ORIGINAL ARTICLE

The phytopharmacological evaluation of the methanol root extract of *Azanza Garckeana* (Malvaceae) on isolated Wistar rat uterine smooth muscles

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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 the methanol crude root extracts to possesses the highest uterotonic potential.

Aim: To evaluate the phytopharmacological activity of the Methanol root extract of *Azanza garckeana*.

Methods: This was a laboratory-based study designed using the uterus isolated from estrogenised adult non-gravid female virgin Wistar rats. The methanol crude extract was obtained by continuous maceration. The crude was partitioned using hexane, chloroform, ethyl acetate and n-butanol in the increasing order of polarity. The most active fraction was fractionated using silica gel column chromatography . Fourier Transform Infrared Spectroscopy and High-Performance Liquid Chromatography were used to identify the major uterotonic compound. One-way ANOVA, Bonferroni post hoc tests were used to analyze data using STATA version 13. Bar charts and dose-response curves were done using Graphpad Prism version 5.00 for Windows (San Diego California USA).

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.

Conclusions: The study suggests the presence of a major uterotonic phytochemical constituent in the methanol root crude extract of *Azanza garckeana,* which was indicated to be related to the family of glycosides. Further pharmacological and toxicological studies need to be undertaken on the plant.

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Key words: *Azanza garckeana*, plant root extract, uterotonic activity, EC_{50}, bioactivity-guided fractionation

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INTRODUCTION

Herbal plant extracts have been suggested to induce uterine smooth muscle contractions. The screening of these plants for uterotonic properties have been done in different countries using animal models both *in vivo* and *in vitro* [2, 5, 12, 13, 17, 24]. Uterotonic activity is the ability of an agent to induce contractions or to increase the tonicity of uterine smooth muscles [7].

Different types of uterotonic herbal plants are used to induce or accelerate labour by pregnant women in sub-Sahara Africa; this is especially common in rural areas and low-income populations [22]. The uterotonic activity of some of these plants have been screened using *in vivo and in vitro* animal models with positive results. Few studies have been done to isolate uterotonically active phytochemical compounds from these plants [8].

In Zambia, pregnant women use *Azanza garckeana* (F.Hffm.) Exell & Hillc (family Malvalceae) to induce or accelerate labour [14, 25]. A study done in 2020, to screen *Azanza garckeana* crude root extracts for uterotonic activity on isolated 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 [4].

Therefore, this study aimed to perform a phytopharmacological evaluation of the methanol crude root extract of *Azanza garckeana* on isolated non-gravid estrogenised Wistar rat uterine smooth muscles.

METHODOLOGY

Study design

This laboratory based Experimental study was designed using non-gravid estrogenised female virgin Wistar rats. Baseline contractions and Oxytocin were used as negative and positive controls, respectively. The experiments were carried out in triplicates.

Materials and methods

Azanza garckeana plant collection and identification

The fresh leaves and roots of the plant were collected from 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 of 22209) was deposited in the Herbarium (UZL).

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 (1.75Litres Satin Russell hubb Blender). The crushed root materials were packed in airtight Ziploc plastics and stored in a refrigerator at 4 °C until required. The roots weighing 3kg were extracted using methanol solvent by continuous maceration using a magnetic stirrer for 72hours hours, and then the extract was stippled and filtered with Whitman filter paper (No. 1). The Methanol crude extract was then dried in the vacuum at the temperature of 40ºC to obtain the powder whose yield was calculated. The extract was put in Ziploc containers and stored in the refrigerator at 4 °C until required.

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 200g and aged from 5 to 6 months old were used as specimens for the experiment [4].

Isolated Wistar rat uterine smooth muscle mounting

24hours before the experiment, the Wistar rats were pre-treated with 0.2mg/kg Diethylstilbestrol. On the day of the experiment, the rats were sacrificed by cervical dislocation, 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 25ml organ bath (AD Instruments) using a cotton thread in the organ bath containing De Jalon's physiological solution (9g/l of sodium chloride, 0.5g/l Sodium hydrogen carbonate, D. Glucose, 0.402g/l potassium chloride and 0.08g/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 which was connected to the power lab (AD Instruments) and computer installed with Lab tutor software. The tissue tension was adjusted using the transducers to the resting uterine smooth muscle contractions of 5mN, and then the force of contraction was zeroed (0mN) using the PowerLab. The suspended uterine smooth muscle strips connected to the transducers were allowed to equilibrate for at least 30 minutes.

