

Full Length Research Paper

Isolation, fractionation, chemical constituents and activities of polysaccharides of stem and root of *Cissus adenocaulis* (Steud).

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Polysaccharide from *Cissus adenocaulis* (Steud) was isolated, fractionated and purified with a view to investigate the nature and biological properties of the polysaccharide and to compare their activities with polysaccharides of medicinal value. Crude polysaccharide was isolated from the stem and root of *C. adenocaulis* by a procedure that consisted of extraction (with cold and hot water) and precipitation with cold absolute ethanol. The polysaccharide was fractionated into four fractions by selective precipitation with Cetyltrimethyl ammonium bromide (CTAB). Biological properties of the polysaccharides of *C. adenocaulis* were also investigated by determining the haemolytic and immunostimulating properties. The results of the study revealed that the polysaccharide from the stem and root of *C. adenocaulis* was a glycoprotein. Cetyltrimethyl ammonium bromide (CTAB) fractionated the polysaccharides into neutral, basic and acidic sugars. Homogeneity of each fraction was confirmed by thin layer chromatography. Polysaccharides of *C. adenocaulis* were adjudged to be a heteropolysaccharide, individual sugars in the polysaccharide include glucose, galacturonic acid and galactosamine. Studies on the biological properties of the polysaccharide revealed that it has antihaemolytic properties and exhibited weak antigenic properties.

Key words: *Cissus adenocaulis*, Cetyltrimethylammonium bromide (CTAB), haemolytic properties, anti-inflammatory, antigenic.

INTRODUCTION

Recent studies revealed that polysaccharides apart from playing significant roles as structural elements (cellulose and chitin), are also involved in a number of biological, physiological, defensive and protective processes (Mathew et al., 2007). Quite a number of them have found good uses in pharmaceuticals and medicine. Roles of polysaccharides as medicinal macromolecules are indispensable. Polysaccharides from *Echinops* have been used in traditional Chinese medicine as drugs with anti-

inflammatory effects and antitumor promotion action (Horvath et al., 1998). The fungus *Gonoderma lucidum* has attracted considerable attention because its polysaccharide has antitumor and hypogenic activities (Miyazaki et al., 1980). Immunomodulating polysaccharides from the spores of *G. lucidum* have equally been reported (Pinilla and Luu, 1999; Kwon et al., 2002). A wide range of antitumor or immunomodulating polysaccharides of different chemical structure extending

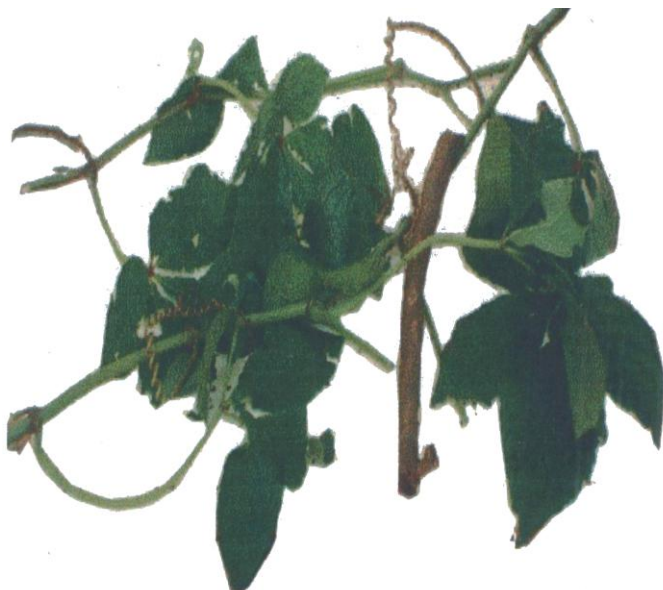


Figure 1. Photograph of *Cissus adenocaulis* (Steud.).

from homopolymers have also been isolated from higher *Basidiomycetes* mushroom. Although stimulation of antibody production is most common with proteins, studies have shown that some polysaccharides are also antigenic (Bartelt et al., 1994) and as such form an integral component of vaccines usually when coupled to a suitable protein. Meningitis vaccines have been prepared in this way and multivalent polysaccharides vaccines have been formulated against *Streptococcus Pneumonia* and *Klesbsiella* species (Sutherland 1999, 2002).

