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SMOOTH MUSCLE RELAXANT ACTIVITY OF ESSENTIAL OIL OF *PISTACIA INTEGERRIMA* J.L. STEWART EX BRANDIS GALLS ON RAT UTERUS

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ABSTRACT

Ethnopharmacological relevance: *Pistacia integerrima* J.L. Stewart ex Brandis is valued in traditional medicine used in India for the treatment of asthma, chronic bronchitis, diarrhoea, and fever and incorporated in Ayurvedic formulations like 'Chvyanprash Avaleha', 'Kumari Asava', 'Kumari Kalp' prescribed in weakness as rejuvenating agent and tonic. However, *in vitro* studies providing new insights into its pharmacological properties on isolated rat uteruses have not been thoroughly investigated. Aim: The present investigation aimed to elucidate the effect of the essential oil of *Pistacia integerrima* on isolated rat uterus and to find the probable mechanism. Methods: The essential oil of *Pistacia integerrima* was characterised by GCMS analysis and tested using *in vitro* studies to relax the contraction of rat uterus rings induced by KCl (60 mM) and oxytocin. We also investigated the effect of the essential oil of *Pistacia integerrima* on extracellular calcium uptake. In order to study the effect of β-adrenergic antagonist and NO-synthase inhibitor in the relaxing effect of essential oil of *Pistacia integerrima*, the uterine rings were incubated with L-NAME (100 µM) and propranolol (10µM) prior to addition of essential oil of *Pistacia integerrima*. The effect of the essential oil of *Pistacia integerrima* on extracellular calcium uptake was studied in a Ca^{2+} free medium. Results: The essential oil of *Pistacia integerrima* (10, 30 and 100µg/mL) showed a relaxant effect on both KCl and oxytocin-induced tonic contractions of isolated rat uterus separately. Preincubation of L-NAME, a classical NO-synthase inhibitor (100 µM), did not alter the relaxation induced by the essential oil of *Pistacia integerrima*. Pre-treatment of the uterine rings with essential oil of *Pistacia integerrima* (5, 10 and 20 µg/mL) and Nifedipine (10^{-7} M) inhibited the cumulative response curve of $CaCl_2$ (10^{-4} to 10^{-2} M) in Ca^{2+} free medium. Conclusion: The present investigation indicates that the essential oil of *Pistacia integerrima* J.L. Stewart ex Brandis shows uterine relaxant effect *in vitro* in a concentration-dependent manner attributed to tetracyclic triterpenoids. The effect is mediated inhibition of the cumulative Ca^{2+}-induced contraction in the depolarised uterus rings and does not depend on NO or/and β-adrenergic receptors. These findings suggest that the essential oil of *Pistacia integerrima* acts on voltage-sensitive calcium channels.

Keywords: *Pistacia integerrima* J.L. Stewart ex Brandis, tocolytic, essential oil

INTRODUCTION

Tocolytic agents are used to treat preterm labour, which is a significant cause of morbidity and mortality, by decreasing the uterine contractility for postponing labour and improving neonatal outcomes[1-4]. Preterm labour has been conventionally treated with β_2 agonists, magnesium sulphate, oxytocin antagonists and prostaglandin synthetase inhibitors. However, sometimes they are insufficient, and some areas are associated with side effects like hyperglycemia, increased cardiac output, tachycardia, pulmonary oedema, cardiac depression, and inhibition of neuromuscular transmission[5]. It is, therefore, necessary to search for effective and safe alternative drugs for the treatment of preterm labour.

Pistacia integerrima JL Stew ex Brandis (Family Anacardiaceae) galls are found during the autumn season and widely distributed on the steps of the Western Himalayas from Indus to Kumaon and regularly cultivated in Punjab plains. The galls of *P. integerrima* Stew ex brandis are used in some of the Ayurvedic formulations like 'Chvyanprash Avaleha', 'Kumari Asava', 'Kumari Kalp' prescribed in weakness as rejuvenating agent and tonic[6]. It has been used in folk medicine as an antispasmodic, antiasthmatic, antiarthritic, anticancer, immunomodulator, antidiabetic and hepatoprotective properties[7,8].

Pistacia integerrima JL Stew ex Brandis galls have been the subject of various previous pharmacological reports that have demonstrated CNS depressant[9], analgesic, anti-inflammatory[10-12], hyperuricemia effect, anti-hyperuricemic[13], and anti-angiogenic[14] activities. Additionally, an essential oil study has demonstrated in vitro relaxant action of rat ileum[15].

