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The bioactivity-guided fractionation of the methanol root extract of *Azanza garckeana* (Malvaceae) on isolated Wistar rat uterine smooth muscles

Alfred Chanda, Angela Gono-Bwalya and Lavina Prashar

Abstract
Background: Pregnant women in Chongwe, Zambia, traditionally use the root of *Azanza garckeana* (F.Hffm.) Exell & Hillc (Malvalceae) to induce or accelerate labour. A previous study on the plant showed that the crude root extracts possess uterotonic potential on isolated Wistar rat uterine smooth muscles. In addition, the methanol crude root extract was the most potent extract.
Aim: The study aimed to isolate by Bioactivity-guided fractionation method the compound with the significant uterotonic activity in the root of *Azanza garckeana*.
Materials and Methods: This was a laboratory-based study designed using the uterus isolated from adult non-gravid female virgin albino Wistar rats estrogenised with 0.2 mg/kg Diethylstilbestrol. The fresh plant materials (leaves and roots) were collected from Chongwe district. The methanol crude extract was prepared by continuous maceration, was concentrated to powder, and reconstituted with water. The crude extract was then partitioned with Hexane, Chloroform, Ethyl acetate, and n-butanol in the increasing order of polarity, respectively. Fractionation of the most active fraction was performed with column chromatography.
The uterine smooth muscle strips isolated from estrogenised adult non-gravid female virgin albino Wistar rats were attached to the signal transducer connected to the power lab (A.D. Instruments, UK) and computer installed with Lab tutor software. Non-cumulative doubling concentrations of fractions and sub-fractions from the *Azanza garckeana* Methanol crude extract were added one at a time to the De Jalon's physiological solution in the organ bath where the uterine smooth muscle strips were suspended. Each sample was allowed to act for 10 minutes, and the amplitude of contraction was measured. One-way ANOVA, Bonferroni post hoc tests were used to analyze data using STATA version 13. Bar charts and dose-response curves were designed using Graphpad Prism version 5.00 for Windows (San Diego, California U.S.A.).
Results: The final aqueous suspension demonstrated the highest uterotonic potency (EC_{50} = 2.49 x 10^{-3} mg/ml; 95% CI 1.19 x 10^{-3} to 5.23 x 10^{-3} mg/ml, p=0.0001), while sub-fraction pool number 2 (sub-fractions number 41 to 61) demonstrated the highest uterotonic potency (EC_{50} at 2.05 x 10^{-3} mg/ml; 95% CI 2.09 x 10^{-3} to 3.85 x 10^{-3} mg/ml, p=0.0001). Sub-fraction pool number 2 demonstrated highest uterotonic activity, and the compound was suggested to be related to the family of glycosides after High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR) analysis.
Conclusions: The study suggests the presence of a significant uterotonic phytochemical constituent in the methanol root crude extract of *Azanza garckeana*, which may be related to the family of glycosides. The study has provided scientific evidence suggesting the root of *Azanza garckeana*, a plant used traditionally for inducing or accelerating labor, possesses uterotonic activity. Further pharmacological and toxicological studies need to be undertaken on the plant.

Keywords: *Azanza garckeana*, plant root extract, uterotonic activity, EC_{50}, Bioactivity-guided isolation

1. Introduction
Pharmacological and chemical methods are used to screen plants for biological activity and isolate active phytochemical compounds from these plants. Active phytochemical compounds are then considered models or scaffold compounds for drug design or synthesis (Koparde *et al.*, 2019) [12]. Uterotonic activity is the ability of an agent to induce contractions or to increase the tonicity of uterine smooth muscles. The uterus is a thick, pear-shaped, muscular organ approximately 7 cm long and 4-5 cm wide at its widest point. The myometrium is the middle muscular layer that makes up a significant proportion of the uterine body. Smooth muscle contraction by different agonists results in a rapid increase in intracellular calcium. Intracellular calcium binds to four binding sites of calmodulin, causing a conformational change that allows the calmodulin-calcium complex to interact and activate the inactive myosin light chain kinase (MLCK).

MLCK rapidly phosphorylates the 20 kDa myosin light chain (M.L.C). The phosphorylates of the 20 kDa myosin light chain lead to conformational changes in the myosin head that causes actin activation of myosin ATPase, resulting in force generation and contraction of the muscle fibers (Goodman *et al.*, 2010) [7]. Myometrial contraction and relaxation are influenced by cell membrane receptors such as prostaglandin, Oxytocin, beta 2, muscarinic, and histamine receptors.

Different types of uterotonic herbal plants are used to induce or accelerate labor by pregnant women in sub-Sahara Africa; this is especially common in rural areas and low-income populations (Tripathi *et al*., 2012) [23]. The uterotonic activity of some of these plants has been screened using *in vivo and in vitro* animal models with positive results (Nworu *et al*., 2007; Amiera *et al*., 2014; Bello *et al*., 2010; Watcho *et al*., 2010; Lwiindi *et al*., 2015) [18, 2, 5, 25, 13].

In Zambia, pregnant women use *Azanza garckeana* (F.Hffm.) Exell & Hillc (family Malvaceae) to induce or accelerate labor (Maluma *et al*., 2017; www.theplantlist.org) [14]. A study by Chanda *et al.* (2020) [4] to screen *Azanza garckeana* crude root extracts for uterotonic activity on isolated albino Wistar rat uterine smooth muscles found the methanol crude root extract to be the most potent, followed by the hot aqueous and cold aqueous extracts, respectively.