The plant *C. adenocaulis* belong to the botanical family Vitaceae (Metcalfe and Chalk, 1957). It is commonly found in an undisturbed level location in open Guinea savanna wood land. *C. adenocaulis* is a climber usually on shrubs. The leaves are alternate and serrated at margins (Figure 1). It has a calyx with about 4 to 5 petals. Opposite the petals are about 4 to 5 stamens that are inserted at the base of the disk. The plant has pink flowers and green fruits. Other *Cissus* species include *Cissus archoides* (Welw. Ex. Bak) *Cissus cornifolia* (Bak), *Cissus adeopoda* (Sprahue), *Cissus Caesia*, *Cissus erinita* (planch), (Dalziel, 1959). *C. adenocaulis* has high therapeutic potential. The entire plants found in East Africa have been used to heal wound while the fresh leaves have been used for swellings, pneumonia, as purgative and in the treatment of swollen abdomen (Hedberg et al., 1983). The root of *C. adenocaulis* in Tanzania have been used for treatment of hernia, appendicitis, uvulities, enlarged spleen, stomach ache, migrane, mental diseases, syphilis and to prevent abortion (Chhabra et al., 1993). However, there had been

little or no scientific information on *C. adenocaulis* found in Nigeria and West African countries. Neither is there any information on the biological activities of extracts of *C. adenocaulis* as well as on the phytochemical constituents of the plant. Therefore this study aimed at providing information on the nature and the biological properties of polysaccharides of *C. adenocaulis* found in Nigeria especially South - Western Nigeria.

MATERIALS AND METHODS

Plant Materials

Fresh stems and roots of *C. adenocaulis* were collected at a location in Osun State Nigeria. The plant was identified and authenticated by Mr B.A. Daramola, Department of Botany, Obafemi Awolowo University Ile-Ife, Nigeria. The plant materials were cut into bits and homogenized in absolute ethanol at room temperature. The filtrate was collected and the residue was further washed with absolute ethanol until the organic layer became colourless. The residue was air dried at 25°C over a period of seven days.

Reagents and Chemicals

All reagents and chemicals used in this study were of analytical grades procured from different sources such as British Drug House (BDH) Poole, U.K., Sigma Chemical Company, Louis, U.S.A., Fluka Chemical Company and Pharmacia Fine Chemicals, Uppsala, Sweden. All the solutions, reagents and buffers were prepared with glass distilled water.

Methods

Preparation of crude polysaccharides of *C. adenocaulis*

The extraction of crude polysaccharide of *C. adenocaulis* was carried out using cold water (25°C) and hot water (85°C) by a procedure that was based on those earlier described by Nosedá et al. (1999) and Pinilla and Luu (1999). Dried plant residue (30.0 g) was suspended in distilled water (0.2 L). The suspension was stirred mechanically at 25°C for 6 h followed by filtration through cheese cloth. The process was repeated two times, the supernatants were combined and concentrated to a reduced volume and termed cold water soluble polysaccharide (CWP). The residue was subjected to further extraction at 85°C in about 0.3 L of distilled water in a water bath for 4 h. The suspension was stirred mechanically and the supernatant was collected by decantation. This process of extraction was repeated four times. The supernatants were combined, concentrated to smaller volume under reduced pressure at 40°C and termed hot water soluble polysaccharide (HWP).

Precipitation of Polysaccharides

Cold and hot water soluble polysaccharides (0.1 L) were precipitated with the addition of 3 volumes of cold absolute ethanol to the concentrated fluids as described by Liu et al. (2002). The precipitates were collected by centrifugation at 5,000 rpm for 10 min

on Gallekamp Junior Bench centrifuge. The precipitates were redissolved in distilled water (0.05 L) and dialysed extensively at room temperature against water. The dialysate was collected and termed *C. adenocaulis* crude polysaccharide (CADC).

Fractionation of polysaccharide of *C. adenocaulis*

Polysaccharides of *C. adenocaulis* were fractionated based on selective precipitation with Cetyltrimethyl ammonium bromide (CTAB) using the method described below by Yamada et al. 1982.

Equal volume of 80% (w/v) CTAB was added to 25 ml of dissolved polysaccharides with continuous stirring on magnetic stirrer. The suspension was kept in ice for 24 h in the deep freezer. The precipitate was collected by centrifugation and dissolved in 5 ml of 10% (w/v) NaCl followed by addition of concentrated potassium acetate. Cold absolute ethanol (25 ml) was then added and the precipitate was collected by centrifugation at 5000 rpm for 10 min. This was taken up in water (5 ml) and dialysed extensively against water to yield *C. adenocaulis* fraction 1 (CADF₁).

Boric acid (1% w/v, 35 ml) was added to the supernatant collected above and the pH was adjusted to 8.8 with 2M NaOH. The suspension was kept in ice bath for 90 min, after which the precipitate was collected by centrifugation at 5,000 rpm for 10 min and rinsed with 0.5% Borate buffer pH 8.8. This was later redissolved in 2% (v/v) acetic acid (5 ml). Absolute ethanol (50 ml) and concentrated potassium acetate (2 ml) were later added. The precipitate was collected and dialysed extensively against water to yield *C. adenocaulis* fraction 2 (CADF₂).

The pH of the supernatant collected above was adjusted to 9.5 with 2M NaOH. The suspension was centrifuged at 5,000 rpm for 10 min to collect the precipitate which was taken up in water and dialysed extensively against water to give *C. adenocaulis* fraction 3 (CADF₃).

Finally, the pH of the supernatant obtained above was adjusted with glacial acetic acid to pH 4.4. The precipitate was collected and dialysed extensively against water to yield *C. adenocaulis* fraction 4 (CADF₄).