However, it has not been investigated potential actions on the uterus muscle, which is related to the popular use on menstrual disorders or as an abortifacient. Chemical investigations of the galls led to the characterisation of compounds α-pinene, β-pinene, alpha-phellandrene, δ-carenetriterpenoids, catechins and flavonoid glycosides. Recently[16] isolated pistaciaphenyl ether and pisticiphloro-glucinyl ether from the galls.

Based on the ethnobotanical and pharmacological reports, we postulate that the essential oil of *P. integerrima* Stew ex Brandis galls could protect uterine spasms. The present investigation was conducted *in vitro* studies of rat uterine rings to test this hypothesis.

MATERIALS AND METHODS

Drugs and standards

L-NAME was purchased from Sigma Aldrich, USA, Oxytocin (Pitocin, Parke Davis). Propranolol purchased from M/s. Loba Chemie, Mumbai, Maharashtra, India. Nifedipine was a generous gift sample from Sun Pharmaceuticals India. All the chemicals for the De-Jalon solution were of analytical grade and purchased from Thomas Baker and Sd Fine-Chem Limited, India.

Procurement and Extraction of Essential oil of *P. integerrima* J.L. Stewart ex Brandis

Dried galls of *Pistacia integerrima* J.L. Stewart ex Brandis were obtained in April 2010 from a local distributor in Mumbai, India, and verified by Dr. Ganesh Iyer. A voucher specimen (number 007) was added to the Ramnarain Ruia College herbarium at Bombay University in India. Using Clevenger's apparatus, the essential oil of *Pistacia integerrima* J.L. Stewart ex Brandis galls (EOPI) was extracted from powdered and dried galls through a process known as hydrodistillation[17].

Characterisation of *P. integerrima* J.L. Stewart ex Brandis galls

Pycnometers and refractometers were used to measure the specific gravity and refractive index of the EOPI's essential oil. GC/MS analysis using a Hewlett-Packard 5970A mass selective detector was used to assess the chemical composition of EOPI as described earlier[14].

Animals

Female Sprague–Dawley rats (175 ± 15 g) were purchased from Haffkine's Institute, Mumbai, India. All animals were housed in standard polypropylene cages with wire mesh top and husk bedding and maintained under standard temperature conditions (23 ± 2 ˚C) and relative humidity (60 ± 5%) with 12 h light: dark cycle. Animals were fed with commercially available standard rodent pellet diets. Water was provided *ad libitum* to the animals. Animals were acclimatised to laboratory conditions before the tests. All experiments were carried out between 09:00 and 17:00 h. For all animal experimentation protocols (Protocol No. 25/2010), prior approvals were obtained from 'Institutional Animal Ethics Committee', of Bombay College of Pharmacy, Mumbai (registration number CPCSEA-BCP/2010/22) and all studies were performed in accordance with 'Committee for the Purpose of Control and Supervision on Experiments on Animals' (CPCSEA) guideline, Government of India on animal experimentation.

Pharmacological studies

Tissues were obtained from virgin female Wistar rats (200–250 g body weight) pre-treated with estradiol-17-benzoate (40 µg/kg s.c.) 48 h prior to the experiments. The oestrus stage was confirmed by vaginal smear microscopic examination.

Preparation of isolated rat uterus: Estradiol benzoate (5 mg/kg) was subcutaneously administered to female rats to induce the oestrous cycle. The oestrous stage was verified by microscopic analysis of vaginal smears after 24 hours. The uterine horns of the sacrificed animals were carefully removed from the surrounding tissue and fat. They were then placed in an organ bath that contained the following components of the De-Jalon Solution (mM): NaCl-154, KCl-5.63, $CaCl_2$-0.648, NaHCO3-5.95, and glucose-2.77; the mixture was kept at 31°C and continuously bubbled (1 bubble/s) using a gas mixture consisting of 5% CO_2 and 95% O_2.

The tissues employed in the investigation had a length of around 2 cm. Every horn had its connective tissues and adherent lipids removed. Before recording isotonic contractile responses, the tissues (2 cm) were placed in a 20 mL organ bath, kept at a tension of 1 g, and allowed to equilibrate for 1 hour. In between, the tissues were washed with 20 mL of fresh De-Jalon every 10 minutes. Using LabChart 7 software and a force transducer (MLT 050/D) from AD Instruments, Australia, the mechanical responses in the ileum were recorded isometrically.

At the peak deflection, the contractile amplitude was measured. Only preparations with a plateau contraction that lasted at least 10 minutes were chosen for the EOPI-induced relaxation of preparations pre-contracted with 60 mM K+ solutions. The first research findings were used to determine the EOPI dosages. The LD_{50} of EOP I in Swiss albino mice's acute toxicity was found to be 229.1 mg/kg i.p.[15]

Experimental design: The following methodology was used to test *P. integerrima* J.L. Stewart ex Brandis galls essential oil (EOPI) on uterine ring contractions: Twenty minutes after the stimulus, the uterine rings were contracted with a depolarising solution, and the test chemicals were injected during tonic contraction and this time roughly corresponded to a plateau phase in previously utilised procedures[18-20].

