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Research Article

Evaluation of uterotonic activity of ethanol leaf extract of *Tridax procumbens* on uterine strips isolated from gravid and non-gravid rats

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Tridax procumbens L, Uterine smooth muscle, uterotonic activity.

SUMMARY

Objective: In order to provide pharmacological rationale for folkloric use, this study was designed to investigate the effect and possible mechanisms of action of the ethanol leaf extract of *Tridax procumbens* (ELETP) on uterine smooth muscle.

Methods: Isometric contractions of uterine muscle strips (UMS) preparations of non-gravid (NPRGR), early (EPRGR) and late gravid (LPRGR) rats, mounted in 50 ml organ bath were studied under an initial tension of 1 g, at 37^{0}C and pH of 7.4. Uterine muscle strips (UMS) were pre-contracted with phenylephrine (PE) (10^{-7} M) before being treated with various doses of ELETP (0.2-1.0 mg/ml). Mechanism of ELETP-induced contraction was further examined using atropine, L-NAME, indomethacin and nifedipine.

Results: The results showed that ELETP significantly increased contraction of UMS in a dose-dependent manner with the gravid having higher responses. Also in the presence of ELETP, the responses of UMS to acetylcholine and oxytocin–induced contraction were significantly ($p<0.05$) increased. The uterotonic effects caused by ELETP were significantly reduced when uterine strips were pre-incubated with calcium blocker (Nifedipine) by 94.6 %, 98.1% and 87.9%; Nitric oxide synthase inhibitor (L-NAME) 71.1 %, 41.4% and 51.6%; prostacyclin inhibitor (indomethacin) 57.7 %, 98.1 % and 87.9 %; and muscarinic blocker (atropine) 82.4 %, 80.5 % and 81.9 % in NPRGR, EPRGR and LPRGR groups respectively.

Conclusion: We conclude that ELETP enhanced the contraction of uterine smooth muscle and this activity may probably involve calcium exchange as well as NO and prostacyclin pathways.

INTRODUCTION

According to World Health Organization (WHO) estimates, 80 % of the population living in rural areas in developing countries depend on traditional medicine for their health needs.[1] While the use of herbal medicines in pregnancy varies considerably between countries, evidence from the African continent suggests wide variability in use of herbal medicines during pregnancy, from a high of about 68% as reported in studies[2,3] to a low of 12 % in another study from Nigerian.[4] In Lusaka, Zambia, 21 % of pregnant women seeking care in public health system used traditional medicines during pregnancy.[5] Research on medicinal plants is crucial, as it often provides a "starting points" for discovery of novel remedies and natural drugs for the management and treatment of pregnancy and birth related problems.[6]

Several plants species and parts are reportedly used by the traditional birth attendant (TBA) practitioners during pregnancy, birth and post-partum period due to their uterotonic properties. These include root of *Hydrastis Canadensis* L, and *Actaea racemosa* [Nutt.] L, fruit of *Vitex agnus-castus* L, leaves of *Rubus wilkensiana* L, seed oil from *Ricinus communis* L, leaf of *Acalypha idaeus* and *Cassia italic*.[7-9] Others include leaf and stem bark of *Citroposis articulate*, stem bark of *Chenopodium ambrosioides* and *Oldenlandia affinis*.[9] Scientific validation for the use of most of these plants parts and species in the management and treatments of antenatal and postnatal complications have been reported in various animal models.[10]

Tridax procumbens Linn. (Asteraceae) is a plant that is native to tropical America and naturalized in tropical Africa. It is a wild herb distributed throughout the world.[11] The extracts of *Tridax procumbens* is reported to possess significant pharmacological activities like anti-oxidant,[12] analgesic,[13] hypoglycemic,[14] hypotensive,[15,16] hepatoprotective,[17] and anti-microbal activity.[18] The leaves of *Tridax procumbens* is one of the medicinal plants routinely used by traditional birth attendant in Badagry, South Western part of Nigeria during pregnancy. It was claimed that the extract of this plant was routinely used during labour and management of post-partum haemorrhage as well as to expel retained placenta. However, its potency as uterotonic agent has not been scientifically validated and documented. In view of this dearth of information, the present study was undertaken to investigate the possible contractile effect and mechanisms of action of ELETP on isolated uterine muscle strips of non-gravid and gravid female rats.

MATERIALS AND METHODS

Plant material and preparation of ethanol leaf extract of *T. procumbens* (ELETP)

Samples of fresh *Tridax procumbens* plants were collected from open grassland of Lagos State University, Ojo, Lagos, Nigeria. Identification of the plant was carried out by a Taxonomist of the Forestry Research Institute, Ibadan, Nigeria. Following identification, a specimen with voucher number FHI 1008877 of the plant was deposited in the herbarium of the Forestry Research Institute. Fresh *T. procumbens* leaves (1 kg) were air-dried in a well-ventilated and shaded room at 26 ± 1^0C for 2 weeks. The air-dried leaves were milled into fine powder in a warring commercial blender. 500 g of the resultant powder was soaked inside 1.5 litres of 95% ethanol for 72 hours at room temperature. The solvent was evaporated at 40^0C under vacuum (Rotavapor) and final ethanolic extract was lyophilized to give a yield of 13.6% of ELETP. The stock solution was prepared as aqueous suspension with 4 g/100 ml of normal saline for this study.