Fractionation of polysaccharide on Sephadex G-100.

The procedure of Andrews (1964) for protein fractionation by gel filtration was also employed for the purification and fractionation of cold and hot water soluble polysaccharide. Sephadex G-100 column (1.5 cm x 90 cm) was equilibrated with 1% acetic acid. Then 15 ml (87.5mg) of dialysed CWP was loaded onto the column and eluted with 1% (v/v) acetic acid at a flow rate of 20ml/hr. Fractions (4ml) were collected and the sugar content was monitored at 490 nm according to the procedure of Dubois et al. (1956). The elution profile was obtained by plotting the absorbance of each fraction at 490 nm against fraction number. The fractions with carbohydrates were pooled, concentrated to smaller volume and stored for further analyses.

Biochemical assays

Hydrolysis of polysaccharides

The method of complete acid hydrolysis was employed to hydrolyse the polysaccharide (cold and hot water soluble polysaccharide) and the four CTAB fractions (CADF₁, CADF₂, CADF₃ and CADF₄).

Typically, 3 ml of each sample were mixed separately with 6M

HCl (3 ml) in quick fit (100 ml) capacity flask. The mixture was then refluxed for 4h and the resulting hydrolysate was allowed to cool at room temperature. The pH of each hydrolysate was adjusted to 7 with 2M NaOH. The neutralised hydrolysates were concentrated to about 5 ml on a rotatory evaporator and the constituent sugars in each concentrated hydrolysates were identified by thin layer chromatography.

Thin layer chromatography of sugars.

The identification of individual sugars in the hydrolysates was carried out by thin layer chromatography. The materials to be spotted were reconstituted in 10% isopropanol (1 ml). The solutions were carefully spotted on thin layer chromatographic plates using capillary tubes at the origin (1.5 cm from the base of the plate) along with sugar standards. The plates were left to dry for at least 5 min after spotting and then transferred to a chromatographic tank which has been equilibrated with the solvent system used for development (EtoAC:H₂O:MeOH:HoAC 65:5:5:25 v/v/v/v). The plates were air dried and sprayed with detecting reagent that consisted of aniline hydrogenphthalate in H₂SO₄.

Estimation of total sugar concentrations.

Total sugars were estimated by phenol/sulphuric acid reaction method as described by Dubois et al. (1956) with slight modification by Oyedapo and Araba (2001). This involved pipetting cold water soluble polysaccharides (0.2 ml) in triplicates into clean dried test tubes. The volume in each tube was adjusted to 1 ml by the addition of appropriate volumes of distilled water. To each test tube was added 5% (w/v) phenol (1 ml) followed by concentrated sulphuric acid (2.5 ml) through a burette. The reaction mixture was incubated at room temperature for 15 min for colour formation after which they were cooled down in cold water. The absorbance was read at 470 nm against the reagent blank. Hot water soluble polysaccharides, (CADF₁, CADF₂, CADF₃ and CADF₄) were treated as above and their sugar concentrations determined by extrapolation from the standard calibration curve of α-D glucose.

Estimation of total protein concentrations.

The total protein concentrations in CADF₁, CADF₂, CADF₃ and CADF₄ were estimated according to the phenol/Folin reaction method as described by Lowry et al. (1951).

Estimation of total hexosamine, hexoses and fucose concentrations.

Hexosamine, hexose and fucose concentrations in the polysaccharide hydrolysates were estimated by the method of Winzler (1958) as described by Anoop and Jegadensan (2003).

Total uronic acid estimation

Uronic acid concentrations in the cold and hot water soluble polysaccharides and the four CTAB fractions were determined by modified uronic acid-carbazole reaction method as described by Bitter and Mur (1962). Typically, polysaccharides (hot and cold water soluble), CADF₁, CADF₂, CADF₃, and CADF₄ (0.2 ml) were

Table 1. Mode of administration of polysaccharide of *Cissus adenocaulis* (Steud).

Number of shot	Day	Volume (ml) administered	Mode of administration
1 st	1	0.1	Intravenous
2 nd	4	0.2	Intravenous
3 rd	8	0.1	Subcutaneous
4 th	11	0.2	Subcutaneous
5 th	15	0.5	Intravenous
6 th	19	0.5	Intravenous

pipetted in triplicates into clean dried test tubes and the volume in each tube was adjusted to 1 ml with appropriate volumes of distilled water. To each of the tubes was added 0.025M Sodium tetraborate: H₂SO₄ (5 ml) and the mixtures were heated at 100°C for 5 min in boiling water bath. This was followed by the addition of 0.125M carbazole reagent (0.2 ml) and the mixtures were thoroughly shaken before heating in a boiling water bath for additional 15 min. The mixtures were allowed to cool at room temperature and the absorbance read at 540 nm against the blank. Uronic acid concentration was estimated by extrapolation from the standard calibration curve of galacturonic acid.

