http://dx.doi.org/10.434/jpb.v13i2.9
Vol. 13 no. 2, pp. 134-147 (September 2016)
http://ajol.info/index.php/jpb

Journal of
PHARMACY AND BIORESOURCES

In vitro response of isolated non-pregnant mouse uterus to the methanol extract of *Emilia coccinea* (Sims) G. Dons leaf

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Received 29th July 2016; Accepted 9th September 2016

Abstract
The leaves of *Emilia coccinea* (Sims) G Dons are used in Southern Nigeria for birth control and in other parts of West Africa for treating female infertility issues. However, no scientific data are available for its effect on uterine contraction. Therefore, this study is aimed at evaluating the effect of the methanol extract of *Emilia coccinea* leaves on uterine contractility. The cumulative concentrations (0.0004-4.884 mg/ml) of the methanol extract (EM) were tested on rhythmic spontaneous-, oxytocin (OT)-and high potassium chloride (KCl)-induced uterine contractions, OT-induced uterine contractions in calcium-deprived state as well as on OT-induced uterine contractions in the absence and presence of glibenclamide, amiodarone and propranolol. The extract, EM produced significant *(P<0.05)* decrease in the frequency and amplitude of spontaneous contractions with IC_{50} of 0.4694±0.07 and I_{max} of 2.031±0.32, as well as OT (0.1 µg/mL)-induced myometrial contractions and OT-induced contractions in calcium-deprived state containing ethylenediaminetetraacetic acid (EDTA), while it exerted no significant changes on high KCl (80 mM)-induced myometrial contractions. The inhibitory effects of EM were significantly increased *(P<0.05*) in the presence of glibenclamide (7.5 ng/mL) and propranolol (3.0 ng/mL) while with amiodarone (65.0 ng/mL), EM elicited no observable significant changes in the inhibition of contractions. These observations may explain some of the mechanisms involved in the activity of EM and may explain its folkloric use as birth control, however further studies are advised to characterize and isolate specific bio-constituents responsible for the observed effects.

Keywords: *Emilia coccinea*; Mouse uterus; Contraception; Oxytocin; Glibenclamide.

INTRODUCTION

From history, Africans have been known to use plants in management and treatment of diseases and ailments. Some African communities still depend solely on the use of plants and other remedies as sources of medicine (Sofowora, 1982; Burkill, 1984, 1985). *Emilia coccinea* (Sims) G Dons is commonly used as a herbal medicine in tropical parts of Africa such as Nigeria, Democratic Republic of Congo, Ghana, Sierra Leone, Liberia, Togo, Angola, Cameroon, and Gambia (Burkill, 1985; Olorode, 1984). It is of the family Compositae (Olorode, 1984; Ayitey-Smith, 1989) and is known as "yellow tassel flower" in English language, as "ṇtị̀-ènē see", in Igbo Language, as "ọ̀kọ́lọ́mátọ̀rú ededе" in Ijo Language, and as "ọ̀dúndún-odò" in Yoruba Language (Burkill, 1985).

This plant has been reported to be used for the treatment of ulcerated body rashes, fever, abscesses, wounds, sores,

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ISSN 0189-8442

sinusitis, ringworm, jaundice, abdominal pains, gastritis, convulsions, epilepsy in children and vertigo (Burkill, 1984; Teke *et al.,* 2007; Erhabor *et al.,* 2013; Foyet *et al.,* 2014). *Emilia coccinea* (Sims) G Dons has been identified as one of the plants used by traditional medical practitioners in treating female infertility in humans in Cameroon (Adjanohoun *et al.,* 1996; Telefo *et al.,* 2011; Fongod *et al.,* 2013) and in Congo, the leaf-sap is given as an antiabortifacient (Telefo *et al.,* 2011; Fongod *et al.,* 2013) and for menstrual troubles while in Southeastern Nigeria, the leaves of the plant together with *Ageratum conyzoides* are boiled and the hot decoction taken for birth control especially after delivery (Jain *et al.,* 2005).

Contraception also known as birth controls or fertility controls are ways, methods, or devices used to prevent or eliminate pregnancy in family planning processes (Hanson and Burke, 2010). And these practices are as old as the ancients; however, safe and effective methods became available since the 20th century and onwards (Black *et al.,* 2012). Some plants have been reported to possess contraceptive properties and were used in Ancient Greece from the 7th century onwards (Ridlle *et al.,* 1995) and documented by numerous ancient writers on gynaecology, such as Hippocrates (Gediya *et al.,* 2011). Till date the use of plants has been useful in birth control methods (Adjanohoun *et al.,* 1996; Telefo *et al.,* 2011; Fongod *et al.,* 2013).

From previous reports, the phytochemical compounds extracted from *Emilia coccinea* (Sims) G Dons include alkaloids, tannins, saponins, steroids, terpenoids, flavonoids and cardiac glycosides (Sofowora, 1982; Edeoga *et al.,* 2005; Teke *et al.,* 2007; Gediya *et al.,* 2011; Okiei *et al.,* 2009; Idu *et al.,* 2010; Mensah *et al.,* 2013). The presence of these compounds in the leaves suggest that this plant might possesses properties that can have effects on reproduction. However, no scientific data are available for its effects on uterine activity. Therefore, this current study is aimed at evaluating the effects of the methanol leaf extract on uterine contractions using animal models in order to investigate possible antifertility effects.

