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ORIGINAL RESEARCH

In vivo investigation of female reproductive functions and parameters in nonpregnant mice models and mass spectrometric analysis of the methanol leaf extract of *Emilia Coccinea* (Sims) G Dons

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Keywords

Contraception, *Emilia coccinea*, female reproductive cycle.

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Abstract

In Southern Nigeria, the leaves of *Emilia coccinea* (Sims) G Dons are used traditionally for birth control. This study was therefore aimed at evaluating the activities of the methanolic leaf extract of *Emilia coccinea* (EM) on parameters that affect reproduction as well as the acute toxic effects of the plant using nonpregnant female mice models. Leaves of EM were extracted by maceration with 99.8% methanol. Oral acute toxicity profiles were examined. The effects of EM on female reproductive cycle were determined after oral treatment with EM at 1000 and 100 mg/kg/day daily for 6 days using stilbesterol (1 mg/kg/day) and normal saline as controls. The activities of EM (1000 mg/kg/day and 100 mg/kg/day p.o) on reproductive hormones and organs were also studied using estradiol valerat (100 mg/kg/day p.o), progesterone (10 mg/kg/day s.c.), and normal saline as controls. The extract did not induce any observable toxic effect after 24 h. At 1000 mg/kg, the extract significantly shortened the estrus cycle ($P < 0.05$) while prolonging the estrus phase which were comparable to that observed with stilbesterol. The extract also increased uterine weight and altered the histology of uterine and ovarian tissues. The female reproductive hormones were additionally altered at 1000 mg/kg and the effects were comparable to that of estradiol valerat such as to indicate possible antifertility effects. LC-HRFTMS analysis showed 9 putatively identified compounds with pyrrolizidine alkaloid occurring at the highest intensity among the identified compounds. In conclusion, the leaf extracts of EM has been shown in this study to exhibit antiovarulatory and estrogenic activities which would support the traditional use of the plant in Nigeria.

Introduction

For centuries, the use of plants to maintain and manage human health and wellbeing has been a common practice among the African communities. To date, about 90% of

the African population still depends solely on this practice (Cunningham 1993). Some of these preparations have, however, been associated with toxic effects which can lead to mortality or morbidity therefore necessitating careful monitoring.

Emilia coccinea (Sims) G Dons (EM) known as “yellow tassel flower” in the English language is an edible plant commonly used in traditional medicine within tropical parts of Africa such as Nigeria for birth control. *E. coccinea* is of the family Compositae (Asteraceae) (Olorode 1984). It is considered and described as a ubiquitous weed of the waste place and fallow land, widely distributed in tropical Asia and in tropical rain forests of West Africa (Edeoga et al. 2005; Chillendon 1956).

Phytochemical compounds such as alkaloids, terpenoids, flavonoids, tannins, saponins, steroids, and cardiac glycosides have been previously reported in EM (Edeoga et al. 2005; Okiei et al. 2009; Idu et al. 2010; Sofowora 1982; Teke et al. 2007; Mensah et al. 2013). These constituents are known to have medicinal and varied physiological effects in humans (Sofowora 1993; Kubmarawa et al. 2007; Addae-Mensah 1999; Okoegwale and Omefez 2001; Okoegwale and Olumese 2001; Ogunlesi et al. 2008). Previous studies also reported EM to possess antioxidant, antimicrobial, antidiabetic, anti-inflammatory, antidiarrhea, anxiolytic, antidepressant as well as antihepatotoxic activities (Erhabor et al. 2013; Agoha 1981; Burkill 1984; Odugbemi 2006; Telefo et al. 2011; Foyet et al. 2014).

However, no scientific data or reports are available on the activity of the plant on the female reproductive system. This study is therefore aimed at investigating potential antifertility properties of the plant by examination of the plant effect on the female reproductive cycle, organs, and hormones using mouse models. This is necessary to provide a proof of concept for the use of the plant as a contraceptive by traditional healers in Nigeria. The acute toxicity effects of the plant were also investigated in this study in order to determine the safety profile of the plant.

The choice of animal model has great impact on the outcome of results. The mice model is an established model to study effects of agents on humans as they are easy and flexible to handle and manipulate (Groothuis et al. 2007). Many authors have shown that similarities exist between mouse and human reproductive organs and cycle (Nelson et al. 1981; Kurita et al. 2005) thus, allowing careful extrapolation of findings to human. The reproductive cycles of both mice and humans are under the control of the endocrine system and are responsible for reproduction. The reproductive cycle is an endocrine clock that transmits time through regular periodic fluctuations in the concentration of mean values of reproductive hormones of the hypothalamus, anterior pituitary, and ovaries and transduce the information in a timely dependent manner to the uterus and ovaries. Mice models have been used to demonstrate the roles of two estrogen receptor (ER) isoforms in endometrial regulation (Hewitt et al. 2005, 2003).

Materials and Methods

Plant samples collection and extract preparation

Fresh samples of *Emilia coccinea* (Sims) G Dons leaves were collected in July within the locality of the Obingwa Local Government Area of Abia State, Nigeria between 6.00 A.M and 9.00 A.M and 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_a302 was prepared and deposited for future references. The leaves were then cleaned and dried for 10 days at room temperature 24–26°C.

The dried samples were blended into powder with the aid of an electric blender. The powdered sample was then macerated in 100% methanol and stirred repeatedly for 24 h. After the 24 h maceration process, the macerate was filtered through a Whatman No. 1 filter paper. The residue was discarded and the filtrate was evaporated to dryness using a water-bath set at 60°C. The concentrate was further dried to a constant weight in a Hotbox oven (Gallenramp[®], England) set at 40°C. The dried extract was kept in a refrigerator until usage. The given powder yielded 11.35% of a dark green extract (EM).

Animals used

Adult female Swiss Albino mice (25–35 g) aged 3–4 months, were used in all experiments. The animals were bought from a local Animal Center in Benin City, Nigeria, and kept in the Animal House of the Department of Pharmacology and Toxicology, University of Benin, Nigeria for the duration of the experiments. The mice were handled according to standard guidelines for the use and care of Laboratory experimental animals as stated by the Ethical committee, Faculty of Pharmacy, University of Benin, Nigeria as well as the standard guidelines for use of laboratory animals (National Institute of Health, Bethesda, MD: Public Health Service Policy on Humane Care and Use of Laboratory animals, 2002) and acclimatized for 2 weeks before the experiment proper.

Acute toxicity study

The oral acute toxicity study was carried out according to methods of Miller and Tainter (Miller and Tainter 1944). The animals were randomly divided into five (5) groups of five (5) mice each for the treatments. A single oral dose of 2000, 1000, 500, or 100 mg/kg of the extract was administered to groups A–D, respectively, by means of an orogastric tube connected to a syringe. Group E orally

received distilled water (1 mL/kg) and served as the control. The animals in all the groups were observed during the first 4 h after the single oral administration of the extracts for behavioral, neurological, and autonomic profiles and for any lethality or death up to 24 h after administration. These include locomotion, reaction to noise, tail activities and the appearance of feces. After the first 4 h of observation, all the animals were allowed free access to food and water. The surviving animals after 24 h were sacrificed and their blood samples were collected with 27G needles on 1 ml syringe into EDTA and lithium heparinized bottles for hematological and biochemical tests, respectively.

The biochemical analysis was performed on serum obtained after centrifugation of whole blood (without anticoagulant) at 1680 g for 5 min. Standardized diagnostic kits (Randox[®] by Randox Laboratories LTD, Crumlin, UK) were used for spectrophotometric (UNICO[®] S1200 spectrophotometer, UNICO Suite E, Dayton, NJ) determination of the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, bicarbonate, total proteins, albumin, total and conjugated bilirubin, sodium, chloride, and potassium. ALT and AST were analyzed by adopted method of Reitman and Frankel (1957). The biuret method of protein estimation of Kaplan (1972) was used in the assay for the total amount of protein in each sample. The assay for the total amount of albumin present in each sample was carried out using a method adopted from Young *et al.* (1975) using bromocresyl purple dye. And the assay for the total amount of bilirubin and the amount of conjugated bilirubin present in each sample was estimated by the adopted method of Jendrassik and Grof (1938).

