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Proximate analysis and evaluation of total phenolic compounds in *Azanza garckeana* fruit for use as gelatin crosslinking agent

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ABSTRACT

Azanza garckeana (A. garckeana) is a wild edible fruit native in Africa, widely distributed in the East and Southern Africa. The species grow naturally in semi-arid areas. The consumption of the fruit is linked to many phytochemicals present within the fruit, one of these being phenolic compounds. Several studies have been conducted on A. garckeana, but, study on the phenolic content of the pulp and the seed coat has been overlooked. However, to link the effect of these phenolics, the complete quantification of the whole fruit is mandatory. The objective of this research was therefore to conduct proximate analysis and to determine the Total Phenolic Content (TPC) in different constituents of A. garckeana fruit for use as a natural crosslinking agent in gelatin resin. Firstly, the phytochemical yield was determined and this was done through separating the fruit into different components (i.e., pulp, seed and the seed coat) defatting and extracting using methanol. The extracts were filtered, concentrated and the yield was calculated. Secondly, phytochemical screening for phenolics and clean-up was done prior to the determination of TPC. TPC was estimated based on Folin Ciocalteu's method using SpectraMax M2 UV-vis spectrophotometer. The linearity of the Folin Ciocalteu method was verified through the method of least squares which was applied through different concentrations of standard gallic acid. TPC was calculated from the gallic acid standard curve and the results expressed as mg/g gallic acid equivalents (Standard curve equation: $y = 2.9503x + 0.0348$; Correlation coefficient: $R^2 = 0.997$). The extraction yield was found to be 0.57, 0.18 and 0.14g/g for the pulp, seed and the seed coat, respectively. All phytochemical screening tests revealed a positive test for phenolics. The TPC varied from 18.14 to 20.78 and 24.92mgGAE/g in the seed coat, seed and the pulp, respectively. High TPC (24.92mgGAE/g) was found in the fruit pulp.

Keywords: Phenolic compounds, Azanza garckeana, phytochemicals, maceration, methanol Extract

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1.0 INTRODUCTION

A. garckeana is a wild edible fruit and is an excellent source of nutrients and phytochemicals which have medicinal properties (Nkafamiya, Ardo et al. 2016, Maroyi 2017). These phytochemicals play a significant role in pharmacology. Phytochemicals are naturally-occurring secondary metabolites that protect plants from environmental factors and possible diseases. These secondary metabolites contribute to the plant's colour, aroma and flavor (Rao 2003, Paredes-López, Cervantes-Ceja et al. 2010).

Among fruit plants that has been exploited for its phytochemicals is the A. garckeana fruit (Figure 1). A. garckeana is variously called snot apple or African chewing gum (English); uxakuxaku (Ndebele); mutohwe (Shona). It is native in Africa, widely distributed in the Eastern, Western and Southern Africa. The species grow naturally in open woodlands, grasslands, thickets, riverine vegetation and rocky places.

The generic name "Azanza" is derived from the word "Azania", a word meaning black and originating from Zanzibar whereas the specific name "garckeana" is in honour of Professor August Garcke (1819-1904), a German botanist and plant collector who specialized in pharmacology (Orwa, Mutua et al. 2009). Although being used widely in nutrition, *A. garckeana* remains one of the underutilized species, that grow naturally in the woodlands (Ochokwu, Dasuki et al. 2015, Dikko, Khan et al. 2016, Mshelia, Watirahyel et al. 2016).

Figure 1: Azanza garckeana fruit

Studies have been done on *A. garckeana* root, leaf, bark, pulp and seed to determine its nutritional value and pharmacological activity. Adamu et al (2013) performed a phytochemical screening of the fruit and Jacob et al (2016) did a proximate analysis of the fruits' primary metabolites and metal ions. In another study, Michael et al (2015) evaluated the phytochemicals found in the seed. Among the phytochemicals, 2.6% polyphenols were found within the seed. Mshelia et al (2016) performed phytochemical screening to determine the antimicrobial activity of the bark extracts of Azanza garckeana plant. In an almost similar study, Dikko et al (2016) determined the antimicrobial activity of the fruit and also isolated its betulinic acid. Maroyi (2017) took a step further and determined the fruits chemical, nutritional and toxicological properties. All results obtained from the studies gave evidence that A. garckeana is a rich source of phytochemicals and has a possibility for commercial utilisation.

