-dependent mechanism [53].

273    Hypertension is the most common medical disorder of pregnancy, and it is possible that

274    activation of the TRPV1 receptor in the uterine vasculature could control such hypertension

275    during pregnancy.

276    Our study demonstrate that  vasorelaxation to capsaicin is attenuated by15% in uterine

277    artery of pregnant as compared to that of non pregnant goat. Eugenol-induced vasorelaxation

278   in PE-precontracted arteries was 31% greater in nonpregnant goats and 68% greater in

279   pregnant goats than that of capsaicin (100%) in nonpregnant goats. These results clearly

280   indicate that the contribution of Capsaicin-sensitive TRPV1 channels to vasorelaxation of

281   uterine artery is  reduced in pregnancy .

282    The TRPV1 antagonist CAPZ is a chemical derivative of capsaicin that has been used

283   extensively as a pharmacological tool to assess the role of TRPV1 in inflammatory

284   hyperalgesia [54]. Similarly, RR, a water soluble polycationic dye, has been found to block

285   the pores of the capsaicin-responsive cation channel TRPV1, thus interfering with all

286   polymodal ways of TRPV1 activation [55]. TRPV1 channel blocker has been reported to

287   inhibit TRPV1-mediated vasorelaxation in human coronary artery at 0.1 mM by 94%, rat skin

288   saphenous nerve preparation at 50 μM, and rat mesenteric artery at 3 μM by about 40% [56].

289   RR at 10 μM inhibited capsaicin-induced vasorelaxation in goat superior mesenteric artery by

290   83%, indicating that the TRPV1 channel in this tissue possesses antagonist binding sites with

291   greater sensitivity to RR [34]. TRPV1-induced vasorelaxation may be mediated by activation

292   of endothelial TRPV1 channels, causing increased $Ca^{2+}$ influx and consequent

293   phosphorylation of protein kinase A (PKA) and eNOS, leading to release of the vasorelaxing

294   factors cAMP and nitric oxide [28]. We demonstrate that CAPZ and RR reduced maximal

295   capsaicin-induced uterine artery vasorelaxation by 73% and 83%, respectively, in

296   nonpregnant and by 41% and 34%, respectively, in pregnant goats. The reduced blocking

297   effect of CAPZ and RR at capsaicin sensitive TRPV1 channels in pregnant as compared to

298   that of nonpregnant uterine arteries, RR having a greater inhibitory effect than does CAPZ in

299   nonpregnant goats and CAPZ having a greater inhibitory effect than RR in pregnant goats

300   clearly indicates that there is a significant reduced sensitivity of capsaicin sensitive TRPV1

301   channels to both the antagonists in uterine artery of pregnant goat. The reduced sensitivity of

302   TRPV1 channels could be resulted from reduced function arising from down-regulation or

303   expression of capsaicin sensitive TRPV1 channels or expression of other members of the

304 TRP families (e.g., TRPM8 and TRPA1) in these remodelled arteries whose roles in female

305 reproductive tract is yet to be determined.

306 Eugenol has numerous pharmacological effects, including inhibition of voltage-gated

307 sodium channels and activation of TRPV1 [56]. Electrophysiological studies show that

308 eugenol activates $Ca^{2+}$-permeable ion channels [57]. Both the in vitro and in vivo actions of

309 eugenol are reversed by CAPZ, suggesting that its effects are mediated by TRPV1. However,

310 eugenol is a nonselective TRPV1 agonist, as it is also able to activate other TRP channels,

311 such as TRPA1 and TRPM8 [58], and TRPV4 [59]. In this study we observed that eugenol

312 vasorelaxation increased from 31% in nonpregnant goats to 68% in MUA of pregnant caprine

313 when compared to that of capsaicin-induced vasorelaxation (100%). Furthermore, CAPZ and

314 RR inhibited eugenol vasorelaxation (100%) in uterine artery by 90% and 82%, respectively,

315 in nonpregnant and by 67% and 79%, respectively, in pregnant caprine indicating that

316 eugenol vasorelaxation in the MUA of nonpregnant goats is predominantly mediated by

317 capsaicin sensitive TRPV1 channels. This reduced inhibitory effect of CAPZ on eugenol-

318 induced vasorelaxation compared to that of RR clearly suggests that, in pregnant uterine

319 artery, eugenol vasorelaxation could be mediated in part by both CAPZ sensitive channels

320 and CAPZ-resistant TRPV1 channels. Eugenol in MUA of gravid uteri could be activating

321 other TRP channels such as TRPA1 and TRPM8 and this could explain the CAPZ-resistant

322 eugenol vasorelaxation observed in uterine arteries from pregnant animals.

