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Phytochemistry and Uterine Contractile Effect of VLC Fractionated Bi-herbal Formulation "Makann" in Mice

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Abstract

This study evaluate the phytochemistry, uterine activity of a bi-herbal formulation of *G. kola* and *C. papaya* roots in rodents. Identification of phytochemical compounds in BH was done using high performance liquid chromatography (HPLC) and fractionated with vacuum liquid chromatography and thin layer chromatography. *Ex-vivo* BH elicited cumulative concentrations response on spontaneous contractions of oxytocin-induced contraction in the presence and absence of calcium and potassium induced contraction. The Phytochemicals showed alkaloids (Hordenine, Cytisine, Methyl Jasmonate, Galanthamine and an unidentified compound), flavonoids (benzoic acid, isoquercetin, rutin, chlorogenic acid and an unidentified compound), phenols (eugenol and ferulic acid) and cardiac glycosides (metildigoxin, cymarin and alpha-acetyldigoxin). The fractions were tested for spontaneous uterine contractions. Uterine contraction had an isncrease in amplitude spontaneous contractions with a steady decrease in frequency. In conclusion, the results obtained from this study adhere to the folklore report, validating its myometrial contractile response with the implicated phytoconstituents.

Keywords: Phytochemistry, Uterine Contraction, Bi-herbal Formulation, Mice

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INTRODUCTION

Phytochemicals are plants secondary metabolites, they are natural, small molecular compounds that are important in plant protection and self-defence (Hanson, 2003). Classification of secondary metabolites is based on their chemical structure and functional groups. The major groups are; terpenes (plant volatiles, cardiac glycosides, carotenoids and sterols). The phenolics (phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (alkaloids and glucosinolates) (McMurry, 2010; Velu *et al*., 2018). These compounds are bioactive ingredients in nutripharmaceuticals and modern medicine, with a wide range of therapeutic activity and direct interaction with receptors, cell membrane and nucleic acid (Velu *et al*., 2018). Plant extract activity could be as a result of synergistic interactions of various phytocompounds present in the extract, this activity may not be seen in the evaluation of a single compound. The synergistic activities of bioactive compounds in extracts are the reasons they are effectively used in the treatment of wide ranges of diseases and other disorders (Mulyaningsih *et al*., 2010; Hamoud *et al*., 2012; Eid *et al*., 2013).

The membranes of living organisms act as a barrier to prevent the leakage of cellular metabolites from the cell. They contain numerous proteins; ion channels, receptors, and transporters which communicate or exchange materials with other cells and the cell's immediate environment. Calcium ion channels have been reported to be modulated by Lipophilic phytocompounds of mint oil. Herbal drugs are rich in phenolics with antioxidant activity, which modulate proteins and biomembranes (VanWyk and VanWink, 2004; VanWyk and Wink, 2015).

Carica papaya fruit, seed, latex and root are good sources of bioactive compounds and the quantity of phytocompounds in the different parts differ and vary with the extraction methods, plant age, cultivation and gender (Sentilkumaran and Shalini**,** 2014). *Carica papaya* fruit is an excellent source of vitamins A, C and E, a good source of vitamins B and G, rich in iron, calcium, minerals, magnesium and potassium, folate and fiber (Sentilkumaran and Shalini**,** 2014). Phytochemical researches showed that *Carica papaya* contains mainly alkaloids carpaine and pseudocarpaine, tannins, flavonoids, carcin, gamma terpine, glycoside, carposides and sugars (Asha *et al*., 2014). The analysis of the leaves of *Carica papaya* revealed the following elements: oxygen 87 %, calcium (4.47 %), magnesium (3.37 %) and potassium (1.49 %). Other elements in lesser quantity were silicon, (0.805 %),

chromium (0.0129 %), aluminum, phosphorus, chloride, sulphur, stannous, strontium (Ram *et al.,* 2014). Flavonoids, alkaloid, tannins, saponins, cardiac glycosides, anthroquinones, steroids, carbohydrates and proteins were reported to be present in aqueous extract of *Carica papaya* leaves (Sherwai *et al*., 2013). Unripe fruit of *C. papaya* extracts contain terpenoids, alkaloids, flavonoids, carbohydrates, glycosides, saponins, steroids and fibrin (Aravind *et al*., 2013). *Carica papaya* fruit has many phenolic groups which scavenge free radicals. The latex main constituents are papain and chymopapain and the injection of papain extract in dog increased prothrombin and coagulation threefold. Phytochemical study of fresh *Carica papaya* unripe and ripe fruit pulp adding no solvent or chemical revealed that unripe *Carica papaya* is rich in papain and other cystein endopeptidases (chymopapain, caricain and glycyl endopeptidase). These four endopeptidases are found in latex, fruit, leaves and roots in differing quantity (Azarkan *et al.,* 2003).

Phytocompounds isolated from *Garcinia kola* are tannins, saponins, alkaloids, cardiac glycosides (Ebana, *et al*., 1991), biflavonoids (kolaflavone and 2-hydroxybi-flavonols), tocotrienol and chromanols (garcioic and garcinal) are two new phytocompounds reported in *Garcinia kola* (Terashima *et al*., 2002). Methanol extract of *Garcinia kola* seed revealed the presence of these secondary metabolites: tannins, saponins, flavonoids, alkaloids and cardiac glycosides (Essien and Effiong, 2014). Adesuyi *et al.* (2012) detected the presence of magnesium, zinc, iron, manganese, copper, lead, phosphorus, sodium, potassium and calcium in *Garcinia kola* seed.