Characterization of purified polysaccharides.

Haemolytic activity

The haemolytic assay was carried out using modified method of Oyedapo and Famurewa (1995). This method involved pipetting varying volumes of purified polysaccharides (0.0, 0.25, 0.5, 0.75 and 1.0 ml) into clean dried test tubes and the volumes were adjusted to 1 ml with distilled water. Phosphate buffered saline (0.1M, 2.5 ml) and freshly prepared 2% (v/v) red blood cells (0.5 ml) were added into each tube. The reaction mixtures were incubated at 37°C for 1 h, followed by centrifugation at 5000 rpm for 15 min. The supernatants were collected and the absorbance was read at 540 nm. The above procedure was repeated for other purified polysaccharides (CADF₁, CADF₂, CADF₃, CADF₄) and Triton X-100. Percentage haemolysis was calculated using the expression:

$$\frac{Abs_{540\text{ nm in the polysacc harde}} - Abs_{540\text{ nm in PBS}}}{Abs_{540\text{ nm 0.5\% TritonX}} - 100 - Abs_{540\text{ nm in PBS}}} \times 100$$

Red blood cells treated with 0.5% Triton X-100 serve as control and represented 100% lysis.

Immunostimulating activity

Immunostimulating activity of polysaccharides of the stem and root of *C. adenocaulis* was evaluated as described by Yeung et al. (1986) with slight modifications. Anti-CADC (Anti- polysaccharide) was raised by immunizing rabbits with purified polysaccharides (10 mg/ml) as described by carpenter (1975). Typically, purified polysaccharide (1%) was injected into the experimental animals (rabbits). Table 1 shows the mode of administration. Control rabbits received normal saline instead of test dose. The animals were sacrificed 7 days after the last injection and blood samples were collected into unheparinized tube and the anti serum was obtained by centrifugation.

Antibody precipitating activity of the polysaccharide was tested by agar gel diffusion method as described by Ouchterlony (1964). 1.0 g of dextrose agar was added to 100 ml of distilled water and warmed. This gives 1% agar solution that was autoclaved for 6 h. The agar was then poured into six petri dishes and was allowed to solidify. Holes were bored in a pattern that consists of a central hole with six circumferential holes using a cork borer (7mm in diameter). These holes were spaced so that the distance from the center hole to that of the peripheral holes was 1 cm. The central well was filled with antiserum while the outer wells were filled with the polysaccharide that was serially diluted (2⁰ to 2¹⁴). The plates were later kept in humid box and observed daily for lines of precipitate between the antiserum and polysaccharide wells.

The above outlined procedure was repeated with CADF₁, CADF₂, CADF₃, CADF₄, Glycogen, starch conancavalin A (Lectin).

RESULTS AND DISCUSSION

Extract yield (amount of extractable polysaccharide)

C. adenocaulis polysaccharides were extracted by cold and hot water extraction methods. The yield of polysaccharide obtained by cold water extraction method was 39.06% while hot water extraction was 23.44% of the starting material. The variation in the yield of polysaccharide obtained was due to the extraction methods that were employed. Various methods of extractions have been employed by investigators which include boiling of materials in water (Goldstein and Hayes, 1978), cold water extraction (Pinilla and Luu, 1999) as well as alkali extraction. Observations have shown that cold water extraction method was simple and gave a higher yield than hot water extraction method.

Fractionation of Polysaccharide by Gel Filtration

Fractionation of cold water and hot water soluble polysaccharide by gel filtration on Sephadex G – 100 with 1% acetic acid as eluant gave three distinct peaks from cold water soluble polysaccharides (Figure 2A) while two peaks were obtained from the hot water soluble polysaccharides (Figure 2B). Peak I (fractions 8 to 10) and peak III (fractions 18 to 21) from cold water soluble

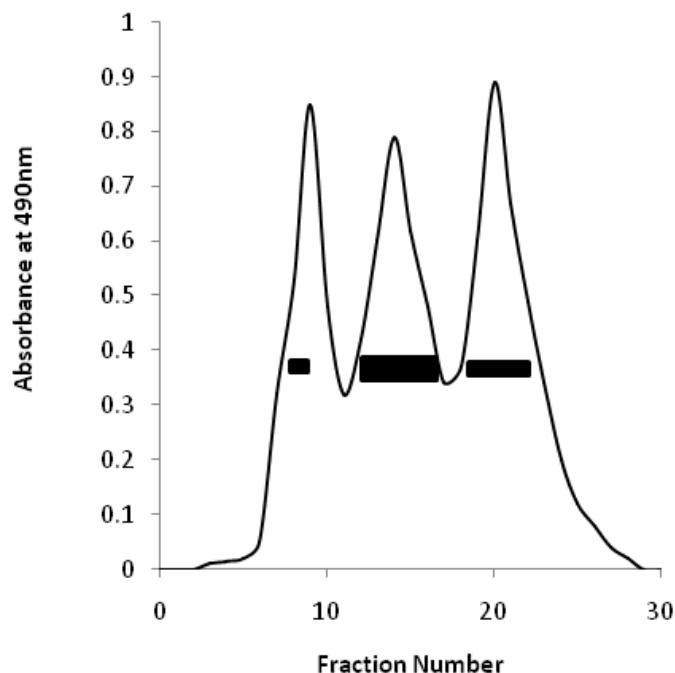


Figure 2A. Elution profiles of Cold water soluble polysaccharide *Cissus adenocaulis* (Steud).