The hematological parameters were analyzed at the University of Benin Teaching Hospital (UBTH), Nigeria using Sysmex (Kx-21N) automated hematological analyzer for *in vitro* diagnostic use in clinical laboratories. The samples were manually diluted with a diluting factor of 1–200 before loading into the automated hematological analyzer.

The effect of EM on ovulation/female reproductive (Estrus) cycle

The stages of the estrus cycle for each mouse was determined daily between 9 A.M and 12 Noon. The daily determinations were performed for 2 weeks (14 days) by both the visual method (Macroscopic Observation) and the swab method (microscopic observation) of obtaining vaginal smears (Snell 1941). Both procedures were performed independently. Animals found to be pseudopregnant were excluded from the experiments. Animals with similar cycles were placed together in a group.

In the Visual Examination (Macroscopic) method of observation, a modified method of Elvis-Offiah and Bafor (2014) was employed. This method utilized the changes in the appearance of the vagina that occur during the estrus cycle; the degree of vaginal swelling (particularly with respect to the dorsal lip), the color, and moistness were examined. The size of the vaginal opening and the presence or absence of cellular debris in the vagina was also examined. The mammary glands were observed to detect changes in appearance. The abdominal distension was also measured in order to ascertain the possible size of the uterus.

The Swabbing Method of Vaginal Smear Examination (Microscopic Observation) utilized a slight modification of the technique of Stockard and Papanicolaou (1917) and it basically involved identification of cell types and their relative quantities present in the preparation obtained from the vagina by swabbing the vaginal walls. Attributions of these characteristics to specific cycles were ascribed and scored as described by Elvis-Offiah and Bafor (2014).

Vaginal smears were collected from the animals daily by flushing the vagina with distilled water using a micropipette. The mouse was placed in a position which reduced animal's movement as much as possible and the swabbing pipette was introduced gently at a depth of approximately 0.1 cm into the mouse vagina. The swab pipette was released to flood the vaginal epithelium and a small quantity of vaginal fluid was drawn. The fluid containing the cells was gently transferred onto a clean, pre-labeled glass slide, below the relevant animal number. Care was taken to minimize pseudopregnancy. The smears were fixed with 95% ethyl alcohol and stained with Gentician Violet (GV) for 2 min. The GV was then gently rinsed off with water and allowed to dry. The smears were observed under LABO Optical Binocular Microscope, model AXL, (Haryana, India) attached to digital camera (1.3 M pixel) connected to a computer using $\times 10$ magnification.

Extract administration

After complete estrus cycles of the animals were ascertained, the animals with similar estrus cycles were placed in the same group of five animals per group, whereas animals found pseudopregnant were eliminated. Group I animals were in their proestrus phase and were given stilbesterol (1 mg/kg p.o.) thus serving as the positive control, Groups II (diestrus phase) and IV (metestrus phase) were given 1000 mg/kg (p.o.) and 100 mg/kg (p.o.) of the extract, respectively, whereas Group III (estrus phase) were given normal saline (p.o) and served as the negative control. The administration was carried out once daily for

6 days (6 days had been previously determined to cover one complete regular estrus cycle). Macroscopic and microscopic observations were carried out for the 6 days of treatment and subsequently for 10 days after treatment.

Determination of the activity of EM on hematological parameters and on reproductive cycle, hormones, and organs

Five experimental groups were used for this study. Group I was treated with estradiol valerat 100 mg/kg (p.o.), Groups II and III were treated with EM (1000 and 100 mg/kg p.o.) of the extract, Group IV was treated with progesterone 10 mg/kg (s.c.), whereas Group V was given normal saline (p.o) and served as the negative control. All the treatments were carried out for 6 days. On the 7th day, the animals were quickly sacrificed under anesthesia, and their blood samples collected with 27G needles on 1 mL syringe into lithium heparinized bottles for hormonal assay. The uteri were also carefully dissected out, surrounding tissues removed, then rapidly blotted on filter papers and quickly weighed on a sensitive balance. The uterine tissues were then fixed in 10% formalin and submitted for histological observations.

Histological examination was carried out in order to evaluate the effect of EM on the gross morphology of the whole uterus. The tissue sections were processed with an automated tissue processor (Citadel, 2000 Fischer Scientific, Loughborough, UK) and embedded in paraffin. Sections of 6 μm thicknesses were cut with a rotary microtome (LEICA RM2235; Leica Biosystems Inc., Buffalo Grove, IL) and stained with hematoxylin and eosin (HE) for morphology assessment. Histological sections were examined using the light microscope ($\times 100$) and digital images were taken.

The hormonal assay was done by an automated qualitative test utilizing the Minividas Analyzer, (VIDAS Kit, France) on serum or plasma (lithium heparin), using the Enzyme Linked Fluorescent Assay (ELFA) technique (Yolken and Stopa 1979). Briefly, the assay combines an enzyme immunoassay sandwich method with ELFA. The Solid Phase Receptacle (SPR[®]) served as the solid phase as well as the pipetting device for the assay. Reagents for the assay were ready-to-use and predispensed in a sealed reagent strip. The results were automatically calculated using calibration curves (4-parameter logistics model) and then printed out.

The assay procedure for all the hormones was the same but reaction and testing time varied. The assay for luteinizing hormone (LH), and follicle-stimulating hormone (FSH) was completed within approximately 40 min; the progesterone assay was completed within 45 min while that for estrogen was completed within 1 h.

LC-HRFTMS identification of constituents in extract

LC-HRFTMS analysis was performed on a Dionex Ultimate-3000 (DIONEX, Sunnyvale, CA) coupled to a Thermo Scientific Exactive Orbitrap system (Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany). The column used was an ACE 5 C18 75 \times 3.0 mm column from Hichrom Ltd., Reading, UK. Compounds were eluted with a flow rate of 300 $\mu\text{L}/\text{min}$ using water (A) and acetonitrile (B), both of which contained 0.1% formic acid, by a gradient starting with 10% B and increasing to 100% B in 30 min. The mobile phase was maintained at 100% B for 5 min after which the column was equilibrated with 10% B. The files were sliced into positive and negative datasets using ProteoWizard (Kessner et al. 2008) prior to data mining using MZmine 2.10 (Pluskal et al. 2010). Peak detection was accomplished using the centroid mass detector and a noise level of 1000. The chromatogram builder generated peak lists from the mass lists obtained from the previous step. The minimum time span was 0.2 min, minimum height was 10,000, and the m/z tolerance was set to 0.0001 m/z or 5 ppm. Chromatogram deconvolution was accomplished using the local minimum search algorithm with the following parameters: threshold (90%), search minimum in RT range (0.4 min), minimum relative height (5%), minimum absolute height (10,000), minimum ratio of peak top/edge (2), and peak duration range (0.2–5.0 min). The peak lists were deisotoped using the isotopic peaks grouper with an m/z tolerance of 0.001 m/z or 5 ppm, retention time tolerance of 0.1 minutes (absolute), and maximum charge of 2. The representative isotope was the most intense. The peak lists were then merged using the Alignment function. The weight for m/z and for RT was 20, and the RT tolerance was 5%. The aligned peak lists were gap-filled using the Peak Finder, with an intensity tolerance of 1% and RT tolerance of 0.5 min (absolute). The adducts were identified, together with other complexes that may have formed. The chemical formulas of each peak were predicted using the formula prediction tool developed by MZmine. An algorithm was employed to use the molecular formula data set from Dictionary of Natural Products[®] (June 2015). The database is customized to be curated and embedded into the MACRO. The monoisotopic exact masses for each metabolite were then calculated to be used for the customized library. The processed data from MZmine was incorporated into the customized library through the built-in Excel macro for peak identification and dereplication. “Hits” and unidentified peaks were double checked against the MS raw data in Xcalibur 2.2. Hits from the database were accessed using ChemBioFinder version 13 (PerkinElmer Informatics, Cambridge, UK).

