

EMILIA COCCINEA: PHYTOCHEMICALS, PROXIMATE ANALYSIS, GC-MS PROFILING, AND *IN-VITRO* EVALUATION ON THE UTERUS IN NON-PREGNANT FEMALE MICEEmmanuel E. Odion^{1*}, Sunday P. Luwoye¹, Uchechi J. Ude¹ and Adaeze P. Uchendu²

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DOI: [njcpt.com.ng/101.1.2.41](https://doi.org/10.1177/10112412031241241)

Abstract

Background: *Emilia coccinea* is a source of naturally occurring plant compounds, used traditionally in the treatment of reproductive disorders and prevention of conception. **Objectives:** The objective of this study was to evaluate the phytoconstituents in the leaf, and evaluate the proximate parameters and effects that the extract and fractions will have on the uterine contractility of adult non-pregnant female mice.

Methods: The phytochemical constituents were evaluated using standard methods and gas chromatography-mass spectrometry (GC-MS), proximate analysis was determined by the method described by the Association of Official Analytical Chemists while the spontaneous uterine contractility was determined by measuring the concentration-responses relationship following the administration of the extract and fractions.

Results: Phytochemical screening revealed flavonoids, saponins, glycosides, terpenoids, tannins, alkaloids, and steroids, while GC-MS analysis identified forty-four compounds, belonging to different classes of phytochemicals. Some of these compounds are 2-nonynoic acid; 2,5-cyclooctaden-1-one; 2-myristynoylglycinamide; 1,9-decadiyne; 2-methyl-2-oxiranylcyclobutanone; N, N-di-2-propynyl-2-propyn-1-amine; formic acid hydrazide and 6-(1-pyrolidinyl)furazano[3,4-6]pyrazin-5(4H)-one. Proximate analysis gave the moisture content (10.24±0.09) %, ash value (7.65±0.41) %, crude fibre content (10.38±0.38) %, crude protein content (5.07±0.09) % and total fat content (4.39±0.57) %. The methanol extract and dichloromethane fraction significantly decrease the amplitude and frequency of uterine contraction in a concentration-dependent manner with associated IC₅₀ of 0.1023 mg/ml and 0.2715 mg/ml (methanolic extract) and 0.2509 mg/ml and 0.1411 mg/ml (dichloromethane fraction) respectively.

Conclusion: This thus establishes that *Emilia coccinea* contains steroids, alkaloids and minerals that cause uterine smooth muscle relaxation in albino mice. Hence, this report supports its use as a possible tocolytic agent.

Keywords: *Emilia coccinea*, Proximate analysis, Phytochemicals, GC-MS analysis, Uterine contractility

Introduction

Emilia coccinea (Sims) G. Don; family Asteraceae, is a fast-growing erect herb^[1] known as Scarlet tasselflower. Though endemic to Africa, have been established in both North and South America and some parts of Asia^[2,3]. In Africa, it has been reported in Kenya, Nigeria, and Cameroon^[4]. *Emilia coccinea* (EC), is a weed of about 0.4 m to 1.5 m high that colonizes roadsides, riverbanks, and fallowed lands^[5,6]. It survives at an elevation of 2 km^[7], in humid, properly drained soils, pH limit between 6.1 to 7.8 with great vulnerability to light. Once established, it can tolerate drought^[7,8]. The leaves are simple and alternately arranged, while the lower regions are petiolate. The leaf blade is between spatulate and elliptical, up to 0.12 m x 0.05 m in dimension. Higher leaves are sessile, and the blade is spatulate to lanceolate, up to 0.20 m x 0.06 m^[1]. Leaves of EC have found application in local delicacies as vegetables in both Kenya and Nigeria^[9,10]. Extract from the leaf is used by traditional medicinal practitioners in the treatment of jaundice^[11], measles and skin rash^[12], gonorrhoea and syphilis^[7,8],

sinusitis and ringworm [13]. It is used in the management of convulsions and epilepsy in children and as a potent sedative for the treatment of some neurological disorders [14]. Ethno-medicinally EC plant preparation has been reported to be used as a contraceptive and treatment of diseases of the reproductive system [15,16].

These activities associated with *EC* could be ascribed to the presence in the leaf of phytochemicals. Phytochemicals are naturally occurring plant chemicals, produced, and released to either defend the plant from predators or aggressors or protect the plant from harsh environmental conditions. The presence of these compounds may not show any visual pharmacological effect in the plant. Extraction, partial or total purification may result in compounds with different pharmacological potentials either in human or experimental animals. The need to identify these groups of phytochemicals has given rise to qualitative tests that will streamline the search for the required activity. Most of these tests for the analysis of various groups of phytochemicals are standardized, so also for proximate analysis.