323 Collectively, our results indicate that capsaicin sensitive TRPV1 channels show reduced

324 sensitivity in the MUA in pregnant goats as compared to those in nonpregnant. We

325 demonstrate that reduced blocking effects and/or binding affinities of both the selective and

326 nonselective TRPV1 antagonists in the MUA in pregnancy could be due to decreased

327 function or expression of capsaicin sensitive TRPV1 during vascular remodelling. Our

328 findings show greater differential increase in vasorelaxation between eugenol and capsaicin

329 in the MUA from pregnant than from nonpregnant animals. Though mechanism is yet to be

13

330     discovered, eugenol-induced selective vasorelaxation during pregnancy show high impact on

331     the therapeutic potential of this compound. A further investigation to characterize affinity of

332     eugenol to both TRPV1 and other related members of the TRP family in caprine model is

333     needed. Likewise, a greater inhibitory effect by both CAPZ and RR on eugenol-induced

334     vasorelaxation in MUA as compared to that on capsaicin vasorelaxation clearly indicates that

335     the TRPV1 channel has a greater sensitivity to eugenol that is further augmented upon

336     vascular remodelling in pregnancy. TRPV1 shows wide tissue distribution with highest

337     expression in dorsal root ganglion, trigeminal ganglia, and nodose ganglia [20] and is

338     associated with inflammatory conditions and nociception. TRPV1 expression occurs in

339     epidermis [60], polymorphonuclear granulocytes [61], smooth muscles [62], mast cells [63],

340     dendritic cells, and macrophages [64]. Several studies have shown its expression in neuronal

341     tissues brain regions [65, 66], and in non-neuronal tissues Similarly, it is sensitized in the

342     presence of inflammatory mediators such as prostaglandins and bradykinins, physical and

343     chemical stimuli [67-69]. Capsaicin and other related compounds are lipophilic and share

344     structural similarity with endogenous fatty acid derivatives that have been identified as

345     TRPV1 agonists such as anandamide (an endocannabinoid) interact at intracellular regions of

346     TRPV1 [70]. Mild acidic pH at extracellular side of the channel [71] also activates TRPV1.

347     Various activators of TRPV1 potentiate the effect leading to synergistic and enhanced

348     activity [72] which is important in the context of inflammation due to wide variety of

349     inflammatory agents generated in inflamed conditions.

350     In the context of pregnancy where an inflammatory condition ensues producing cytokines

351     and recruitment of leukocytes to the uterine site for implantation, growth, development, and

352     progression of pregnancy. Hormonal, cytokine, and immune milieu during pregnancy would

353     provide a continuous synergising and activating stimulus to TRPV1 and thus eugenol and

354     capsaicin could impact on this ion channel and induce vasorelaxation during hypertensive

14

355  pregnancy disorders including preeclampsia. Thus, these compounds can be promising

356  vasorelaxation inducers; however, further mechanistic studies are needed.

# Conclusions

358  Our results indicate that eugenol and capsaicin can be used as selective vasorelaxant and

359  TRPV1 may be involved in this phenomenon. These results are supported by evidence of

360  induced vasorelxation in the pregnant middle uterine arteries by the agents. We also showed

361  that eugenol is more potent and stronger inducer of vasorelaxation as shown by greater

362  inhibitory effect of CAPZ and RR on eugenol-induced vasorelaxation in pregnant MUA

363  compared to that in nonpregnant animals. Increased sensitivity of TRPV1 to eugenol during

364  pregnancy could be due to increased sensitivity of TRPV1 and/or activation of other TRP

365  channels, such as TRPV4, TRPA1, and TRPM8. Eugenol could be therapeutically useful in

366  treatment and prevention of hypertension during pregnancy (i.e., preeclampsia). Selective

367  vasorelaxation in the pregnant Caprine MUAs in novel spiral artery model by eugenol

368  mediated by TRP channels makes it a potent therapeutic vasorelaxant and begs a further

369  investigation of the underlying mechanisms.

# Acknowledgement

# Author Contributions

378  Conceptualization: Jandhyam H, Parija SC, Nayak NR

379  Data curation: Jandhyam H, Parija SC

380  Formal analysis: Jandhyam H

381     Funding acquisition: Jandhyam H, Parija SC, Nayak NR

382     Investigation: Jandhyam H, Parija SC

383     Methodology: Jandhyam H, Parija SC

384     Project Administration: Parija SC

385     Resources: Parasar P, Nayak NR, Mohanty BP

386     Software: Parija SC, Jandhyam H

387     Supervision: Parija SC, Mohanty BP

388     Validation: Parija SC, Parasar P, Nayak NR

389     Writing: Parija SC, Jandhyam H,Parasar P, Nayak NR,Mohanty BP

# References

391    1.   Vest AR, Cho LS. Hypertension in pregnancy. Cardiol Clin. 2012;30(3):407-23.