We, therefore, set out to do the Bioactivity-guided isolation of the methanol crude root extract of *Azanza garckeana* on isolated non-gravid Wistar rat uterine smooth muscles estrogenised with 0.2 mg/kg Diethylstilbestrol. The findings of this study provide scientific evidence on the significant uterotonic constituent in the crude root extract of *Azanza garckeana*. This study provides scientific evidence on the traditional use of *Azanza garckeana* to induce or accelerate labor by pregnant women in Zambia. The bioactivity-guided fractionation of the root extract of *Azanza garckeana* yielded the major uterotonic constituent as the preliminary step in drug discovery and development. Uterotonic plants have been associated with Postpartum hemorrhage and uterine rupture in pregnant women, therefore this study also provides further information on the safety of using the plant to induce or accelerate labor and on its traditional use for other diseases during pregnancy (Ngwenya and Bulawayo, 2016) [16].

2. Methodology

2.1 Study design

This laboratory-based Experimental study was designed using non-gravid female virgin albino Wistar rats estrogenised with 0.2 mg/kg Diethylstilbestrol. Diethylstilbestrol was used on non-gravid female Wistar rats to promote the thickening of the uterine endometrial layer so that the uterus horns can be easily isolated from the pelvic cavity and so as to enable the uterine smooth muscles to contract as a unit. The rats were sacrificed, uterus horn was isolated, cut into longitudinal strips, and suspended in the 50ml organ bath (A.D. Instruments, UK). The uterine smooth muscle strips were connected to the signal transducer which was connected to the power lab (A.D. Instruments, UK) and computer installed with Lab tutor software. The baseline contractions and Oxytocin were used as negative and positive controls, respectively. The experiments were carried out in dose (concentration) triplicates on the same uterine smooth muscle tissues with tissue washings between the doses.

2.2 Data collection tools

The quantitative data were collected using the Lab-tutor software designed to collect data on the contraction amplitude produced during the experiment. In addition, the data collection tables were used to collect data on the various concentrations applied to the organ bath and to record the corresponding amplitude of contraction produced.

2.3 Materials and methods

2.3.1 *Azanza garckeana* plant collection and identification

The fresh leaves and roots of the plant were collected from the Chongwe district of Zambia (15°16'17.3' South 28°45'53.0' East) with the aid of a known local herbalist. The plant was taken for identification at the University of Zambia, and its specimen (accession number 22209) was deposited in the Herbarium (U.Z.L.).

2.3.2 *Azanza garckeana* Methanol crude root extract preparation

Azanza garckeana roots were washed clean, cut into small pieces with a Laboratory axe, shade dried for 14 days in a well-ventilated place and crushed with a blender (1.75 Litres Satin Russell hubb Blender). The crushed root materials were packed in airtight Ziploc plastics and stored in a refrigerator at four °C until required. The roots weighing 3kg were extracted using methanol solvent by continuous maceration using a magnetic stirrer for 72 hours, and then the extract was stippled and filtered with Whitman filter paper (No. 1). The Methanol crude extract was then dried in a vacuum at the temperature of 40 ºC to obtain the powder whose yield was calculated. The extract was put in Ziploc containers and stored in the refrigerator at four °C until required.

2.4 Experimental Animals (Specimen)

Animals were housed in the animal house at the University of Zambia, School of Medicine. Female Wistar rats were separated from male rats after birth as soon as they could be identified as female. The selected rats were kept in plastic cages at room temperature and on a 12 h light/dark cycle with access to pellet food and water ad libitum. The adult female virgin Wistar rats weighing from 150 to 200 g and aged from 5 to 6 months old were used as specimens for the experiment (Chanda *et al.*, 2020) [4].

2.5 Isolated Wistar rat uterine smooth muscle mounting

24 hours before the experiment, the 5 selected Wistar rats were pre-treated with 0.2 mg/kg Diethylstilbestrol. On the experiment day, the rats were sacrificed; uterus horns were dissected out, cleaned of excess fat and connective tissues, and cut into longitudinal strips of about 2cm. The uterine smooth muscle strips were suspended in the 25 ml organ bath (A.D. Instruments) using a cotton thread in the organ bath containing De Jalon's physiological solution (9 g/l of sodium chloride, 0.5 g/l Sodium hydrogen carbonate, D. Glucose, 0.402 g/l potassium chloride and 0.08 g/l hydrated calcium chloride). The suspended uterine smooth muscle strips suspended in the organ bath were maintained at 37 °C and aerated with a mixture of 95% Oxygen (O_2) in 5% Carbon dioxide (CO_2) using an aquarium air pump (Model No: 9905). The uterine smooth muscle strips were connected to the signal transducer connected to the power lab (A.D. Instruments, UK) and computer installed with Lab tutor software. The tissue tension was adjusted using the transducers to the resting uterine smooth muscle contractions of 5 mN, the force of contraction was zeroed (0 mN) using the PowerLab. The suspended uterine smooth muscle strips connected to the transducers were allowed to equilibrate for at least 30 minutes

to obtain the baseline uterus contractions before the samples were applied.

2.6 Uterotonic evaluation

2.6.1 Standard drugs used for the experiment

The drugs that were used in this study are as follows

1. **Diethylstilbestrol** (Kunj Pharma Pvt. Ltd) was used in this study to promote thickening of the adult female Wistar rat's uterine endometrial layer so that the uterus horns can be easily isolated from the pelvic cavity and so as to enable the uterine smooth muscles to contract as a unit.
2. **Oxytocin** (Mylan Health pty Ltd, Australia) was used in this study as a positive control for screening fractions and sub-fractions for uterotonic activity.

2.6.2 Exposure assessment

Non-cumulative doubling concentrations of fractions (1.60 x 10^{-4} mg, 6.40 x 10^{-4} mg, 2.56 x 10^{-3} mg, 1.02 x 10^{-2} mg, 4.10 x 10^{-2} mg, 1.16 x 10^{-1} mg and 6.55 x 10^{-1} mg) and sub-fractions (1.00 x 10^{-5} mg, 4.00 x 10^{-5} mg, 1.60 x 10^{-4} mg, 6.40 x 10^{-4} mg, 2.56 x 10^{-3} mg, 1.02 x 10^{-2} mg and 4.10 x 10^{-2} mg) of *Azanza garckeana* and Oxytocin (2.00 x 10^{-8} mg, 8.00 x 10^{-8} mg, 3.20 x 10^{-7} mg, 1.28 x 10^{-6} mg, 5.12 x 10^{-6} mg, 2.05 x 10^{-5} mg and 8.20 x 10^{-5} mg) were added one at a time to the De Jalon's physiological solution in the organ bath where the uterine smooth muscle strips were suspended. Each sample was allowed to act for 10 minutes, and the amplitude of contraction was measured. The experiment was done in dose (concentration) triplicate for each sample.