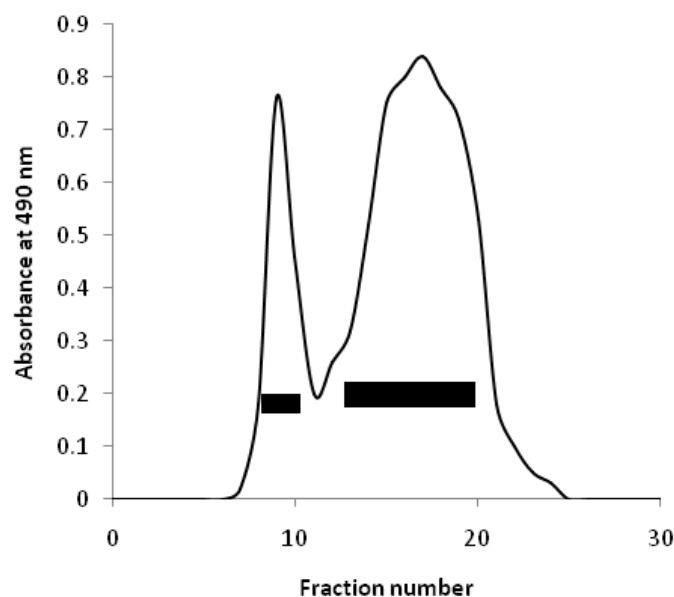


Figure 2B. Elution profile of hot water soluble polysaccharides of *Cissus adenocaulis* (Steud).

polysaccharide were similar to peaks I (fractions 8 to 10) and II (fractions 18 to 21) in hot water soluble polysaccharides respectively. It was apparent that peak II in cold water soluble polysaccharide might have been lost

in hot water soluble polysaccharide due to the method of extraction (heating) that was employed. The absence of peak II in hot water soluble polysaccharide further supported the reduction in yield that is always associated with hot water extraction method (Goldstein and Hayes, 1978).

Gel filtration techniques have equally been employed in fractionation and purification of polysaccharides using various gel types such as Sephadex G-50 (Pinilla and Luu, 1999), Sephadex G-100 (Yamada et al., 1989), Sephacryl S-300 (da Silva and Parente, 2002). Elution can be conducted with different solvent systems such as water (Yamada et al., 1989; 1988, Paik et al., 2003), 0.1M Tris-HCl buffer pH 7.0 (da Silver and Parente, 2002), 1% (v/v) acetic acid (Pinilla and Lui, 1999).

Fractionation of polysaccharide with CTAB

The fractionation of polysaccharide of *C. adenocaulis* carried out with Cetyltrimethyl ammonium bromide (CTAB) gave four fractions namely, CADF₁ (neutral), CADF₂ (basic, pH 8.8), CADF₃ (basic, pH 9.2) and CADF₄ (acidic pH 4.4) which were comparable with the four fractions that were obtained from the fractionation of crude polysaccharide from the roots of *Bupleurum falcatum* (Yamada et al., 1988). A frequently used method for fractionation of water extracts from plant tissue or culture is the treatment to saturation with ammonium sulphates which does not usually precipitate arabinogalactans due to their high solubility (Fincher and stone, 1974; Mau et al., 1982; Wydra, 1991). Ion-exchange chromatography has also been used for initial fractionation of extra cellular polysaccharides (Paik et al., 2003; Diallo et al., 2003). This procedure removed many contaminants including protein and neutral sugar such as glucose (York et al., 1985). As such, selective precipitation was a preferred method of fractionation of polysaccharides. This was based on selective precipitation of polysaccharides using non-covalent binding of polysaccharides with lectins, formation of salt or complexes of pectic acid with neutral cupric acetate (Aspinall et al., 1969, Aspinall 1982), cation detergents such as cetyltrimethylammonium bromide (CTAB) and Cetylpyridium bromide (Yamada et al., 1988). CTAB have been employed in the fractionation of polysaccharides from *Abisidia cylindrospora*, *Angelica acutiloba* (Yamada et al., 1982, 1989). The quaternary complexes that were formed might be fractionated by varying the salt concentration or pH (Aspinall, 1982).