Despite numerous studies performed on A. garckeana fruit, there is still a gap i.e there is need to both qualitate and quantify its bio-active compounds specifically its phenolic compounds. Such information may be useful for further studies and for the application of A. garckeana bio-active compounds in other industries besides the pharmaceutical industry. More and more convincing evidence suggests that the benefits of phytochemicals in fruits can be even greater than is currently understood (Ames and Gold 1991).

Studies on plants have revealed that plant phenols contain hydroxyl (-OH) functional groups that are responsible for forming hydrogen bonds with different substrates leading to formation of strong interactions. (Adamu, Ushie et al. 2013, Michael, Onyia et al. 2015, Maroyi 2017). These phenolic compounds have been confirmed to have antioxidant activity, hence their use in pharmacology. Besides the use of these phenolic compounds in pharmacology, plant phenolics have been reported to have excellent properties as food preservatives, natural food colourants as well as crosslinking agents in the production of paper, paints, dyes, photography and in cosmetics (Valenzuela, Nieto et al. 1991, Waterman and Mole 1994, Yasin, Babji et al. 2017). Their use is based on the ability of polyphenols to form covalent and non-covalent interactions that results in the formation of a complex structure, the presence of aromatic ring which results in the formation of a stable chemical compound and also the ability to form hydrophobic interaction that improves water resistance e.g. with amino acids functional groups (Maqsood, Benjakul et al. 2014, Michael, Onyia et al. 2015, Altemimi, Lakhssassi et al. 2017, Yasin, Babji et al. 2017).

This work was therefore aimed at opening the possibility of utilisation of *A. garckeana* fruit's polyphenolic compounds for the crosslinking of gelatin resin. This is because plant phenolics are chemically stable due to the presence of an aromatic ring and have an interaction with gelatin that improves water resistance (Altemimi, Lakhssassi et al. 2017). Also, they have great affinity for proline amino acid in gelatin which improves gel strength (Yasin et al., 2017, Altemimi et al., 2017).

To achieve the aim of this study, the proximate analysis, the phytochemical yield of the fruit was determined and the fruit constituents (i.e. for the pulp, seed and the seed coat) screened for phenols, TPC determined using UV-visible spectrophotometry and the functional groups determined using FT-IT spectroscopy.

2.0 MATERIALS AND METHODS

2.1 Materials

All chemicals used were of analytical grade from Sigma-Aldrich.

2.2 Methods

2.2.1 Preparation of *A. garckeana* fruit

Ripe *A. garckeana* fruits were collected in September 2019 from Mbembesi area in Lupane, Zimbabwe. To prepare the fruit, its carpels (pulp) was separated from the seed. Drying was done for 90 days under direct sunlight at temperatures between 26- 33°C. After 90 days the fruit was dry and this was measured by the ability of the fruit to be coarsely ground by pestle and mortar. After grinding, the seed was separated from its seed coat. However, most characterisation was mainly based on fruit pulp due to its high phenolic content.

2.2.2 Proximate Analysis of A. garckeana fruit

The moisture, fat and ash content of A. garckeana were determined using gravimetric method.

2.2.2.1 Determination of Moisture content

Moisture content of the dried fruit was determined using the gravimetric method according to Official Methods of analysis of the Association of Official Analytical Chemistry (Chemists and Chemists 1925, Chemists and Analysis 1965). Using this method, fruit constituents (i.e. pulp, seed and seed coat) were placed in separate ceramic pans and the samples were weighed to the nearest 0.001g. The samples were then placed in a drying oven set at 105°C for a minimum of 2 hours and they were heated until they reached a constant weight. Weight differences of samples after heating was considered as the moisture content of sample and was calculated based on equation [1]:

$$Mc\ (\%) = \frac{Change\ in\ mass\ of\ the\ sample}{(Initial\ mass\ of\ the\ sample)}\ x\ 100 \qquad [1]$$

2.2.2.2 Determination of PH

PH measurements was performed using a calibrated pH meter at 25°C (Webster 2003). The A. garckeana pulp was weighed and mixed with distilled water in the ratio of 1:1. The slurry was covered and allowed to stand for an hour. After an hour the pH reading was taken (Pansu and Gautheyrou 2006).