EXPERIMENTAL

Collection of plant samples and preparation of extracts. Fresh leaves of *E. coccinea* (Sims) G Dons were collected in July, 2015 within the environment of Obingwa Local Government of Aba, Abia State, Nigeria between 6 A.M and 9 A.M in the morning and between 5.00 P.M and 6.30 P.M in the evening. The plant specimen was authenticated by Dr. H. A. Akinnibosun of the Department of Plant Biology and Biotechnology, University of Benin, Nigeria. The herbarium sample with voucher number, UBH_a 302 was processed and deposited for future references. The leaves were then cleaned and dried at room temperature (24-26 °C for) 10 days.

The dried fresh leaves were blended into powder with an electronic blender (Power Deluxe, PDB-8231-F). The resulting powdered leaves (280.2 g) were macerated in 2500 ml of 100% methanol at room temperature and were constantly stirred for 24 h. After a 24 h maceration, the mixture was filtered with Whatman filter paper, the residue was discarded and the filtrate was evaporated to dryness with the aid of a water bath set at 60°C in order to obtain the concentrate which was further dried to a constant weight with Hotbox oven size one (Gallenkamp®, England) set at 40°C. The dried extract was kept in a refrigerator until needed. The given powdered extract yield was 11.35% with a dark green colour (EM).

Drugs/ physiological salts. Stilbestrol, amiodarone, AM (TEVA UK. Ltd)**,** propranolol, P (Sigma-Aldrich, UK)**,** glibenclamide, G (Daonil®, Swiss

Pharmaceutical Nigeria, Ltd.), and oxytocin, OT (Pantocyn®, Jiangsu Ruinian Qianjin Pharmaceutical Co. Ltd. China), Sodium Chloride- NaCl (Guangdong GuanghuaSci-Tech Co. Ltd. China), Sodium bicarbonate- $NaHCO_3$ (Sigma-Aldrich, Inc.), D-Glucose- $C_6H_{12}O_6.H_2O$ (Guangdong GuanghuaSci-Tech Co. Ltd. China), Potassium Chloride- KCl (Guangdong GuanghuaSci-Tech Co. Ltd. China), and Calcium chloride -$CaCl_2$ (XL®). Ethylenediaminetetraacetic acid (EDTA), all of analytical grade.

Animals/ isolated uterine tissue preparation. All experiments were performed using adult female Swiss Albino mice (25-35 g) aged 3-4 months. The animals were purchased from a local Animal Center in Benin City, Nigeria, and housed in the Animal Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Nigeria. The mice were acclimatized for a period of two weeks before commencement of these studies. All studies were as approved by the Animal Care and Use of the Faculty of Pharmacy, University of Benin, Nigeria and handled according to standard guidelines for the use and care of Laboratory experimental animals (National Institute of Health, USA: Public Health Service Policy on Humane Care and Use of Laboratory animals, 2002). The animals were housed in stainless-steel cages, containing a bedding of wood shavings (which were changed regularly as needed) and were fed regularly with mouse diet and water *ad libitum.*

The mice were administered 1 mg/kg stilboesterol p.o constituted in Tween 80 and distilled water (1:1) 24 h before the start of the experiment. This procedure was determined in our laboratory to be an effective priming procedure for induction of oestrous. Oestrus was ascertained by visual assessment of the vulva and by microscopic assessment of vaginal smears. The animals were humanely sacrificed and the uterine horns were completely and immediately excised and kept in previously aerated and warmed physiological salt solution. A section of the uterine horn, 0.5 mm in length, was cut with the mesenteries and fat trimmed off. The sections were securely affixed longitudinally in a 10 mL organ bath containing physiological salt solution (PSS) of the following composition in g/5L distilled water: NaCl 45.0; $NaHCO_3$ 2.5; D-glucose 2.5; KCl 2.1; $CaCl_2.2H_2O$ 1.32. The lower end of the tissue was attached to a tissue holder and the upper end to an isometric force-displacement transducer (Model 7003-E) connected to a Data capsule digital recorder system (Model 4050 Ugo Basile, Italy). The physiological salt solution was kept constant at 37^oC with continuous aeration. Each section of the uterus was kept under optimum resting tension between 0.5 – 1.0 g and equilibrated for 30 min before the commencement of an experimental protocol.

Study on the Effect of EM on Spontaneous Uterine Contractions. To evaluate the activity of EM on rhythmic spontaneous uterine contractions, the baseline (100%) amplitude (force) and frequency were recorded in the first 5 min after 30 min equilibration according to methods of Bafor *et al.*, (2011). This was followed by subsequent exposure of the tissue to increasing cumulative concentrations of the extract from 0.0004 – 4.8884 mg/mL for each concentration and the responses were observed. The first 3 min after each extract concentration was recorded.

Study of the Effect of EM on OT-, and High KCl-Induced Uterine Contractions. To assess the effect of EM on agonist-induced contractions and also determine possible involvement of extracellular calcium channel with the extract activity, the effects of single concentrations of OT (0.1 µg/mL) and KCl (80 mM) were investigated respectively. The effects of the agonists were investigated in the absence of the extract within a 5 min interval

and then in the presence of cumulative concentrations of EM (0.0004 – 4.8884) mg/mL at 3 min intervals for OT and 2 min intervals for KCl. Experiments for KCl or OT were separately performed.

Study of the EM on OT-induced Uterine Contractions in Calcium-deprived State. To ascertain the effect of EM on OT-induced contraction in calcium-deprived state (Ca^{2+} -free medium), after equilibration of the tissue in normal PSS for 30 min, the tissue was re-equilibrated in calcium-deprived medium containing EDTA for 15 min. The effect of OT (0.1 µg/mL) was investigated for 5 min interval and without flushing cumulative concentrations of EM were added from 0.0004 – 4.8884 mg/mL and responses recorded.