Herbal drugs are readily available in developing countries, cheap and are believed to be without side effects. Unfortunately, they are yet to be properly harnessed and their ethno-medicinal claims are yet to be verified. A literature search though revealed that the methanolic extract of *EC* significantly altered uterine contraction. The search for the group of phytochemicals responsible for this observed activity is lacking. Therefore, this study aimed to determine the phytoconstituents in *EC* using standard methods and evaluate the extract and fractions for tocolytic potential.

Methods

Collection, preparation, extraction, and concentration of plant materials

EC was collected in the month of January, around the new Pharmacy building, Ugbowo campus of the University of Benin, Benin City. It was immediately taken for identification in Prof. MacDonald Idu Herbarium in the Department of Plant Biology and Biotechnology, University of Benin. The plant specimen was with the following specimen number UBA-E132.

Leaves were carefully dislodged from the stuck, and air dried under shade away from direct sunlight for 14 days before it was placed in a warm air oven (40 °C) for 2 hours. This was then pulverized using an electric milling machine (Galiham) to a fine powder and then packed into an air-tight container until used.

Two-hundred-gram powder of *EC* was macerated with 700 ml of methanol (Scharlar 99.99 %) for 1 week with initial agitation for 2 hours (30 min intervals for 4 times) immediately after soaking, during this period the macerating extract was kept in an air-tight jar to prevent evaporation of the extracting solvent (methanol) and in a dark compartment away from light.

After 7 days, the extract was decanted into a large beaker (1L) with the aid of a funnel and cotton wool. Immediately the filtrate was passed through filter paper (size no. 1), collected, and concentrated using a rotary evaporator (ST150SA) *in-vacuum* at 40 °C. The resultant crude extract was stored in a refrigerator at a temperature of 4 °C until used for analysis.

Solvent Partitioning

Crude extract (16.0 g) was dissolved in methanol and water in a ratio of 1:4 and poured into a separating funnel. N-hexane (300 ml) was added to the mixture in a funnel and the contents of the separating funnel were gently rocked to prevent the formation of emulsion at the interphase with the cap securely placed. The separating funnel was returned to an upright position (tripod stand) until the phases were completely separated with continuous venting of the separating funnel to avoid pressure building up. The n-hexane phase was collected via the opening of the stopcock. This was repeated until the n-hexane fraction was colourless. The solvent collected was bunk-up and concentrated using a rotary evaporator at 4 °C in a vacuum. This process was repeated using dichloromethane and ethyl acetate. The concentrates for the extract and fractions were kept in the refrigerator at 4 °C until used.

Phytochemical screening

The powdered leaf was evaluated for phytochemicals using the methods described by Sofowora, Treas and Evans [17,18]. The phytochemicals investigated include tannins, glycosides, flavonoids, alkaloids, triterpenoids, saponins and phenols.

Proximate analysis of the powdered leaf of Emilia coccinea

This analysis was carried out by using the methods described by The Association of Official Analytical Chemists (AOAC) [19] using the powdered leaf of EC. The parameters analysed are the moisture content, crude fibre content, ash value, crude protein content and total fat content.

Plant extract preparation for GC-MS analysis

Fifty milligrams of the crude extract were placed in a 100 ml beaker and 10 ml of the solvent mix (1:1 - Hexane: Dichloromethane) was added before it was homogenized. This was cleaned up with 100 - 200 mm mesh silica gel and 3 g of anhydrous sodium sulfate in a well-packed column, conditioned with hexane to form a slurry. The cleaned-up extract (1 microlitre) was injected through the injection port into the Gas Chromatography (Agilent 6890N) equipped with an autosampler connected to an Agilent Mass spectrophotometric detector.

GC-MS analysis

The pulsed splitless mode was used to inject 1 μ L of cleaned-up crude extract onto a DB 5MS coated fused silica column with length, internal diameter and film thickness 30 m x 0.25 mm x 0.15 mm, with Helium gas as the carrier gas and head pressure maintained at 20 psi to give a constant pressure of 1 ml/min. Operating conditions were set as follows: column temperature was initially held at 55 °C for 0.4 min, increased to 200 °C at a rate of 25°C/mins, then to 280 °C at a rate of 8°C/mins and to a final temperature of 300°C at a rate of 25°C/mins, held for 2mins. Identification of the compounds was based on the retention time.

Animals used

Non-pregnant adult female albino mice (20-27g) were utilized in this work. The mice were housed in the Department of Pharmacology and Toxicology Animal House, University of Benin, for the duration of the research. Mice were acclimatized for a period of two weeks before the commencement of the study, they were kept in plastic cages containing beddings of wood shaving which were regularly replaced as needed and were regularly fed with rodent pellet feed and clean water. Animal use and experiment were approved by the University of Benin, Faculty of Pharmacy Ethics Committee. The mice were handled by standard protocols of use and care of laboratory animals [20,21].