2.2.2.3 Determination of Fat Content

Fat content was determined using maceration method ofr Dikko's and Edeoga. (Edeoga, Okwu et al. 2005, Dikko, Khan et al. 2016). Using this method, coarsely powdered A. garckeana was defatted with hexane and water solvents in the ratio of 1:5 using maceration apparatus. The apparatus were placed in a dark cupboard for 5 hours in-order to remove waxes and lipophilics. After 5 hours the solvent was vaporised and the change in mass recorded as fat content.

2.2.2.4 Determination of Ash Content

The ash content will be determined based on ASTM E1755-01. A 10g sample will be used. To prevent sample spattering during ashing, the sample will be oven dried first prior to extraction. A high temperature muffle furnace capable of maintaining a temperature of 550-600°C will be used. The furnace will burn the sample in the presence of oxygen for 24 hours. After ashing the weight of the sample will be noted and the ash content determined according to the equation [3]: *(reference this standard).*

$$\%\ Ash = \frac{Mash}{Mdry}\ x\ 100 \qquad [3]$$

Where:
M_{ash} is the mass of ashed sample and
M_{dry} is the original mass of the dried sample.

2.2.3 Extraction of phytochemicals

The coarsely ground samples were defatted according to Dikko and Edeoga's method (Edeoga, Okwu et al. 2005, Dikko, Khan et al. 2016). Phytochemicals were extracted using the cold extraction method (i.e. maceration). To prepare the stock solution, 99% aqueous methanol solution was prepared and acidified with 2M HCl (Harborne 1973, Biesaga and Pyrzyńska 2013, Dikko, Khan et al. 2016). The mixture was reacted in the sample to solvent ratio of

1:10 and the mixture was incubated at room temperature for 72 hours with 5 minutes agitation after every 24 hours until the soluble matter was dissolved (Harborne 1998, Dikko, Khan et al. 2016). After 72 hours, the crude extracts were filtered using a funnel and Whatman No.1 (150mm) filter paper.

2.2.4 Sample concentration

The filtrate was concentrated using a rota vapor R-3000 using a pressure of 0.42 bars, 60°C temperature and 50 rotations per minute. The concentrated extracts were stored in the refrigerator at 4°C awaiting further use (Harborne 1973).

2.2.5 Determining phytochemical yield

The phytochemical yield of the methanolic extracts was determined using Equation [2]:

$$\%\, Yield = 100(1 - \frac{Wextract}{Wmaterial}) \qquad [2]$$

Where:
*Wextrac*t is the weight of the isolated phenolics from the column, and
*Wmateria*l is the weight of the methanolic extract fed into the column

2.2.6 Screening for polyphenols

The crude extracts were subjected to chemical test (Ferric chloride test, Folin Ciocalteau's reagent test and sodium chloride test) using standard procedures (Harborne 1973, Dikko, Khan et al. 2016, Mshelia, Watirahyel et al. 2016). A positive (+) test was shown by a blue-black colour after the addition of the test solution to the crude extracts (Harborne 1973, Trease and Evans 1983, Jeong, Miyamoto et al. 2000).

2.2.7 Solid Phase Extraction (SPE) of polyphenols

SPE of phenols was done using a simple syringe method demonstrated by (Ranatunge, Adikary et al. 2017). Using this method, Polypyrrole (Ppy) was used as a sorbent. Ppy was synthesized with ferric chloride as an oxidant (Chitte, Shinde et al. 2011).After the synthesis of Ppy sorbent, syringe was plugged with cotton wool and this was followed by the packing of the syringe with polypyrrole (PPy) sorbent. The syringes were then placed in respective cartridges. According to Bagheri (2004), PPy offers about 80-100% recovery of phenols, has proven high retaining property for phenols as well as low consumption of desorbing solvent (Bagheri, Mohammadi et al. 2004).