MATERIALS AND METHODS

Plant Material and Authentication

Garcinia kola and *Carica papaya* roots were harvested from Egbekwe and Ovbiogie respectively in Ovia North East Local Government Area of Edo State in the month of November. The samples were authenticated in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State and were allotted voucher specimen numbers; *Garcinia kola* UBH-365 and *Carica papaya* UBH-C505.

Plant Extraction

The roots of the two plants were properly rinsed, cut into small pieces and dried under a shade for two weeks. The dried roots were further dried in hot air oven at 60 °C for 6 hr. before pulverizing separately into powder using a laboratory milling machine. *Garcinia kola* (100 g) and *Carica papaya* (100 g) roots were macerated separately in boiled water, absolute methanol, ethyl acetate, n-hexane. In the bi-herbal formulation, 50 g of *Garcinia kola* with 50 g of *Carica papaya* roots were combined (100 g) and was macerated in boiled water and absolute methanol. They were left at room temperature (30 °C ± 2 °C) with frequent shaking for 72 hr. They were filtered using glass funnel tightly plugged with cotton wool and the filtrates were concentrated in a hot air oven at 60 °C. They were properly labeled and kept in the refrigerator at 4 °C for use.

Experimental Animals

Albino adult female mice (20 – 30 g) and immature female albino rats (18 days) were used. The animals were maintained at the Animal Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Edo state, Nigeria. Ethical approval (EC/FP/021/20) was obtained from the Ethics Committee on the Use of Animals for Experimental Procedures, Faculty of Pharmacy, University of Benin. The animals were housed in well ventilated cages and fed with animal pellets (Top feed) with free access to clean water *ad libitum*. All animals were handled carefully according to the Guide for the Care and Use of Laboratory Animals (2011). All animals were weighed on S-Mettler electronic compact balance model K- 500BH (max=500, d=0.01g), weight was recorded in grams. Anal temperature was taken using C-Tone digital thermometer (Mode: GF502) in degree Celsius (°C).

Phytochemistry

High Performance Liquid Chromatography (HPLC)

HPLC identification of phytocompounds present in BH extract was carried out using Hewlett Packard HPLC (series 1050) having a UV detector, an isocratic pump, a reversed phase C18 column.

Alkaloids were eluted with gradient elution of two solvents, Solvent A (Methanol) and Solvent B (acetic acid:water, 1:25) mobile phase. The gradient program was started with 100 % (B) for the first 4 minutes. This was followed by 50 % eluent (A) for the next 6

minutes, concentration of (A) was increased to 80 % for the next 10 minutes and then reduced to 50 % for 2 minutes, a column temperature of 30 °C, frequency of 270 nm, flow rate of 0.5 $mlmin^{-1}$, injection volume of 10 µl and run time of 10 min were employed.

Flavonoids and phenols elution were carried out in a mobile phase consisting of 2 % (v/v) acetic acid:water (A) and 100 % (v/v) acetonitrile (B), followed by 4 - 15 % (B) for 0 - 4 min, 25 - 50 % (B) for 4 - 15 min and 50 - 95 % (B) for 15 - 30 min, column temperature of 25°C, flow rate was 1 ml/min. Flavonoids frequency was 320 nm, injection volume of 20 µl and run time was 30 min. Phenol frequency was 280 nm, injection volume of 10 µl, and run time was 30 min was employed.

In cardiac glycosides analysis, the mobile phase was acetonitrile: methanol: water (l:2:l), flow rate was 1.0 ml/min, injection volume of 10 µl, run time was 10 min. Cardiac glycosides were detected at 340 nm, and column temperature of 25 °C.

The identification of the chromatographic peaks was achieved by comparing the retention times and spectral characteristics (nm) of the eluting peaks with those of reference standards (Celeghini *et al.,* 2001).

Vacuum Liquid Chromatography (VLC)

Six grams of BH extract was finely mixed with 10 g of silica gel (10 – 40 µm) and was uniformly layered on a 500 ml sintered glass funnel fritted with disk grade 3 packed with silica gel 60 – 120 µm and 10 – 40 µm sizes. The sintered glass funnel was fixed to a receiving flat bottom flask and connected to a vacuum pump. The column was run with 100 ml of hexane:ethylacetate (70:30 – 0:100), ethylacetate:methanol (90:10 – 0:100) and methanol:water (50:50) see Table 3.1, under 50 mmHg pressure and fractions were collected into separate tubes (Maurya *et al.*, 2018).

Table 1: Vacuum liquid chromatograph (VLC) solvent ratio

TUBE NUMBER	HEXANE	ETHYLACETATE	METHANOL	DISTILLED WATER
1	70	30		
2	60	40		
3	50	50		
4	40	60		
5	30	70		

6	20	80		
7	10	90		
8	0	100		
9		90	10	
10		80	20	
11		70	30	
12		60	40	
13		50	50	
14		40	60	
15		30	70	
16		20	80	
17		10	90	
18		0	100	
19			50	50
20			0	100

Hexane, ethyl acetate, methanol and distilled water were used in a vacuum liquid chromatography separation method to fractionate aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* on silica gel. Twenty fractions were obtained.