Study of the Effect EM on OT-induced Uterine Contractions in the Absence and Presence of Antagonists (Amiodarone, Propranolol and Glibenclamide). To ascertain the specific interactions of EM on K^+ -, K_{ATP}^+-, β-, and Ca^{2+}- channels, OT (0.1 µg/mL) was added to the bath for 5 min and without flushing EM (4.0 mg/mL) was added for 3 min which was immediately followed by addition of amiodarone (65.0 ng/mL) or propranolol (3.0 ng/mL) or glibenclamide (7.5 ng/mL) in separate experiments.

Data analysis. All statistical analysis was carried out using the GraphPad Prism, (version 6.0; GraphPad software Inc, San Diego, CA, USA). Contractions occurring at the last 3 min of the phasic contractions were used to calculate the mean frequency and amplitude. The results in some cases were displayed as percentages of control applications (absence of extract, control = 100%).

In data sets with numerous data points, mean log concentration-response curves were analyzed by fitting data to a 4-parameter Logistic non-linear regression model with the following equation values (Y = Bottom)(1 + $10^{\wedge}$(LogIC$_{50}$-X)*HillSlope). Where Y = response which starts at the bottom and goes to the Top in sigmoid shape, X = logarithm of concentration and IC_{50} is the concentration that produces half the maximal inhibitory responses.

All data were shown as mean ± standard error of mean (SEM) where 'n' represents the number of samples each from different animals. Significance was evaluated using appropriate t-tests, and where necessary, One-way analysis of variance followed by Tukey's multiple range tests with P values ≤ 0.05 considered statistically significant in all cases.

RESULTS AND DISCUSSION

The cumulative concentrations of the extract from 0.0004 to 4.884 mg/mL was observed to decrease ($p < 0.05$) the amplitude (force) of uterine contraction in a concentration-dependent manner with maximal effect observed at the highest concentration and an associated IC_{50} of 0.4694 ± 0.07 and I_{max} of 2.031 ± 0.32 (Figures 1 & 2). However, it showed no changes on frequency of spontaneous uterine contraction compared to baseline (control) value (Figures 1 & 2). The extract, at cumulative concentrations from 0.0004 to 4.884 mg/mL caused a significant ($p< 0.05$) decrease in the amplitude and frequency (Figures 3 & 4) of oxytocin (0.1 µg/mL)-induced uterine contraction in a concentration-dependent manner with effects more obvious at higher concentrations.

That the extract reduced the amplitude of spontaneous contraction suggests possible interaction with either extracellular voltage-operated calcium channels leading to inhibition of the channels or decrease in intracellular stores of calcium as well as interaction with prostaglandins which are known to play a major role in the regulation of spontaneous uterine contractions (Kupittayanant *et al.,* 2002). The effect of EM

on one parameter of spontaneous contractions while not affecting the other is suggestive that there might also be involvement of myometrial gap junctions which regulates the frequency and amplitude of contractions (Mackler *et al.,* 1999; Garfield *et al.,* 1980).

At all concentrations of the extract used in this study, it was observed that the extract slightly decreased the amplitude (Figures 5 & 6) of KCl-induced uterine contraction, but this was considered statistically insignificant compared to the control (KCl alone) value. Contraction of the isolated uterine smooth muscle by high K^+ in extracellular fluid is known to occur via smooth muscle depolarization and subsequent opening of the voltage-operated calcium channels (VOC) in particular, the L-type calcium channel (Hollingworth *et al.,* 2008). This results in influx of Ca^{++} into the smooth muscle cells and finally contraction. Therefore, this suggests that EM plays no significant direct role on extracellular voltage-gated calcium channels and its interaction with calcium appears to be more related to the intracellular channels as shown in the calcium-free studies.

In calcium-deprived medium with EDTA, the cumulative concentrations of EM from 0.0004 to 4.884 mg/mL significantly decreased ($p < 0.05$) both the amplitude (Figures 7 & 8) and frequency (Figures 7 & 8) of oxytocin (0.1 µg/mL)-induced uterine contraction. This effects displayed by the extract occurred in a concentration-dependent manner which was more obvious at higher concentrations. This therefore suggests possible interaction of EM with the release of calcium from intracellular stores, where the only available calcium originates from intracellular stores (Batra, 1986).

OT induces contraction by elevating intracellular calcium concentration by phospholipase C (PLC)-mediated myoinositol 1,4,5-triphosphate (IP_3) induced release of Ca^{2+} from internal stores (Anwer *et al.,* 1993), this causes influx of extracellular Ca^{2+} through voltage-operated and receptor-operated Ca^{2+} channels (Garfield *et al.,* 1980), or capacitative calcium entry through the receptor–operated calcium channel (ROC) (Monga *et al.,* 1999). It may therefore be that the extract interacts with the mechanisms involved in the responses of the uterus to oxytocin via either the blockade of OT receptors or inhibition of one of the mechanisms through which OT elicits its contractile effects on the uterus. This however remains to be further verified.

The inhibitory activities of EM (4 mg/ml) on both the amplitude and frequency (Figures 9, 10 & 11) of OT (0.1 µg/mL)-induced uterine contractions were significantly increased ($p < 0.05$) in the presence of glibenclamide (7.5 ng/ml) as compared with control (OT alone). The percentage decrease of amplitude of OT-induced contraction was 53.67 % while the frequency was 41.7 %.