Experimental procedure

Uterine tissue preparation

Stages of the oestrus cycle for each mouse were determined by both macroscopic and microscopic methods as described by Byers and co-workers [22] and were confirmed by the presence of cornified cells [23]. The mice were then humanely sacrificed by cervical dislocation and immediately the uterus was abstracted and set in a Petri dish holding previously warmed and aerated physiological salt solution of the constitution: NaCl (4.5 g), (NaHCO₃) 0.1 g, D-glucose (0.25 g), (KCl) 0.21 g, CaCl₂. 2H₂O (0.16 g) in 500 ml of distilled water to prevent tissue death. Connective and adhering tissues were carefully removed from the isolated uterus tissue and each uterine horn was dissected into segments of 1-2 mm length with rust-free dissecting scissor and forceps.

To each uterine segment, a loop was made at one end and a long thread attached to the other end of each uterine strip obtained using a needle and thread and with the aid of a tissue holder mounted onto an organ bath containing aerated physiological solution (10 ml), maintained at 37 °C. The uterine tissue strips were mounted vertically in the organ bath and activity was recorded using isometric force transducers (Panlab AD Instruments, Spain) connected to bridge amplifiers which in turn was connected to the PowerLab data acquisition system consisting of a recording unit (Powerlab 2/26 Model ML826 AD Instruments, Australia) coupled to a Labchart software (AD Instruments). The tissue was equilibrated under resting tensions of 4.90 mN for 30 - 45 minutes or until regular contractions were obtained [24].

Spontaneous uterine contractility determination

After equilibration, the uterus tissue was allowed to contract for 10 minutes before the administration of the extract, this served as the control experiment. Methanol extract of EC was added cumulatively

(0.01-12.21 mg/ml) to the isolated uterine tissue of a mouse to obtain the concentration-response relationships. Each concentration was allowed a contact time of 5 minutes ^[25] before washing with a physiological salt solution. The tissue was then allowed about 20 minutes to recover, indicating that the concentrations of the extract used were not toxic to the tissue. This was carried out on 5 different animals (n=5). This experiment was repeated on another uterine tissue using dichloromethane and ethyl-acetate fractions.

Data analysis

All data were analysed using GraphPad Prism, (version 8.1.1; GraphPad Software Inc, San Diego, CA, USA). Contractions occurring at the last 5 or 10 minutes of the phasic contractions were used to calculate the mean frequency, amplitude, and area under the curve.

Results

Alkaloids, saponins, tannins, terpenoids, flavonoids, steroids and glycosides were the phytochemicals associated with the leaf of EC (Table 1).

Table 1: phytochemical screening of the leaf powder of *Emilia coccinea*

Phytochemicals	Inference
Alkaloids	+
Saponins	+
Tannins	+
Terpenoids	+
Flavonoids	+
Steroids	+
Glycosides	+

Proximate parameter evaluation indicated moisture, ash, protein, fibre, and fat contents as 9.94±0.49 %, 7.65±0.41 %, 5.07±0.09 %, 10.38±0.38 % and 4.39±0.57 % respectively (Table 2).

Table 2: Proximate analysis of *Emilia coccinea* leaf powder

Proximate parameter	(%)
Moisture content	9.94±0.49
Total ash value	7.65±0.41
Crude protein content	5.07±0.09
Crude fibre content	10.38±0.38
Total fat value	4.39±0.57

Forty-four compounds were identified from the GC-MS analysis following the comparison of the chromatogram obtained with the NIST 14 [30] reference spectral library (Table 3), these compounds belong to different classes of alkaloids and steroids. Eight of the compounds showed a total percentage area of 69.98 %, with a breakdown of 2-nonynoic acid (31.76 %), 2,5-cyclooctaden-1-one (20.93 %), 2-myristynoylglycinamide (4.96 %), 1,9-decadiyne (3.49 %), 2-methyl-2-oxiranylcyclobutanone (2.44 %), N-di-2-propynyl-2-propyn-1-amine (2.17 %), formic acid hydrazide (2.12 %) and 6-(1-pyrolidinyl)furazano[3,4-6]pyrazin-5(4H)-one (2.12 %).