Thin layer chromatography (TLC)

The twenty fractions from VLC were subjected to thin layer chromatography. Fractions were spotted on activated commercial thin layer chromatography plates silica gel GF_{254} (Merck) and placed in a trough of DCM: MEOH (9:1) solvent and sprayed with conc. HCL, the distance traveled by the solvent and the compounds present were measured and the retention factor (R_f) calculated (Gujjetil and Mamidala, 2013);

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent front}}$$

Spontaneous Uterine Contraction of VLC fractions

5 mg/ml of BH VLC Fractions on spontaneous uterine contraction were studied using the method of Bafor *et al.* (2010). The concentration–response relationships was obtained with a contact time of 5 min per concentration. At the end of each experiment, the tissue was washed 3 times with PSS and left to recover. The effect of 5 mg/ml BH fractions on spontaneous uterine contractility were also evaluated, (n = 5).

Data Analyses

The results obtained were subjected to relevant statistical analyses. Data were presented as percentage of control in mean ± S.E.M (standard error of mean) of frequency (cycles/5 min) and amplitude (g) of uterine contractions. Comparisons were made using one-way repeated measures ANOVA with Dunnett's correction for multiple comparison or student's t-test where appropriate. $P \leq 0.05$ was used to indicate statistical significance. Graph pad prism 7.00 (California, USA) and Microsoft office excel 2013 were used.

RESULTS

Percentage yield of extracts

The different solvents used for extraction had effect on the percentage yield of *Garcinia kola* and *Carica papaya* roots individually and when combined. *Carica papaya* root aqueous extract had the highest yield of 33.15 % followed by aqueous extract of the bi-herbal formulation 20.41 %; and *Garcinia kola* root methanol extract had higher yield (9.83 %) compared to the bi-herbal methanol extract (5.68 %). *Garcinia kola* root lowest yield was in hexane extract (0.39 %) while *Carica papaya* lowest yield was in ethyl acetate extract (0.21 %) as shown in Table.

Phytochemistry

HPLC analysis of aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH) revealed five alkaloids; Hordenine, cytisine, methyl Jasmonate, galanthamine and an unidentified compound (Figure 4.36). Galanthamine was the most abundant alkaloid with an area of 61.9 $^{0}/_{0}$ followed by Hordenine 13.9 $^{0}/_{0}$ and the least abundant alkaloid was Cytisine 7.5 $^{0}/_{0}$ (Table 4.7).

Benzoic acid, isoquercetin, rutin, chlorogenic acid and an unidentified compound were the flavonoids identified in BH extract (Figure 4.37). The most abundant flavonoid was chlorogenic acid 37.5 $^{0}/_{0}$ followed benzoic acid 11.5 $^{0}/_{0}$ with isoquercetin 5.5 $^{0}/_{0}$ been the least abundant (Table 4.8).

Table 2: Effect of solvent on extraction and percentage yield

PLANT	SOLVENT	EXTRACT APPERANCE	% YIELD
Garcinia kola root	Distilled water	Light brown, sticky	3.38
	Methanol	Light brown, gum	9.83
	ethyl acetate	Light brown, gum	9.29
	Hexane	Light brown, oil	0.39
Carica papaya root	Distilled water	Dark brown, thick	33.15
	Methanol	Brown, slightly thick	5.38
	ethyl acetate	Brown, slightly powdery	0.21
	Hexane	Brown, oil	0.28
Garcinia kola and *Carica papaya* combination	Distilled water	Dark brown, thick	20.41
	Methanol	Brown, slightly thick	5.68