392    2.   Mancia G, Fagard R, Narkiewicz K, Redón J, Zanchetti A, Böhm M, Christiaens T, Cifkova R, De Backer G, Dominiczak A, Galderisi M, Grobbee DE, Jaarsma T, Kirchhof P, Kjeldsen SE, Laurent S, Manolis AJ, Nilsson PM, Ruilope LM, Schmieder RE, Sirnes PA, Sleight P, Viigimaa M, Waeber B, Zannad F; Task Force Members. 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens. 2013;31(7):1281-357. doi: 10.1097/01.hjh.0000431740.32696.cc.

400    3.   Witlin AG, Sibai BM. Magnesium sulfate therapy in preeclampsia and eclampsia. Obstet Gynecol. 1998;92(5):883–9.

402    4.   Lambert G, Brichant JF, Hartstein G, Bonhomme V, Dewandre PY. Preeclampsia: an update. Acta Anaesthesiol Belg. 2014;65(4):137–49.

404    5.   World Health Organization. WHO Guidelines Approved by the Guidelines Review Committee. WHO Recommendations for Prevention and Treatment of Pre-Eclampsia and Eclampsia; 2011. Available from: www.ncbi.nlm.nih.gov/ pubmed/23741776.

407    6.   Quan A. Fetopathy associated with exposure to angiotensin converting enzyme inhibitors and angiotensin receptor antagonists. Early Hum Dev. 2006;82(1):23-8.

409    7.   Al-Maawali A, Walfisch A, Koren G. Taking angiotensinconverting enzyme inhibitors during pregnancy: is it safe? Can Fam Physician. 2012;58(1):49-51.

411    8.   Meads CA, Cnossen JS, Meher S, Juarez-Garcia A, ter Riet G, Duley L, Roberts TE, Mol BW, van der Post JA, Leeflang MM, et al. Methods of prediction and prevention of pre-eclampsia: systematic reviews of accuracy and effectiveness literature with economic modelling. Health Technol Assess. 2008;12(6):iii–v. 1-270.

9.  Rolnik DL, Wright D, Poon LC, et al. Aspirin versus placebo in pregnan-cies at high risk for preterm preeclampsia. N Engl J Med. 2017;377:613–22.

10. Caritis S, Sibai B, Hauth J, Lindheimer MD, Klebanoff M, Thom E, VanDorsten P, Landon M, Paul R, Miodovnik M, Meis P, Thurnau G. Low-dose aspirin to prevent preeclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med. 1998;338:701-5.

11. Brown CM, Garovic VD. Drug treatment of hypertension in pregnancy. Drugs. 2014;74(3):283–96. doi:10.1007/s40265-014-0187-7

12. Al Khaja KA, Sequeira RP, Alkhaja AK, Damanhori AH. Drug treatment of hypertension in pregnancy: a critical review of adult guideline recommendations. J Hypertens. 2014;32(3):454-63.

13. Say L, Chou D, Gemmill A, Tuncalp O, Moller AB, Daniels J, Gulmezoglu AM, Temmerman M, Alkema L. Global causes of maternal death: a WHO systematic analysis. The Lancet. Global health. 2014;2(6):e323–33.

14. McBride CA, Bernstein IM, Badger GJ, Horbar JD, Soll RF. The effect of maternal hypertension on mortality in infants 22, 29weeks gestation. Pregnancy hypertension. 2015; 5(4):362–66.

15. Lain KY, Roberts JM. Contemporary concepts of the pathogenesis and management of preeclampsia. Jama. 2002;287(24):3183–6.

16. Rosenberg TJ, Garbers S, Lipkind H. Chiasson M. A. Maternal obesity and diabetes as risk factors for adverse pregnancy outcomes: Differences among 4 racial/ethnic groups. American J of Public Health. 2005;95(9):1545–51.

17. Boghossian NS, Yeung E, Mendola P, Hinkle SN, Laughon SK, Zhang C,  Albert PS. Risk factors differ between recurrent and incident preeclampsia: A hospital-based cohort study. Annals of Epidemiology. 2014;24(12):871–7e3.

18. Lim, K.-H., Steinberg, G., & Ramus, R. M. (2018). Preeclampsia. Retrieved November 14, 2018, from http://emedicine.medscape.com/article/1476919-overview.

19. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: Systematic review of controlled studies. British Medical Journal.2005;330(7491):565.

20. Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod. 2003;69(1):1-7.

21. Brosens I. A study of the spiral arteries of the decidua basalis in normotensive and hypertensive pregnancies. J Obstet Gynaecol Br Commonw. 1964; 71():222-30.

17

450  22. Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. Hypertension. 2007; 50(6):1142-7.

453  23. Makris A, Thornton C, Thompson J, Thomson S, Martin R, Ogle R, Waugh R, McKenzie P, Kirwan P, Hennessy A. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. Kidney Int. 2007 May; 71(10):977-84.