Statistical analysis

All data shown were expressed as mean \pm standard error (SEM) and “*n*” represents the number of samples per animal. Significance was evaluated using an appropriate *t*-test, and where necessary, one-way analysis of variance followed by Tukey’s multiple range tests. $P \leq 0.05$ was considered statistically significant in all cases.

Results

Acute toxicity studies

The results of the acute toxicity study of EM in mice after 24 h administration showed no visible signs of toxicity in the animals (Tables 1, 2). Although there were slight increases in some hematological and biochemical parameters, they were considered statistically insignificant when compared with the control. No mortality, neurological, autonomic and behavioral changes were recorded within the dose range up to 2000 mg/kg body weight.

Studies on the reproductive cycle

The results of the effect of EM on the female reproductive cycle were examined in several ways. The number of metestrus periods experienced was taken as a measure of the number of cycles completed during the observation. From the macroscopic (visual) and the microscopic

(vaginal smear) methods, it was observed that before drug administration the animals had a range of 5–6 day estrus cycles. Four types of cells were distinguished in the vaginal smears, namely: leukocytes, non-nucleated epithelial cells, nucleated epithelial cells, and cornified cells. The presence and absence of these cell types, and the relative proportion of each cell type, was used to determine the stage of the estrus cycle. The treatment of mice with 1000 mg/kg of the extract for six (06) days, significantly prolonged the estrous cycle ($P < 0.05$) with an accompanying prolonged duration of the estrus phase with 90% cornification of vaginal wall cells (Fig. 1) and shortened duration of the diestrus phase and metestrus phases; whereas the 100 mg/kg dose showed a prolonged metestrus phase (Fig. 1) compared with the control. The effects seen with the higher dose (1000 mg/kg) were similar to the effects observed with the standard drug (stilbesterol, 1 mg/kg), (Table 3). Withdrawal of the treatment did restore the four phases of the estrus cycle and the duration of the cycle.

Effect of EM on reproductive hormones and organs

EM was further studied for activities on some female reproductive hormones and organs. EM induced a significant increase ($P < 0.01$) in uterine weight at 1000 mg/kg (Table 4) without any significant change observed at the lower dose.

Table 1. Effect of EM on hematological parameters after 24 h.

Parameters	Group 1 (2000 mg/kg)	Group 2 (1000 mg/kg)	Group 3 (500 mg/kg)	Group 4 (100 mg/kg)	Group 5 (Control)
WBC $\times 10^3/\mu\text{L}$	9.52 \pm 1.45	10.67 \pm 1.51	11.60 \pm 1.88	8.74 \pm 2.35	5.60 \pm 1.41
LY $\times 10^3/\mu\text{L}$	6.28 \pm 0.92	7.37 \pm 1.44	8.16 \pm 1.53	5.92 \pm 1.77	3.62 \pm 0.93
MO $\times 10^3/\mu\text{L}$	1.26 \pm 0.22	1.2 \pm 0.15	1.44 \pm 0.17	0.96 \pm 0.26	0.74 \pm 0.18
GR $\times 10^3/\mu\text{L}$	1.98 \pm 0.5	2.07 \pm 0.23	2.00 \pm 0.22	1.80 \pm 0.39	1.26 \pm 0.45
LY %	65.8 \pm 3.70	67.83 \pm 3.94	69.04 \pm 2.30	66.98 \pm 2.16	65.72 \pm 3.65
MO %	13.14 \pm 0.65	11.4 \pm 0.44	12.94 \pm 0.83	10.82 \pm 0.18	13.24 \pm 1.19
GR %	21.06 \pm 3.06	20.77 \pm 3.84	18.02 \pm 1.49	22.20 \pm 2.06	21.04 \pm 4.27
RBC $\times 10^6/\mu\text{L}$	9.65 \pm 0.69	11.04 \pm 0.51	10.74 \pm 0.38	9.00 \pm 1.38	7.55 \pm 1.25
Hgb g/dL	12.5 \pm 0.74	14.6 \pm 1.08	13.72 \pm 0.70	12.96 \pm 0.81	10.12 \pm 1.50
HCT %	38.24 \pm 2.2	47.43 \pm 5.95	41.86 \pm 1.98	41.80 \pm 3.74	29.26 \pm 5.0
MCV fl	38.38 \pm 0.88	42.63 \pm 3.39	38.90 \pm 0.87	38.00 \pm 1.77	38.70 \pm 1.36
MCH pg	12.92 \pm 0.79	13.13 \pm 0.38	12.68 \pm 0.30	11.84 \pm 0.63	14.08 \pm 2.35
MCHC g/dL	32.78 \pm 1.57	31.07 \pm 1.51	32.72 \pm 0.26	31.26 \pm 1.09	37.42 \pm 8.06
PLT $\times 10^3/\mu\text{L}$	383.2 \pm 177.7	501.33 \pm 156.1	624 \pm 92.7	454 \pm 145	211 \pm 79.5

Values are expressed as mean \pm SEM ($n = 5$); control group received 0.5 mL/kg normal saline. Although there are slight increases in blood hematological parameters, however, they are considered statistically insignificant when compared with the control.

WBC, white blood cells; LY, lymphocytes; MO, monocytes; GR, granulocytes; RBC, red blood cells; Hgb, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; PLT, platelets; EM, *Emilia coccinea*.

Table 2. Effects of EM on biochemical blood plasma parameters of mice after 24 h.

Parameters	Group 1 (2000 mg/kg)	Group 2 (1000 mg/kg)	Group 3 (500 mg/kg)	Group 4 (100 mg/kg)	Group 5 (Control)
Total bilirubin (mg/dL)	0.34 ± 0.04	0.38 ± 0.05	0.34 ± 0.06	0.28 ± 0.04	0.36 ± 0.10
Conjugated bilirubin (mg/dL)	0.125 ± 0.025	0.140 ± 0.024	0.12 ± 0.02	0.10 ± 0.00	0.20 ± 0.04
Total protein (g/dL)	4.98 ± 0.30	4.82 ± 0.24	4.42 ± 0.46	3.96 ± 0.33	3.70 ± 0.34
Albumin (g/dL)	3.96 ± 0.33	2.42 ± 0.19	3.96 ± 0.78	2.58 ± 0.38	4.68 ± 0.62
AST (IU/dL)	21.4 ± 1.99	17.6 ± 1.86	29.0 ± 1.52	22.0 ± 2.70	20.4 ± 1.03
ALT (IU/dL)	16.2 ± 1.99	12.8 ± 1.46	19.8 ± 2.42	15.6 ± 1.72	15.6 ± 0.98
Urea (mg/dL)	33.4 ± 1.4	27.8 ± 2.24	37.8 ± 2.44	34.2 ± 3.09	35.8 ± 1.83
Sodium (mMol/L)	72.6 ± 4.76	88.6 ± 7.64	59.6 ± 10.27	58.8 ± 8.74	47.0 ± 2.24
Potassium (mMol/L)	3.56 ± 0.17	3.68 ± 0.18	3.32 ± 0.10	3.80 ± 0.19	3.18 ± 0.07
Bicarbonate (mMol/L)	19.8 ± 0.58	19.0 ± 0.63	19.8 ± 1.24	18.4 ± 0.51	18.6 ± 0.6
Chloride (mMol/L)	21 ± 1.0	21.8 ± 1.07	22.6 ± 1.54	20.6 ± 0.87	20.6 ± 0.81
Creatinine (mg/dL)	0.80 ± 0.045	0.66 ± 0.09	0.82 ± 0.09	0.72 ± 0.107	0.88 ± 0.04
Urea (mg/dL)	33.4 ± 1.4	27.8 ± 2.24	37.8 ± 2.44	34.2 ± 3.09	35.8 ± 1.83

Values are expressed as mean ± SEM ($n = 5$ mice); control group received 0.5 mL/kg normal saline. Although there are slight increases in some blood biochemical parameters, however, they are considered statistically insignificant when compared with the control.