Effect of EOPI on KCl-induced tonic contraction: After stabilisation of uterine tissues, they were challenged with KCl 60mM (final organ bath concentration), which induced a rapid phasic contraction, followed by slight relaxation and a sustained tonic contraction. EOPI (0.1 to 30 µg/mL) was added to the organ bath after this steady-state contraction, and the relaxant effect was recorded as described above.

Effect of EOPI on oxytocin-induced rhythmic contractions of isolated rat uterus: As described earlier[21] the uterine horn pieces were mounted and allowed to equilibrate for one has mentioned earlier. Addition of Oxytocin 0.5 mU/ mL (final concentration) induced rhythmic contractions. After washout, the tissues were challenged with OT. After the rhythmic contractions were stabilised, the effects of cumulative amounts of EOPI (10, 30 and 100 µg/mL) were studied and recorded similarly, as mentioned earlier.

Effect of EOPI on extracellular calcium uptake: After a conditioning period in normal De-Jalon solution, uterine rings were incubated in calcium-free solution for 10 min. Subsequently, cumulative concentrations of $CaCl_2$ (10–100 mM) were added to the organ bath to get the concentration-response curve. The preparation was stabilised with normal De-Jalon solution for 60 min, and a similar concentration-response curve was repeated after 10 min preincubation of EOPI (5, 10 and 20 µg/mL). The effect of solvent was assessed. The data were expressed as a percentage of the maximal tonic response induced by KCl 60mM (100%).

Effect of EOPI on adrenergic antagonist and NO synthase inhibitor: In order to study the effect of β-adrenergic antagonist and NO-synthase inhibitor in the relaxing effect of EOPI, the uterine rings were incubated for 20 min with L-NAME (100 µM) and propranolol (10 µM) prior to addition of EOPI. To obviate the effect of biological variability of tissues, the test was repeated five times, adding tissue control in each experiment[22-23].

STATISTICAL ANALYSIS

All data are expressed as Mean ± SEM. The significance ($p < 0.05$) of the results was assessed by means of paired or unpaired Student's t-test and ANOVA, followed by a multiple comparison test, where appropriate. IC_{50} values were calculated by GraphPad Prism 5 (GraphPad Software Inc).

RESULTS

Characterisation of EOPI

The essential oil was discovered to be a colourless liquid with a pronounced astringent taste and terebinthine odour. The results showed that the EOPI had a specific gravity of 0.889 g/mL and a refractive index of 1.215. It was discovered that 1.73 percent of the essential oil extracted from *Pistacia integerrima* J.L. Stew. Ex Brandis galls were yielded. The pH of 1 mg/mL EOPI was discovered to be between 6 and 6.5.

GC-MS was used to analyse the leaf essential oil's constituent parts. The following compounds made up the EOPI (percentage of oil weight): L-4-terpineol (11.93), Borneol (8.90), Tetra-hydrocarvone (10.27), 4-Carvomenthenol (17.06), Levo-bornyl acetate (13.99), Cymene (11.54), and (-)-Spathulenol (6.35).

Effect of EOPI on KCl-induced tonic contraction

EOPI antagonised KCl-induced tonic contractions of isolated rat uterus at 10, 30 and 100 µg/mL concentrations. IC_{50} was found to be 13.78 µg/mL (Figure 1).

Effect of EOPI on oxytocin-induced tonic contraction

The maximum response evoked by oxytocin was reduced by the EOPI and was found to be 75.34, 51.48, and 14.62% at doses of 10, 30 and 100 µg/mL, respectively (Figure 2).

Effect of EOPI on extracellular calcium uptake

Uterine rings were pre-incubated with different concentrations of EOPI (5, 10 and 20 µg/mL) under a calcium-free solution. Subsequent cumulative addition of calcium (10^{-4} to 10^{-2} M) induced contraction was significantly inhibited by EOPI (Figure 3). These results may imply that effects induced by EOPI on contractions provoked by KCl are due to changes in the ability of tissues to uptake external calcium.

Effect of EOPI on adrenergic antagonist and NO synthase inhibitor

Propranolol, a conventional β- adrenergic antagonist (10 µM), was also unable to inhibit the spasmolytic effect. Preincubation of L-NAME, a classical NO-synthase inhibitor (100 µM), did not alter the relaxation induced by EOPI.