456  24. Baylie RL, Brayden JE. TRPV channels and vascular function. Acta Physiol (Oxf). 2011;203(1):99–116. doi:10.1111/j.1748-1716.2010.02217.x

458  25. Waning J, Vriens J, Owsianik G, Stüwe L, Mally S, Fabian A, Frippiat C, Nilius B, Schwab A. A novel function of capsaicin-sensitive TRPV1 channels: Involvement in cell migration. Cell Calcium. 2007;42:17–25.

461  26. Sharma SK, Vij AS, Sharma M. Mechanisms and clinical uses of capsaicin. Eur J Pharmacol. 2013;720:55-62.

463  27. Reyes-Escogido ML, Gonzalez-Mondragon EG, Vazquez-Tzompantzi E. Chemical and pharmacological aspects of capsaicin. Molecules. 2011; 16:1253-70.

465  28. Yang D, Luo Z, Ma S, Wong WT, Ma L, Zhong J, et al. Activation of TRPV1by dietary capsaicin improves endothelium-dependent vasorelaxation and prevents hypertension. Cell Metab. 2010;12:130-41.

468  29. Garcia-Sanz N, Fernandez-Carvajal A, Morenilla-Palao C, Planells-Cases R,Fajardo-Sanchez E, Fernandez-Ballester G, Ferrer-Montiel A. Identification of a tetramerization domain in the C terminus of the va-nilloid receptor. J Neurosci. 2004;24:5307–14.

471  30. Lo YC, Hsiao HC, Wu DC, Lin RJ, Liang JC, Yeh JL, et al. A novel capsaicin derivative VOA induced relaxation in rat mesenteric and aortic arteries: involvement of CGRP, NO, cGMP, and endothelium-dependent activities. J Cardiovasc Pharmacol. 2003;42:511-20.

474  31. Fujimoto S, Mori M, Tsushima H, Kunimatsu M. Capsaicin-induced, capsazepine-insensitive relaxation of the guinea-pig ileum. Eur J Pharmacol. 2006;530:144-51.

476  32. Gupta S, Lozano Cuenca J, Villalon CM, de Vries R, Garrelds IM, Avezaat CJ, et al. Pharmacological characterisationof capsaicin-induced relaxations in human and porcineisolated arteries. Naunyn chmiedebergs Arch Pharmacol. 2007;375:29-38.

479  33. Chen Q, Zhu H, Zhang Y, Zhang Y, Wang L, Zheng L. Vasodilating effect of capsaicin on rat mesenteric artery and its mechanism. J Zhejiang Univ Med Sci. 2013;42:177-83.

481  34. Mohanty I, Nayak NR, Parija SC. Augmentation of capsaicin-induced vasorelaxation of superior mesenteric artery (Capra hircus) in acidosis: role of TRPV1 channels. Indian Journal of Traditional Knowledge. 2016;15:625-31.

484   35. Leal-Cardoso JH, Coelho de Souza AN, Souza IT, Figueiredo IM. Effects of eugenol on
485       excitation– contraction coupling in skeletal muscle. Arch. Int. Pharmacodyn Ther.
486       1994;327:113–24.

487   36. Leal-Cardoso JH, Lahlou S, Coelho-de-Souza AN, Criddle DN, Pinto Duarte GIB,
488       Santos MIV. Inhibitory actions of eugenol on rats isolated ileum. Can J Physiol
489       Pharmacol. 2002;80:901–6.

490   37. Damiani CE, Rossoni LV, Vassallo DV. Vasorelaxant effects of eugenol on rat thoracic
491       aorta. Vascul Pharmacol. 2003;40:59–66.

492   38. Nishijima H, Uchida R, Kameyama K, Ohkubo T, Kitamura K. Mechanisms mediating
493       the vasorelaxing action of eugenol, a pungent oil, on rabbit arterial tissue. Jpn J
494       Pharmacol. 1999;79:327–34.

495   39. Szallasi A, Cruz F, Geppetti P. TRPV1: a therapeutic target for novel analgesic drugs?
496       Trends Mol Med. 2006;12:545-54.

497   40. Boominathan. Natural product-based therapy for Hypertension: Eugenol, a natural
498       compound isolated from herb Ocimum sanctum Linn (Tulsi), relaxes vascular smooth
499       muscle cells, and decreases blood pressure by increasing the expression of SERCA and
500       BKCa       Channels.       Genome-2-Bio-Medicine       Discoverycenter.       2015.
501       http://genomediscovery.org.

502   41. Peixoto-Neves D, Leal-Cardoso JH, Jaggar JH. Eugenoldilates rat cerebral arteries by
503       inhibiting smooth muscle cellvoltage-dependent calcium channels. J Cardiovasc
504       Pharmacol. 2014;64:401–6.

505   42. Mulvany MJ, Aalkjaer C. Structure and function of small arteries. Physiol Rev.
506       1990;70:921–961.