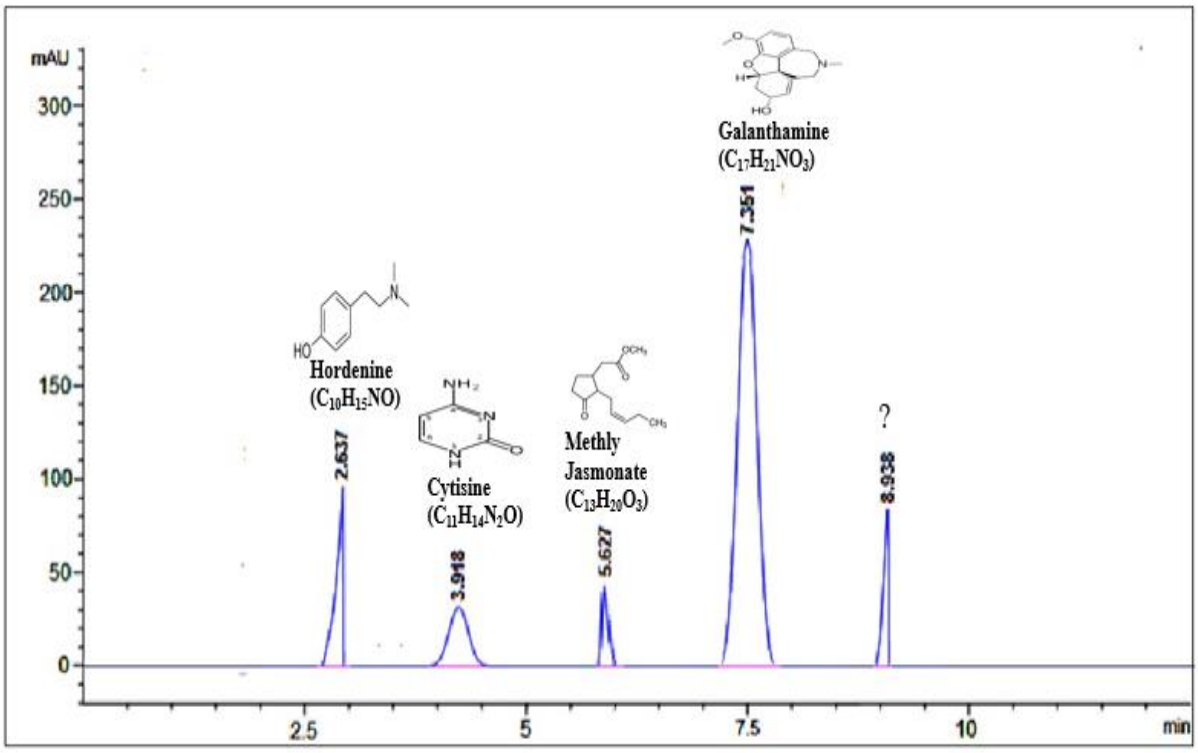


Figure 1: HPLC Chromatogram of alkaloids identified in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Table 3: HPLC identified alkaloid compounds in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Peak (#)	RetTime (min)	Width (min)	Height (mAU)	Area (%)	Compound
1	2.637	0.394	97.320	13.938	Hordenine
2	3.918	0.641	32.033	7.464	Cytisine
3	5.627	0.528	45.032	8.643	Methyl Jasmonate
4	7.351	0.742	229.571	61.920	Galanthamine
5	5 8.938	0.261	84.831	8.048	?

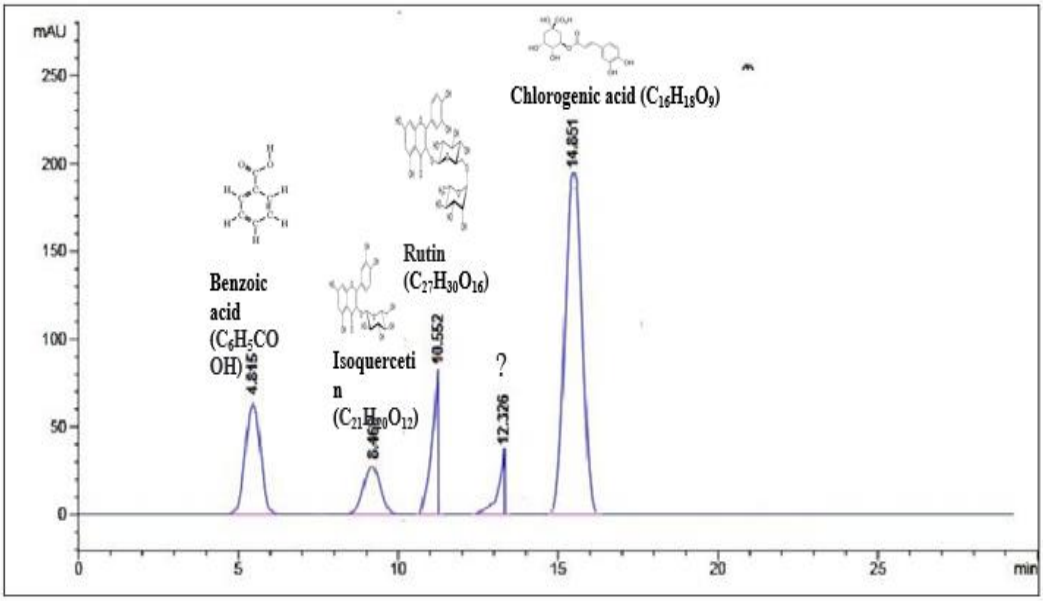


Figure 2: HPL chromatogram of flavonoids identified in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Table 4: HPLC identified flavonoid compounds in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya*

Peak (#)	RetTime (min)	Width (min)	Height (mAU)	Area (%)	Compound
1	4.815	1.479	60.004	11.465	Benzoic acid
2	8.469	1.474	28.736	5.472	Isoquercetin
3	10.552	0.871	9.563	9.563	Rutin
4	12.326	1.153	7.250	7.250	?
5	14.851	1.481	37.500	37.500	Chlorogenic acid