Animals and Experimental design

Healthy young adult female Wistar albino rats, between 8 and 10 weeks of age and weighing 150 - 200g, were used. The animals were kept and maintained under conventional laboratory conditions of temperature, humidity and light, and were allowed standard pellet diet (Livestock Feeds Nig., Ikeja, Nigeria) and water *ad libitum*. The animals were divided into three groups as follows: Group I: Non-pregnant rats (NPRGR). Vaginal smears were monitored from young adult female rats before the animals were sacrificed in order to ascertain that they were in oestrous state. Group II: Early pregnancy rats (EPRGR) and Group III: Late pregnancy rats (LPRGR). Animals in groups II and III were mated when in oestrous stage with male rats and monitored every day for the presence of sperm positive vaginal smear or vaginal plug.[19] The day on which sperm positive vaginal smear or plug was first observed was taken as Day 1 of pregnancy of the female rat.[19] The period from Day 1 to Day 8 was taken as early pregnancy/gestation, while from Day 16 to Day 21 was taken as late pregnancy/gestation period.

Experimental Procedure

Experimental protocols and procedures used in this study were approved by the animal ethics committee of the Lagos State University College of Medicine, Ikeja and conformed to the 1985 Guidelines for Laboratory Animal Care of the National Institute of Health (NIH).

The animals were anaesthetized with urethane at a dose rate of 1mg per gram body weight and sacrificed by cervical dislocation. The abdomen was surgically opened and the uterine horns exposed by means of blunt dissection and then freed of connective tissue. Each horn was cut out separately, and transferred to a petri dish containing physiological salt solution (De Jalon). Strips of 2-3 mm long were suspended with cotton thread to the base of a 50 mL isolated organ bath containing the De Jalon solution. The other end of the strip was attached to an isometric force transducer (Ugo Basile model 7004). This was coupled to a Data Capsule Acquisition System Model 17400, for isometric tension recording. The De Jalon solution contained the following composition (mM): NaCl (154), $NaHCO_3$ (1.7), $NaHCO_3$ (1.7), $MgCl_2$ (1.4), KCl (5.6), $CaCl_2$ (0.3) and glucose (5.55) (E. Merck, Darmstadt, Germany). The solution was bubbled with a 95% O_2 and 5 % CO_2 gas mixture. The temperature and pH was maintained at 37°C and 7.4 ± 0.2 respectively. The mounted uterine strips preparations was subsequently left to equilibrate for 60 min, under a passive tension of 1 g during which time the bathing physiological solution was changed after every 10 minutes.[20]

The effects of the ELETP were tested at concentrations of 0.2 – 1.0 mg/ml. Each experiment was replicated five times. Standard uterotonic drugs used were Oxytocin and Acetylcholine which were administered at concentrations of 10^{-6} -10^{-2} and 10^{-9} -10^{-5} M respectively. Probable synergies or antagonisms between the ELETP and oxytocin or acetylcholine were investigated while the effects of atropine, L-NAME; indomethacin, and Nifedipine (Sigma-Aldrich Chem., St. Louise, MI, USA) on the activities of the extract were examined by pre-treating the uterine smooth muscle with 2 and 1 μM of these drugs respectively.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Concentration-response curve to extract was expressed in grams. Statistical analysis of differences between the means of non-gravid, early and late gravid groups were analyzed (version 5.00, GraphPad Software, Inc., San Diego, California, USA) using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. A value of $p \leq 0.05$ was considered significant.

RESULTS

Effect of ELETP on contractile responses of uterine muscle strip (UMS) isolated from non-pregnant and pregnant rats

Figure 1 shows typical tracings of contractile responses to ELETP recorded in uterine strips isolated from non-pregnant and pregnant rats. The results showed that ELETP elicits a dose-dependent contractile activity in the uterus of both gravid and non-gravid, having higher effect in the gravid (Figure 2). At 0.2 mg/ml, ELETP elicited a uterine contraction equivalent to 0.197 ± 0.05 g force and this increased to 0.473 ± 0.11 g (EC_{50} 0.4 ± 0.01 mg/ml) when ELETP dose was 1.0 mg/ml in non-pregnant rats. Similarly, administration of 0.2 mg/ml ELETP generated a contractile tension of 0.302 ± 0.10g and 0.780 ± 0.24g (EC_{50} 0.8 ± 0.02 mg/ml) in both early and late pregnancy groups respectively. These were significantly ($p<0.05$) higher than that in non-pregnant rats (Figure 2).