2.7 Bioactivity-guided isolation of the bioactive compound with the highest uterotonic activity in *Azanza garckeana*

The Bioactivity-guided isolation was done by separating *Azanza garckeana* phytochemical compounds according to the order of polarity using the solvent partitioning method. First, the methanol crude root extract was reconstituted with water, and it was partitioned with n-hexane, chloroform, ethyl acetate and n-butanol, respectively. After solvent partitioning, the most potent fractions were fractionated by column chromatography to obtain two plant sub-fractions, one of which contained the primary uterotonic compound (Figure 1).

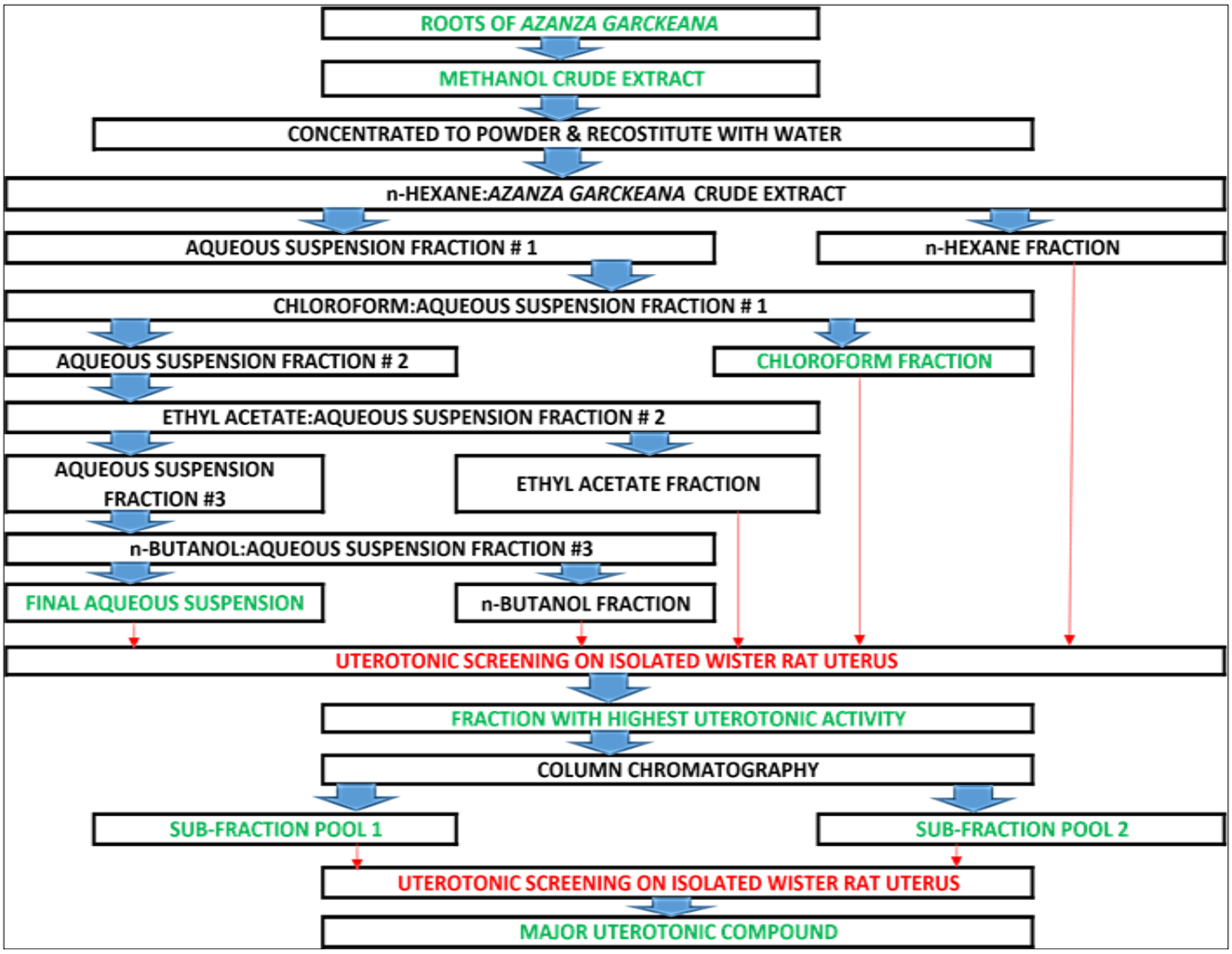


Fig 1: Stages of the Bioactivity-guided isolation process of *Azanza garckeana* methanol crude root extract on isolated Wistar rat uterine smooth muscles. The portioned solvents were agitated continuously for 2 minutes and were allowed to settle for 60 minutes to form 2 layers. The two layers of fractions were separated using a separating funnel. All fractions and sub-fractions were dried to powder before being screened for uterotonic activity. Thin Layer Chromatography (T.L.C.) was done for each sub-fraction, and sub-fractions with similar T.L.C. profiles (spots) were pooled. Sub-fraction pool one was obtained by pooling sub-fraction numbers 4 to 12 and 70 to 82, while Sub-fraction pool 2 resulted from the pooling of sub-fraction numbers 41 to 61.