Homogeneity test of the CTAB fractions

The homogeneity tests of the four CTAB fractions: CADF₁, CADF₂, CADF₃ and CADF₄ on Sephadex G-100

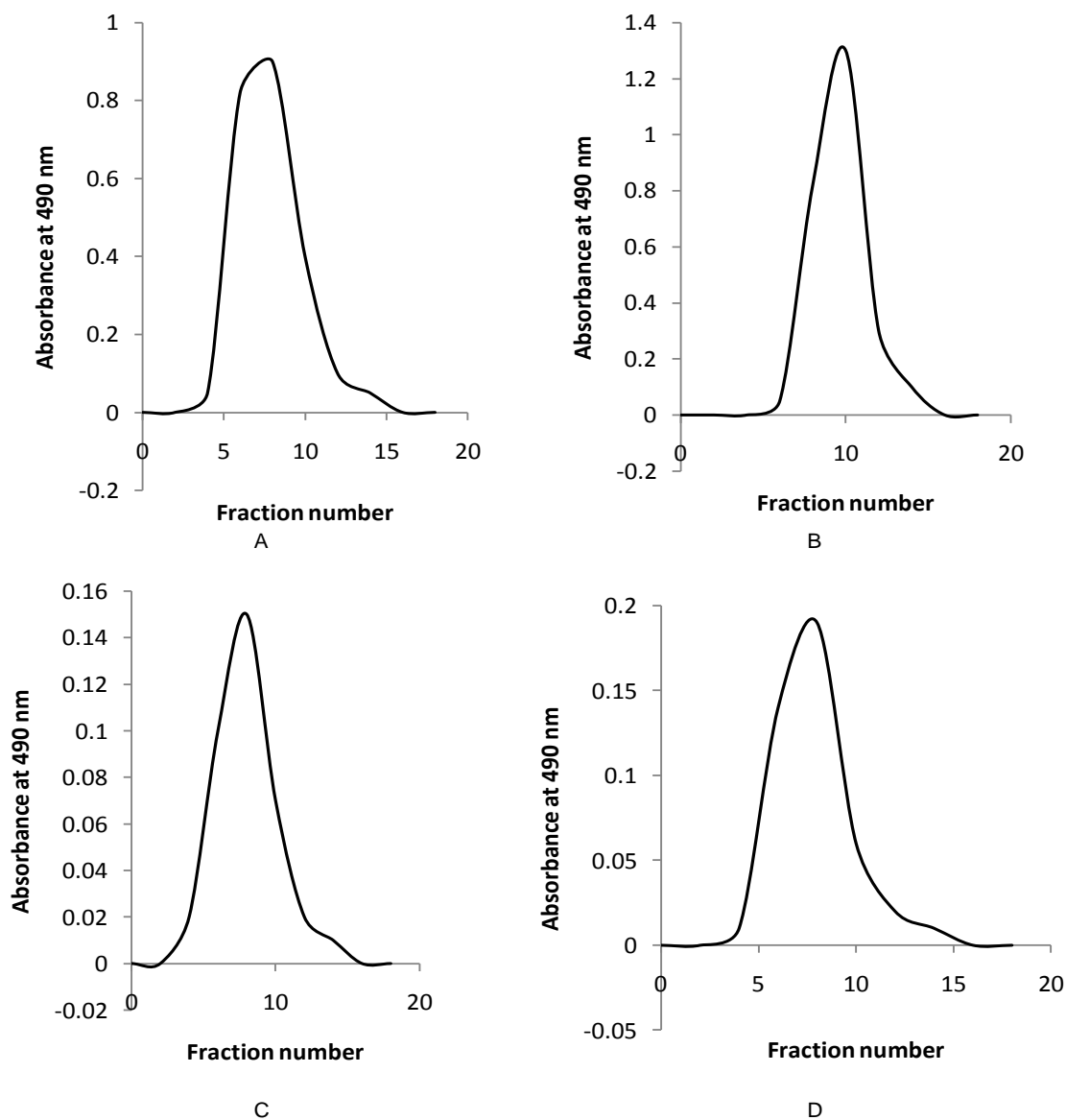


Figure 3. Elution profiles of CADF₁, CADF₂, CADF₃ and CADF₄ (A, B, C, D) respectively.

resulted in one distinct peak each (Figure 3), which implied that each of the fractions contained one sugar hence homogenous in preparation.

Hydrolysis of polysaccharide

The purified polysaccharides and the four CTAB fractions were hydrolysed with 6N HCl in boiling water for 2 h. The resulting hydrolysates were later neutralized with 2M NaOH. Studies have shown that several solvents have been employed in hydrolyzing polysaccharides which include Trifluoroacetic acid (TFA), sulphuric acid, periodic

acid as well as perchloric acid. Hydrolysis with these strong acids has been demonstrated to result in loss of aglycosidic groups that might be present in sugars hence a mild method is preferred.