Glibenclamide is known to bind to and inhibit the ATP- sensitive potassium channels (K_{ATP}) (Luzi and Pozza, 1997). The intriguing increased inhibitory effect of the extract in the presence of glibenclamide may suggest a lack of involvement of the extract with K_{ATP} channels, it also suggests that blockade of K_{ATP} may not always promote contraction of the uterus as observed in previous studies. Further investigations are therefore recommended on the K_{ATP} regulation of myometrial contractility.

The inhibitory activities of EM (4 mg/ml) on the amplitude of OT (0.1 µg/ml)-induced uterine contractions were observed to be unaffected in the presence of amiodarone (65.0 ng/ml) (Figures 12 & 13) whereas the frequency of OT-induced uterine contractions significantly decreased (* $p < 0.05$) with percentage decrease of 47.16 % (Figures 12 & 14) as compared with control (OT alone). Amiodarone, acts primarily by blocking potassium channels (Gessner *et al.,* 2010;

Kiehn *et al.*, 1999; Dorian *et al.*, 2002). That the effect of the extract was maintained in the presence of amiodarone suggests a lack of involvement of the extract with potassium channels. The effect observed with glibenclamide supports this argument.

The extract (4 mg/mL) in the presence of propranolol (3.0 ng/mL), caused significant ($p<0.05$) decrease in amplitude (Figures 15 & 16) of OT (0.1 µg/mL) - induced uterine contractions with percentage decrease of 52.17 % compared with control (OT alone). However, EM produced no significant effects in the frequency of oxytocin-induced contractions (Figures 15 & 17). Beta-adrenoceptor signals through a downstream (the class C) L-type calcium channel interaction, this receptor-channel complex is coupled to the G_s G-proteins, which activates adenylyl cyclase, catalyzing the formation of cyclic adenosine monophosphate (cAMP) which then activates protein kinase A, and the counterbalancing of protein phosphatase 2A mediating physiologic responses such as uterine smooth muscle relaxation. However, the β-adrenoceptor blockade facilitates the effect of an agonist by removing the effect of the beta receptor to aid uterine contraction (Nesheim *et al.*, 1975; Perez-Hernandez *et al.*, 2008). This study therefore suggests that EM exerts no interaction with beta- adrenergic receptor.

The myometrium of the uterus regularly contracts all through the length of the oestral or menstrual cycles and appear to be seen only in the sub-endometrial layer of the uterus (Kissler *et al.*, 2004a).The waves of uterine contractions normally show the highest amplitude and frequency towards the ovulatory phase and contraction waves with lower frequency and amplitude at other phases while peristalsis appears mostly to be directed towards the cervix during menstruation (Lyons *et al.*, 1991; Fukuda and Fukuda, 1994). Myometrial contraction has been shown to be decreased by oral contraceptives (Akerlund, 1979; Chan and Dawood, 1980) and are therefore used as primary medications to treat primary dysmenorrhea (Creatsas *et al.*, 1990; Davis *et al.*, 2005; Aki *et al.*, 2007).

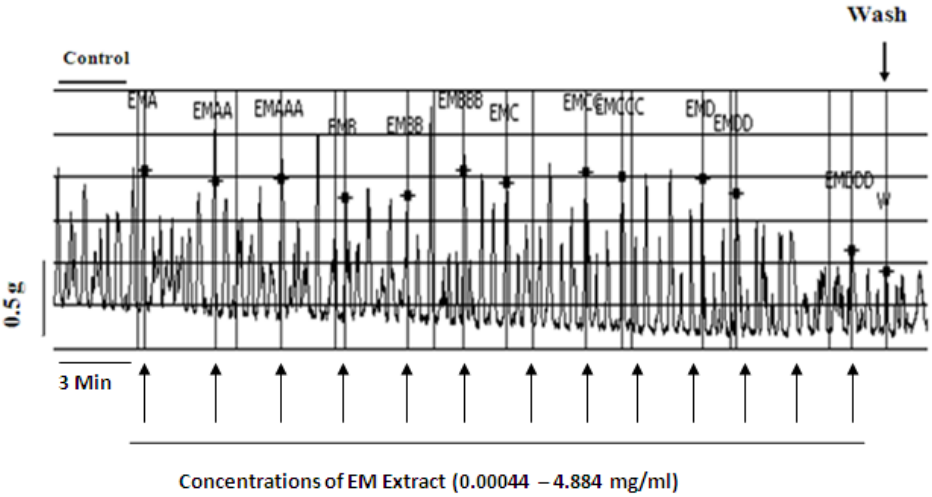

Figure 1: Original recording showing the effect of EM on spontaneous uterine contractions. EM displayed inhibitory properties on the amplitude of spontaneous uterine contractions which were observed at higher concentrations.

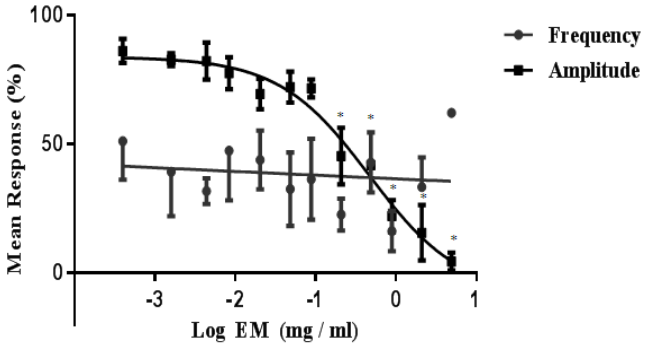

Figure 2: Concentration-response curve showing the effect of EM on the amplitude and frequency of spontaneous uterine contractions. EM significantly inhibited the amplitude of spontaneous contractions which were more pronounced at higher concentrations. **P < 0.05* compared to control, n = 5 experiments.