2.7.1 Solvent partitioning fractionation

The powder of the methanol crude root extract was reconstituted with distilled water and was partitioned with organic solvents in the increasing order of polarity using a separating funnel. The crude extract with the highest activity was partitioned three times with 200 ml of hexane,

chloroform, ethyl acetate, and n-butanol (HIMedia Laboratories Pvt. Ltd, India) in the increasing order of polarity to help with the fractionation of *Azanza garckeana* phytochemical compound as stated in Satyajit *et al.* (2006) [20] and Sudha & Srinivasan (2014) [22].

2.7.2 Preparation of the hexane fraction
In order to separate nonpolar phytochemical compounds from the crude extract, the extract was reconstituted with 200 ml of distilled water, partitioned with hexane (3 x 200 ml), and was agitated continuously for 2 minutes and was allowed to settle for 60 minutes to form 2 layers. All nonpolar compounds, such as lipids and chlorophyll, were in the hexane fraction (layer). This process is sometimes referred to as "defatting". Next, the hexane fraction was dried under vacuum to powder and was screened for uterotonic activity.

2.7.3 Preparation of the chloroform fraction
The remaining aqueous suspension layer was partitioned three times with 200 ml chloroform, which was carried out by agitating the mixture continuously for 2 minutes. The mixture was allowed to settle for 60minutes to form 2 layers. More minor polar phytochemical compounds were in the chloroform fraction, dried to a powder and then screened for uterotonic activity. The more polar compounds were in the remaining second aqueous suspension layer, which was carried forward for the following procedure.

2.7.4 Preparation of the ethyl acetate fraction
The second aqueous suspension layer from the previous procedure was partitioned three times with 200 ml ethyl acetate. Partitioning was done by continuously agitating the mixture for 2 minutes and allowing it to settle for 60 minutes to give two layers. More minor polar phytochemical compounds were in the ethyl acetate fraction, dried to a powder and then screened for uterotonic activity. The remaining third aqueous suspension layer was carried forward for the following procedure.

2.7.5 Preparation of the n-butanol fraction
The third aqueous suspension layer from the previous procedure was partitioned three times with 200 ml n-butanol. Partitioning was done by agitating the mixture continuously for 2 minutes, after which it was allowed to settle for 60minutes to form 2 layers. More polar phytochemical compounds were in the n-butanol fraction. The fraction was dried to a powder and was then screened for uterotonic activity. The remaining final aqueous suspension layer, which contained the most polar phytochemical compounds in *Azanza garckeana*, was dried to a powder and was then screened for uterotonic activity.

2.8 Silica gel filtration of *Azanza garckeana* fractions
The potent fractions of *Azanza garckeana* were further fractionated by silica gel column chromatography (silica gel: HIMedia Laboratories Pvt. Ltd, India) to obtain sub-fractions. The Thin Layer Chromatography (T.L.C.) was done for each sub-fraction, and the sub-fractions with similar T.L.C. profiles (spots) were pooled.

2.9 Thin Layer Chromatography (T.L.C.)
The Thin Layer Chromatography was conducted on a glass sheet coated with a thin layer of adsorbent material, which, in this case, was silica gel impregnated with a fluorescent material. Each component on the T.L.C. appeared as spots separated vertically, and each had a retention factor (R_f).

2.10 Analysis of the compound with the highest uterotonic activity
The isolated primary uterotonic phytochemical compound was analyzed using High-Performance Liquid Chromatography (Shimadzu, Japan). Ultraviolet (U.V.), wavelength 220nm was used as a detector for High-Performance Liquid Chromatography (HPLC), the mobile phase used was Acetonitrile: Water (28:72) and the column used was C18 2.7 µm, 3.0x100 mm (Cortecs part number 1860007372). The HPLC oven temperature was at 20 °C and the flow rate used was 0.03 ml/min. The isolated compound was also analyzed using Fourier Transform Infrared Spectroscopy (Shimadzu, Japan) to identify the functional groups present on the compound. The wavenumbers (1/cm) obtained from the Fourier Transform Infrared Spectroscopy (FTIR) spectra were compared to the standard wavenumbers to help identify the suggested present functional groups on the isolated compound (Socrates, 2004; Ognyanov *et al*., 2018) [21, 19].

2.11 Display of data
This study displayed data using tables, dose-response curves (concentration vs amplitude of contraction) and figures. In addition, all data were presented as mean±standard error of the mean (S.E.M.), 5% level of significance, and 95% confidence interval.

2.12 Data analysis
The independent variable was the milligram concentration per millilitre (mg/ml), while the dependent variable was the amplitude of contractions in millinewtons (mN). To determine the differences in uterotonic activity within the various concentrations, one-way ANOVA was used. Bonferroni post hoc test was used to test at which concentration significant contractions were observed. STATA version 13 and Graphpad Prism version 5.00 for Windows (San Diego, California U.S.A.) were used to analyze data. Origin software was used for FT-IR spectra. 5% level of statistically significant and 95% confidence interval were used.

2.13 Ethical considerations
The animals were treated humanely and were given access to standard nutrition, water and the environment. The animals were sacrificed by cervical dislocation before the isolation of the uterus.

The study was conducted according to the guidelines for the design and statistical analysis of experiments using laboratory animals (Festing & Altaman, 2018; Council for International Organization of Medical Sciences and the International Council for Laboratory animal science, 2012) [6]. Furthermore, approval was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) before the study was conducted (REF. No. 006-12-18).

3.1 Description of the *Azanza garckeana* methanol extracts
The crude root methanol extract of *Azanza garckeana* was dark brownish-black in colour with a percentage yield of 6.51% the finding which was corresponding to a similar study where the methanol crude root extract had the percentage yield of 6.26% but was lower than the hot aqueous (22.26%) and cold aqueous (11.32%) extracts (Chanda *et al.*, 2020) [4]. Alawode *et al.* (2020) [3] looked at the effect of extraction solvents on the phytochemical yield of the pulp and shaft of the plant and reported the methanol extract to have the highest percentage yield. The variations in the percentage yield obtained in the two studies could be due to the Differences in

the geographical location from which the plants were collected and the parts used during the extraction method.