Concentration–response of isolated uterine muscle strip to acetylcholine and oxytocin in the absence and presence of ELETP

The dose-dependent response of uterine muscle strip to acetylcholine (10^{-9} M - 10^{-5} M) in the presence and absence of ELETP is shown in Figures 3a and 3b. Administration of acetylcholine at 10^{-9} M produced a contraction of 0.109 ± 0.01g, 0.618 ± 0.1g and 1.162 ± 0.02g). This increased to 0.485 ± 0.02g, 2.02 ± 0.1 and 2.98 ± 0.3g at concentration of 10^{-5} M for non-pregnant, early and late pregnancy groups respectively (Figure 3a and 3b). Pre-incubation of uterine muscle strip with 0.4 or 0.8 mg/ml of ELETP in both non-

pregnant and pregnant groups with various concentration of acetylcholine produced significantly enhanced ($p<0.05$) contractile effects (Figure 3b).

Oxytocin at concentration of 10-6 M generated a corresponding contraction of 0.334 ± 0.03g, 0.509 ± 0.3g and 2.62 ± 0.6g. This increased to 0.96 ± 0.05g, 2.51 ± 0.2g and 4.69 ± 1.2g at concentration of 10^{-2} M in non-pregnant, early and late pregnancy groups respectively (Figure 4a). While pre-incubation of the uterine muscle strip with ELETP at 0.4mg/ml and 0.8mg/ml in non-pregnant and pregnant groups with various concentration of oxytocin, produced a significant ($p<0.05$) increase in contractile response when compared to oxytocin alone (Figure 4b).

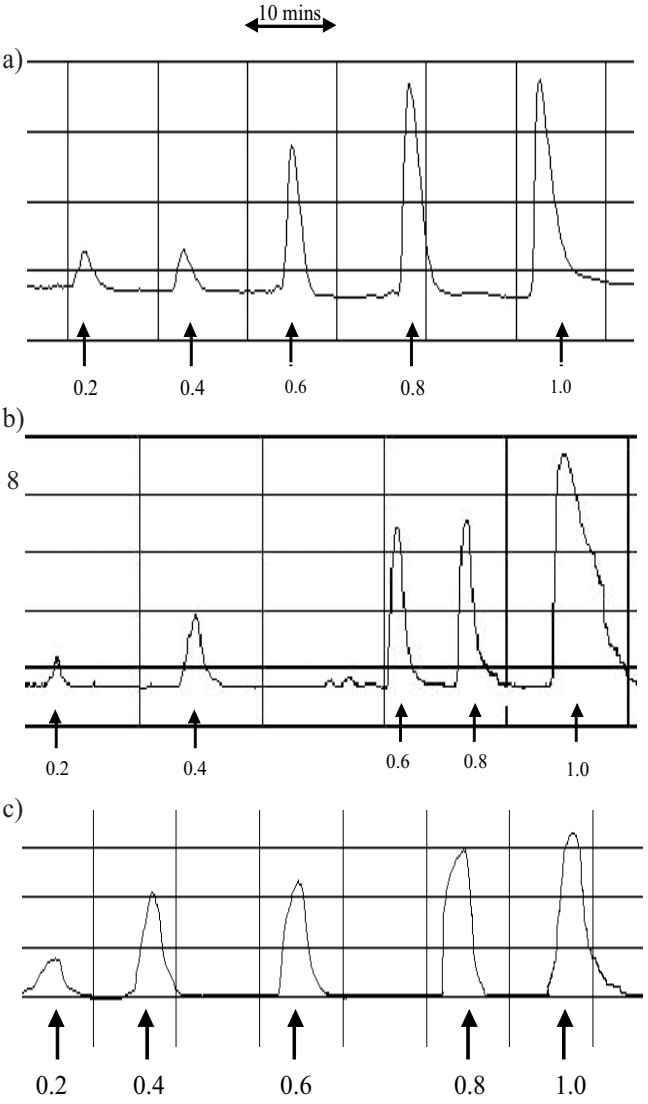

Figure 1: Typical tracing showing the effects of graded concentration of ELETP on contraction of isolated uterine muscle strip preparation obtaining from (a) non-pregnant (b) early pregnancy (c) late pregnancy rats. Arrows represent cumulative administrated ELETP (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml respectively).