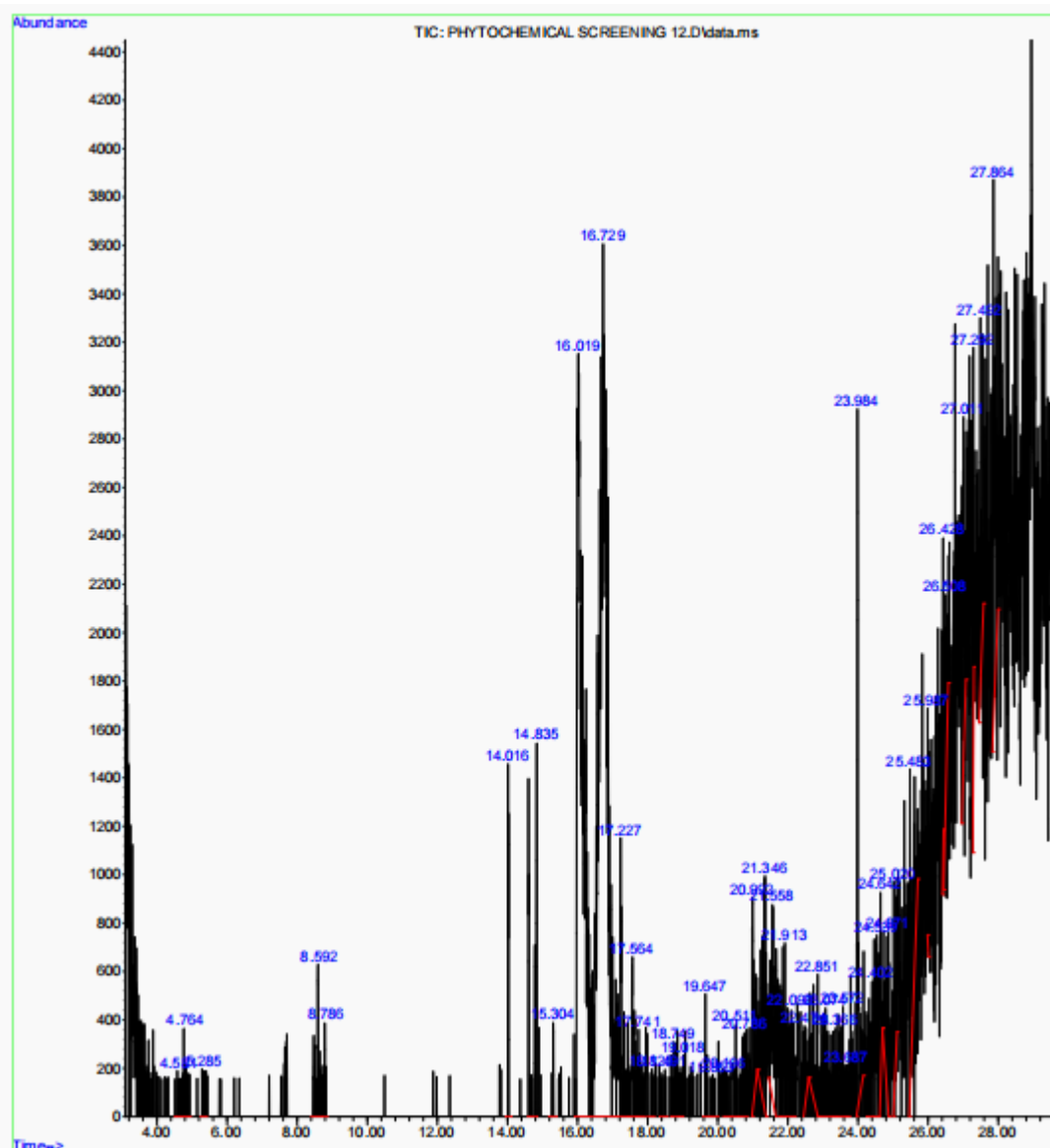


Figure 1: Chromatogram of methanol extract of *Emilia coccinea*

Table 3. GC-MS Analysis of the methanolic extract of *Emilia coccinea* leaf extract

S/N	Retention Time (Min.)	Area%	Compounds
1	4.581	0.20	Propargylamine
2	4.764	0.40	(S)-methyl oxirane,
3	5.285	0.15	2,4-pentadienenitrile
4	8.592	1.16	5-Methyl-2(5H)-furanone
5	8.786	0.43	2-Propenenitrile
6	14.016	1.12	5-methyl-6-keto-bicyclo(2.2.0)-5-aza-hex-2-ene
7	14.835	2.17	N,N-di-2-propynyl-2-propyn-1-amine
8	15.304	0.22	Amphetamine
9	16.019	20.92	2,5-cyclooctadiene-1-one
10	16.729	31.76	2-nonanoic acid
11	17.227	2.44	2-methyl-2-oxiranylcyclobutanone
12	17.564	1.40	Pyrrole
14	18.491	0.18	1-Propanenitrile
15	19.018	0.27	3-hydroxy propane nitrile