3.2 Solvent partitioning of the Methanol crude root extract of *Azanza garckeana*

The methanol crude root extract was selected for solvent partitioning because a previous similar study demonstrated it to be the most potent extract (EC_{50} = 1.28 x 10^{-2} mg/ml) as compared to the hot (EC_{50} =0.02792 mg/ml) and cold (EC_{50} =0.4884 mg/ml) aqueous extracts. These findings from the previous study suggested that the Methanol crude root extract may contain the highest concentration of the phytochemical compound with the major uterotonic activity (Chanda *et al.*, 2020) [4]. The dose-response curve for oxytocin gave the best fit sigmoidal slope. The EC_{50} was at 1.17 x 10^{-7} mg/ml with the confidence interval of 3.18 x 10^{-8} to 4.32 x 10^{-7} mg/ml (p-value = 0.0001) and the E_{max} was at 4.10 x 10^{-5} mg/ml. The EC_{50} for Oxytocin was similar to that of Amiera *et al.*, 2014 [2] (EC50, 0.060 ± 0.011 µg/ml) and Watcho *et al.*, 2010 [25] (EC_{50}, 0.02 nM). The amplitude of uterine smooth muscle contraction for Oxytocin increased linearly with an increase in concentration and reached a plateau at 1.28 x 10^{-6} mg/ml.

3.3 Uterotonic activity of *Azanza garckeana* fractions

The amplitudes of contractions produced by the Hexane fraction (p= 0.2045), ethyl acetate fraction (p=0.2341) and n-Butanol fraction (p=0.5847) were not statistically significant. The amplitude of contraction produced by the chloroform fraction (p = 0.0001) and final aqueous fractions (p <0.0001) were statistically significant. The final aqueous fraction was not only more potent (EC_{50} = 2.56 x 10^{-3} mg/ml; 95% CI 1.19 x 10^{-3} to 5.23 x 10^{-3} mg/ml, p<0.0001) than the chloroform fraction (EC_{50} = 1.09 x 10^{-2} mg/ml; 95% CI 4.517 x 10^{-3} to 2.640 x 10^{-2} mg/ml, p=0.0001), but it was also more efficacious. The maximum amplitude of contraction produced by the fractions were 12.88 ± 0.52**mN for chloroform and 21.14 ± 0.15**mN for the final aqueous fraction, respectively (Figure 2).

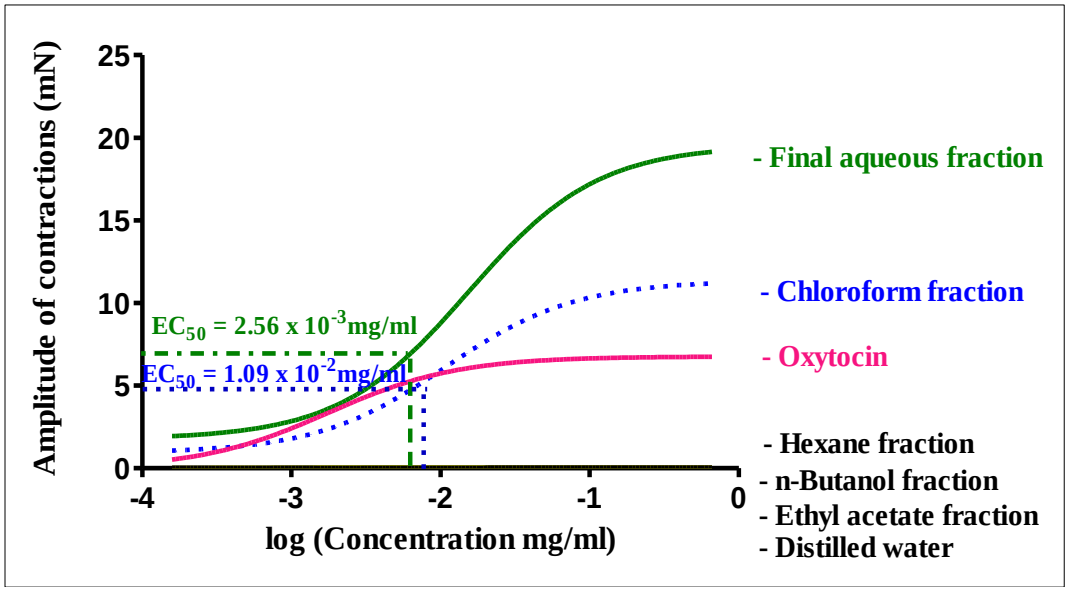


Fig 2: Concentration-dependent contraction of the uterine smooth muscle strips to 5 *Azanza garckeana* fractions. Points represent means _ S.E.M. for the number of experiments (n=3). Distilled water, negative control; Oxytocin, positive control.

The activity of the Chloroform fraction increased linearly at 2.56 x 10^{-3} mg/ml and reached a plateau after 4.10 x 10^{-2} mg/ml. The activity of the final aqueous fraction increased linearly after 6.40 x 10^{-4} mg/ml and reached the plateau after 4.10 x 10^{-2} mg/ml. The t-test analysis of the potency for each of the *Azanza garckeana* fractions with that of Oxytocin also showed that the potency of the fractions was different from that of Oxytocin (Chloroform fraction EC_{50} 1.09 x 10^{-2} mg/ml, 95% CI 9.42 x 10^{-4} to 1.24 x 10^{-2} mg/ml, p<0.0001; Final aqueous fraction EC_{50} 2.56 x 10^{-3} mg/ml 95% CI 2.30 x 10^{-3} to 2.68 x 10^{-3} mg/ml, p<0.0001).