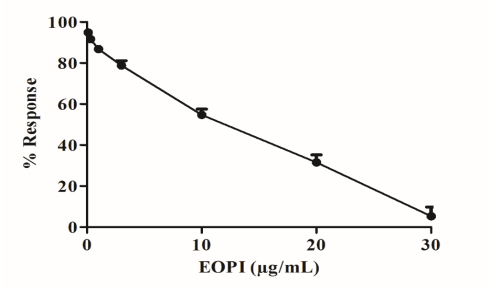

Figure 1: Relaxant Effect of EOPI on KCl-induced tonic contraction.

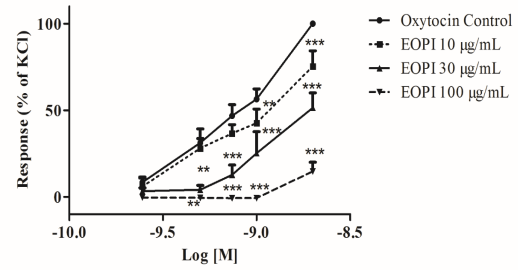

Figure 2: Inhibitory effect of EOPI on oxytocin-induced rhythmic contractions of isolated rat uterus. Each value represents the Mean ± SEM of six determinations. **p < 0.01, ***p < 0.001 Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

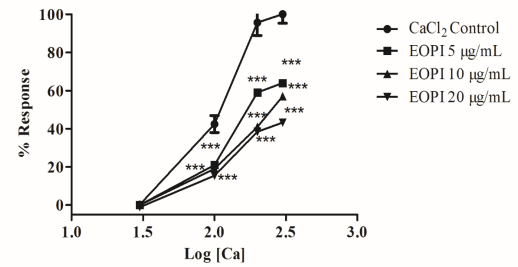

Figure 3: Inhibitory effect of EOPI on $CaCl_2$-induced contraction of isolated rat uterus preparation. Each value represents the Mean ± SEM of six determinations. **p < 0.01, ***p < 0.001 Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

DISCUSSION

Several mechanisms have been reported for smooth muscle relaxation, like β_2 adrenoceptors stimulation and phosphodiesterase (PDE) inhibition receptor antagonism like antioxytocics[5], antihistaminics and antimuscarinics[24]. Oxytocin causes uterine contractions by increasing the intracellular Ca^{2+} level by releasing Ca^{2+} from both sarcoplasmic reticulum via inositol-1,4,5-triphosphate (IP3) pathway and extracellular fluid through voltage-operated calcium channels[25]. Oxytocin-induced rhythmic contractions have been known to be inhibited by calcium channel blockers[26], β_2-agonists, oxytocin receptor antagonists[27] and papaverine[25].

For the first time, the present investigation demonstrated that EOPI was effective in ameliorating the uterine smooth muscle spasm attributed to the presence of tetracyclic triterpenoids. EOPI showed relaxation of contractions evoked by high K^+, which is related to the influx of Ca^{2+} into the cell, specifically through the L-type channel. It suggests that EOPI has a different mechanism of action since the potassium channel openers do not inhibit high K^+ depolarised contractions[28].

The inhibitory effect of EOPI on high K^+ depolarised contractions and oxytocin-induced rhythmic contractions did not elucidate whether it possessed intracellular activity. In order to ascertain this hypothesis, the influence of EOPI was studied on contractions induced by Oxytocin in Ca^{2+} free PSS. The contractions in these conditions were related only to calcium release from intracellular stores[25]. Pre-treatment of the uterine rings with EOPI and Nifedipine showed an inhibitory effect on the cumulative response curve of $CaCl_2$. These results may imply that effects induced by EOPI on contractions provoked by KCl are due to changes in the ability of tissues to uptake external calcium. In addition, the relaxant effect of EOPI on uterine relaxation was independent of Nitric oxide (NO) and adrenergic antagonist, similar to our earlier reports on ileum[15].

The present investigation strongly supports using *Pistacia integerrima* J.L. Stewart ex Brandis as a tocolytic agent. GCMS analysis of EOPI revealed the presence of tetracyclic triterpenoids, which exhibit uterine relaxant effect[29].

CONCLUSION

The present investigation indicates that the essential oil of *Pistacia integerrima* J.L. Stewart ex Brandis (EOPI) shows a concentration-dependent uterine relaxant effect *in vitro* attributed to tetracyclic triterpenoids. The effect is mediated inhibition of the cumulative Ca^{2+}-induced contraction in the depolarised uterus rings and does not depend on NO or/and β-adrenergic receptors. These findings suggest that EOPI acts on voltage-sensitive calcium channels.

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