2.2.8 Determination of Total Phenol Content (TPC)

TPC was determined using Folin- Ciocalteu reagent (FC) method with some modifications. Briefly, the vaporised samples were reacted with ethanol to make a 10mg/ml stock solution. 0.1ml sample was taken from the stock solution and reacted with 0.5ml of 10% FC reagent and kept for 5 minutes in the dark. 0.4ml of 2% sodium carbonate was added into the reaction mixture and distilled water added to a 10ml mark. Triplicate samples were prepared for each sample type. The blank was concomitantly prepared with 0.1ml ethanol, 0.5ml of 10% FC reagent, 0.4ml of 2% sodium carbonate and diluted with distilled water to a 10ml mark. The phenolics in the study samples were isolated by the SpectraMax M2 UV-vis spectrophotometric assay at 765 nm wavelength and at a temperature of 37°C.

Linearity of the method was verified through the method of least squares. In this method, gallic acid was used as the reference standard. To prepare of standard gallic acid stock solution**,** gallic acid was reacted with ethanol in the ratio of 1:20 and the mixture diluted with water to a 100ml mark. From the stock solution, standard concentrations of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30mg/ml were formulated. An aliquot of 0.1ml from the standard concentrations was mixed with all reagents used in the preparation of the sample. The standard curve was then plotted as a function of absorbance against sample concentration. The concentration of the phenolics in A. garkeana were obtained from the standard curve and expressed in milligrams equivalent gallic acid (GAE) per gram of each fraction using the formula:

$$C = C_1 {}^{V}/_{m} \qquad [2]$$

Where C = total phenolic content in mg/g in GAE (Gallic acid equivalent), C_1 = concentration of sample established from the calibration curve in mg/ml, V = volume used during TPC assay in ml, and m = mass of the plant extract used in grams.

2.2.9 Determining the Functional groups in *A. garckeana* fruit pulp

Shimadzu Fourier Transform Infrared (FT-IR) spectroscopy was used to determine the functional groups present in A.garckeana fruit pulp. Happ-Genzel apodization function was used and the measurement mode used was transmittance. 45 scans were run per sample. When infrared light is passed on a sample, light that corresponds to a specific wave number is transmitted through a sample. The amount of light transmitted corresponds to a specific wave number and functional group.

3.0 RESULTS AND DISCUSSIONS

3.1 Proximate Analysis results

3.1.1 Moisture Content

The moisture content affects the Water Holding Capacity (WHC) of a material, i.e. the higher the moisture content of the raw material, the higher is its WHC. However, the hydrophobic interactions may reduce this phenomen depending on the level of crosslinking formed (Zhao, Li et al. 2016).

Table 1: *A. garckeana* Moisture Content

Fruit constituent	Moisture Content
1. Pulp	34.06%
2. Seed	11.92%
3. Seed Coat	14.31%

The highest Moisture content (Mc) of *A. garckeana* fruit was found in the fruit pulp (34.06%) and the lowest was in the seed (11.92%). The high number of hydroxyl groups found in fruit pulp contributes to this high moisture content value. According to Table 1 and 2, moisture content is proportional to the number of phytochemicals, the higher the moisture content, the higher is the phytochemical yield. However, moisture content values obtained in this research are higher than those obtained by other reserchers e.g. Alfred and Nkafamiya found a lower moisture content (6.7%) (Nkafamiya, Ardo et al. 2016, Maroyi 2017). The reasons for this deviation could be: geographical differences and differences in test conditions.

3.1.2 pH

The pH of the fruit pulp was found to be 5.6. The pH value is almost similar to the pH value found by (Maroyi 2017). According to the French pedological reference base (Muller, Dean et al. 1995) the classification for this pH is “acidic” as the value lies between 5 and 6.5. This acidity is due to the presence of organic and inorganic acids within the fruit pulp.

3.1.3 Fat Content

The fat content of the fruit was found to be 1%. This is in-line with other findings (Saka and Msonthi 1994, Nkafamiya, Ardo et al. 2016, Maroyi 2017) in literature. The fat content is significant enough hence the fruit can be consumed to provide energy to the body.

3.1.4 Ash Content

The average Ash of *A. garckeana* was found to be 4.36%. According to (Saka and Msonthi 1994) most fruits have ash content ranging between 3-5% hence the ash content obtained is within the range of other researches. The ash content value obtained shows that A. garckeana has prominent amount of minerals that can be utilized for nutritional purposes.

3.2 Phytochemical yield and Screening

Table 2 shows the phytochemical yield and chemical screening test results for different components of *A. garckeana* fruit.