The uterotonic verification of five fractions from the fractionation of the methanol crude extracts of *Azanza garckeana* showed that only the chloroform soluble fraction and the final aqueous suspension fraction possessed significant uterotonic activity. The final aqueous suspension was more potent and efficacious than the chloroform fraction. The higher potency of the final aqueous suspension suggested that the compound with the highest uterotonic activity might have been in this fraction. When organic solvent partition fractionation is performed, the final aqueous fraction contains glycosides with long sugar chains. Hence the compound with the highest uterotonic activity was thought to be a glycoside which has also been previously isolated from *Azanza garckeana* (Satyajit *et al.*, 2006) [20]. The final aqueous suspension demonstrated less potent activity than the standard uterotonic drug Oxytocin, but it exhibited higher contraction amplitudes at both the Emax and EC_{50} concentrations. The higher contraction amplitude produced by the final aqueous suspension fraction of *Azanza garckeana* suggested that the compound with the highest uterotonic activity may not have been lost during the solvent partitioning fractionation process.

The other fractions obtained from the methanol extract of *Azanza garckeana* were hexane, chloroform, ethyl acetate, and butanol fractions, respectively. The n-hexane fraction may have contained nonpolar compounds, such as lipids and chlorophylls. The process of removing fats and other nonpolar compounds from a plant extract using n-hexane is sometimes referred to as defatting. Defatting resulted in an aqueous suspension, clear of nonpolar compounds. This suspension was inactive as far as the uterotonic activities were concerned. More minor polar compounds were present in the chloroform soluble fraction. Polar compounds probably up to monoglycosides were present in the ethyl acetate soluble fraction. The n-butanol soluble fraction contained polar compounds, which were mainly glycosides. The final aqueous

suspension fraction contained polar glycosides with polysaccharides (Visht & Chaturvedi, 2012; Satyajit *et al.*, 2006) [24, 20].

3.4 Silica gel filtration of *Azanza garckeana* fractions

Thin Layer Chromatography (T.L.C.) of the methanol extract of *Azanza garckeana* yielded three (3) spots suggesting three herbal phytochemical compounds in the plant. The T.L.C. results are in line with the findings of Nkafamiya *et al.* (2015) [17], who, in their phytochemical screening of the root crude extract of *Azanza garckeana* using chemical methods, found that the plant contained Alkaloids, Phenols and Saponins (Nkafamiya *et al.*, 2015) [17]. However, it must be noted that although comparisons have been made to other studies, the T.L.C. spots yield depend on factors such as the extract, mobile and stationary phases and the concentration of the sample applied to the T.L.C. plate.

The amplitudes of contractions produced by the sub-fraction pool number 1 were significant (F =20.29, p <0.0001). The activities were significantly observed at 2.56 x 10^{-3}mg/ml (p<0.0001). The sub-fraction had the EC_{50} at 1.47 x 10^{-2} mg/ml (95% CI 5.59 x 10^{-3} to 3.88 x 10^{-2} mg/ml) and the Emax (3.11 ± 0.31**mN) at 4.10 x 10^{-2} mg/ml. The amplitudes of contractions (mN) produced by the sub-fraction pool number 2 were significant (p<0.0001). The activities were significantly observed at 1.60 x 10^{-4}mg/ml (p<0.0001). The pool had the EC_{50} at 2.05 x 10^{-3}mg/ml (95% CI 2.09 x 10^{-3} to 3.85 x 10^{-3} mg/ml, p=0.0001) and the Emax (9.46 ± 0.59**mN) at 4.10 x 10^{-2} mg/ml. The pool of sub-fractions 41 to 61 which was named sub-fraction pool number 2 was more potent than sub-fraction pool number 1 (pool of sub-fraction 4 to 12 and 70 to 82), but it was also more efficacious. Both pools of sub-fractions were less potent but more efficacious than the standard uterotonic drug Oxytocin (Figure 3).