507   43. Phipps E, Prasanna D, Brima W, Jim B. Preeclampsia: Updates in Pathogenesis,
508       Definitions,   and   Guidelines.   Clin   J   Am   Soc   Nephrol.   2016;11(6):1102–13.
509       doi:10.2215/CJN.12081115

510   44. Ananth CV, Keyes KM, Wapner RJ. Pre-eclampsia rates in the United States, 1980-
511       2010: age-period-cohort analysis. BMJ. 2013;347:f6564.

512   45. Sharp A, Cornforth C, Jackson R, Harrold J, Turner MA, Kenny LC, Baker PN,
513       Johnstone ED, Khalil A, von Dadelszen P, Papageorghiou AT, Alfirevic Z, STRIDER
514       group. Maternal sildenafil for severe fetal growth restriction (STRIDER): a multicentre,
515       randomised,   placebo-controlled,   double-blind   trial.   Lancet   Child   Adolesc   Health.
516       2018;2:93–102

517   46. Podymow T, August P. Update on the Use of Antihypertensive Drugs inPregnancy.
518       Hypertension. 2008;51:960-969.

19

519    47. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH,
520        Romero R, Thadhani R, Karumanchi SA; CPEP Study Group. Soluble endoglin and
521        other circulating antiangiogenic factors in preeclampsia. N Engl J Med. 2006;355:992–
522        1005.

523    48. Venkatachalam    K.,    and    Montell    C,    "TRP    channels,"AnnualReview    of
524        Biochemistry,vol.76,pp.2007; 387–417.

525    49. Christoph T, Bahrenberg G, De Vry J, Englberger W, Erdmann VA, Frech M, Kogel B,
526        Rohl T, Schiene K, Schroder W, Seibler J, Kurreck J. Investigation of TRPV1 loss-of-
527        function phenotypes in transgenic shRNA expressing and knockout mice. Molecular and
528        Cellular Neuroscience. 2008; 37:579–89. [PubMed: 18249134]

529    50. Vilceanu D, Honore P, Hogan QH, Stucky CL. Spinal Nerve Ligation in Mouse
530        Upregulates TRPV1 Heat Function in Injured IB4-Positive Nociceptors. The Journal of
531        Pain. 2010;11:588–99. [PubMed: 20015699]

532    51. Tingaker BK, EkmanOrdeberg G, Facer P, Irestedt L, Anand P. Influence of pregnancy
533        and labor on the occurrence of nerve fibers expressing the capsaicin receptorTRPV1in
534        human corpus and cervix uteri. Reprod Biol Endocrinol. 2008;6:8.

535    52. Yan T, Liu B, Du D, Eisenach JC, Tong C. Estrogen amplifies pain responses to uterine
536        cervical distension in rats by altering transient receptor potential-1 function. Anesth
537        Analg. 2007;104:1246–50.

538    53. Watanabe M, Ueda T, Shibata Y, Kumamoto N, Ugawa S. The role of TRPV1 channels
539        in carrageenan-induced mechanical hyperalgesia in mice. Neuroreport. 2015;26:173–8.

540    54. Pierre MS, Reeh PW, Zimmermann K. Differential effects of TRPV channel block on
541        polymodal activation of rat cutaneous nociceptors in vitro. Exp Brain Res. 2009;196:31-
542        44.

543    55. Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Högestätt ED, et al.
544        Pungent products from garlic activate the sensory ion channel TRPA1, Proc Natl Acad
545        Sci USA. 2005;102:12248-52.

546    56. Park CK, Kim K, Jung SJ, Kim MJ, Ahn DK, Hong SD, et al. Molecular mechanism for
547        local anesthetic action of eugenol in the rat trigeminal system. Pain. 2009;144:84–94.

548    57. Ohkubo T, Shibata M. The selective capsaicin antagonist capsazepine abolishes the
549        antinociceptive action of eugenol and guaiacol. J Dent Res. 1997;76:848–51.

550    58. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, et al. Noxious cold
551        ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron.
552        2004;41:849–57.

553  59. Peixoto-Neves D, Wang Q, Leal-Cardoso JH, Rossoni LV, Jaggar JH. Eugenol dilates
554      mesenteric arteries and reduces systemic BP by activating endothelial cell TRPV4
555      channels. British Journal of Pharmacology. 2015; 172:3484–94

556  60. Choi TY, Park SY, Jo JY, et al. Endogenous expression of TRPV1 channel in cultured
557      human melanocytes. J Dermatol Sci. 2009;56(2):128–30

558  61. Heiner I, Eisfeld J, Halaszovich CR, et al. Expression profile of the transient receptor
559      potential (TRP) family in neutrophil granulocytes: Evidence for currents through long
560      TRP channel 2 induced by ADP-ribose and NAD. Biochem J. 2003;371:1045–53.