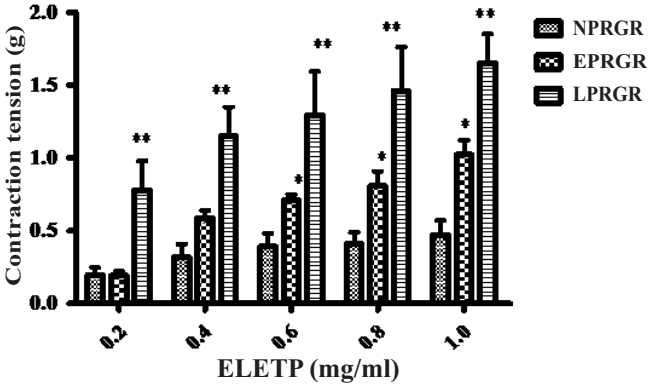

Figure 2: Dose-response curve showing the effect of ethanolic leaf extract of *Tridax procumbens* (ELETP) on the contraction of uterine muscle strip isolated from non-pregnant (NPRGR), early pregnancy (EPRGR) and late pregnancy (LPRGR) rats. Each point represents mean ± SEM $^{*}(p<0.05)$; $^{**}p<0.01)$ (n=6)

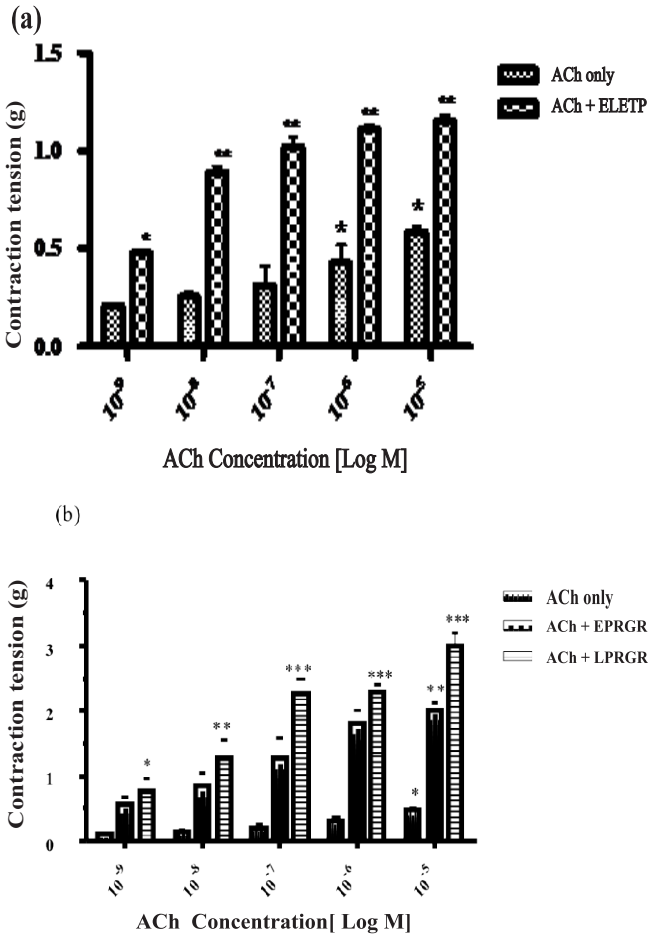

Figure 3: Cumulative concentration–response curve for acetylcholine (10^{-9}-10^{-5}M) on isolated uterine muscle strip from (A) non-pregnant and (B) pregnant rat in the absence and presence of (0.4 or 0.8 mg/ml) ELETP. ACh: Acetylcholine, EPRGR: Early Pregnancy Rat and LPRGR: Late Pregnancy Rat. Each point represents mean±SEM $^{*}(p<0.05)$; $^{**}(p<0.01)$ (n=6)

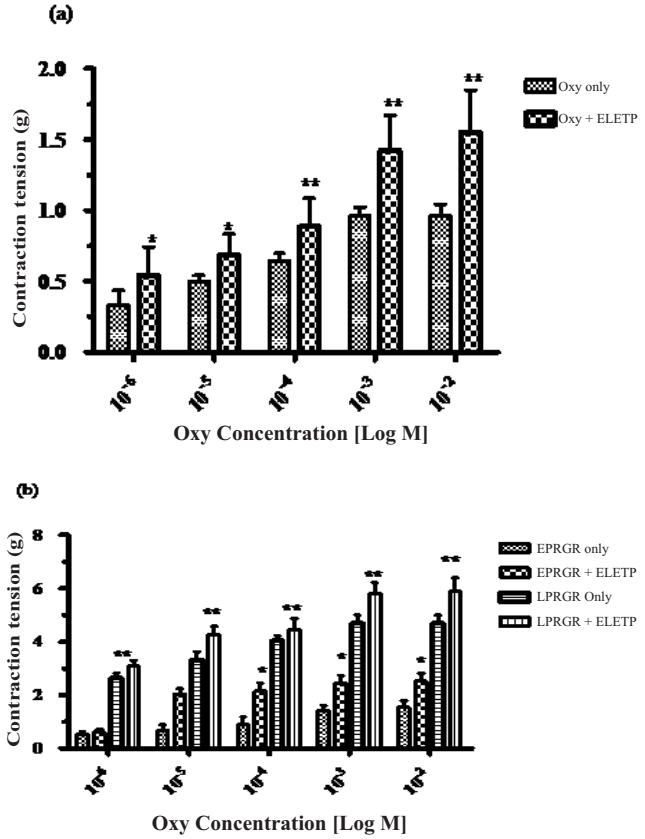

Figure 4: Cumulative concentration–response curve for Oxytocin (10^{-6} - 10^{-2}M) in isolated uterine muscle strip from (a) non-pregnant and (b) pregnant rat in the absence and presence of (0.4 or 0.8 mg/ml) ELETP. Oxy: Oxytocin, EPRGR: Early Pregnancy and LPRGR: Late Pregnancy. Each point represents mean±SEM *($p<0.05$); **($p<0.01$) (n=6)