AST, aspartate aminotransferase; ALT, alanine transaminase; EM, *Emilia coccinea*.

Effect of EM on the female reproductive hormones

The assay of the female reproductive hormones indicated that the lower EM dose of 100 mg/kg lacked significant effect on the hormones (Table 5) except for progesterone where a significant reduction ($P < 0.05$) was observed. At the high dose of 1000 mg/kg, EM induced an estrogen-like effect when compared with the standard drug (estradiol valerat, a synthetic 17β -estradiol). EM (1000 mg/kg) also significantly reduced ($P < 0.05$) serum levels of FSH and LH with no observable effects on estrogen and progesterone hormones (Table 5).

Effect of EM on the uterus and ovary

The histological analysis of the uterus and ovary of EM-treated mice showed that the high dose of EM (1000 mg/kg) exerted significant changes similar to the effects of the standard drug (estradiol valerat) (Figs. 2, 3) on both the tissues of the ovaries and uterus, whereas the low dose (100 mg/kg) showed mild changes in the structure of these tissues. The high dose EM caused maturation of ovarian follicles and luteinization of ovarian stroma as well as uterine cystic glandular hyperplasia with moderate luminal and stromal infiltrates of inflammatory cells.

Identification of phytochemical constituents

The LC-MS spectral results and database search enabled the detection of nine (9) major compounds (Table 6) and eleven (11) unidentified major compounds (Table 7) from

the methanol crude extract (Tables 6 and 7). The unidentified compounds possibly represent new compounds and will require further purification procedures coupled with 1D and 2D NMR analyses for proper identification. The nine major compounds identified include: 3-ethyl-4-methyl-1,3,4-pentanetriol (1), 5,6-epoxy-5-(hydroxymethyl)-1,2,3,4-cyclohexanetetrol (2), 1,4-butanediol Di-Ac (3), 2-butene-1,4-diol; (E)-form (4), Di-Ac, hexahydro-7-hydroxy-1H-pyrrolizine-1-methanol; (1S,7S,7aS)-form, 1'-angeloyl (6), cyclobuxoxazine C; 16-O-(4-hydroxy-3,5-dimethoxybenzoyl) (6), martinelline (7), isoharderoporphyryrin (8), 12-O-a-L-arabinofuranoside-alnustic acid (9) (Table 6). The structures of identified compounds are shown in Figure 4.

Of the compounds identified, the LC-HRFTMS chromatogram showed compound 5 to be the most intense compound present, whereas compound 6 was present in very low quantities (Fig. 5).

Discussion

The result obtained from the acute toxicity studies showed a reasonable safety profile of the extract up to the dose of 2000 mg/kg after a 24 h test. Preclinical toxicity testing is one of the basic screening required to generate important information about potential damage of agents or new compounds to humans and is of utmost importance in drug development processes (Parasuraman 2011; David and Enevide, 2013). The US Food and Drug Administration states that the screening of new molecules for pharmacological activity and toxicity on animals is essential as this reveals similar incidence of occurrence

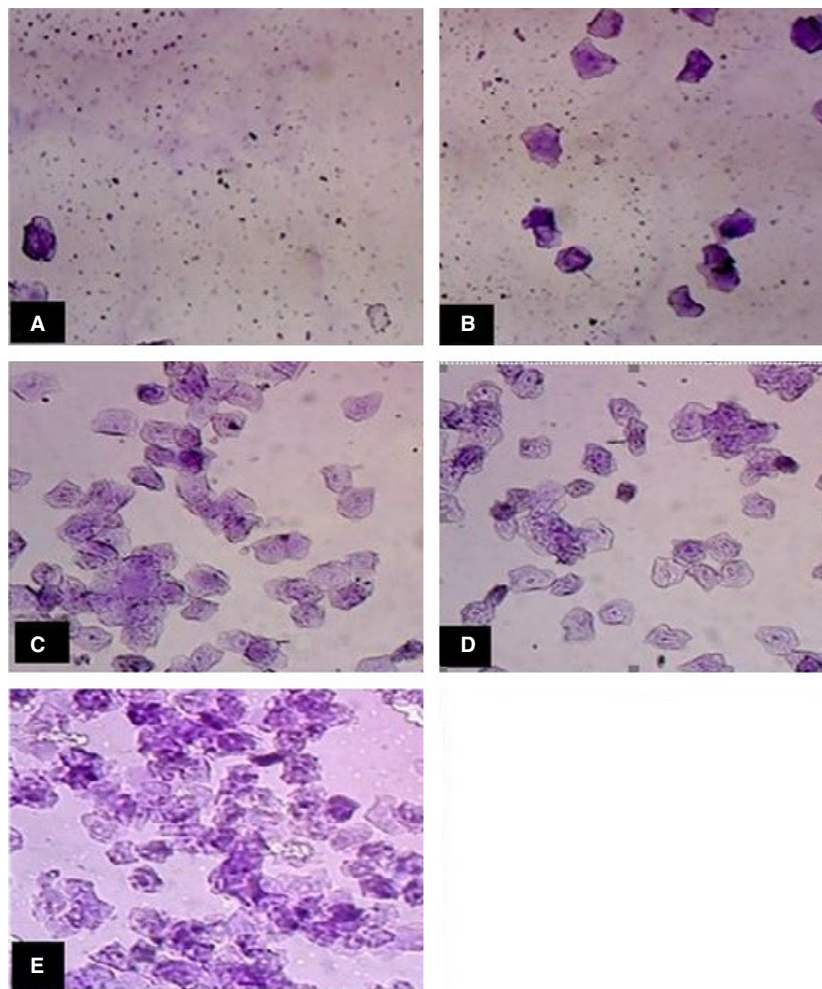


Figure 1. Representative of mouse vaginal smears on 5-days estrus cycle. LABO AXL binocular microscope $\times 100$. EM (1000 mg/kg), day 1 (A), day 2 (B), day 3 (C), day 4 (D), day 5 (E). EM, *Emilia coccinea*.

Table 3. effect of em on ovulation/female reproductive (estrus) cycle.

Treatment	Dose (mg/kg)	Length of cycles (days)	Length of different stages of estrus cycle (days)			
			Proestrus	Estrus	Metestrus	Diestrus
Control	—	4.50 \pm 0.20	1.00 \pm 0.00	1.00 \pm 0.20	1.50 \pm 0.20	1.00 \pm 0.20
EM	1000	6.50 \pm 0.2*	1.10 \pm 0.20	5.02 \pm 0.49**	0.20 \pm 0.2	0.18 \pm 0.25
EM	100	4.50 \pm 1.18	0.80 \pm 0.25	1.20 \pm 0.29	1.50 \pm 0.28	1.00 \pm 0.26
Estrogen	1	5.80 \pm 0.58*	0.50 \pm 0.24	4.00 \pm 0.49**	1.0 \pm 0.22	0.30 \pm 0.10

Values are expressed as mean \pm SEM ($n = 5$ mice). Control group received 0.5 ml/kg normal saline. P -value $*(P < 0.05)$ was considered to be statistically significant when compared with the control using one-way analysis of variance (ANOVA), $**P < 0.01$. EM, *Emilia coccinea*.

and severity in humans (United States Code Sections, Statutes at Large, Public Laws, and Presidential Documents, Code 1992).