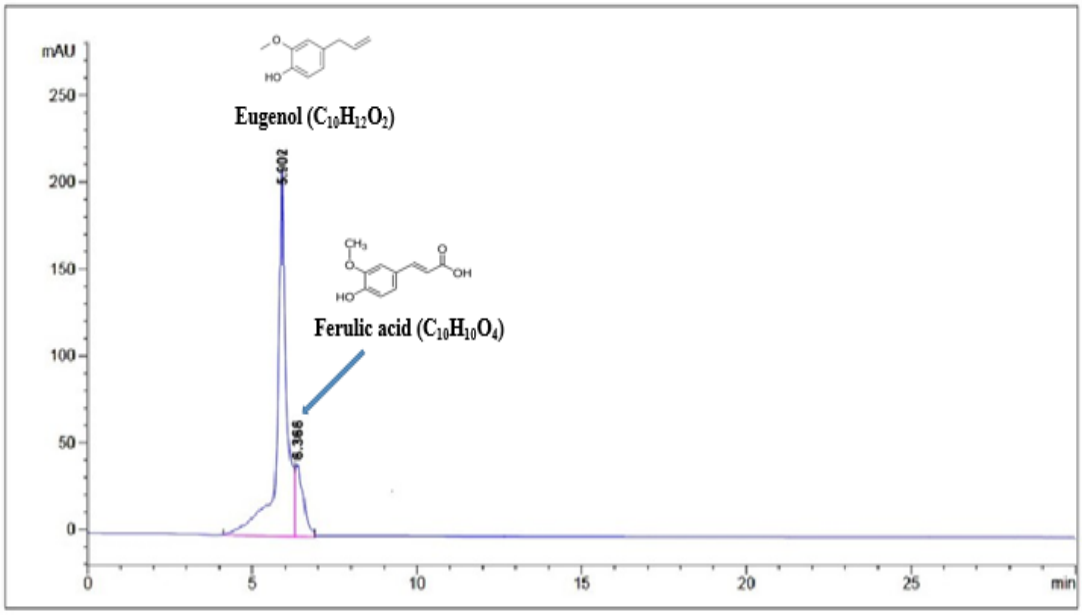


Figure 3: HPLC chromatogram of phenols identified in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Table 5: HPLC identified phenol compounds in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Peak (#)	RetTime (min)	Width (min)	Height (mAU)	Area (%)	Compound
1	5.902	0.199	208.746	85.292	Eugenol
2	6.366	0.211	41.512	14.708	Ferulic acid

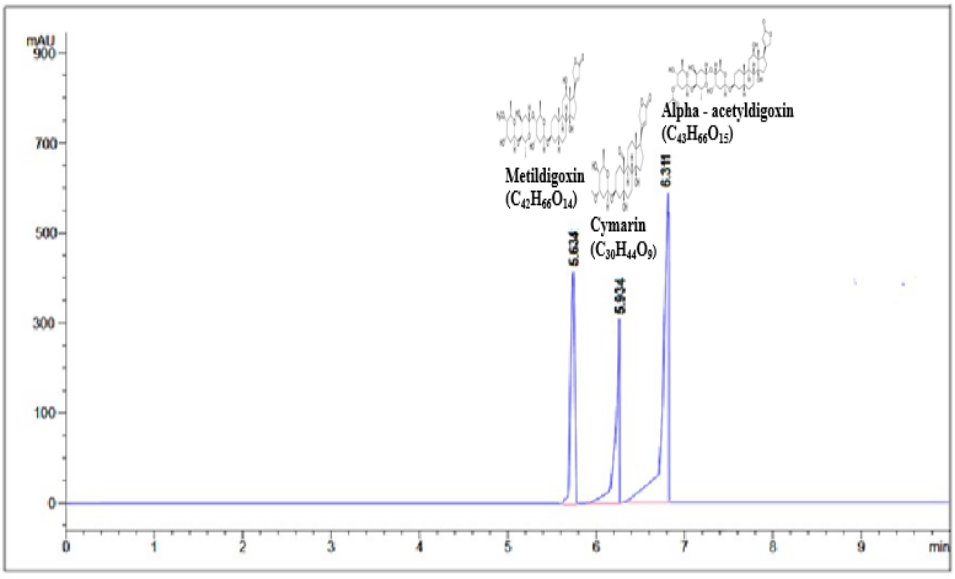


Figure 4: HPLC chromatogram of Cardiac Glycosides identified in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Table 6: HPLC identified cardiac glycoside compounds in aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

Peak (#)	RetTime (min)	Width (min)	Height (mAU)	Area (%)	Compound
1	5.634	0.087	546.095	20.6021	Metildigoxin
2	5.934	0.172	217.176	16.1795	Cymarin
3	5.934	0.250	63.339	63.3386	a-acetyldigoxin

Table 7: VLC fractions of aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH) and weight.

FRACTION CODES	BULKED FRACTIONS	WEIGHT (mg)
NF1	1	27.99
NF2	2, 3	18.00
NF3	4, 5	53.00
NF4	6, 7	53.99
NF5	8, 9	90.99
NF6	10	90.00
NF7	11	210.00
NF8	12	177.00
NF9	13	166.99
NF10	14, 15, 16	458.99
NF11	17	107.99
NF12	18, 19	1031.99
NF13	20	1486.99

Thirteen fractions (NF1 - NF13) were obtained based on TLC result. The fractions with similar migration on TLC plate were bulked.

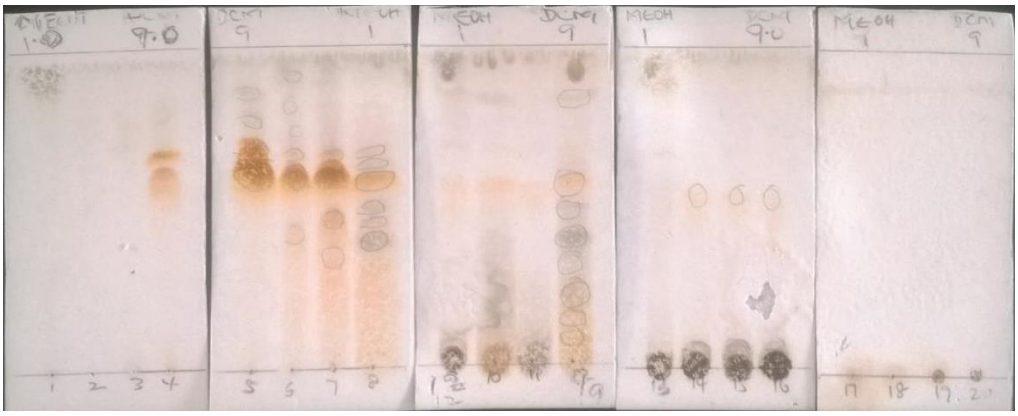


Plate 1: Thin Layer chromatography of 20 VLC fractions of aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH).