Contractile response of the isolated uterine muscle strip to ELETP after inhibition by atropine, L-NAME, Indomethacin and nifedipine

Figures 5a and 5b show the effect of ELETP (0.2 - 1.0 mg/ml) on the contraction response of isolated uterine muscle strip in the presence and absence of atropine (10^{-4} M). In the presence of atropine, the contractile response of the uterus was inhibited by 82.4 % in the non-pregnant group, 80.5 % in the early pregnancy group and 81.9 % in the late pregnancy group. Comparison between the groups with and without atropine showed that there was a significant decrease ($p<0.05$) in the maximum contraction response of the uterine muscle strip with atropine when compared with the one without atropine. The uterotonic effects of the ELETP in the presence and absence of L-NAME is shown in Figures 6a and 6b. In the presence of L-NAME (10^{-4} M) the contraction response of the uterine muscle strip was reduced by 71.1 % in the non-pregnant group, 41.4 % in early pregnancy group and 51.6 % in the late pregnancy group so that the inhibitory effect of L-NAME was significantly higher ($p < 0.05$) in the non-pregnant group when compared to the pregnant groups.

Contractile effect of ELETP (0.2- 1.0 mg/ml) on uterine muscle strip after incubation in indomethacin (10^{-4} M) is shown in Figures 7a and 7b. The uterine muscle strip contraction was reduced by 57.7 % in the non-pregnant group, 98.1 % in early pregnancy group and 87.9 % in the late pregnancy group. Pregnant groups showed more pronounced inhibition with effect higher in early pregnancy group (Figure 7b).

Similarly, the effect of the ELETP on the uterine muscle strip contraction in the presence and absence of nifedipine (a calcium blocker) is shown in Figures 8a and 8b. In the presence of nifedipine (10^{-4} M) the reduced contraction observed was up to 94.6 % in non-pregnant group, 98.1 % in early pregnancy group and 87.9 % in the late pregnancy group. There was a significant reduction in contractile responses in late pregnancy when compared to non-pregnant and early pregnancy groups.

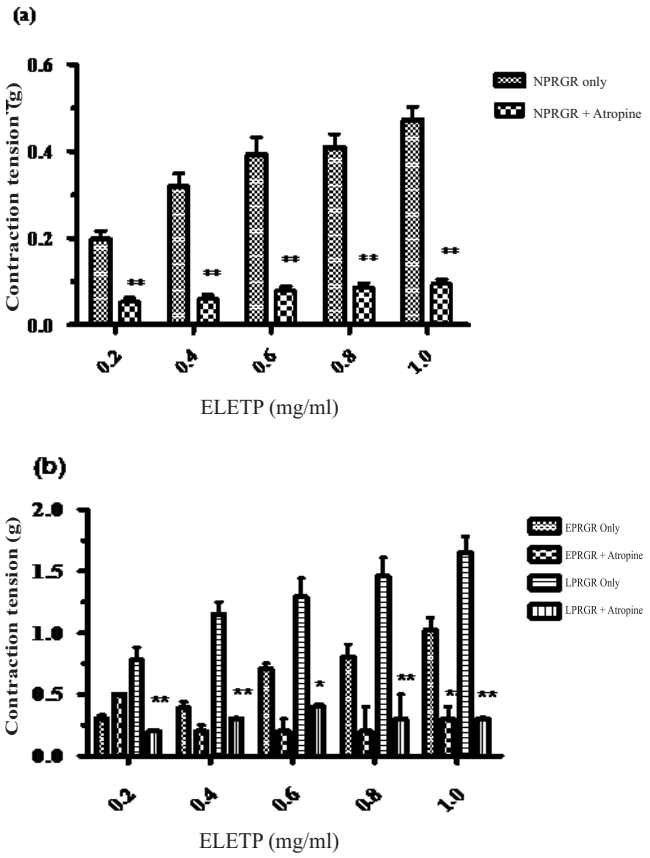

Figure 5: Cumulative concentration–response curve for ELETP (0.2 - 1.0 mg/ml) in isolated uterine muscle strip from (a) non-pregnant and (b) pregnant rat in the absence and presence of (10^{-4}M) atropine. EPRGR: Early Pregnancy and LPRGR: Late Pregnancy. Each point represents mean±SEM *($p<0.05$); **($p<0.01$) (n=6)