The study of the effect of EM on the reproductive cycle revealed that high dose of EM prolonged the estrus cycle by prolonged or persistent estrus phase in mice. During

normal estrus cycle of mice, increase in estrogen level coincides with proestrus phase and results in cornification of cells of the vaginal walls. Increased cornification up to 90% induces a new phase (estrus phase). At the onset of estrus, estrogen levels begin to drop with increase in levels of progesterone. This increase in progesterone levels leads

to LH surge and consequently ovulation. Estrogen levels continue to fall and this leads to loss of cornification of vaginal cells and consequently, the next phase of the cycle (diestrus).

However, persistent estrus most often associated with antioviulatory effects of agents exhibited by high dose of EM indicated significant increase in estrogen levels which could not decline during the estrus phase. This shows that the antioviulatory effects of EM could be by mechanisms that either the block or prevent ovulation.

This report is consistent with previous studies of Huang and Meites (1975) and Brown-Grant et al. (1973). They showed that an extended vaginal estrus often indicates that the female cannot spontaneously achieve the ovulatory surge of LH and that constant or persistent vaginal estrus (or cornification) may result if the vaginal epithelium becomes cornified and remains so in response to certain agents particularly those with estrogen-like properties as found with diethylstilbesterol.

The clinical signs and symptoms of prolonged estrus include cornification of vaginal epithelial cells, tail flagging, and mating receptivity, as well as redness and swelling of the vulva (Eurell and Frappier 2006). Previous studies had

Table 4. Effect of EM on mouse uterine weight (g).

Groups	Dose (mg/kg body weight)	Weight of Uterus (g)	Weight of Uterus (mg/100 g body weight)
Estradiol valerat	100	0.226 ± 0.028*	10.4 ± 0.001*
EM	1000	0.399 ± 0.10**	14.3 ± 0.036**
EM	100	0.203 ± 0.03	9.0 ± 0.001
Progesterone	10	0.195 ± 0.02	8.4 ± 0.001
Control	—	0.131 ± 0.02	5.5 ± 0.001

Values are expressed as mean ± SEM ($n = 5$ mice). Control group received 0.5 mL/kg normal saline.

P -value $*(P < 0.05)$ was considered to be statistically significant when compared with the control using one-way analysis of variance (ANOVA). $**P < 0.01$. EM, *Emilia coccinea*.

Table 5. Effect of EM on female reproductive hormonal levels.

Hormones (mIU/mL)	Control	EM (100 mg/kg)	EM (1000 mg/kg)	Estrogen (100 mg/kg)	Progesterone (10 mg/kg)
FSH	2.93 ± 0.21	2.58 ± 0.22	1.83 ± 0.09*	1.73 ± 0.12*	2.78 ± 0.33
LH	3.75 ± 0.26	2.60 ± 0.25	1.93 ± 0.03**	2.33 ± 0.45*	2.78 ± 0.19
PROG	1.98 ± 0.13	0.85 ± 0.36*	1.17 ± 0.22	1.13 ± 0.09	1.20 ± 0.15
OEST	20.0 ± 0.57	20.4 ± 0.30	17.6 ± 0.33	18.0 ± 0.43	19.6 ± 0.73

Values are expressed as mean ± SEM ($n = 5$ mice). Control group received 0.5 mL/kg normal saline.

PROG, progesterone; OEST, estrogen. EM, *Emilia coccinea*; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

P -value $*(P < 0.05)$ was considered to be statistically significant when compared with the control. $**P < 0.01$.

Table 6. Putatively Identified Compounds in *Emilia coccinea*.

	Compound Name	Molecular formula	Molecular weight (g/mol)	m/z	R_t (min)
1	3-Ethyl-4-methyl-1,3,4-pentanetriol	$C_8H_{18}O_3$	162.1256	$[M+1]^+$ 163.1329	0.22
2	5,6-Epoxy-5-(hydroxymethyl)-1,2,3,4-cyclohexanetetrol	$C_7H_{12}O_6$	192.0635	$[M-1]^-$ 191.0562	1.19
3	1,4-Butanediol; Di-Ac	$C_8H_{14}O_4$	174.0891	$[M-1]^-$ 173.0818	2.14
4	2-Butene-1,4-diol; (E)-form, Di-Ac (possibly a fragment of compound 3)	$C_8H_{12}O_4$	172.0735	$[M-1]^-$ 171.0663	2.28
5	Hexahydro-7-hydroxy-1H-pyrrolizine-1-methanol; (1S,7S,7aS)-form, 1'-Angeloyl	$C_{13}H_{21}NO_3$	239.1519	$[M+1]^+$ 240.1592	4.87
6	Cyclobuxoxazine C; 16-O-(4-Hydroxy-3,5-dimethoxybenzoyl)	$C_{36}H_{54}N_2O_6$	610.3992	$[M-1]^-$ 609.3919	25.05
7	Martinelline	$C_{33}H_{52}N_{10}O_2$	620.4283	$[M-1]^-$ 619.4210	21.07
8	Isoharderoporphyrin	$C_{35}H_{36}N_4O_6$	608.2638	$[M+1]^+$ 609.2710	24.93
9	Alnustic acid; 12-O- α -L-Arabinofuranoside	$C_{36}H_{60}O_8$	620.4284	$[M-1]^-$ 619.42115	35.33

Table 7. Unidentified compounds detected in *Emilia coccinea*. Double bond equivalence (DBE) indicates number of rings and double bonds in the structure where 1 ring = 1 DBE.

	Double bond equivalence	Predicted formula	Molecular weight	<i>m/z</i>	<i>R_t</i>
10	5.5	C ₉ H ₆ N ₃ O ₂	188.0453	[M-1] ⁻ 187.0381	1.14
11	8.0	C ₂₉ H ₄₀ N ₄ O ₇	556.2911	[M-1] ⁻ 555.2838	1.19
12	30.0	C ₆₁ H ₆₂ N ₂ O	838.4888	[M-1] ⁻ 837.4815	23.5
13	15.0	C ₃₄ H ₄₀ O ₉	592.2689	[M+1] ⁺ 593.2763	29.33
14	6.0	C ₂₉ H ₄₈ O ₂	428.3652	[M+1] ⁺ 429.3725	29.86
15	8.0	C ₄₀ H ₆₅ NO ₃	607.4945	[M-1] ⁻ 606.482	31.19
	2.0	C ₃₅ H ₆₅ N ₃ O ₅			
16	1.0	C ₃₇ H ₇₂ N ₂ O ₇	656.5343	[M-1] ⁻ 655.5270	31.35
	2.0	C ₃₈ H ₆₈ N ₆ O ₃			
17	14.0	C ₄₄ H ₅₈ N ₂ O ₃	662.4468	[M+1] ⁺ 663.4541	35.74
18	13.0	C ₄₄ H ₆₁ N ₃ O ₃	679.4736	[M+1] ⁺ 680.4809	35.74
19	18.0	C ₄₆ H ₅₆ N ₂ O ₃	684.4287	[M+1] ⁺ 685.4360	35.78
20	7.0	C ₃₆ H ₅₅ N ₅ O	573.4389	[M-1] ⁻ 572.4315	35.87

shown that females in persistent or prolonged estrus may be sexually receptive regardless of the mechanisms underlying the altered ovarian condition (Smith and Davidson 1974; Cooper et al. 1993). However, Cooper et al. (1993) reported that if ovulation has been blocked by the treatment, LH surge may be induced by mating which would result in either pregnancy or pseudopregnancy but overall fertility of such mating is reduced (Fugo and Butcher 1966). They also showed that significant delays in ovulation can also result in increased embryonic abnormalities and pregnancy loss (Fugo and Butcher 1966; Kupfer 1987).