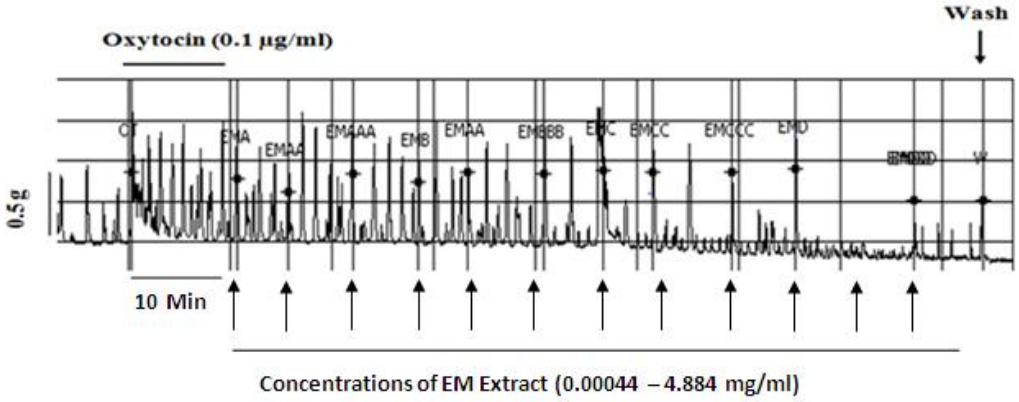

Figure 3: Original tracing of the effect of EM on the OT – induced uterine contractions. EM displayed inhibitory properties observed at higher concentrations compared to control (oxytocin alone). n = 5 experiments

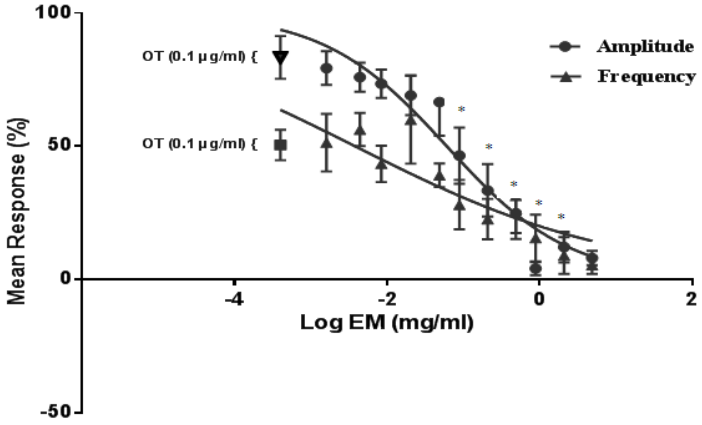

Figure 4: Concentration-response curve of the effect of EM on the amplitude and frequency of OT (0.1 µg/ml)-induced uterine contractions. The extract significantly inhibited both the frequency and the amplitude of contractions which were more pronounced at higher concentrations. **P < 0.05* as compared to control, n = 5 experiments.

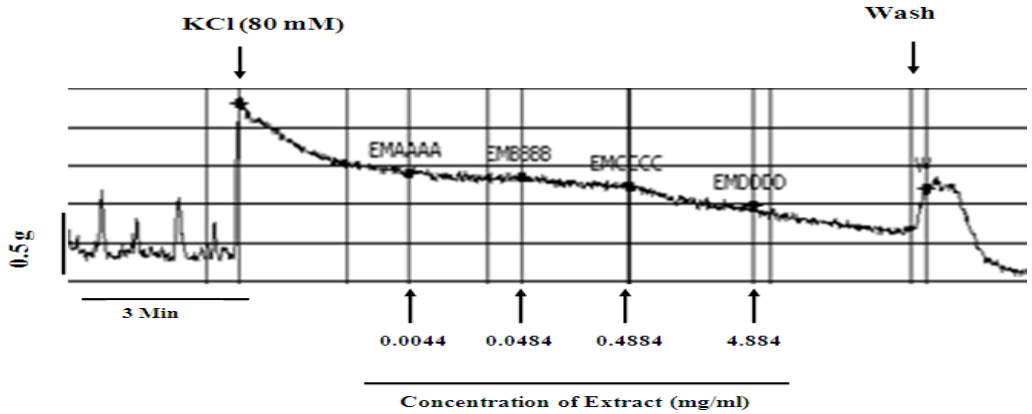

Figure 5: Original tracing of the effect of EM on the High KCl – induced uterine contractions. In the presence of KCl (80 mM) EM displayed a slight decrease in the amplitude of uterine contractions at all concentrations but was not considered statistically insignificant. n = 5

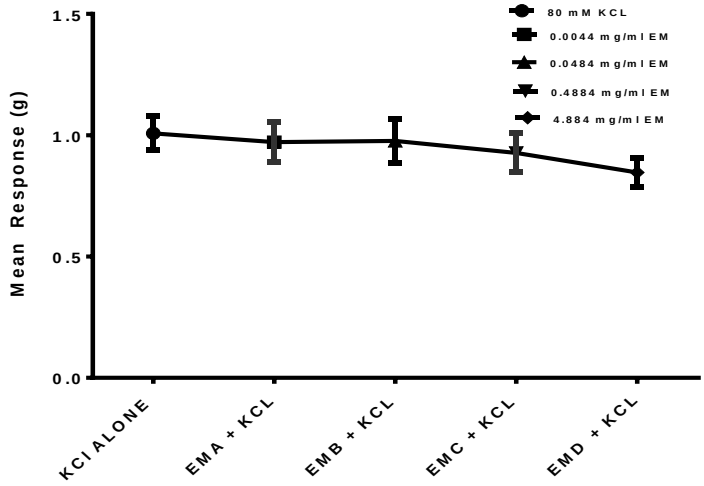

Figure 6: Line graph showing the effect of the EM on the amplitude of High KCl -induced uterine contractions (80 mM). n = 5 experiments.