Table 2: Results for phytochemical yield and screening of phenolics

	Phytochemical yield (g/g)	Screening for Phenolics		
		Ferric Chloride	Folin Ciocalteu	Sodium Hydroxide
Pulp	0.57	(+)	(+)	(+)
Seed	0.18	(+)	(+)	(+)
Seed coat	0.14	(+)	(+)	(+)

Key

(+) positive test

The phytochemical yield was found to be 0.14, 0.18 and 0.57g/g for the seed coat, seed and the pulp, respectively. Further examination of Table 1 reveals that pulp has the maximum extractable matter and the seed coat has the minimum extractable matter. This proves that the pulp besides containing more extractable matter, it is more soluble in methanol solvent as high solubility leads to high extraction. This is in line with findings from Khoddammi et al (2013) which states that, a high polar solvent results in high extractable matter leading to high yield. Also, yield value seems to be directly proportional to polarity i.e. the pulp due to its high polarity (as shown by its high moisture content of 34.06%) gave a high yield as compared to other fruits constituents.

The yield values obtained are in line with the values obtained by Paini Sri (2014) when he calculated the yield of Pluchea, a yield of 0.38g/g was obtained. Other similar results (0.15 g/g) were obtained by Parejo when he studied the yield of Rhodiola rosea (Parejo, Viladomat et al. 2003). High yield values (i.e. 0.14, 0.18 and 0.57g/g) obtained in this research does not only prove high extractable matter in A. garckeana but also the effectiveness of methanol in phytochemical extraction. According to Parekh and Chanda (2017), the high yield value indicates that the fruit can be suitable for use as a gelatin crosslinking agent. Once crosslinked the gelatin can be used in pharmaceuticals, as a matrix for biocomposites, glue for textiles, leather, plastics and paper as well in the production of edible films.

Qualitative test to determine the presence of phenolic compounds in A. garckeana methanolic extracts using ferric chloride, Folin Ciocalteau's reagent and sodium chloride revealed a positive test (+) on phenolics. A positive test was shown by a prominent colour change from green to blue-black for the pulp, dark yellow to blue-black for the seed and light yellow to blue-black for the seed coat. Because of the polarity of the A. garckeana polyphenols, the extracts were effective to donating a hydrogen atomic to the ferric chloride, Folin Ciocalteu's reagent and sodium chloride. This resulted in radical phenoxyl delocalisation resulting in the formation of a blue black complex (Widyawati, Budianta et al. 2014). The positive test reveals that the pulp, seed and the seed coat used are mature enough to contain and propagate the required phenolic compounds thus validating the utilisation of A. garckeana fruit in the crosslinking of gelatin resin.

3.3 Solid Phase Extraction

The UV-vis absorbance values obtained after the Solid Phase Extraction of polyphenols were less than the sensitivity limits of the UV-vis for the waste fractions 1,2,3 and 4 and this proved the absence of polyphenols. The FC assay showed 0.7 absorbance for TPC and 0.005 absorbance for waste fractions. This showed that the Ppy sorbent has high binding capacity for phenols and hence provided the best method for the separation of phenol from other interferences.

3.4 Total Phenolic Content (TPC)

The results in Figure 2 shows the standard curve plotted as a function of absorbance against concentration obtained from UV-vis SpectraMax M2.