561  62. Birder LA, Kanai AJ, de Groat WC, et al. Vanilloid receptor expression suggests a
562      sensory role for urinary bladder epithelial cells. Proc Natl Acad Sci USA.
563      2001;98(23):13396–401.

564  63. Stander S, Moormann C, Schumacher M, et al. Expression of vanilloid receptor subtype
565      1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage
566      structures. Exp Dermatol. 2004;13(3):129–39.

567  64. Chen CW, Lee ST, Wu WT, Fu WM, Ho FM, Lin WW. Signal transduction for
568      inhibition of inducible nitric oxide synthase and cyclooxygenase-2 induction by
569      capsaicin and related analogs in macrophages. Br J Pharmacol. 2003;140(6):1077–87.

570  65. Mezey E, Toth ZE, Cortright DN, et al. Distribution of mRNA for vanilloid receptor
571      subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the
572      rat and human. Proc Natl Acad Sci USA. 2000;97(7):3655–60.

573  66. Storozhuk, Maksim V., Moroz, Olesia F., and Zholos, Alexander V. Multifunctional
574      TRPV1 Ion Channels in Physiology and Pathology with Focus on the Brain, Vasculature,
575      and Some Visceral Systems. BioMed Research International. 2019;5806321.

576  67. Tominaga M, Caterina MJ, Malmberg AB, et al. The cloned capsaicin receptor integrates
577      multiple pain-producing stimuli. Neuron. 1998;21(3):531–43.

578  68. Cromer BA, McIntyre P. Painful toxins acting at TRPV1. Toxicon. 2008;51(2):163–73.

579  69. Alawi K, Keeble J. The paradoxical role of the transient receptor poten-tial vanilloid 1
580      receptor in inflammation. Pharmacol Ther. 2010;125(2): 181-95.

581  70. Jung J, Hwang SW, Kwak J, et al. Capsaicin binds to the intracellular domain of the
582      capsaicin-activated ion channel. J Neurosci. 1999;19(2):529–38.

583  71. Jordt SE, Tominaga M, Julius D. Acid potentiation of the capsaicin receptor determined
584      by a key extracellular site. Proc Natl Acad Sci USA. 2000;97(14):8134–39.

585  72. Latorre R, Vargas G, Orta G, Brauchi S. Voltage and temperature gating in thermoTRP
586      channels. In: Liedtke W, Heller S, editors. TRP Ion Channel Function in Sensory
587      Transduction and Cellular Signaling Cascades.London, UK: CRC Taylor and Francis;
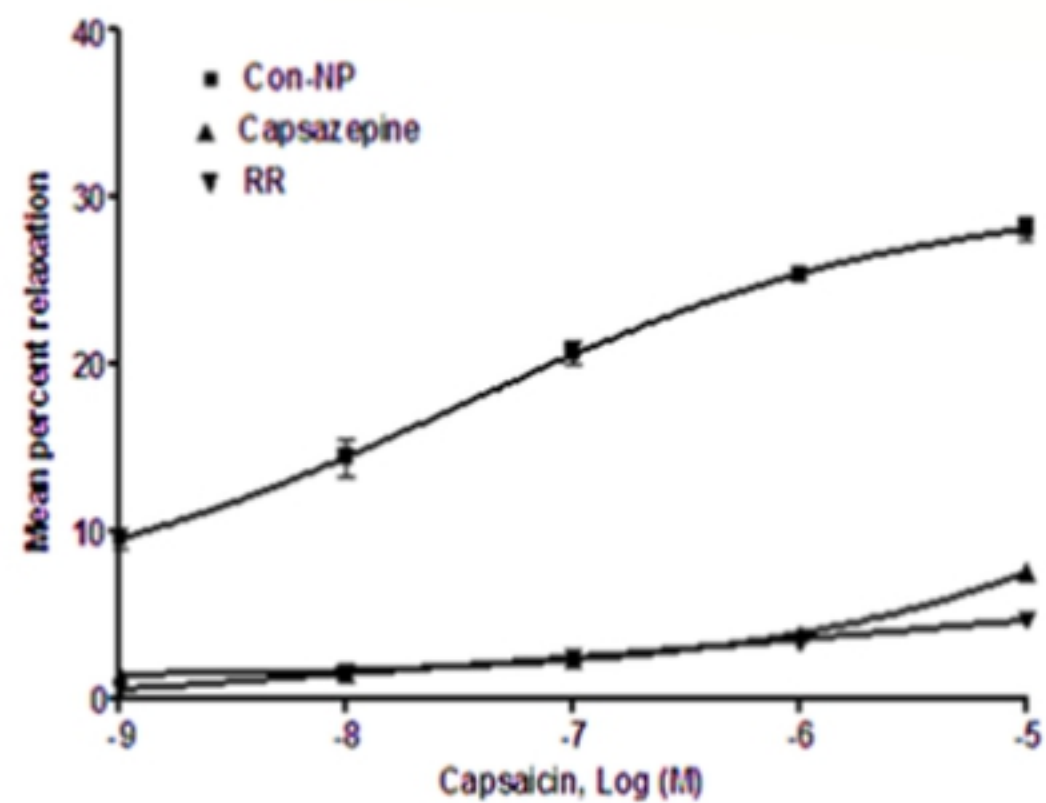
588    2007.