B35-46 Adsorbent: silica gel, Mobile phase: DCM–MEOH 9.0:1.0; Spray reagent: conc. H_2SO_4.

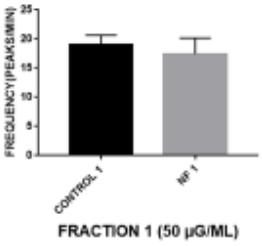
Two phenolic compounds were identified eugenol and ferulic acid (Figure 6). Eugenol with an area of 85.3 $^0/_0$ and ferulic acid 14.7 $^0/_0$ (Table 6)

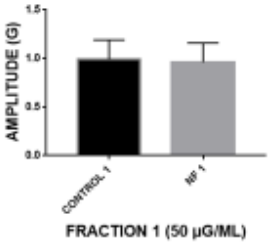
Cardiac glycoside, compounds found in BH extract were metildigoxin, cymarin and alpha-acetyldigoxin (Figure 4.39). The most abundant cardiac glycoside was alpha-acetyldigoxin with an area of 63.3 $^0/_0$ followed by metildigoxin 20.6 $^0/_0$ and cymarin 16.2 $^0/_0$ (Table 6)

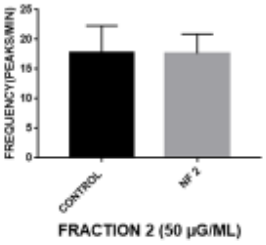
Twenty fractions were obtained by vacuum liquid chromatograph (Table 7) and were reduced to 13 fractions after thin layer chromatography identification (Plate 1)

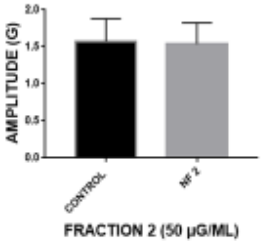
Spontaneous contraction of the 13 VLC fractions showed 2 fractions with higher amplitude and 3 with higher frequency (Figure 5), 5 fractions had lower amplitude, and 9 had lower frequency compared to control (Figure 6)