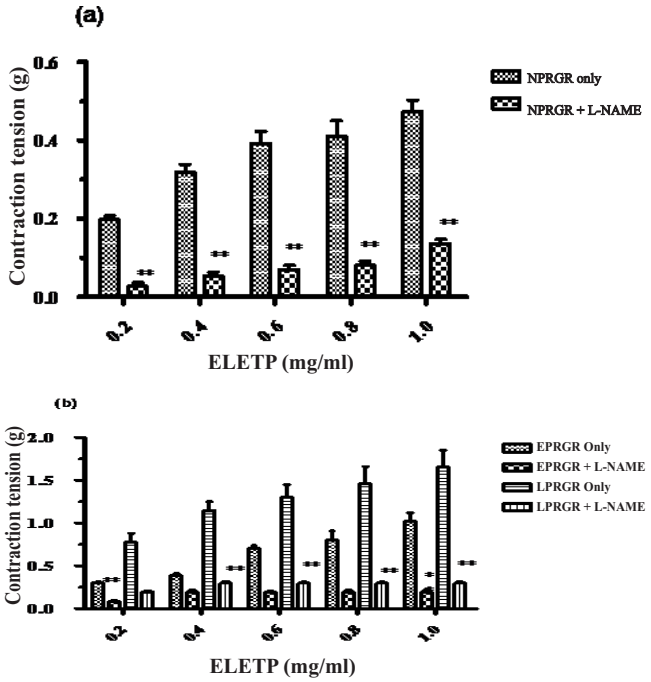

Figure 6: Cumulative concentration–response curve for ELETP (0.2-1.0 mg/ml) in isolated uterine muscle strip from (a) non-pregnant and (b) pregnant rat in the absence and presence of (10^{-4}M) L-NAME. EPRGR: Early Pregnancy and LPRGR: Late Pregnancy. Each point represents mean±SEM $^{*}(p<0.05)$; $^{**}(p<0.01)$ (n=6)

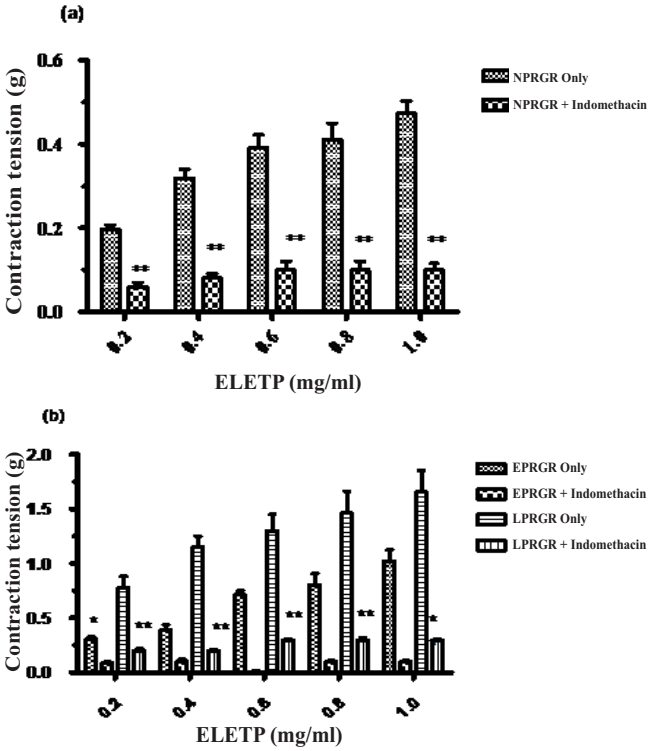

Figure 7: Cumulative concentration–response curve for ELETP (0.2-1.0 mg) in isolated uterine muscle strip from (a) non-pregnant and (b) pregnant rat in the absence and presence of (10-4M) indomethacin. EPRGR: Early Pregnancy and LPRGR: Late Pregnancy. Each point represents mean±SEM $^{*}(p<0.05)$; $^{**}(p<0.01)$ (n=6)