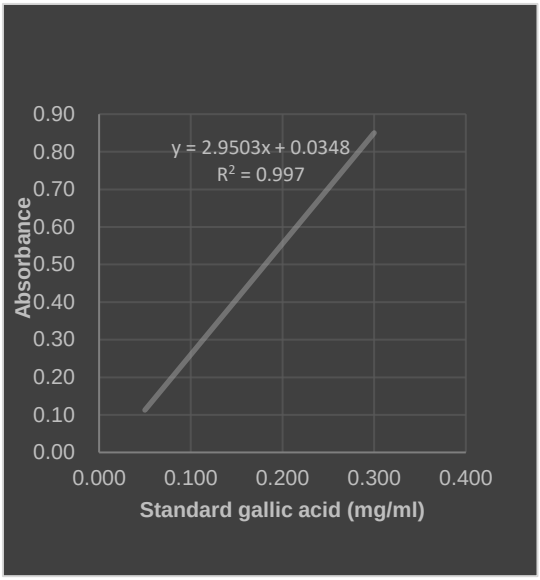

Figure 2: Absorbance recorded for Standard Gallic acid curve at 765nm

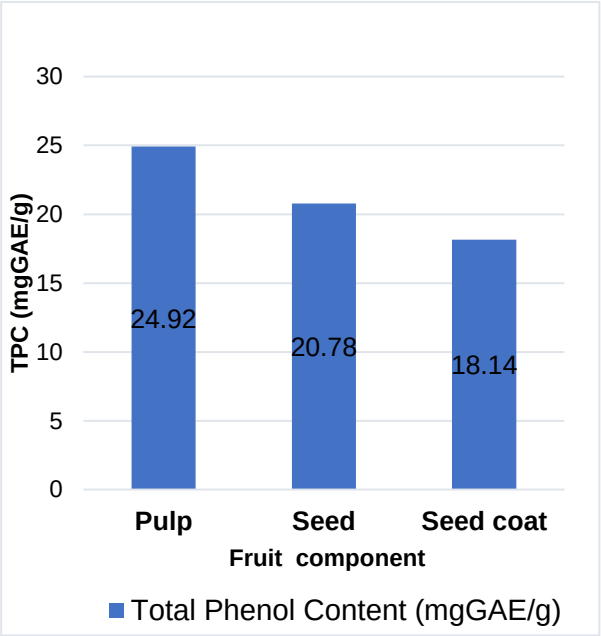

Figure 3: TPC in different components of *A. garckeana* fruit

Figure 2 of absorbance versus concentration obtained for standard gallic acid, y = 2.9503x + 0.0348 (R^2= 0.997) shows that the method for determining the concentration of polyphenols in A. garckeana fruit is linear over the concentrations examined. This is observed from the linear correlation coefficient "R^2" which has a value greater than 0.99, as allowed by RE No. 899 (Guideline 2005, Blainski, Lopes et al. 2013).

Figure 3 shows TPC test results obtained f from the linear equation shown in Figure 2, the TPC was estimated as concentrations of 24.92, 20.78 and 18.14mgGAE/g for the pulp, seed and the seed coat, respectively. The absorbance at the wavelength of 765nm was shown to increase with an increase in the concentration of the solutions. It can therefore be said that all sample solutions followed the Beer- Lambert's Law which states that absorption of light increases exponentially as the concentration of the absorbing substance increases arithmetically (Vogel and Bassett 1989)rom Figure 2 standard curve.

TPC values for the pulp extract (24.92mgGAE/g) were significantly greater than those obtained from the seed (20.78mgGAE/g) and the seed coat. (18.14mgGAE/g). This shows that the polyphenol content in the experimental samples varied according to the type of fruit constituent. Thus, the results clearly shows that the pulp is a rich source of polyphenolic compounds as compared to other fruit constituents. High TPC values might be due to greater extraction efficiency of the pulp extractives as proposed by Butnariu and Coradini (2012). TPC results reveal that there is direct relationship between the yield and the TPC values i.e. high fruit polarity resulted in high yield and TPC value. According to Temitope (2015), TPC is also proportional to number of chromophores within a specific compound, the higher the TPC value, the higher the number of chromophores contained by a given compound. This is because a large number of chromophores increases the absorbance of a solution thereby indirectly increasing the concentration of that particular compound (Abodunrin, Uhuegbu et al. 2015).

The different TPC values also show that the A. garckeana fruit constituents have different number of hydroxyl groups attached to the aromatic ring. This is because the Folin Ciocalteu method used shows a response proportional to the number of hydroxyl groups in the phenolic compounds, forming a blue complex that is proportional to the number of hydroxyl groups on the aromatic ring (Bueno, Machareth et al. 2012).

According to the reporting of several researches around the world on indigenous fruits, fruits are shown to contain different levels of TPC. Judprasong et al (2013) collected and studied several wild fruits for their phenolic contents and the fruit that gave the highest phenolic content was Phyllanthus emblica L. with (37.03mgGAE/g).

The TPC found in *A. garckeana* pulp (24.92mgGAE/g) is close to that found in avocado fruit (25.35mgGAE/g). Avocado fruit is known for its high phenolic content (Segovia, Hidalgo et al. 2018). This shows that all components of *A. garckeana* fruit have high TPC value and potential for commercial utilisation in the crosslinking of gelatin resin.