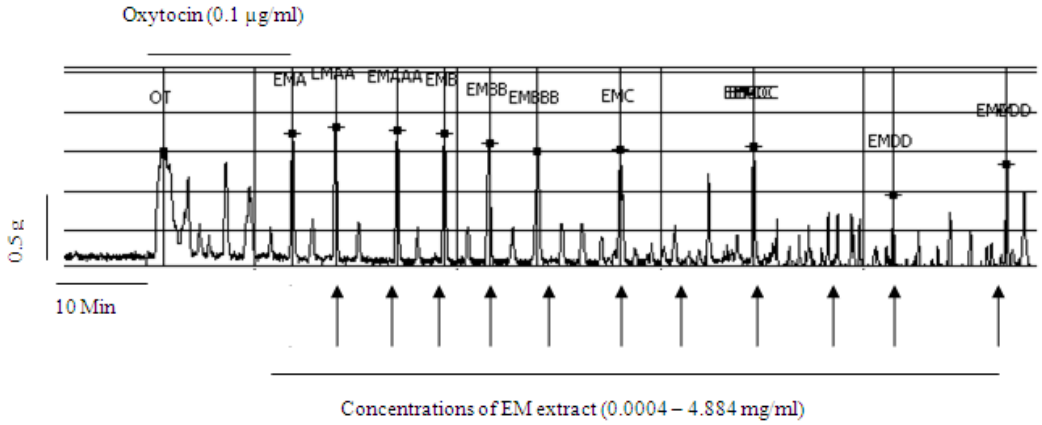

Figure 7: Original recording showing the activities of EM on OT – induced contractions in Ca^{2+}-deprived state. In Ca^{2+}-deprived environment, the amplitude and frequency of OT (0.1 µg/ml) were decreased by EM.

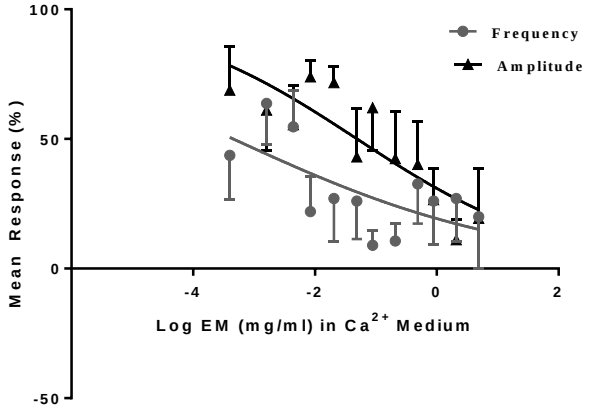

Figure 8: Concentration-response curves showing the activities of EM on OT – induced uterine contractions in Ca^{2+}-deprived state. The extract, EM decreased both the frequency and amplitude of OT-induced uterine contraction in Ca^{2+}-deprived state, n = 5 experiments.

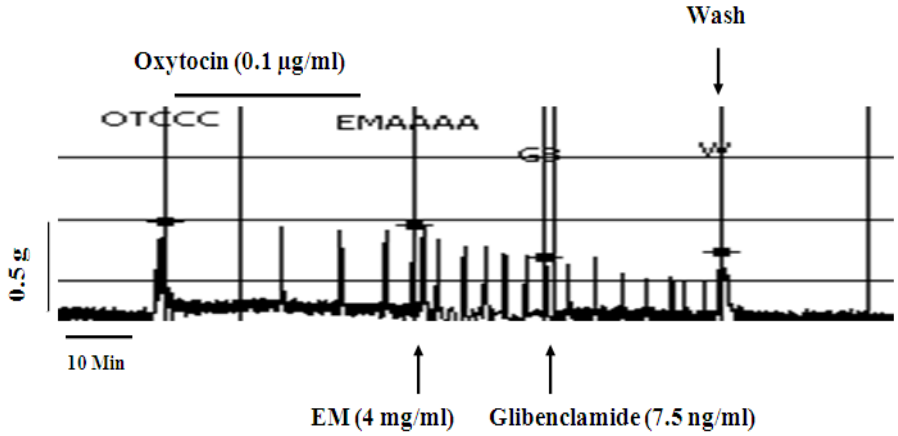

Figure 9: Original recording showing the activity of EM on oxytocin-induced uterine contraction in the absence and presence of glibenclamide (7.5 ng/ml).