16	19.647	0.35	Isobutylamine
17	19.853	0.13	2-fluoroacetaminophen
18	20.196	0.27	2-Chloroethylamine
19	20.511	0.41	Methylguanane,
20	20.786	0.72	1,2-dimethylhydrazine
21	20.992	1.17	2-propenamide
22	21.558	2.12	formic acid hydrazide
23	21.913	1.93	Thiirane
24	22.090	1.51	Acetic acid
25	22.474	0.27	2,3,3-trifluoro-2-propionitrile
26	22.851	0.77	Formic acid Hydrazine
27	23.074	1.45	N-chloro-2-methyl aziridine,1 2-cis
28	23.366	0.55	2-fluoroacetaminophen
29	23.572	1.07	(S)-2-Butanamine,
30	23.687	0.24	2-(methylthio)ethanamine
31	23.984	3.49	1,9-Decadiyne
32	24.402	1.38	2-1-phenylethylidene-hydrazono-3-methyl-2,3-dihydrobenzothiazole
33	24.539	1.57	4-aminopyripyrimidine
34	24.642	0.29	Trifluoromethyltrimethylsilane
35	24.871	0.79	methyl ester theocratic acid,
36	25.020	1.15	1-(2-Adamantylidene)semicarbazide
37	25.483	1.35	Methyl eicosa-5,8,11,14,17-pentanoate
38	25.987	0.41	Bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl, semicarbazone
39	26.428	0.53	Diazoprogesterone
40	26.508	0.80	Cyclopropane, (-3-chloropropyl)methylene
41	27.011	2.12	6-(1-pyrolidinyl)furazano[3,4-b]pyrazin-5(4H)-one
42	27.292	0.72	1,1-Difluoro-2,2,3-trimethyl-cyclopropane
43	27.492	1.56	1-[.alpha.-(1-Adamantyl)benzylidene]thiosemicarbazide
44	27.864	4.96	2-myristynoylglycinamide

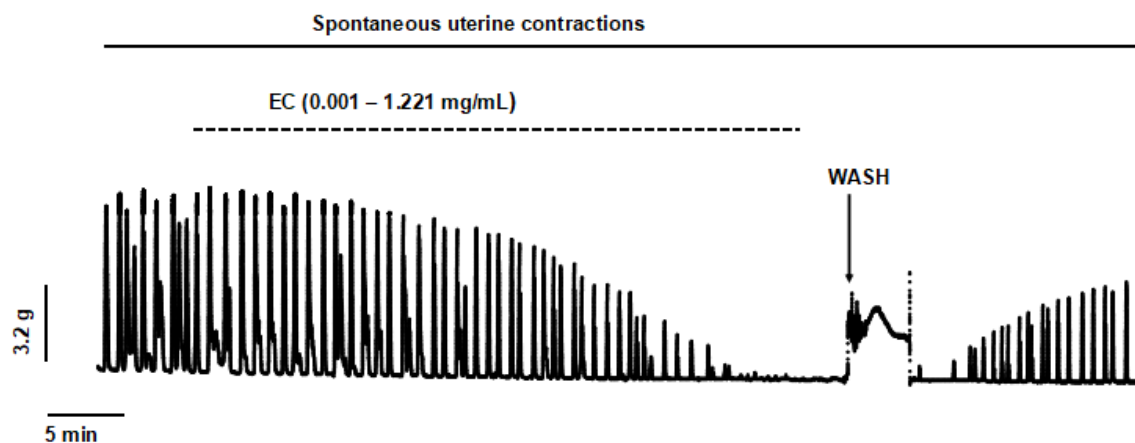


Figure 2: Representative recording showing the cumulative effect of methanol extract of EC on spontaneous uterine contraction. The extract was observed to inhibit uterine contraction. It produced a concentration-dependent decrease in the amplitude and frequency of contraction (Figure 3). The IC₅₀ for the decrease in amplitude and frequency was 0.1023 mg/ml and 0.2715 mg/ml respectively.

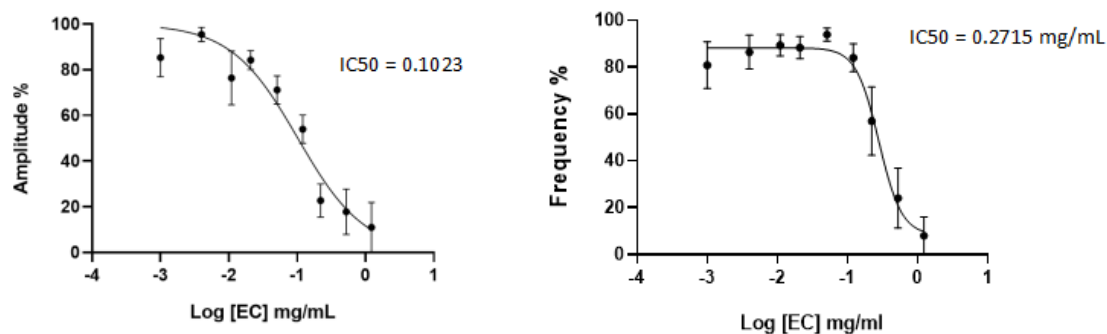


Figure 3: Concentration-response curves showing the effects of EC on the amplitude and frequency of spontaneous uterine contraction. n = 5 animals, EC = *Emilia coccinea*