Uterotonic evaluation

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 promotes thickening of the adult female Wistar rat's uterine endometrial layer so that the uterus horns can be easily isolated from the pelvic cavity.

2. Oxytocin (Mylan Health pty Ltd, Australia) was used in this study as a positive control for the screening of fractions and sub-fractions for uterotonic activity.

Exposure assessment

Non-cumulative doubling concentrations of fractions and sub-fractions of *Azanza garckeana* and standard drugs 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 triplicate (n=3) for each sample.

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

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 was partitioned 3 times with 200ml of hexane, chloroform, ethyl acetate, and n-butanol (HIMedia Laboratories pvt. Ltd, India) in the increasing order of polarity [19, 21]. The stages of the bioactivity-guided fractionation of *Azanza garckeana* methanol crude root extract on isolated Wistar rat uterine smooth muscles is summarized in Figure 1.

Preparation of the hexane fraction

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

Preparation of the chloroform fraction

The remaining aqueous suspension layer was partitioned 3 times with 200ml chloroform which was carried out by agitating the mixture

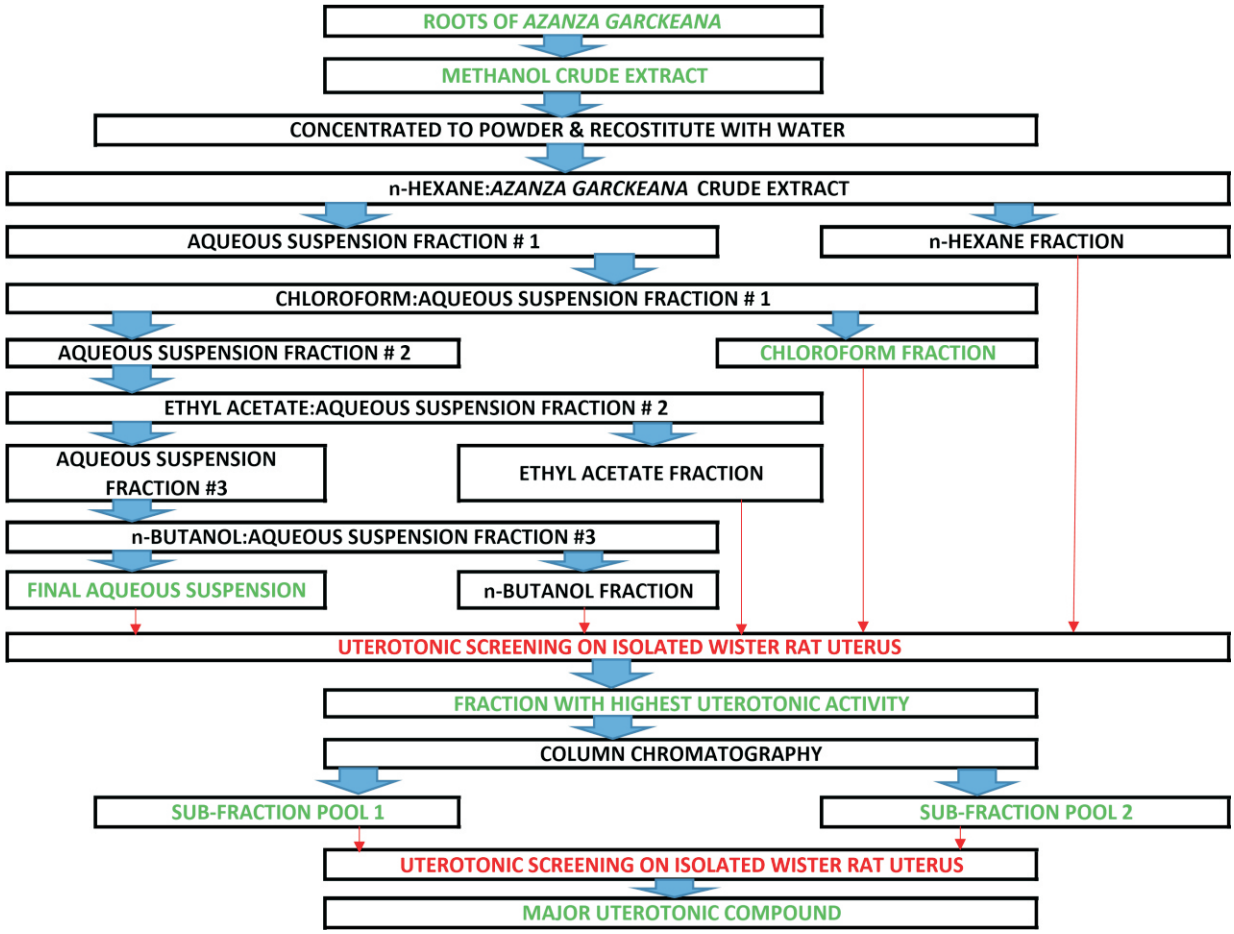

Figure 1: Stages of the bioactivity-guided isolation process of *Azanza garckeana* methanol crude root extract on isolated Wistar rat uterine smooth muscles. The portioned solvents were agitated continuously for 2 minutes and were allowed to settle for 60 minutes to form 2 layers. The 2 layers of fractions were separated using a separating funnel. All fractions and sub-fractions were dried to powder before being screened for uterotonic activity. Thin Layer Chromatography (TLC) was done for each sub-fraction and sub-fractions with similar TLC profiles (spots) were pooled. Sub-fraction pool 1 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.

continuously for 2 minutes. The mixture was allowed to settle for 60minutes to form 2 layers. Less polar phytochemical compounds were in the chloroform fraction, which was dried to powder and was then screened for uterotonic activity. The more polar compounds were in the remaining second aqueous suspension layer, which was carried forward for the next procedure.