589

590

591

592

593

594

595

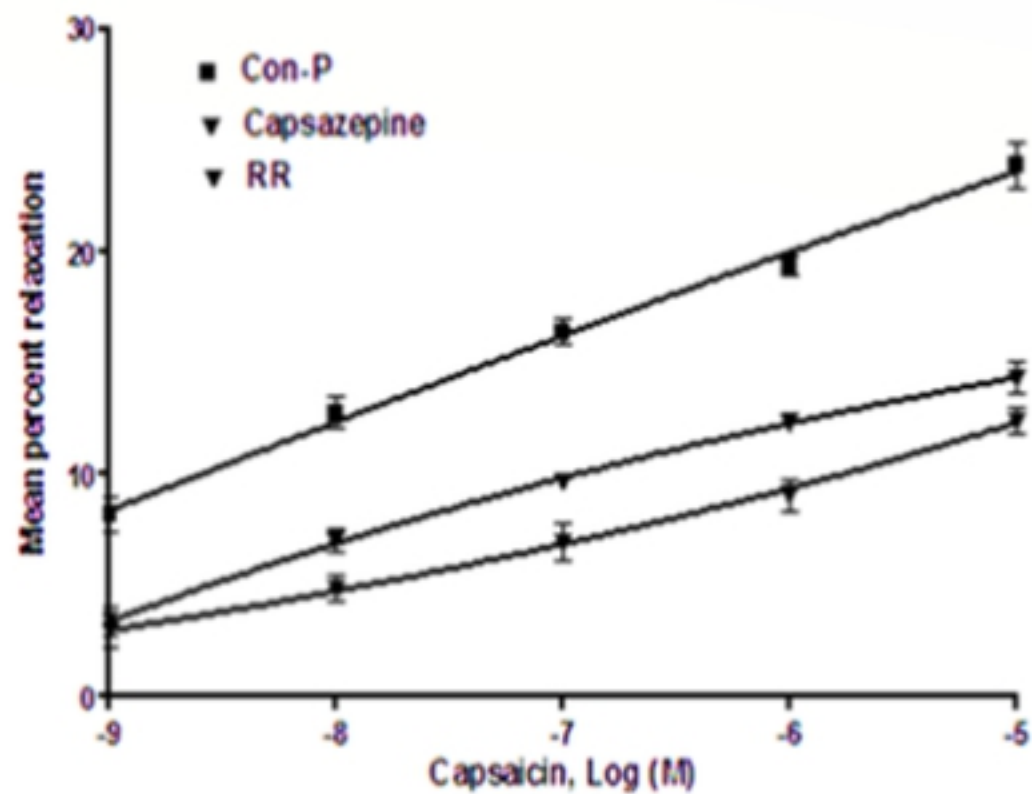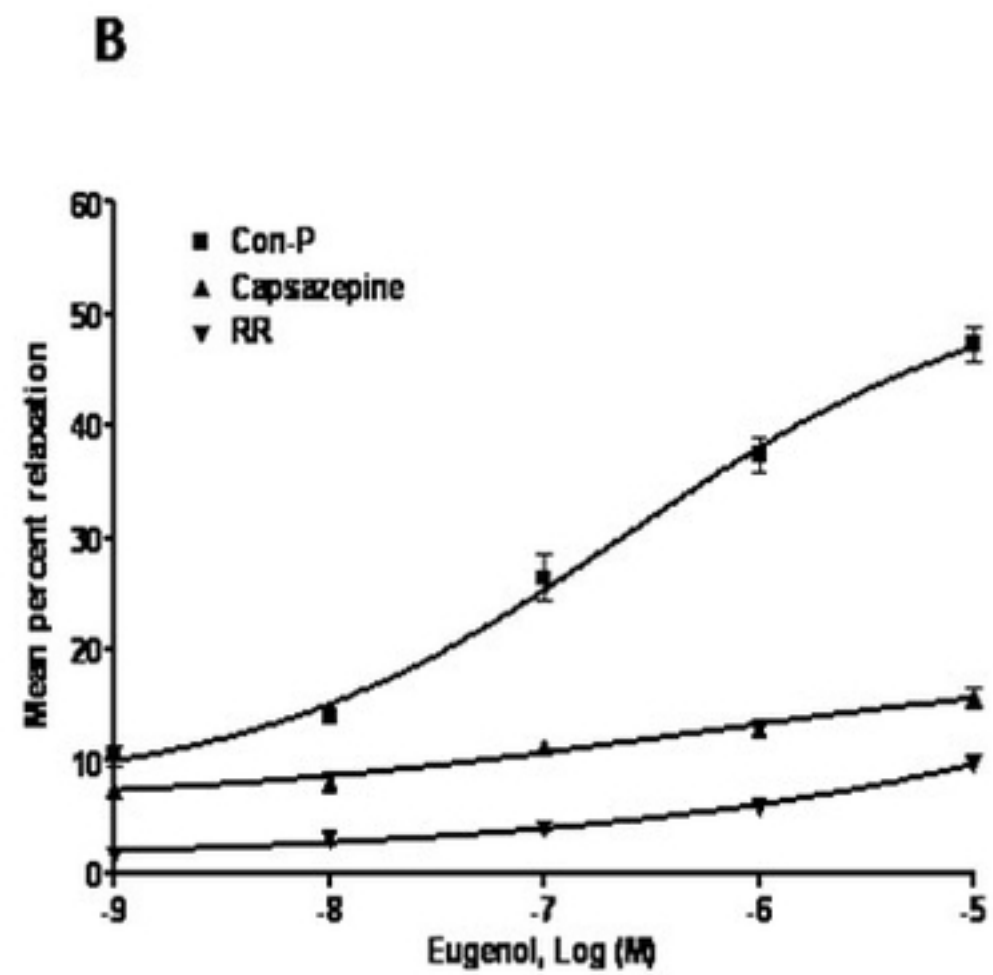