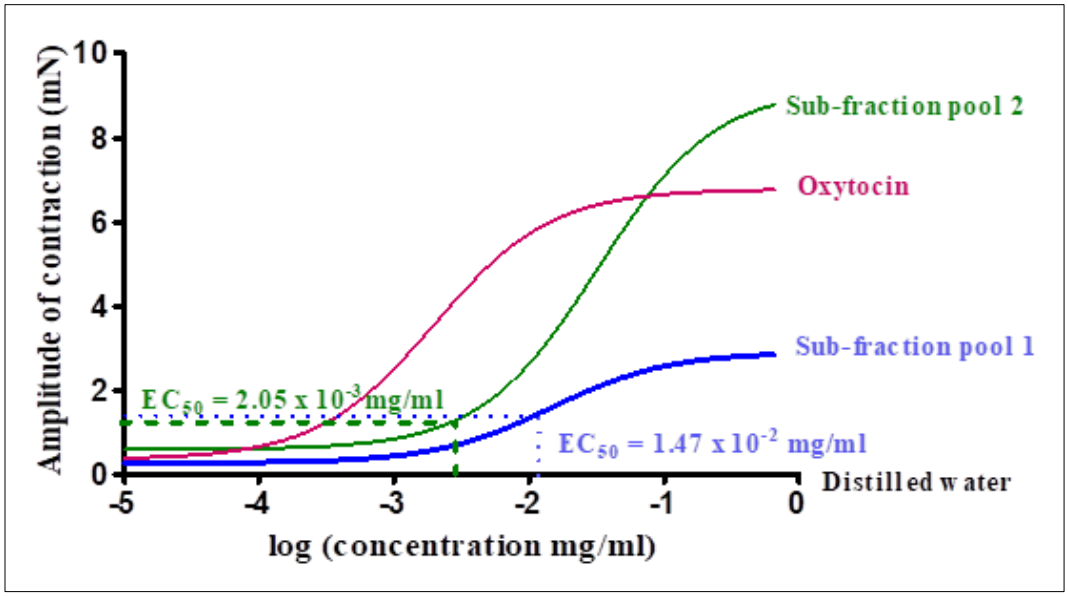


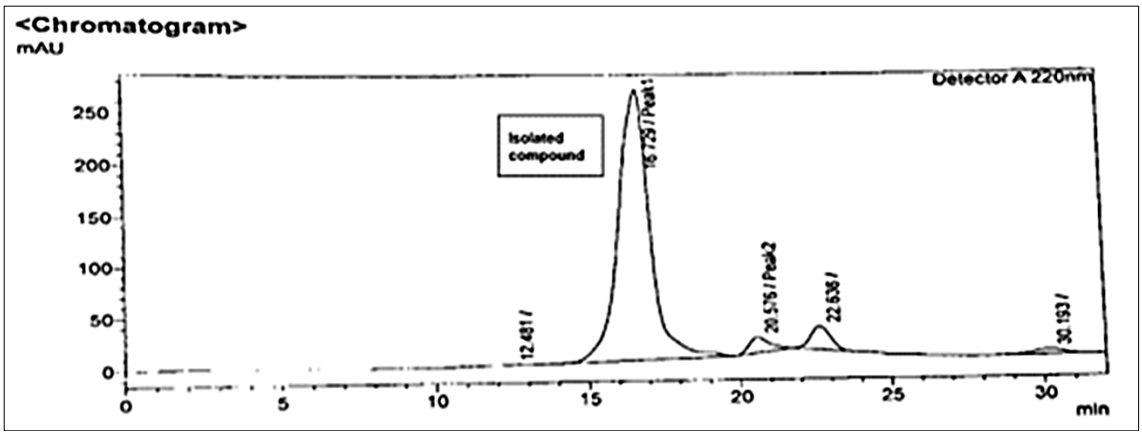
Fig 3: Concentration-dependent contraction of the uterine smooth muscle strips to sub-fraction pools 1 and 2. Points represent means _ S.E.M. for the number of experiments (n=3). Distilled water, negative control; Oxytocin, positive control.

3.5 Analysis of the phytochemical compound with the highest uterotonic activity

3.5.1 High-Performance Liquid Chromatography of the isolated compound

The compound with the highest activity was isolated and sub-fraction pool number 2 (sub-fractions number 41 to 61). The final aqueous suspension, which demonstrated the highest activity, was suggested from literature to be a polar glycoside with polysaccharides (Visht & Chaturvedi, 2012; Satyajit *et al.*, 2006) [24, 20].

The compound was further assessed using the glycoside drug digoxin as the UV-HPLC standard. The UV-HPLC chromatogram for both the isolated compound and the standard glycoside yielded similar peaks with retention times of 16.729 minutes and 16.695 minutes, respectively (Figure 4).



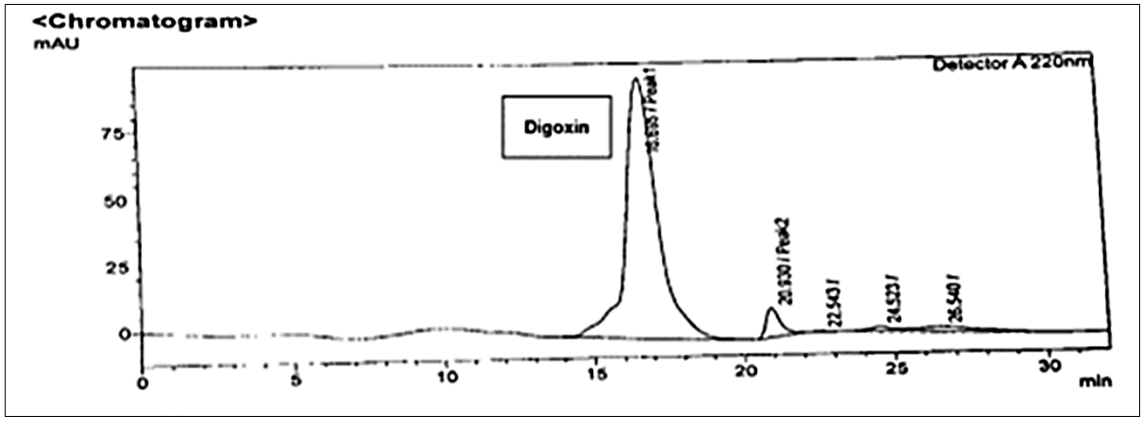


Fig 4: HPLC chromatogram for the isolated compound and Digoxin the standard glycoside.

3.5.2 Fourier Transform Infrared Spectroscopy (FTIR) of the isolated compound

The functional groups of the isolated compound were identified by comparing the obtained wavenumbers to the standard FTIR wavenumber ranges. The FTIR spectra of the isolated compound had a similar FTIR spectrum with pectin, as suggested by the FTIR feedback.

The Fourier Transform Infrared Spectroscopy (FTIR) spectra of the isolated compound with the highest uterotonic activity from *Azanza garckeana* showed the wavenumber at 3400 1/cm, 2900 1/cm, 1600 1/cm, 1400 1/cm, 1025 1/cm, 800 1/cm, 600 1/cm and 425 1/cm, which might be due to the O.H., -C.H.O., C.O.O. −, COO−/−OH, C-O-C), aromatic ring, C-H bending/Polygalacturonic acid and C-O-C torsion deformation in methyl polygalacturonate, respectively (Figure 5).