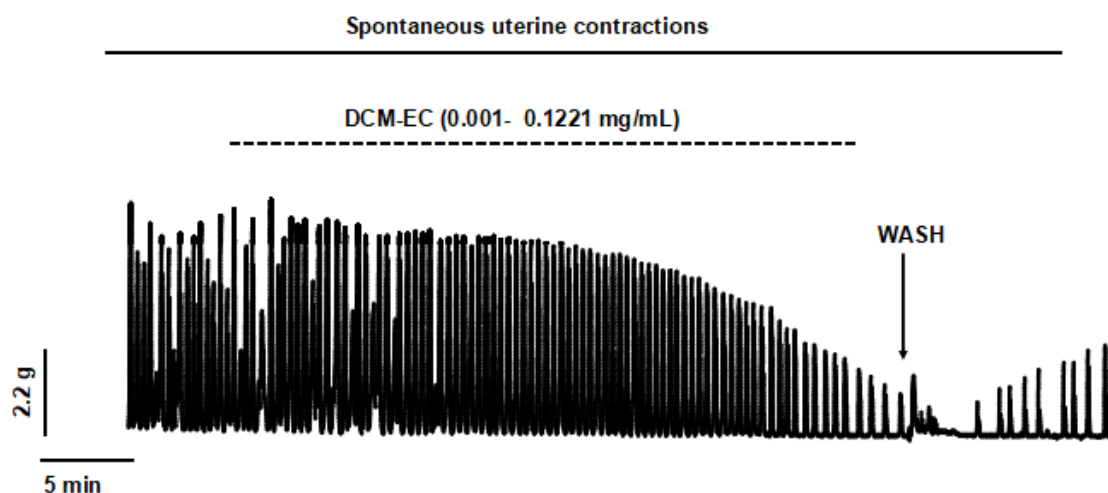


Figure 4: Representative recording showing the cumulative effect of dichloromethane fraction of EC (0.001-0.1221 mg/ml) on spontaneous uterine contraction. The dichloromethane fraction of EC inhibits the amplitude and frequency of uterine contraction (Figure 5). These were observed to be concentration dependent, with IC50 for the amplitude and frequency as 0.2509 mg/ml and 0.1411 mg/ml respectively.

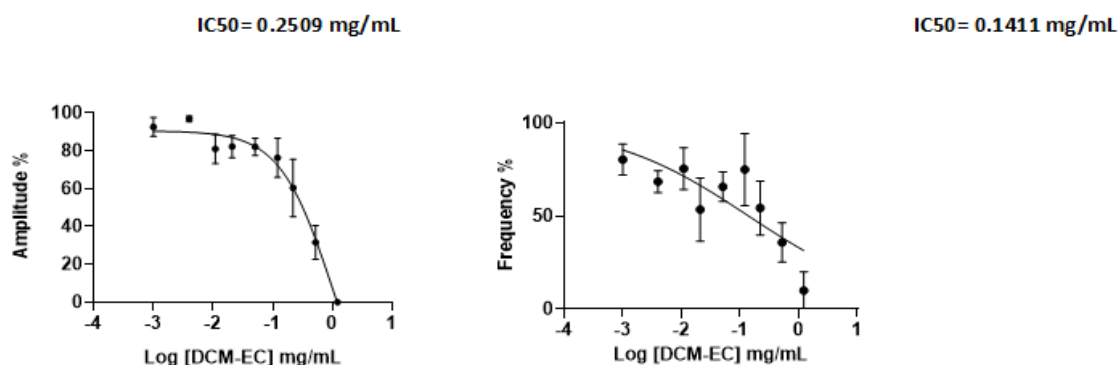


Figure 5: Concentration-response curves showing the effects of DCM-EC on the amplitude and frequency of spontaneous uterine contractions. n = 5 animals, DCM-EC = Dichloromethane fraction of *Emilia coccinea*