Thin layer chromatography

The analysis of the chromatograms of hydrolysates of purified polysaccharide gave three spots (Figure 4) which implied that the polysaccharide could be made up of at least three different sugars. The retardation factor (R_f) of each spot corresponded to R_f of glucose, galactosamine

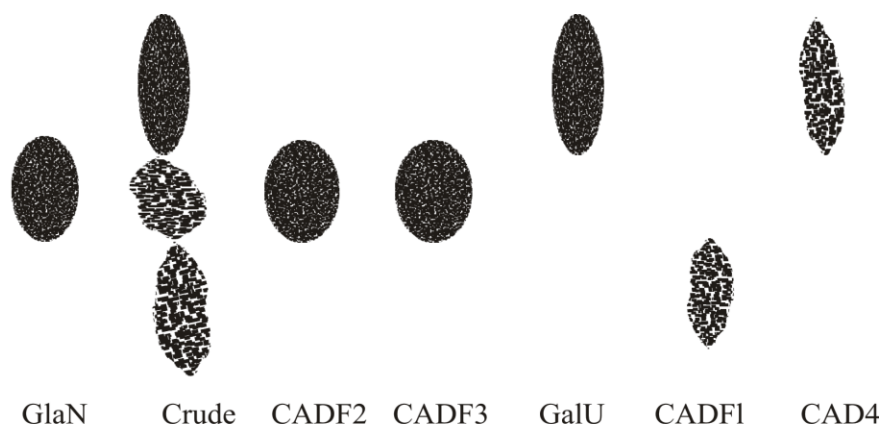


Figure 4. Chromatograms of polysaccharides of *Cissus adenocaulis* and its fractions.

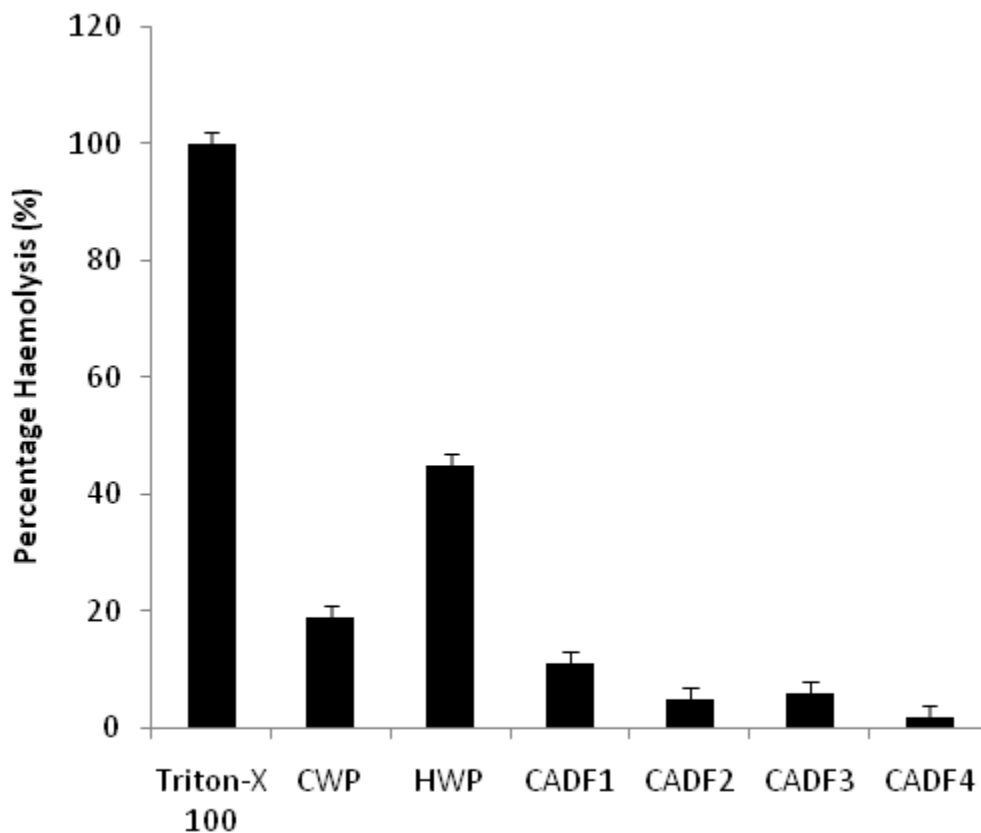


Figure 5. Haemolytic activity of polysaccharides of *Cissus adenocaulis* (Steud). Each value represented the mean \pm SD of 3 readings $P \leq 0.05$ was taken as statistically significant.