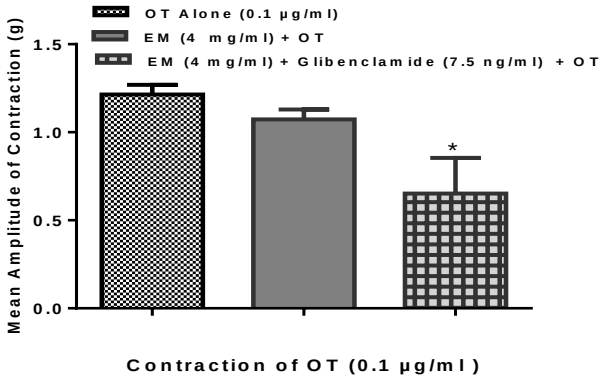

Figure 10: Bar graphs showing the effect of EM (4 mg/ml) on the amplitude of OT –induced contractions (0.1 µg/ml) in the absence and presence of glibenclamide (7.5 ng/ml) n = 5. EM showed significant inhibitory effects on oxytocin-induced contractions in the presence of glibenclamide**,** * $p < 0.05$ compared to control (OT alone).

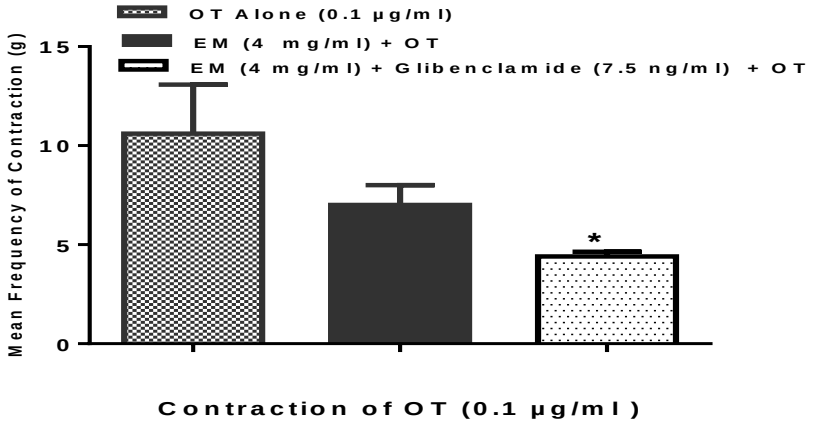

Figure 11: Bar graphs showing the effect of EM (4 mg/ml) on the frequency of OT -induced contractions (0.1 µg/ml) in the absence and presence of glibenclamide (7.5 ng/ml) n = 5. EM showed significant effects in the presence of glibenclamide**,** * $p < 0.05$, compared to control (OT alone).

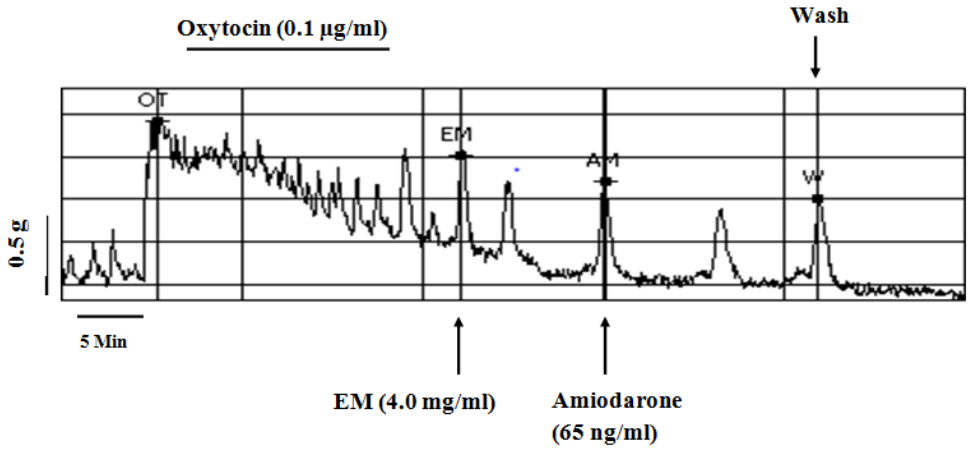

Figure 12: Original recording showing the effect of EM **(**4.0 mg/ml**)** on oxytocin-induced uterine contraction in the absence and presence of amiodarone (65 ng/ml).

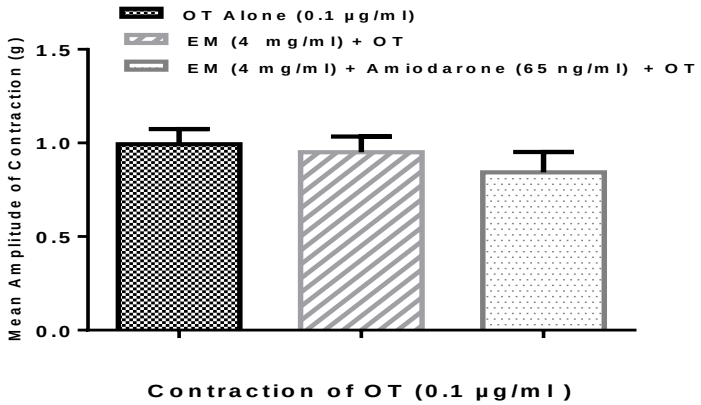

Figure 13: Bar graphs showing the effect of EM (4 mg/ml) on the amplitude of OT -induced contractions (0.1 µg/ml) in the absence and presence of amiodarone (65.0 ng/ml) n = 5 experiments. **EM** showed no statistically significant effects in the presence of amiodarone on OT-induced contractions.