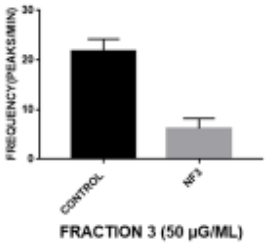
Fractions Contractility

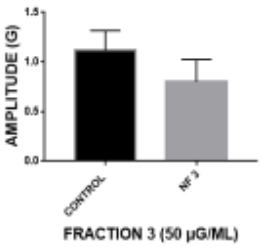


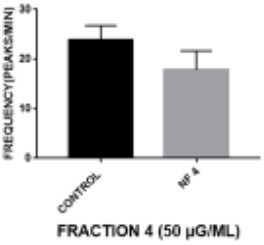


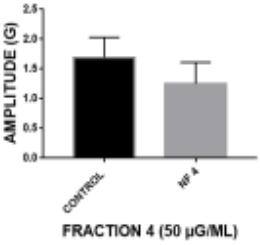


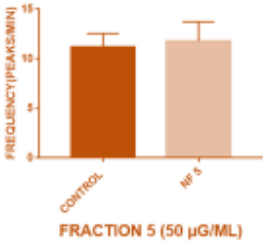


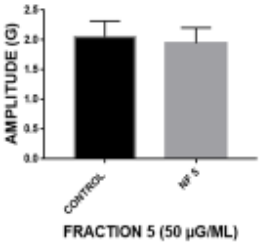


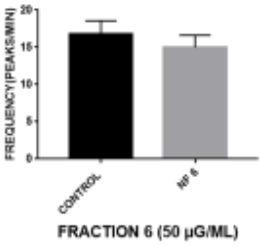


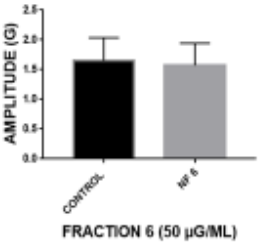


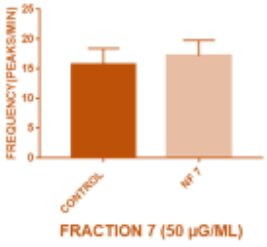


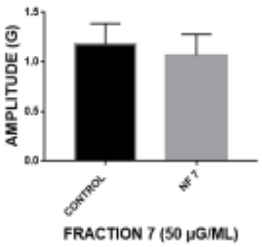


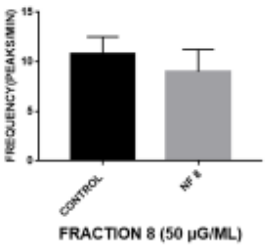


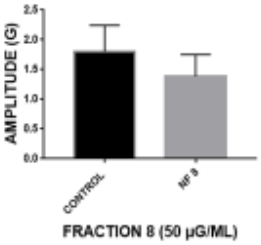












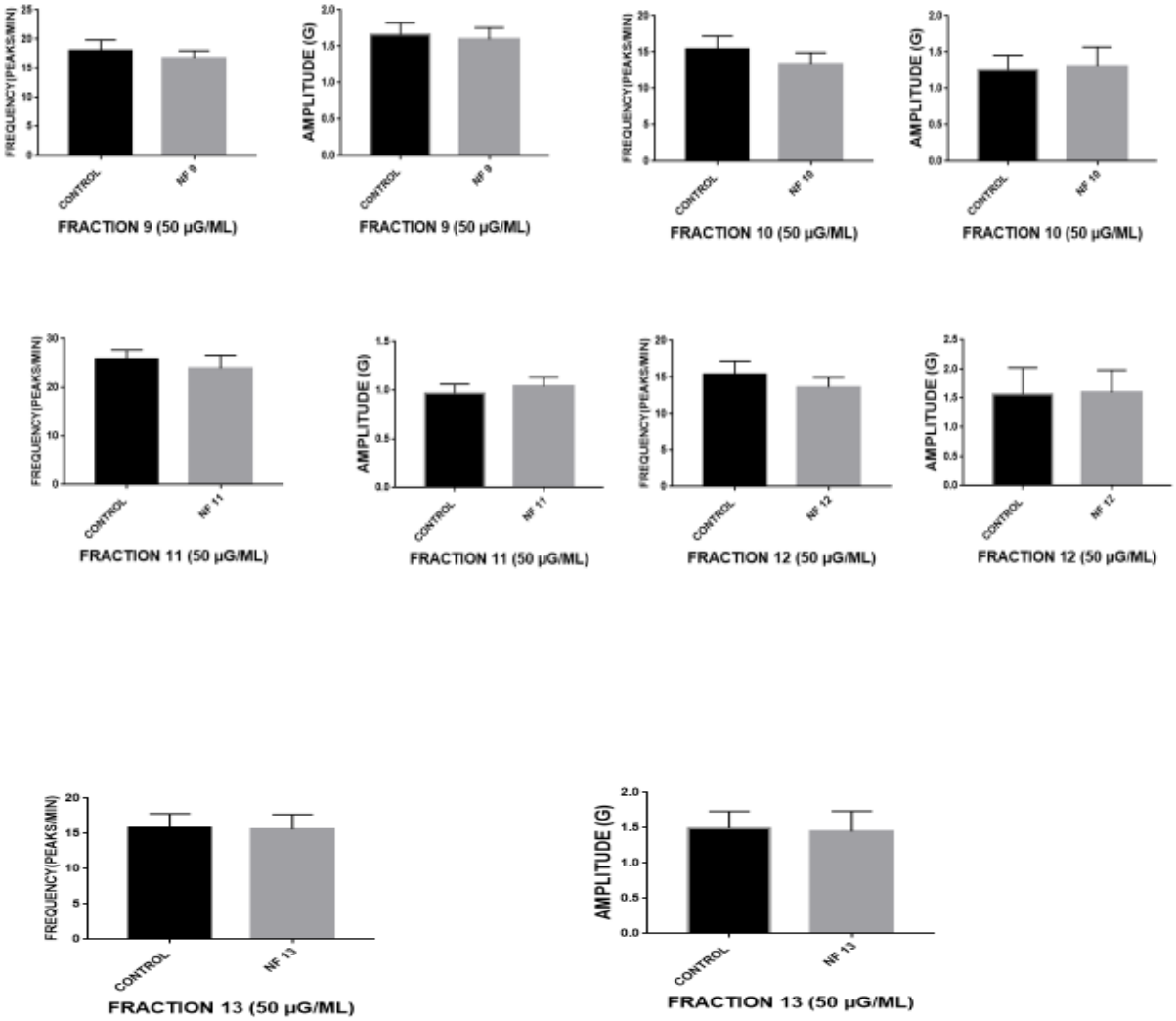


Figure 47:, shows Frequency and Amplitude of VLC 13 fractions of CA extract on virgin female mice uterus *ex vivo*.

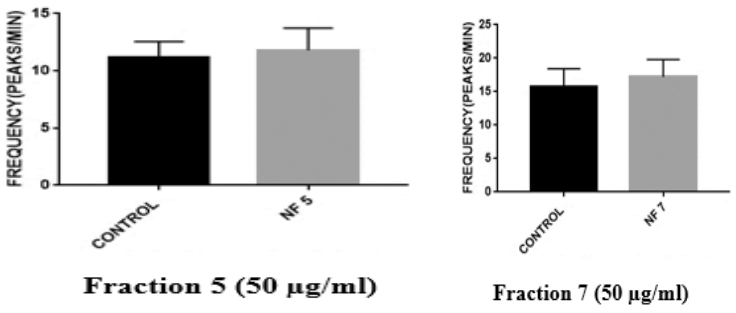


Figure 5: Aqueous bi-herbal root fractions of *Garcinia kola* and *Carica papaya* with higher uterine frequency compared to control in female mice.

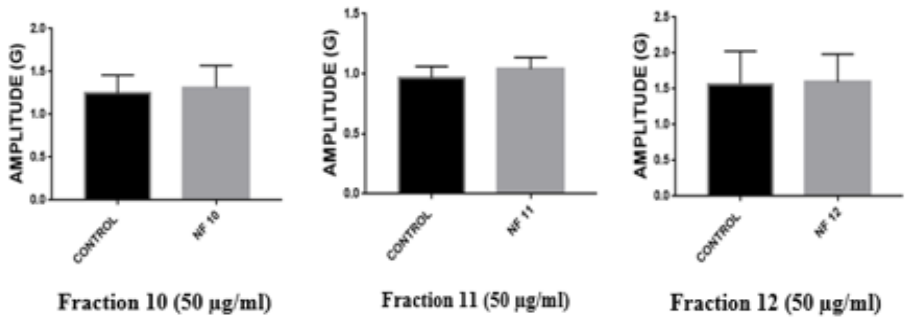


Figure 6: Aqueous bi-herbal root fractions of *Garcinia kola* and *Carica papaya* fractions with higher uterine amplitude compared to control in female mice.