Preparation of the ethyl acetate fraction

The second aqueous suspension layer from the previous procedure was partitioned 3 times with 200ml ethyl acetate. This was done by agitating the mixture continuously for 2 minutes and allowed to settle for 60 minutes to form 2 layers. Less polar phytochemical compounds were in the ethyl acetate fraction, which was dried to powder and was then screened for uterotonic activity. The remaining third aqueous suspension layer was carried forward for the next procedure.

Preparation of the n-butanol fraction

The third aqueous suspension layer from the previous procedure was partitioned 3 times with

200ml n-butanol. This 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 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 powder and was then screened for uterotonic activity.

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 (TLC) was done for each sub-fraction, and the sub-fractions with similar TLC profiles (spots) were pooled.

Thin Layer Chromatography (TLC)

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 TLC appeared as spots which were separated vertically, and each had a retention factor (R_f). This method was conducted to have an overview of the phytochemical compounds which are available in the methanol crude extract of *Azanza garckeana*.

Analysis of the compound with the highest uterotonic activity

The isolated major uterotonic phytochemical compound was analyzed using High Performance Liquid Chromatography (Shimadzu, Japan). High Performance Liquid Chromatography (HPLC-UV) was performed to test the purity of the isolated compound and also to identify it using the given standard compound. Ultraviolent (UV), 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 2.7µm, 3.0x100mm (Cortecs part number 1860007372). The HPLC oven temperature was at 20°C and the flow rate used was 0.03ml/min. The isolated compound was also analyzed using Fourier Transform Infrared Spectroscopy (Shimadzu, Japan) to identify the functional groups present on the compound. The wave numbers (1/cm) obtained from the Fourier Transform Infrared Spectroscopy (FTIR) spectra were compared to the standard wave numbers to help identify the suggested present functional groups on the isolated compound [18, 20].