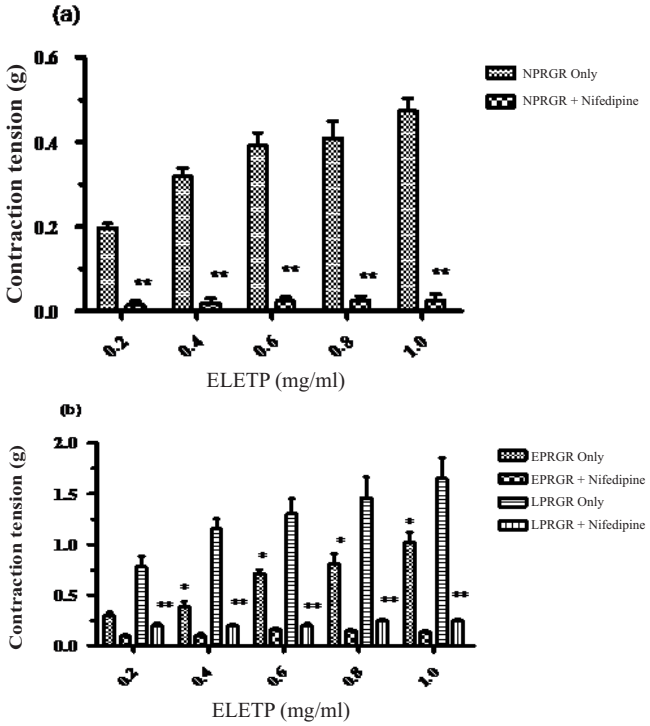

Figure 8: Cumulative concentration–response curve for ELETP (0.2-1.0 mg) in isolated uterine muscle strip from (a) non-pregnant and (b) pregnant rat in the absence and presence of (10^{-4}M) nifedipine. EPRGR: Early Pregnancy and LPRGR: Late Pregnancy. Each point represents mean±SEM $^{*}(p<0.05)$; $^{**}(p<0.01)$ (n=6)

DISCUSSION

The results of this study demonstrate that ELETP induces contraction of isolated uterine tissue of both non-pregnant and pregnant rat in a dose-dependent manner, implying that the extract can initiate contraction in non-contracting uterine muscles. In addition, in the presence of oxytocin and acetylcholine (well-known uterotonins), the contractile effects of ELETP were potentiated. These two drugs (oxytocin and acetylcholine) are known to contract the uterus. Oxytocin binds to oxytocin receptors on the uterus and causes release of inositol -1,4,5-triphosphate from the hydrolysis of phospho-inositide and release of prostaglandins, which increases contraction.[20]

Similarly, acetylcholine binds to and activates acetylcholine receptors on the uterus by initiating a second messenger mechanism that involves the activation of inisitol-1,4,5,-triphosphate and diacylglycerol (DAG) which facilitate contraction. Therefore, the observed contraction of the uterine smooth muscle in this study suggest that ELETP may have some activity on both oxytocin and acetylcholine (mACh) receptors in the uterus.[21] It is interesting to note that the effect of ELETP on the amplitude of contraction of the uterus was less when compared with those of potent oxytotic agents like acetylcholine and oxytocin. The less effect on the force of contraction may be due to the fact that ELETP is still a crude extract containing different components some of which may be antagonistic in maintain tension sustenance.

In this study pre-incubation of the uterine muscle with atropine, a competitive antagonist of acetylcholine at muscarinic receptors inhibited the contractile effect of ELETP. This suggests that ELETP may possess cholinergic-

like effect. It also implies that the extract may contain biological metabolite/ compound(s) which exhibit antagonistic effects on the uterine smooth muscle through muscarinic receptors. Thus, ELETP may have stimulated or activated (mACh) receptors which caused increased calcium release from intracellular stores through IP3 pathway. Furthermore, pre-incubation of uterine tissues with indomethacin, a cyclooxygenase enzyme inhibitor inhibited the effect of ELETP- induced contractions. This suggests that a product of the COX-pathways might have been involved in ELETP–induced uterine contraction. It is well known that indomethacin inhibits both the COX-1 and COX-2 prostaglandin pathway.[22] Prostaglandins (PGDs) are known to play a key role in the maintenance of pregnancy and the onset of labour, whereas their possible role in regulation of uterine contractility in non-pregnant uteri is still controversial.[23]

Prostaglandins are members of the eicosanoid family of proteins. They are lipid-mediators produced by the uterus, foetal membranes and the placenta and are capable of modulating uterine contractions.[24] When prostaglandin (PG) receptors are bound by their specific ligand, distinct intracellular pathways are activated, linked to the contractile (EP1, EP3, FP, thromboxane) or the relaxant (EP2, EP4, IP, DP) receptor isoforms,[25] PGE2 and PGF2α play important roles in myometrial contraction, cervical ripening and the initiation of parturition in humans.[26] The contractile effect of PGs is based on their ability to stimulate Ca^{2+} release from the sarcoplasmic reticulum (SR) of the myometrial cells.[27] Studies indicate that binding of PGF2α to its contractile receptor stimulates G-protein activation of PLC which stimulates production of IP3 and intracellular calcium release.[20,28] The released IP3 mobilizes $[Ca^{2+}]i$ from the SR, resulting in increased uterine contractility.[28]