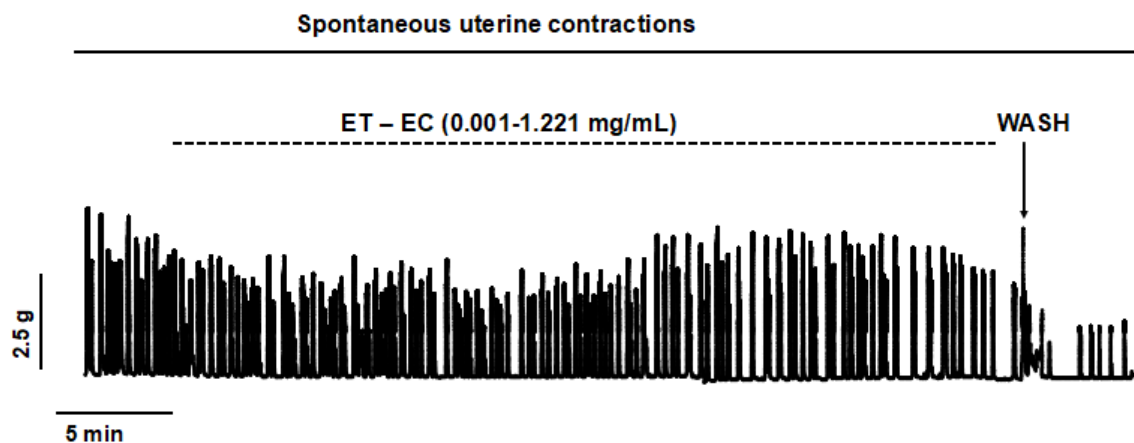


Figure 6: Representative recording showing the cumulative effect of ethylacetate fraction of EC (0.001-1.221 mg/ml) on spontaneous uterine contraction. The ethyl acetate fraction produced no effect on the amplitude and frequency of uterine contraction (Figure 7).

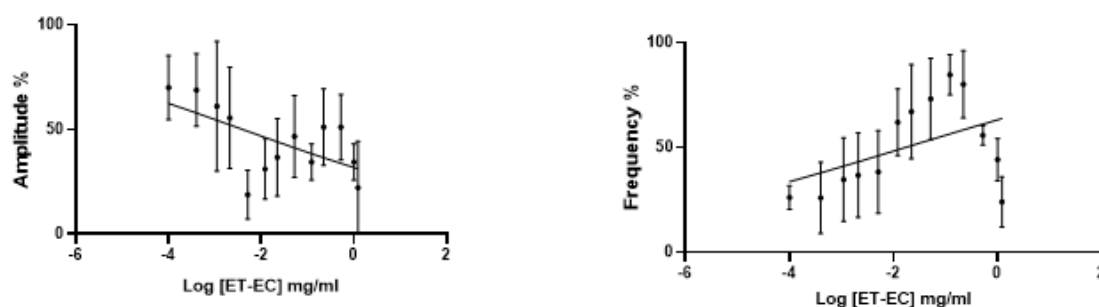


Figure 7: Concentration-response curves showing the effects of EA-EC on the amplitude and frequency of spontaneous uterine contractions. $n = 5$ animals, EA-EC = Ethylacetate fraction of *Emillia coccinea*.

The cumulative concentrations of the methanol crude extract (0.001- 1.221 mg/ml) were observed to significantly decrease the amplitude and frequency of uterine contraction in a concentration-dependent manner with the maximal effect observed at the highest concentration with an associated IC_{50} of 0.1023 mg/ml and 0.2715 mg/ml respectively.

Discussion

Alkaloids, tannins, saponins, flavonoids and glycosides have been reported in previous studies [6,26,27] and implicated in several important pharmacological activities, which include antimicrobial [15] and muscular relaxation [28]. The proximate parameters provide useful information on the stability and nutritional content of the powdered leaf sample. A study by Viola and co-workers [29] revealed low moisture, ash, fibre, and fat contents while the protein content was slightly high when compared to this study. A similar study on the whole plant part of EC gave values that are closely related to a slight decrease in the ash content [9]. This difference could be related to the environmental conditions from where the sample was collected or the mode of processing following the collection of the leaf. Moisture content aids in predicting the shelf life of the powdered sample, since microbes will proliferate in the presence of water. Thus, a high moisture content implies that the powdered sample will have a short life span. A moisture content below 10.00 % is within the accepted limit. The fat and protein contents in the leaf of EC may be viewed as moderate to high, considering the nature of the sample (leaf), since most leaves are not mostly used as fat and protein sources. Fat is an important component of plants and animals; it acts as an insulator against low temperatures and as a source of energy when broken down.

Protein is the product of amino acids that are used to build and replace worn-out tissues. Fibre is needed in the diet to aid digestion and prevent the accumulation of food in the human gastrointestinal tract that may cause discomfort. The ash content in the powdered leaf sample provides information about the level of minerals, which are vital for the proper functioning of the cell. Holistically, different minerals in the human perform various functions thus promoting the well-being of the body. One such mineral is calcium, it facilitates extracellular and intracellular communication, resulting in smooth muscle relaxation in different parts of the body.