In this study, we compared the effects of different doses of EM, estradiol valerat, a synthetic 17 β -estradiol and progesterone on both uterine weight and histology of the uterus of mice to evaluate the estrogenic and antiestrogenic effects of EM. Results obtained showed that higher dose of EM induced significant uterotrophic effects similar to estradiol valerat, an estrogen receptor alpha (ER- α) agonist.

To further evaluate the antifertility and possibly antiovarian effects of EM, we evaluated the effects of EM on reproductive hormones and the histopathology of the ovaries. The extract at high dose was observed to reduce the levels of follicle-stimulating hormone (FSH) and LH in the serum without significant alterations on the levels of estrogen and progesterone. And caused maturation of the follicles with no evidence of ovulation or formation of corpus lutea (postovulatory effects) indicating that ovulation never occurred.

Many authors have shown that monitoring estrogen and progesterone concentration in serum is not a reliable method for diagnosing cytological persistent estrus or antiovarian effects as some species such as rodents and canines do not always show the normal increased levels of

serum estrogen and progesterone (Groothuis et al. 2007; Nelson et al. 1981; Lu et al. 1979).

The increased uterine weight and decreased LH are fundamental responses of females to exposure of increased levels of estrogen and reduced levels of progesterone and can be as a result of the growth of glandular epithelium, increased blood vascularity and fluid accumulation or increase in expression of vascular endothelial growth factor (VEGF) (Kurniawan et al. 2014). Unlike the human uterus, endometrial growth in mice appears to be due to water imbibitions in the uterine lumina and in the tissue rather than cellular amplification (Goldman et al. 1990). This culminates into a hypertrophic response of the uterine tissues. In humans, estrogen dominance is a major cause of infertility in women (Navot and Bergh 1991).

The histological studies of the mouse uterus treated with both high dose of EM and high dose of estradiol valerat also revealed that both agents caused cystic glandular hyperplasia of uterine glands, a state of uncomplicated immoderate enlargement of endometrial glandular cells. This condition has been reported by many authors to result due to increased estrogen levels with accompanying very low levels of progesterone.

In rodents, the effects of exogenous estrogens on the uterus include hypertrophy of the luminal (predominantly) and glandular epithelium (Katsuda et al. 1999), proliferation of the myometrium (Hunter et al. 1999), and stroma (Cook et al. 1999). Several studies have confirmed that these effects are mediated through the estrogen receptor alpha (ER- α) (Buchanan et al. 1999; Ogawa et al. 1999) suggesting that EM exerted its effects either by direct action on ER- α , or it may be involved the mechanisms that might be unrelated to activation of ER- α but by other mechanisms in which estrogen could be involved in.

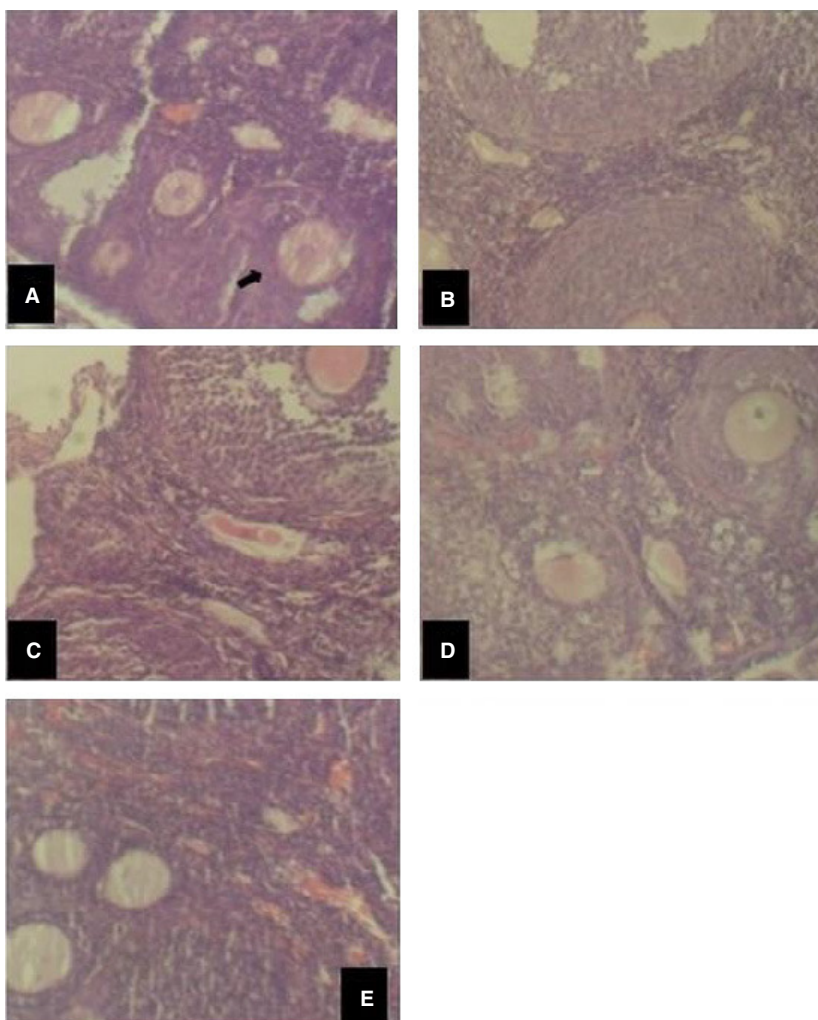


Figure 2. Representative images of hematoxylin and eosin staining of ovarian tissue from mice after 6 days of EM treatment. (A) Control (normal saline), (B) progesterone (10 mg/kg) (C) Estrogen (100 mg/kg), (D) EM (1000 mg/kg), (E) EM (100 mg/kg). Follicles are indicated by arrow in (A). EM, *Emilia coccinea*.

This could possibly be by negative feedback decreases on the pulsatile frequency of GnRH release by the hypothalamus which decreases the release of FSH and LH by the anterior pituitary. The decreased levels of FSH inhibit further follicular development preventing any increase in estrogen levels. Then the insufficient levels of progesterone and lack of estrogen positive feedback on LH release prevent a mid-cycle LH surge. This effect coupled with inhibition of further follicular development prevents ovulation.

This study correlates with several works such as that of Goldman et al. (1991) who reported that chemicals can delay or block ovulation by disrupting the ovulatory surge of LH, or by interfering with the ability of the maturing follicles to respond to the gonadotropic signals. Also, Pang et al. (1977) and Smith (1983) reported that compounds

which increase central opioid receptor stimulation also decrease serum LH and inhibit ovulation in monkeys and rats. Further research on this aspect is, however, beyond the scope of this study and further studies is therefore advised.

Chromatogram and biological effects of identified compounds

Four compounds observed in this study are likely implicated in the antifertility effects of EM. These include, (1*S*,7*S*,7*aS*)-1'-angeloyl hexahydro-7-hydroxy-1*H*-pyrrolizine-1-methanol; (compound 5), 3-Ethyl-4-methyl-1,3,4-pentanetriol (compound 1), 5,6-epoxy-5-(hydroxymethyl)-1,2,3,4-cyclohexanetetrol (compound 2), and isohardoporphyryin (compound 8).