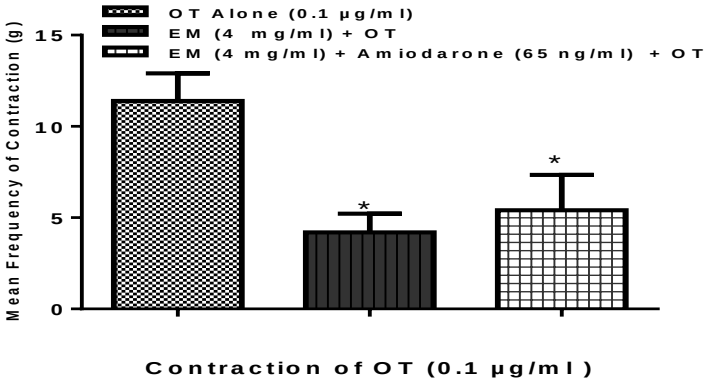

Figure 14: Bar graphs showing the effect of EM (4 mg/ml) on the frequency of OT -induced contractions (0.1 µg/ml) in the absence and presence of amiodarone (65.0 ng/ml) n = 5 experiments. EM showed significant effects in the presence of amiodarone on OT-induced contractions. * $p < 0.05$, compared to control (OT alone).

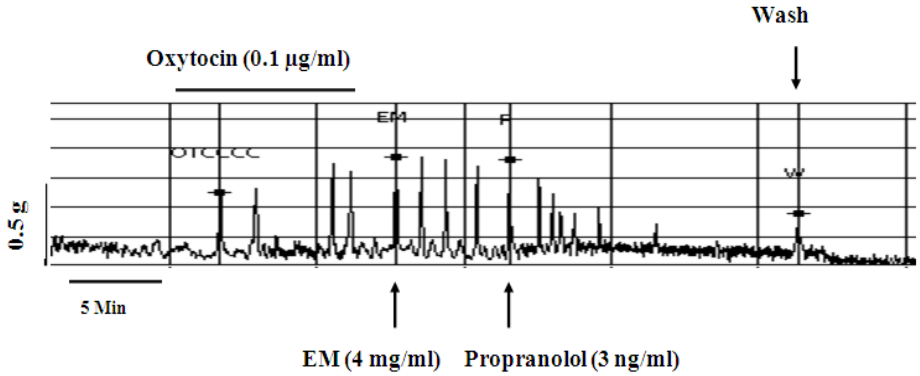

Figure 15: Original recording showing the effect of EM on oxytocin-induced uterine contraction in the presence and absence of propranolol (3 ng/ml).

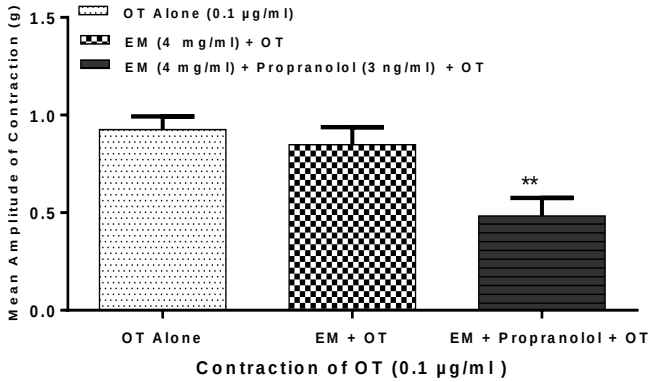

Figure 16: Bar graphs showing the effect of EM (4 mg/ml) on the amplitude of OT -induced contractions (0.1 µg/ml) in the absence and presence of propranolol (3.0 ng/ml), n = 5. EM displayed highly significant effects on OT-induced contractions in the presence of propranolol. **$P < 0.01$

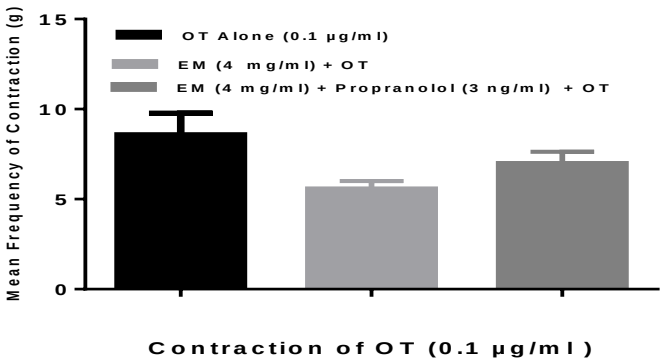

Figure 17: Bar graphs showing the effect of EM (4 mg/ml) on the frequency of OT -induced contractions (0.1 µg/ml) in the absence and presence of propranolol (3.0 ng/ml) n = 5 experiments. EM showed no statistically significant effects in the presence of propranolol on OT-induced contractions.

Although, there exist differences in the structures of rodent and human uterus, the physiological regulatory mechanisms controlling the myometrium contractility are basically the same (Blackburn and Flemming, 2011; Groothuis *et al.*, 2007). Therefore, results from this study can be extrapolated to the plant used by humans and it would therefore seem that the inhibitory effect displayed by the extract on uterine contractility in this study may indeed contribute to the contraceptive use of the plant in in herbal medicine.

Conclusively, this research has displayed that the methanol extract of the leaves of *Emilia coccinea* (Sims) G Dons produces uterine relaxant effects showing possible inhibitory interaction with intracellular calcium stores, prostaglandins and myometrial gap junctions. This study therefore supports the traditional use of the plant for birth control. Further studies are however necessary to identify specific bio-constituents responsible for the observed effects.

Acknowledgements. The authors would like to acknowledge Mrs. Omoruyi Osemelomen, Miss Agbidi Judith, Mr Fejiro Okpilolo and Miss Omogiade Uyi for their laboratory assistance during this research.

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