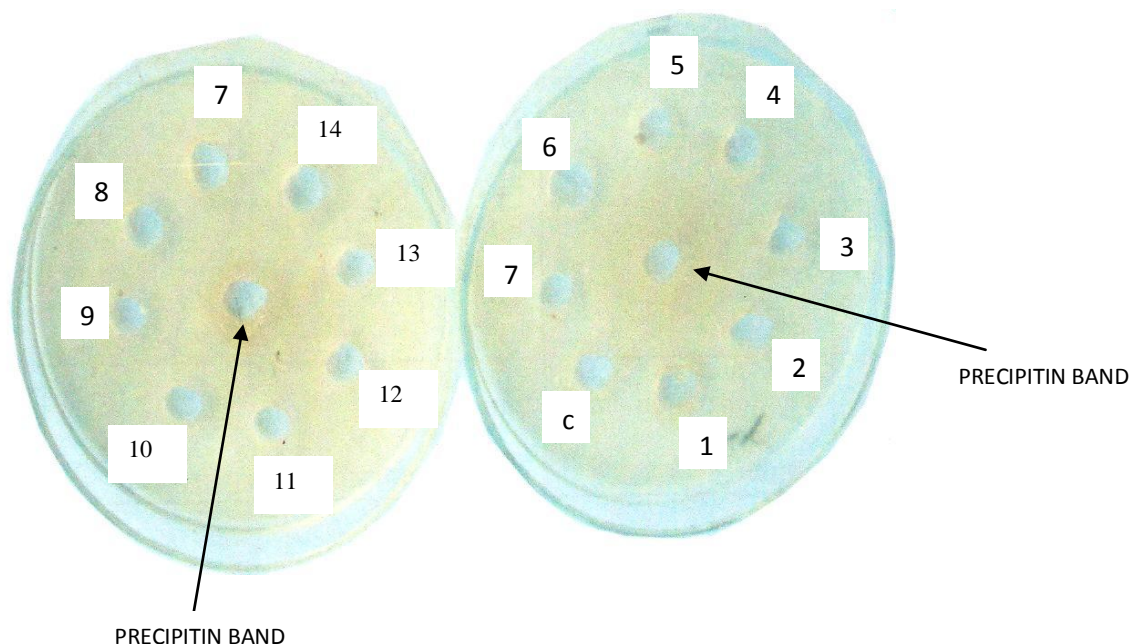
and galacturonic acids (standard sugars). Further analysis of the chromatograms of the hydrolysates of

CTAB fractions (CADF₁, CADF₂, CADF₃ and CADF₄) also gave a single spot each.

Table 2. Chemical analyses of polysaccharides of *Cissus adenocaulis*.

Sample	Total (mg/ml)	Sugar (mg/ml)	Protein (mg/ml)	Fructose (mg/ml)	Hexosamine (mg/ml)	Hexose Uronic acid
CADF ₁	63.13±0.01	36.25±0.03	0.16±0.01	0.07±0.01	6.16±0.02	2.90±0.03
CADF ₂	23.38±0.01	1.10±0.01	-	1.44±0.01	2.75±0.01	0.44±0.01
CADF ₃	6.00±0.01	3.12±0.02	0.17±0.01	0.52±0.00	4.20±0.01	0.45±0.01
CADF ₄	15.00±0.01	3.15±0.01	0.08±0.1	0.04±0.02	2.63±0.02	6.90±0.01

Values are the means of three replicates ± standard deviation (SD).

**Figure 6.** The antibody precipitating activity of polysaccharide of *Cissus adenocaulis*.

The retardation factor (R_f) of the sugar in CADF₁ corresponded to the R_f of glucose, the R_f of CADF₂ and CADF₃ corresponded to the R_f of galactosamine while R_f of CADF₄ fraction corresponded to galacturonic acid (Figure 4).

Chemical Analysis

Table 2 presents the chemical analysis of polysaccharides and the four CTAB fractions. It was observed that CADF₂ and CADF₃ had the highest hexosamine concentrations of 1.44 mg/ml and 0.518 mg/ml respectively.

These results confirmed that CADF₂ and CADF₃ were basic fractions and were precipitated at pH 8.8 and pH 9.5 respectively. It could however be inferred that pH 8.8 would be the best pH for precipitating basic sugars. Also

CADF₄ had the highest uronic acid concentration at acidic pH of 4.4 while CADF₁ had the highest hexose concentration which confirmed that CADF₁ was a neutral fraction.

Haemolytic activity of the polysaccharide

The haemolytic activity or otherwise of the purified polysaccharide was also investigated with 2% (v/v) bovine red blood cells at 25°C. It was observed that HWSP caused highest amount of haemolysis, followed by CWSP, while CTAB fractions exhibited the least haemolytic activity (Figure 5). Haemolytic activity of any substance whether synthetic or naturally occurring is always a measure of the amount of haemoglobin released into the medium when interacted with red blood cells.

Immunostimulating activity

The antibody precipitating activity of polysaccharide of *C. adenocaulis* was tested by agar gel diffusion method. There was the formation of a weak precipitin band (Figure 6). It implied that there were interactions between the purified polysaccharide, CTAB fractions (CADF₁, CADF₂, CADF₃, and CADF₄), Glycogen, Starch, Concanavalin A and blue dextran. Therefore polysaccharide of *C. adenocaulis* could be said to be immunogenic, however there is the need to increase the concentration of the polysaccharide administered to obtain a stronger precipitin band.

Conclusion

Evident from this study, polysaccharides from the stem and root of *C. adenocaulis* is a glycoprotein, a heteropolysaccharide that exhibited different levels of anti-haemolytic and immunostimulating activities.

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