DISCUSSION

Plants are endowed with bio-compounds, which are secondary metabolites with therapeutic and prophylactic properties. In this study *Carica papaya* root was observed to have the highest yield in the aqueous solvent compared to other solvents that may be due to its rich polar compounds that were soluble in water. *Garcinia kola* root recorded more yield in methanol closely followed by ethyl acetate extract, thus suggesting its bio-compounds to be soluble in solvents of less polarity compared to water. The knowledge of the extract yield is important in proper planning of experimental design, to maximize time, and fund. Similar to this result was the work done by Kanadi *et al.* (2019), that investigated the effect of five solvent of different polarity on *Carica papaya* seed and recorded the highest yield in aqueous extract followed by methanol, ethylacetate, chloroform and n-hexane. However, the aqueous extract of *Garcinia kola* and *Carica papaya* roots combination (BH extract) had a higher yield compared with the methanol extract yield of *Garcinia kola* and *Carica papaya* roots combination of 5.68 %. The solvent of extraction also had effect on the type of phytocompounds present in the extracts as observed in our preliminary result, the aqueous extract of the composite formulation contracted mice uterine tissue while, methanol extract inhibited mice uterine tissue contraction. This result corroborated with the work of Truong *et al.* (2019) that reported the differences in polarity of solvents on yield and on level of bioactivity compounds of Severinia buxifolia extracts. Ajanal *et al.* (2012) and Mahdi-Pour *et al.* (2012) reported the yield and bioactivity of any plant or plant part extract to depend on its phytoconstituents, extraction solvent and method of extraction.

A number of separation techniques are available to separate and identify secondary metabolites in different solvent systems and spray reagents (Tânia *et al.,* 2012). High performance liquid chromatography (HPLC), is a chromatographic method that makes use of differences in size, binding affinities, charge, and other properties in separation and identification of compounds with high efficiency, high speed, and high sensitivity (Zhang *et al.,* 1989; Tânia *et al*., 2012). HPLC analysis of BH extract identified the presence of alkaloids, flavonoids, phenolics and cardiac glycosides. The most abundant alkaloid was galanthamine 61.9 0 / 0 and a compound yet to be identified with a peak of 84.831 mAU and a retention time of 58.938 min was recorded. Alkaloids are basic compounds containing one or more heterocyclic nitrogen atoms, derived from amino acids, they are widely distributed in plants and are pharmacologically active. Imperialine-3β-D-glucoside and other alkaloids have been demonstrated to induce smooth muscles contractions (Liu et al., 2018). Alkaloids from the latex of *Carica papaya* have been reported by Cherian (2000) to induce uterine contractions while *Garcinia kola* seeds alkaloid was observed to inhibit uterine contractions. The identification of Flavonoids in BH extract confirmed its abundance in plants including *Carica papaya* parts and *Garcinia kola* seeds was reported by Sherwai *et al.* (2013). Antioxidant and chelating activity of flavonoids were reported by Rijke *et al.* (2006). Liu *et al.* (2018) demonstrated uterine contractions relaxing effect of flavonoids.

Herbal combinations have been reported to be effective at low doses and less toxic at high doses. However, there is a need to determine the safety of herbal plants used as medicine to avoid untold side effects. BH extract increased cumulative spontaneous contractions in mouse uterine tissue at lower concentrations and marked reduction of frequency of contraction at the 1000 mg/mL (FBC 12.21 μg/mL). BH extract increased oxytocin induced contraction, however, in the absence of calcium, frequency was reduced. An increase in amplitude of BH extract in potassium induced contraction was also recorded. These results showed BH extract causes an influx of calcium from the extracellular space into the intracellular cell through the L-type voltage operated calcium channel, mobilize calcium from the internal calcium stores of the endothelia reticulum resulting in uterine tissue contractions this is in agreement with the report of Streb *et al.* (1983) on the role of cellular calcium in tissue contractility. Depolarized myocytes membrane, opens L-type voltage operated calcium channel thereby increasing intracellular calcium ions (Sukwan *et al.,* 2014). Four ions of calcium bind to one calmodulin, forming a calcium-calmodulin

complex (CaCC). Calcium-calmodulin complex activates myosin light chain kinase, which catalysis the phosphorylation of myosin light chain generating force that contracts myometrial smooth muscles. Contraction of the uterus is important in control of bleeding in menstrual flow and after child birth. One of constituents of BH extract is *Carica papaya* root and its latex has been reported to induce uterine contraction (Odoh, 2020). Reduction in uterine contraction at higher concentration is corroborated by the findings of Adebiyi (2004). Alkaloids from the latex of *Carica papaya* have been reported by Cherian (2000) to induce uterine contractions while *Garcinia kola* seeds alkaloid was observed to inhibit uterine contractions. The identification of Flavonoids in BH extract confirmed its abundance in plants including *Carica papaya* parts and *Garcinia kola* seeds was reported by Sherwai *et al.* (2013).

CONCLUSION

In conclusion, the use of *Garcinia kola* and *Carica papaya* bi-herbal formulation by some persons in the treatment of abnormal uterine bleeding due to protracted or heavy bleeding is given scientific backing and validated by the results of this research work

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