However, study of myometrial PG receptor expression, a region-dependent, heterogeneous distribution of contractile and relaxant prostanoid receptors and cyclooxygenase was reported in non-pregnant porcine myometrium.[29] Studies have shown that NO activates both forms of cyclooxygenase by a guanosine 3'-5' cyclic monophosphate –independent mechanism.[30] Also Fronchi *et al*.,[31] found that NO stimulate uterine contraction by increasing cyclic oxygenase production. In the study, it was suggested that NO could upregulate PGE production which can in turn initiate specific receptors coupled to heterotrimetric G-proteins having the heptahelical transmembrane configuration. At low concentration, it raises the intracellular Ca^{2+} levels by a mechanism involving Ca^{2+} influx and by activation of PI –IP3 cycle[32] while at higher concentration PGEs activate PLC and increases the $[Ca^{2+}]i$. Indeed, the observed inhibitory effect of L-NAME a NO synthase in this study confirmed the contribution of endogenous NO on ELETP–induced uterine smooth muscle contraction in this study. This is in agreement with another study that indicated that in rats' endometrium, agonist–induced PG synthesis is mediated by NO and that L-NAME abolished this effect.[33]

Uterine contraction is like any visceral smooth muscle contraction. It is fundamentally controlled and triggered by a transient increase in intracellular calcium $[Ca^{2+}]i$, which initiates uterine action potential, resulting from a transient increase in the cytosolic free Ca^{2+} concentration.[34,35] Calcium is released from the sarcoplasmic reticulum (SR) and from extracellular stores through voltage-gated calcium channels. Therefore, smooth muscle contractility by different agonists or by electrical depolarization results in a rapid increase in $[Ca^{2+}]i$.[36] Calcium ions binds to four binding sites of calmodulin causing a conformational change allowing the calmodulin-calcium complex to interact with inactive myosin light chain kinase (MLCK), thus activating its enzymatic properties.[37] The MLCK rapidly phosphorylates the myosin light chain (MLC).

Phosphorylation of MLC leads to conformational changes in the myosin head that causes actin activation of myosin ATPase, resulting in force generation and/or shortening (contraction) of the muscle fibres.[38] Therefore, the observed ELETP –induced uterine smooth muscle contraction in this study could be due to the opening of calcium channel; or facilitation of the influx of calcium ions from the extra-cellular fluid into the tissue cells, subsequently causing the contraction of the uterine muscle. Although, the extracellular calcium was not measured in this study and the experiment was not performed in a calcium free physiological solution. The inhibitory effect of nifedipine a calcium channel antagonist on ELETP –induced uterine smooth muscle contraction suggests that ELETP might be contracting uterine muscle through calcium- dependent mechanism.

Reported phytochemical analysis of *Tridax procumbens* leave extract revealed the presence of quercetin, kaempferol, (-)-epigallocatechin, Myricetin, oleanolic acid, stigmasterol and β-sitosterol.[39] The exact chemical constituent/s of ELETP that could be responsible for the observed uterine contractile activity of this plant's extract is not yet known. The flavonoids (myricetin and quercetin) commonly found in ELETP have previously been reported to elicit smooth muscle contraction.[39] Myricetin has been observed to elicit smooth muscle contraction via activation of the phospholipase-A2 pathway, resulting in the release of thromboxane A2 by increasing Ca^{2+} concentrations,[40] while Quercetin has been identified as a novel, specific activator of L-type calcium channels and it increases the influx of Ca^{2+},[41] which contradicts its well-known vasodilatory effects. The myorelaxant effect of quercetin in tissue preparations has been suggested to originate from its reaction with a second target beyond the Ca^{2+} channel. In the present study however, it may be argued that the ELETP-induced contractile effects were likely to have been a result of the sudden influx of calcium into the cells, due to quercetin activation of L-type calcium channels. It can therefore be speculated that the pharmacological effects observed with ELETP in this study may be due in part to a synergistic effect of the flavonoids present in ELETP. In addition, ELETP contains a variety of estrogenic compounds as well as other steroids such as stigmasterol and sitosterol.[41] Earlier studies have shown that sitosterol isolated from pomegranate seed extract (*Punica granatum* L., *Punicaceae*) has a uterotropic effect as it increased uterine weight and induced vaginal cornification in ovariectomized animals.[41] Recently, it was found that the pathway by which sitosterol increases uterine contraction occurred via calcium-dependent pathway. It was also reported that this compound could not produce force in the absence of external calcium entry and force produced in the presence of the extract was abolished when calcium entry through L-type calcium channels was

inhibited.[42]

CONCLUSION

This study demonstrated that ELETP possesses uterotonic activity in both non-pregnant and pregnant uterine muscles. This can largely be accounted for by its constituent phytoestrogen (stigmasterol and sitosterol) acting via oestrogen receptor-mediated mechanism. The stimulation of uterine activity by ELETP scientifically support its use by the traditional birth attendants (TBA) to stimulate uterine contraction to enhance the progress of labour, although further studies in human myometrium are required to compare extent of this postulation. Since the contractile force produced in the presence of the extract was abolished with inhibition of calcium entry through L-type calcium channels, it could therefore be concluded that the increase in uterine muscle strip contraction by ELETP occurred via calcium-dependent pathway.

Conflict of Interest

The authors declare no conflict of interest.

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