Display of data

In this study, data was displayed using tables, dose-response curves (concentration vs. amplitude of contraction) and figures. All data was presented as mean±standard error of the mean (SEM), 5% level of significant and 95% confidence interval was also displayed.

Data analysis

The primary variable was concentration (mg/ml) while the secondary variable was the amplitude of contractions (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 USA) were used to analyze data. Origin software was used for FT-IR spectra. 5% level of statistically significant and 95% confidence interval were used.

Ethical considerations

The animals were treated humanely and were given access to standard nutrition, water and 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 animal [6]. Approval was sort and obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) before the study was conducted (**REF. No. 006-12-18)**.

RESULTS

Uterotonic activity of Azanza garckeana fractions

The crude root methanol extract of *Azanza garckeana* was dark brownish-black in colour with a percentage yield of 6.51%.

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 below illustrates the collective dose response curves of the 5 *Azanza garckeana* fractions and their concentration-dependent amplitude of contraction (measured in millinewtons) of the uterine smooth muscle strips.

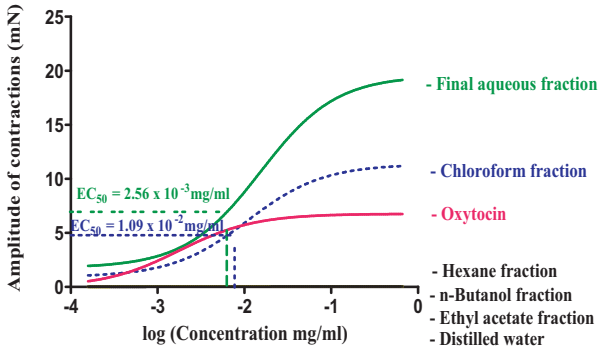

Figure 2: Concentration-dependent contraction of the uterine smooth muscle strips to 5 *Azanza garckeana* methanol crude root extract fractions. Points represent means _ S.E.M. for the number of experiments (n=3). Distilled water, negative control; Oxytocin, positive control.

Silica gel filtration of Azanza garckeana fractions

Thin Layer Chromatography (TLC) of the methanol extract of *Azanza garckeana* yielded 3 spots which suggest the presence of herbal 3 phytochemical compounds in the plant.

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 the bath concentration of 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 the final bath concentration of 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 as sub-fraction pool number 2 was not only 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 below illustrates the collective dose response curves of sub-fraction pool 1 and 2 of *Azanza garckeana* and their concentration-dependent amplitude of contraction (measured in millinewtons) of the uterine smooth muscle strips.

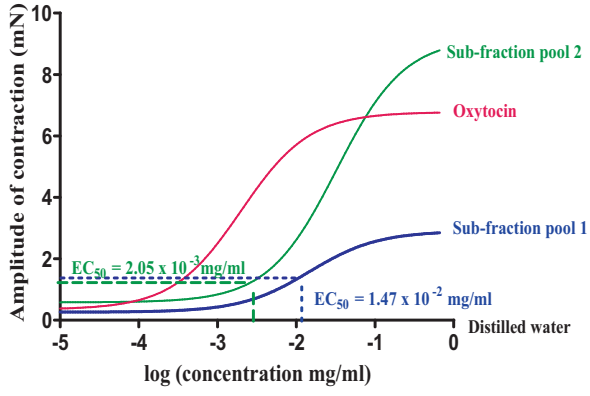

Figure 3: Concentration-dependent contraction of the uterine smooth muscle strips to sub-fraction pool 1 and 2. Points represent means _ S.E.M. for the number of experiments (n=3). Distilled water, negative control; Oxytocin, positive control.

Analysis of the phytochemical compound with the highest uterotonic activity

High Performance Liquid Chromatography (HPLC) of the isolated compound

The compound with the highest activity was in the sub-fraction pool number 2 (sub-fractions number 41 to 61). The UV-HPLC chromatogram for both the isolated compound and the standard glycoside yielded similar peaks with the retention times of 16.729 minutes and 16.695 minutes, respectively.