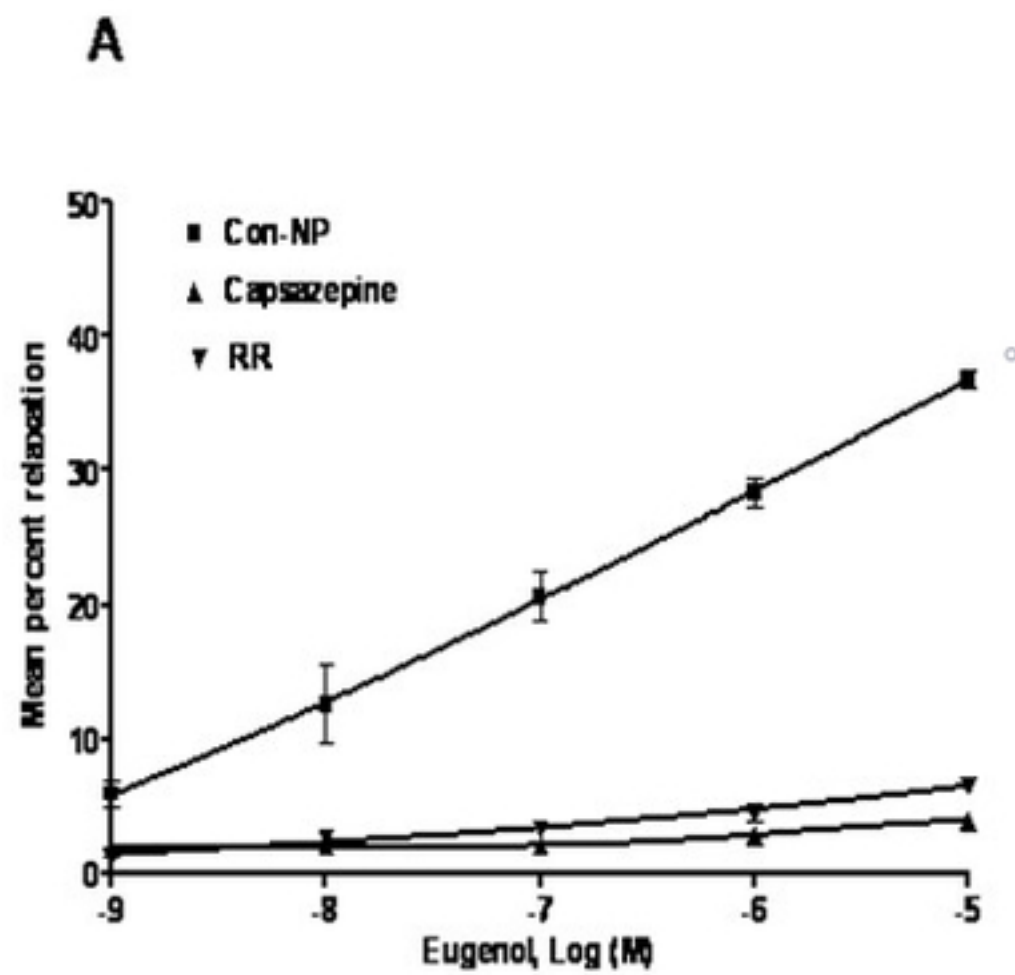596

597

598

599

Fig 1

Fig 2

Fig 3