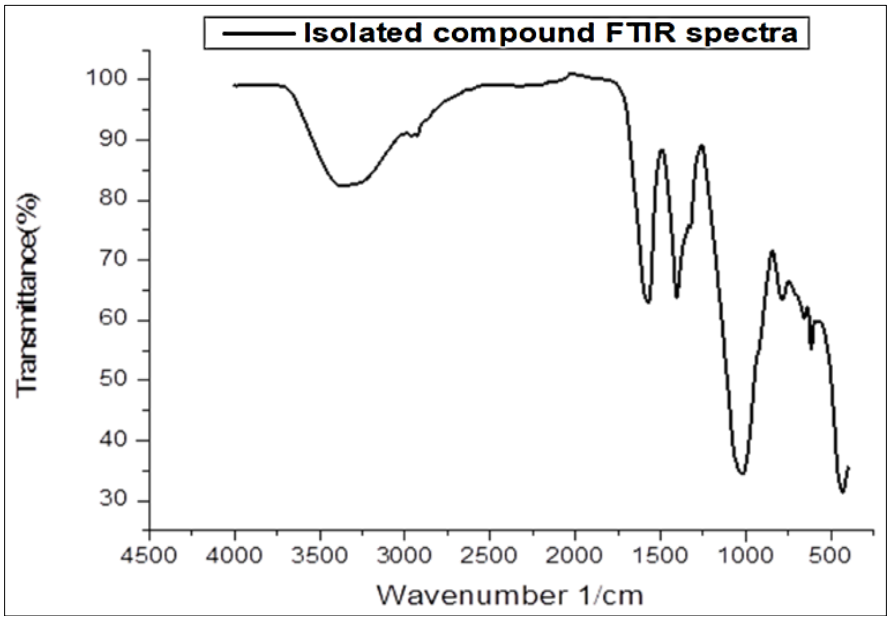


Fig 5: FTIR spectra for the isolated compound

The FTIR spectra for the significant isolated uterotonic compound in *Azanza garckeana* was similar to pectin, a structural hetero-polysaccharide hydrocolloid found mainly in fruit and not in the roots of plants. Pectin is contained mainly in the primary cell walls of many plants and has previously been reported to possess various pharmacological activities (Ognyanov *et al.*, 2018; Joel *et al.*, 2018) [19, 10]. Pectin isolated from the fruits of *Azanza garckeana* by Joel *et al.* (2018) [10] had the FTIR spectra similar to the isolated uterotonic compound in this study. The FTIR spectra done on pectin by Joel *et al.* (2018) [10] showed the broadband at 3415.19 cm-1 which could be due to S.P. hybridized C-H stretching vibration, 2935.40 cm-1 may probably be due to the SP3 hybridized C-H, 2380.32 cm-1 is likely to be C=C stretching vibration and the absorption band at 1639.02 cm-1 through 1737.56 cm-1 may probably be due to the C=O stretching vibration mode. 1533.85 cm-1 absorption band is due to the presence of N-H bending motion of the amine, 1380.87 cm-1 through 1460.29 cm-1 is due to SP3 hybridized CH2 of methylene bridge, 1059.13cm-1 through 1246.46 cm-1 is likely due to the C-O, and 769.51 cm-1 is due to the SP2 hybridized C-H bending vibrations. Pectin naturally occurs with various substituents such as ferulic acid, a compound previously isolated from *Sida acuta* (Malvaceae). *Sida acuta* is a member of the Malvaceae family, a family from which the plant *Azanza garckeana* belong. Abat *et al.* (2017) [1] reported that Ferulic acid is an active substituent that possesses other pharmacological activities such as anti-ageing and Antidiabetic activities (Abat *et al.*, 2017) [1].

Carboxamine group is an essential substituent for the activity of uterotonic drugs such as Oxytocin and Ergometrine. However, ferulic acid does not contain the carboxamine substituent group in its structure, assuming that the isolated compound with the highest uterotonic activity may be pectin with the amidated ferulic acid substituent N-Feruloyltyramine, which has the uterotonic Carboxamine

group in its structure. The N-Feruloyltyramine substituent is synthesized by the plant cell wall to resist or fight infection and may be attached to pectin in the plant's cell wall.

The High Liquid chromatography (HPLC) analysis of the phytochemical constituent with the highest uterotonic activity suggests that it was structurally related to a glycoside called Digoxin which does not possess uterotonic activity. In their study, Guo *et al*. (2008) [9] suggested that Pennogenin glycosides isolated from *Paris polyphylla* possess uterotonic activity. Pennogenin glycosides and Digoxin have similar structures, suggesting that the significant uterotonic phytochemical compound in *Azanza garckeana* might be related to Pennogenin glycoside, but only complete compound structure elucidation can ascertain this assumption.

4. Conclusion

The study showed that *Azanza garckeana* possesses uterotonic activity when evaluated using isolated Wistar rat uterine smooth muscles.

The study indicated the principal uterotonic phytochemical constituent in the methanol crude root extract of *Azanza garckeana* suggested to be related to the family of glycosides. It also provides scientific evidence that the root of *Azanza garckeana*, a plant used traditionally for inducing or accelerating labour, possess uterotonic activity.

Further Pharmacological studies need to be done on the plant to confirm the phytochemical constituents responsible for the uterotonic effect of *Azanza garckeana*. The pharmacological studies should include identifying all the phytochemical compounds responsible for the uterotonic activity of the plant and the structure-activity relationships of all these phytochemical compounds.

5. Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

6. Conflict of interest

All authors declare no potential conflict of interest.

7. Authors' contributions

Alfred Chanda developed a proposal, collected data, analyzed data and prepared a manuscript. Angela Gono-Bwalya provided scientific guidance and reviewed the Manuscript, and Lavina Prashar provided technical support, scientific guidance, review of the proposal and Manuscript.

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9. Supplementary materials

Table 1: The amplitude of contraction produced by non-cumulative concentrations of fractions obtained from *Azanza garckeana* Methanol crude extract. (*Supplementary materials*)

Table 2: *Th*e amplitude of contraction produced by non-cumulative concentrations of sub-fractions obtained from *Azanza garckeana* Methanol crude extract. *(Supplementary materials*)

Table 3: The Fourier Transform Infrared Spectroscopy (FTIR) spectra of the isolated compound with the major uterotonic activity from the *Azanza garckeana* Methanol crude extract. (*Supplementary materials*)

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