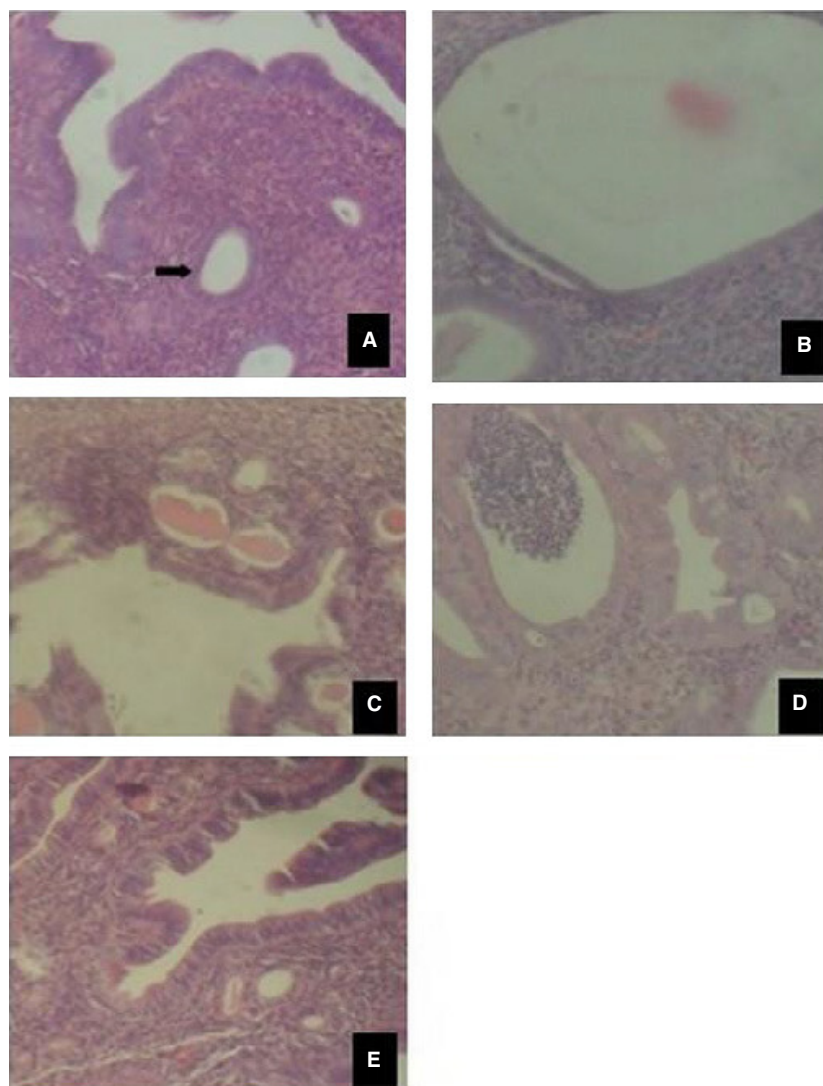


Figure 3. Mouse Uterus H & E $\times 100$: Representative images of haematoxylin and eosin staining of uterine tissue from mice after 6 days of EM treatment. (A) Control (normal saline), (B) Estrogen (100 mg/kg), (C) Progesterone (10 mg/kg), (D) EM (1000 mg/kg), (E) EM (100 mg/kg). Uterine glands are indicated by arrow in (A). EM, *Emilia coccinea*.

1'-Angeloyl-Hexahydro-7-hydroxy-1H-pyrrolizine-1-methanol; (1S,7S,7aS)-form, identified by LC-MS spectral belongs to the class of pyrrolizidine alkaloids (PAs). Some adverse effects which occur on consumption of plants have been associated with the PAs (Roeder 1995). PAs are secondary metabolites which have been known to occur mainly in the families *Asteraceae* (to which the EM in this study belongs), *Fabaceae*, and *Boraginaceae* (Roeder 1995). In particular, PAs have been identified in *Emilia* genus (Roeder 1995). PAs are generally known to exhibit toxic, carcinogenic, and mutagenic effects (Tidjani et al. 2013). Antifertility effects of PAs from *Senecio vulgaris* L. in rats have been reported (Tu et al. 1988).

Similarly PAs from *Heliotropium Indicum* L. (Boraginaceae) have been reported to show significant antifertility, anti-implantation and abortifacient activity in rats (Willaman and Schubert 1961; Das 2011). PAs have also been reported to show weak inhibition of intestinal and uterine smooth muscles (Akinlolu et al. 2006) but smooth muscle stimulatory effects have also been reported (Adelaja et al. 2008). The antifertility effects described for PAs and the identification of PAs in the *Emilia* genus strongly implicates compound 5 as a potential compound for further study as the active constituent responsible for the antifertility effects observed in this study.

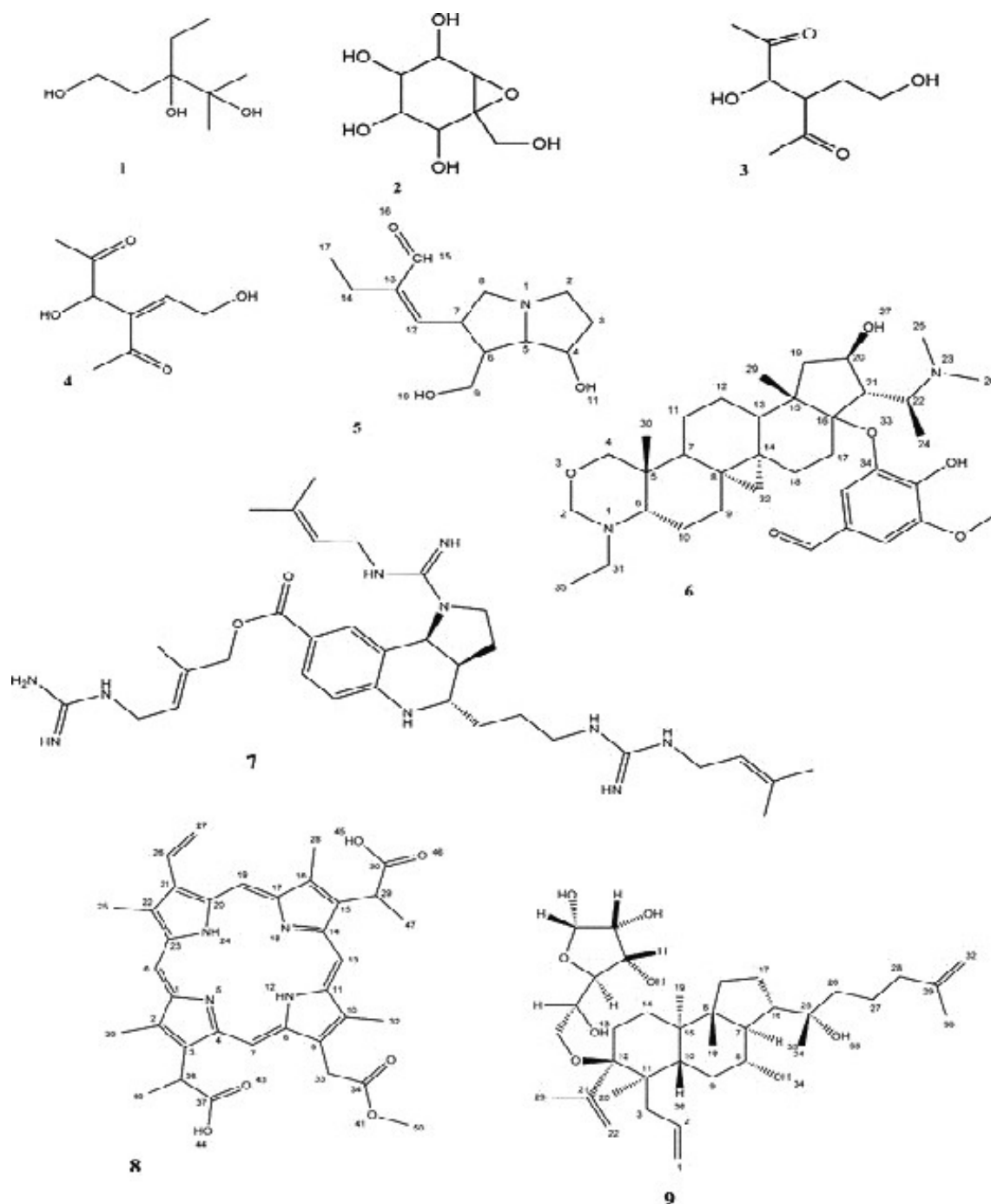


Figure 4. Structure for compound 9 needs to be changed.