Fourier Transform Infrared Spectroscopy (FTIR) of the isolated compound

The 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 OH, -CHO, COO- , COO- /- OH, C-O-C), aromatic ring, C-H bending/Polygalacturonic acid and C-O-C torsion deformation in methyl polygalacturonate, respectively.

DISCUSSION

Solvent partitioning of the Methanol crude root extract of Azanza garckeana

The crude root methanol extract findings were 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 [4].

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} =0.02792mg/ml) and cold (EC_{50} =0.4884mg/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 [4].

Uterotonic activity of Azanza garckeana fractions

The chloroform fraction and the final aqueous fraction possessed significant uterotonic activity. The final aqueous was more potent and efficacious than the chloroform fraction. This 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 contain glycosides with long sugar chains, hence the compound with the highest uterotonic activity was thought to be a glycoside which have also been previously isolated from *Azanza garckeana* [19].

The final aqueous demonstrated activity that was less potent than the standard uterotonic drug Oxytocin, but it demonstrated higher amplitudes of contraction at both the Emax and the EC_{50} concentrations. The higher amplitude of contraction 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 n-hexane, ethyl acetate and n-butanol fractions were inactive as far as the uterotonic activities were concerned. The final aqueous suspension fraction contained polar glycosides with polysaccharides [19, 23].

The TLC results obtained are in line with the findings of a 2015 study, who found the plant to contain Alkaloids, Phenols and Saponins [16]

Analysis of the phytochemical compound with the highest uterotonic activity

High Performance Liquid Chromatography of the isolated compound

The compound with the highest activity was isolated and was in the 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 [19, 23].

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 FTIR spectra for the major isolated uterotonic compound in *Azanza garckeana* was similar to that of pectin which is a structural hetero-polysaccharide hydrocolloid found largely in fruit and not in the roots of plants. Pectin has previously been reported to possess various pharmacological activities [10, 18]. Pectin isolated from the fruits of *Azanza garckeana* has the FTIR spectra similar to the isolated uterotonic compound in this study [10]. Pectin naturally occurs with various substituents such as ferulic acid a compound previously isolated from *Sida acuta* (Malvaleae). *Sida acuta* is a member of the Malvaleae family, a family from which the plant *Azanza garckeana* belong. It is reported that Ferulic acid is an active substituent which possesses other pharmacological activities such as anti-aging and Antidiabetic activities [1].

Carboxamine group is essential for the activity of uterotonic drugs such as oxytocin and Ergometrine, but ferulic acid does not contain the carboxamine substituent group in its structure and this can lead to the assumption that the isolated compound with the highest uterotonic activity maybe a pectin with the amidated ferulic acid substituent called N-Feruloyltyramine which has the uterotonically active Carboxamine group in its structure [10].

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. 1 study suggested that Pennogenin glycosides isolated from *Paris polyphylla* possess uterotonic activity [9]. Pennogenin glycosides and Digoxin have similar structures which may suggest that the major uterotonic phytochemical compound in *Azanza garckeana* might be related to Pennogenin glycoside, but only full compound structure elucidation can ascertain this assumption [11, 15].

STUDY LIMITATIONS

The findings of this study did not completely identify the isolated compound due to financial constraints and the absence of Nuclear Magnetic Resonance spectroscopy in Zambia.

Future investigations should do Nuclear Magnetic Resonance spectroscopy on the isolated compound, as well as perform Pharmacological studies to determine the compound's mechanism of action and pharmacokinetics. Additionally, toxicological studies should be conducted to assess the molecule's potential toxicity.

CONCLUSION

The study showed that *Azanza garckeana* possesses uterotonic activity when evaluated using isolated Wistar rat uterine smooth muscles. The study indicated the presence of the major 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 labor possess uterotonic activity. Further Pharmacological and chemical studies need to be done on the plant.

CONFLICT OF INTEREST

All authors declare no potential conflict of interest. This study was partially supported by the Moses Sinkala research scholarship ward.

AUTHORS' CONTRIBUTIONS

AC developed proposal, collection data, analyzed data and prepared manuscript. AG provided scientific guidance and reviewed of Manuscript. LP provided technical support, scientific guidance, review of proposal and Manuscript.

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