The putative compounds identified in this research are known to possess different pharmacological activities that have been ascribed to EC. 2-nonynoic acid showed some effect in inhibiting the growth of *Mycobacterium tuberculosis* [31]. 2-methyl-2-oxiranylcyclobutanone has been previously reported in *Jatropha curcas* with activity against *Candida albicans* and *Candida tropicalis* [32]. Hydrazide was found to possess an antibacterial effect against *Staphylococcus pneumoniae* [33]. Furazano[3,4-b]pyrazin derivatives have been described as mitochondrial uncouplers that have the potential to be used for the management of Parkinson's disease, obesity and anti-ageing [34]. Myristoylglycine is a lipidated amino acid with a short fatty acyl chain, that induces distinction in brown adipocytes without boosting the differentiation of preadipocytes into white adipocytes excluding the effect on cell viability. This has been recorded as a potential endogenous treatment for obesity and other similar metabolic diseases [35]. Other compounds were identified as alkaloids and steroids.

That the extract reduced the amplitude of spontaneous contraction suggests possible interaction with either extracellular voltage-operated calcium channels or blocking of intracellular calcium mobilization from the sarcoplasmic store as well as interaction with prostaglandins which are known to play a major role in the regulation of spontaneous uterine contractions [36]. At each concentration, the amplitude and frequency of spontaneous contractions were gradually reduced suggesting possible inhibition of the force and frequency of myocyte activity via reduction of intracellular calcium concentrations. An increase in $[Ca^{2+}]_i$ is reported to activate the enzyme myosin light chain kinase (MLCK) increasing the phosphorylation of myosin regulatory light chain-20(MLC20) and subsequent cross-bridge cycling [37]. MLC20 phosphorylation by MLCK is the principal determinant of the amplitude and duration of contraction [38]. Therefore, the inhibition of the amplitude of myometrial contraction markedly observed in this study may be attributed to the prevention of MLC20 phosphorylation by the crude extract of the EC whole plant. Calmodulin translocation of activated MLCK towards the contractile apparatus may be the rate-limiting step of contraction [39] which determines the frequency of contraction. There was more significant inhibition of the amplitude of spontaneous contraction than the frequency, this greater effect of EC on one parameter of spontaneous contraction than the other suggests that the extract may exert less activity on MLCK activation and possibly a greater effect on prevention of MLC20 phosphorylation.

Also, the dichloromethane fraction of EC (0.001- 1.221 mg/ml) was observed to significantly decrease the amplitude and frequency of uterine contraction with an associated IC₅₀ of 0.2509 mg/ml and 0.1411 mg/ml while the n-hexane and ethyl acetate fractions showed no significant change on rhythmic spontaneous uterine contraction. This suggests that the bioactive constituents responsible for this inhibitory effect on uterine contraction were retained in the dichloromethane fraction of the methanol extract of EC after fractionation. These phytochemicals are distributed into various solvents based on their general chemical formula or properties. The process of fractionation has the advantage of partially purifying or grouping the compounds in EC and separating the phytochemicals into different solvents based on their polarity. Methanol, a polar solvent with a hydrophilic and lipophilic part in its molecular structure can extract both the polar and non-polar constituents present in the powdered sample. During the process of partial fractionation, the non-polar compounds are distributed into the n-hexane fraction while the semi-polar constituents are retained in the dichloromethane (alkaloids) fraction and the polar compounds are fractionated into the ethyl acetate fraction.

The myometrium of the uterus regularly contracts all through the length of the oestral and menstrual cycles and appears to be seen only in the sub-endometrial layer of the uterus [40]. 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 [41,42]. The myometrial contraction is decreased by oral contraceptives [43] and is therefore used as a primary medication to treat primary dysmenorrhea [44].

Although the uterus of humans and rodents have structural differences, the physiological regulatory mechanisms controlling the myometrium contractility are quite similar ^[45,46] thus allowing careful extrapolation of findings to humans. Therefore, the inhibitory effect displayed by the plant on uterine contractility in this study can be linked to the use of the plant in birth control and as a tocolytic agent (in the treatment of dysmenorrhea and preterm labour) because of the bioactive constituents distributed into the dichloromethane fraction of the extract.

Conclusion

This study has established that EC is a rich source of nutrients that aid the proper functioning of the human body. In addition, it contains bioactive compounds that were identified in this study. Some of these compounds have documented evidence of important pharmacology activities. The presence of these phytochemicals (alkaloids and steroids) in the plant has been shown to alter the contraction of the uterine of mice. These were shown to be extracted into methanol and subsequently fractionated into dichloromethane which causes relaxant effects on uterine smooth muscles because of their retention in the dichloromethane fraction. Hence, this report supports the use of EC in birth control and suggests its possible use as a tocolytic agent. However, further research is advised to fully isolate/purify specific bioactive compounds responsible for this activity.

Acknowledgement

The authors wish to thank Prof Joseph Kayefor Ebigwai of Mifor Consult Nigeria Limited for running the GC-MS for EC.

Conflict of interest

The authors wish to state that there was no conflict of interest.

Financial Support

The authors received no financial support for this research.

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