2.2.9 Functional groups in *A. garckeana* fruit pulp

FR-IR results prove that the *A. garckeana* fruit has hydroxyl groups, carbonyl and carboxylic groups.

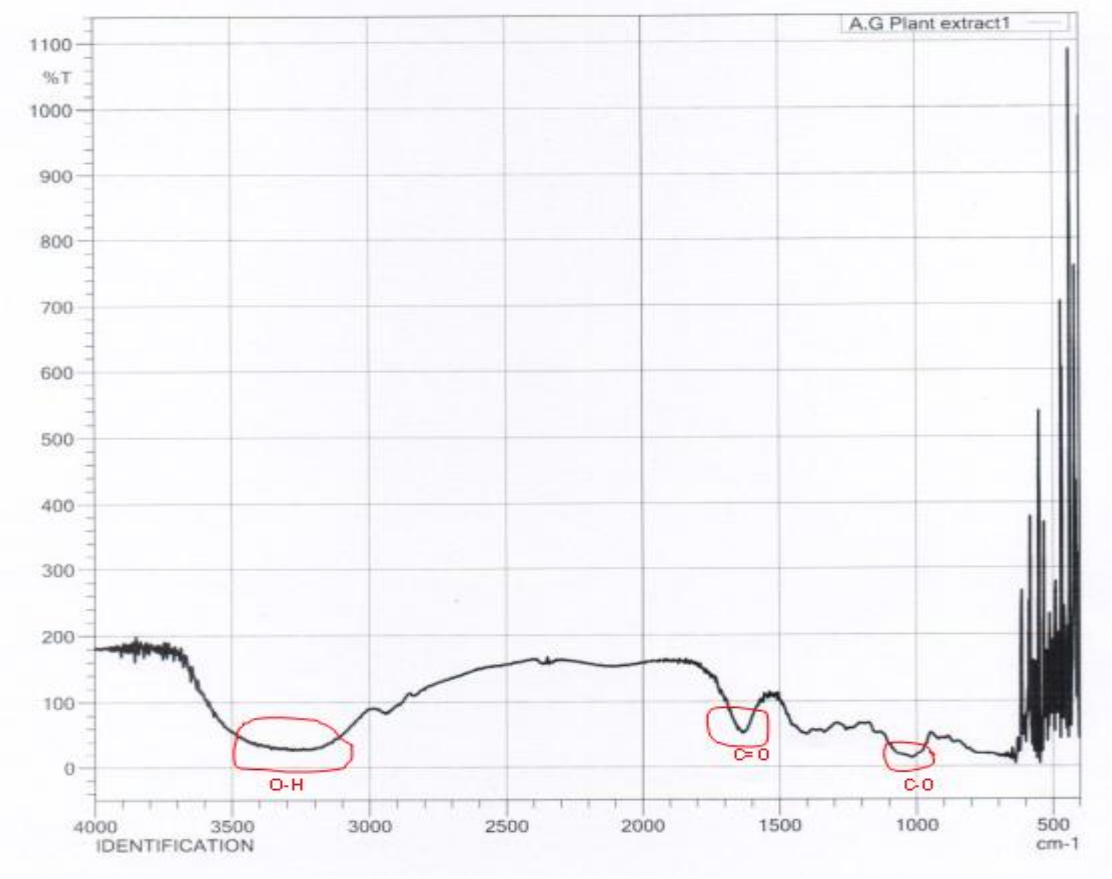

Figure 4: FT-IR Spectrum for A. garckeana fruit extract

Figure 4 shows *A. garckeana* FT-IR spectrum. Hydroxyl groups are shown between 3100cm^{-1}- 3600cm^{-1} whereas the carboxylic group are shown at 1650cm^{-1} and the carbonyl group are shown at 1000cm^{-1} (Amanac 2011). These results are therefore suggestive that these functional groups could most probably be those of phenolic compounds.

This indicates that the fruit can act as a suitable cross-linking agent for gelatin resin. Polyphenols from this research will not only provide a renewable resource but it will also reduces dependence on synthetic phenolics. Moreover, more comprehensive studies on other phytochemicals found in *A. garckeana* needs more exploration.

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