Glycols have been reported to exhibit antitumor effects (Wang *et al.* 1996; Taketa *et al.* 2015), anxiolytic effects (Lin *et al.* 1998), and sedative effects in humans (Singh *et al.* 1981; Forrest and Galletly 1988). Reports have shown polyethylene glycol (PEG) to exert inhibitory effects on liver enzymes and on central nervous system activity (Nishimura *et al.* 1998; Thomsen *et al.*

1995). On effects on smooth muscle, PEG has been reported to exert an inhibitory effect on intestinal smooth muscles (Shah 1969), whereas propylene glycol has been reported to inhibit smooth muscle contractility of the uterus and intestines (Bonnardeaux 1971). Ethylene glycol has been reported to prevent reattachment of contraction filament crossbridges (Sakoda and Horiuti

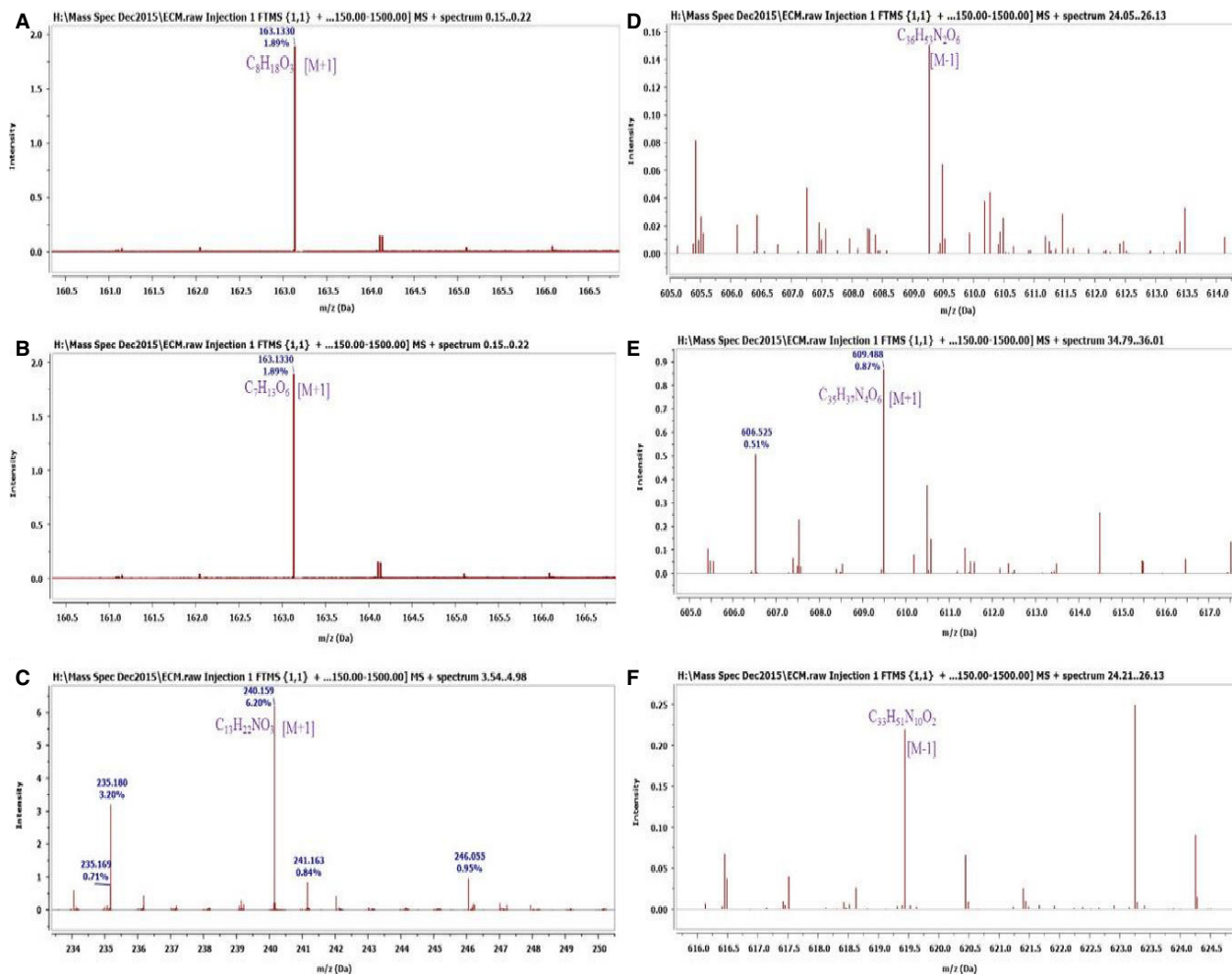


Figure 5. LC-HRFTMS Chromatograms of selected identified compounds (Table 6) showing the mass to charge ratio (m/z) and the intensity. (A) represents the chromatogram of compound 1 with an intensity of 1.89%; (B) represents the chromatogram of compound 2 with an intensity of 1.89%; (C) represents the chromatogram of compound 5 with an intensity of 6.20%; (D) represents the chromatogram of compound 6 with an intensity of 0.16%; (E) represents the chromatogram of compound 7 with an intensity of 0.87%; (F) represents the chromatogram of compound 8 with an intensity of 0.23%.

1992) which would cause and sustain relaxation of smooth muscles. PEG-cholesterol has also been reported to inhibit L-type calcium channels (Ochi et al. 2014) an effect that may also contribute to the smooth muscle inhibiting effect. Glycol ethers are known to prolong gestation in animals possibly through inhibition of gap junctions (Marty and Loch-Carusio 1998). These inhibitory effects on smooth muscles and particularly on uterine smooth muscles may contribute to the effect observed in this study. However, to the best of our knowledge there has been no previous reports on 3-Ethyl-4-methyl-1,3,4-pentanetriol which is also a glycol, on uterine smooth muscle contractility.

Isoharderoporphylin belongs to the porphyrin group of compounds to which heme and chlorophyll belong as

well (Hendry and Jones 1980). A common precursor to heme and chlorophyll is protoporphyrin IX (Hendry and Jones 1980). Harderoporphylin is an intermediate compound between coproporphyrin and protoporphyrin commonly found in animal tissues (Hendry and Jones 1980). However, a plant equivalent exists which is involved in chlorophyll synthesis and metabolism (Hendry and Jones 1980). Until recently, porphyrin derivatives from plants were not associated with biological effects besides effects on photodynamic tumor therapy (Nyman and Hynninen 2004; Pandey et al. 1991). Bafor and colleagues provided the first report on plant-derived porphyrins on uterine contractility (Bafor et al. 2013) where it was reported that different porphyrin derivatives exhibited differing effects of stimulation and inhibition on

uterine contractility (Bafor et al. 2013). This therefore suggests a role for plant-derived porphyrin derivatives on reproductive function and possibly a role for compound 8 in the reproductive effects observed in this study.

Cyclohexanetetrol belongs to the oligosaccharide class of cyclitol carbohydrates or sugars (Ramanathan et al. 1966). Cyclitols which also include polyhydroxylated isocyclic molecules are involved in a number of biological functions often coupled to other functional groups (Lehn et al. 2007). Inositols which are also cyclitols and are referred to as polyhydroxylated cyclohexane derivatives with myo-inositol being the most naturally abundant, also play significant biological roles (Lehn et al. 2007). There has, however, been no report on cyclohexanetetrol or similar nonphosphorylated cyclitol derivative on smooth muscle contractility or signaling. Further studies are therefore required. It is, however, suggested that cyclohexanetetrol may function as a precursor to phosphorylated inositols.

In conclusion, this study has established potential antifertility activities of the methanol leaf extract of *Emilia coccinea* Sims (G) Dons. However, further research is advised to fully establish the mechanism of the active constituent of this plant.

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Conflict of Interests

The authors declare no conflict of interest.

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