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A Comparative Study: The Effects Of Oxalic Acid In The Extraction And Isolation Of Ascorbic Acid (Vitamin C)

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Abstract

Ascorbic acid (Vitamin C) decomposes on exposure to heat, sensitive to light and is destroyed when exposed to atmospheric oxygen. Ascorbic acid was isolated from the aqueous extract of *Casuarina equisetifolia* (Australian pine needles) harvested from the main campus, university of Jos, Nigeria. Before extraction, the ascorbic acid content of the needles (leaves) was analysed to be (61.00±1.60) mg/100g. The extracts were uniformly divided into two portions. Oxalic acid was added to the first portion while the second portion contains only the extract. The first portion was labelled 1.0 and the second portion was 2.0. Isolation of the two samples was carried out gravimetrically and drying (lyophilisation) was carried out with a freeze dryer because Ascorbic acid decomposes at high temperature. The dried isolate was confirmed using UV-Vis, FTIR, GC-MS and H-NMR. The molar concentration of the ascorbic acid (0.02075±0.001, 0.02075±0.00025) mol/dm3 is the same for the extracts using the two methods after 7 days period. This shows that the oxalic acid has no reaction with the ascorbic acid; it creates no interference to the titration process. The weight percents of the lyophilized isolates are (68.04g/500g, 13.608%) and (45.42g/500g, 9.084%) for method 1.0 and 2.0 respectively. The UV-Vis determination gives weak peaks at 240nm and strong peaks at 300nm (λmax) for both methods. The FTIR spectra were analysed. For this research, only the OHs and the lactone C=O (around 1750 cm^{-1}) and C=C stretch (approximately 1680 cm-1) bands will be considered. Based on the results, these are observed only in the isolate 1.0; this may be due to the decomposition of the ascorbic acid in isolate 2.0. The four OH^- bands observed in the isolated ascorbic acid 1.0 can be assigned as follows: C(1) OH^- (3524.13, OH^- stretch, free hydroxyl (alcohol)), C(4) OH^- (3404.48,OH. stretch, H bonded (alcohol)), C(3) OH^- (3303.21, O-H stretch, H bonded (alcohol)), C(2) OH^- (3022.28, OH stretch (carboxylic acid)) and the lactone C=O (1751.98 cm-1) and C=C stretch (1654.63 cm-1) bands. The GC-MS indentified Ethane dioic, dimethyl ester (100%) which has a molecular formula $C_4H_6O_4$ (m/z118.0880) with base peak at m/z 8.0 for the method 1.0, with the possible loss of $C_2H_2O_2$ (Acetolactone, a transient species of mass spectrometer) due to the temperature of the system. The GC-MS also identified isopropyl alcohol, tetramethyl silicate, ethane-dioic, dimethyl ester, and dimethyl fumarate and dimethyl dimalate for the isolate 2.0. The presence of fatty acids and their derivative in an isolate shows the pharmacological properties of the isolate. This research revealed that oxalic acid protected the isolated ascorbic acid from total decomposition. There was partial decomposition from the confirmatory titrimetric analysis (56.88% ascorbic acid) and the GC-MS analysis of 100% ester of oxalic acid, a major decomposition product of ascorbic acid. The FTIR analysis confirms that the compound isolated is ascorbic acid and further confirmation derived from the UV-Vis. The H-NMR revealed the presence of the two essential protons (the most deshielded proton and the most acidic proton) in the structure of ascorbic acid when dissolved in methanol and deuterated DMSO and concentrated by sonication. The application of method 1.0 and 2.0 was carried out to optimize the necessary conditions for maximum productivity.

Keywords:* *Comparative studies; Temperature; Whistling pine; Spectroscopy; Classical analysis; Pine; Qualitative; Quantitative; Oxalic acid; Vitamin C; Ascorbic acid

Introduction

Oxalic acid is an organic compound with the formula $C_2H_2O_4$. It is a white crystalline solid that forms a colourless solution in water. Its condensed formula is HOOCCOOH, reflecting its classification as the simplest dicarboxylic acid. Its acid strength is

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much greater than that of acetic acid.

Ascorbic acid occurs in living tissues; animals and plants products such as citrus fruits, strawberries, hip bones, melons, vegetables, dairy products, meat etc. While plant foods are generally a good source of ascorbic acid, the amount in foods of plant origin depends on the variety of the plant, soil condition, climate where it grew, length of time since it was picked, storage conditions, and method of preparation. Animal-sourced foods do not provide much ascorbic acid, and the provided is largely destroyed by the heat of cooking. For example, raw chicken liver contains 17.9 mg/100 g, but fried, the content is reduced to 2.7 mg/100 g. Chicken eggs contain no Ascorbic acid, raw or cooked. Ascorbic acid is present in human infant milk at 5.0 mg/100 g and 6.1 mg/100 g in one tested sample of infant formula, but cow's milk contains about 1.0 mg/ 100 g. Vitamin C was discovered in 1912, isolated in 1928, and in 1933 was the first vitamin to be chemically produced [1]. It is on the World Health Organization Model List of Essential Medicines, which lists the most effective and safe medicines needed in a health system [2]. Vitamin C is available as an inexpensive generic and over-the-counter medication [3,4,5]. Partly for its discovery, Albert Szent-Györgyi and Walter Norman Haworth were awarded the 1937 Nobel Prizes in Physiology, Medicine and Chemistry, respectively [6]. Foods containing vitamin C include citrus fruits, kiwifruit, broccoli, Brussels sprouts, raw bell peppers, and strawberries. Prolonged storage or cooking may reduce vitamin C content in foods [7].

Ascorbic acid (Vitamin C) is a water-soluble vitamin, with dietary excesses not absorbed, and excesses in the blood rapidly excreted in the urine, so it exhibits remarkably low acute toxicity. More than two to three grams may cause indigestion, particularly when taken on an empty stomach. However, taking vitamin C in the form of sodium ascorbate and calcium ascorbate may minimize this effect. Other symptoms reported for large doses include nausea, abdominal cramps and diarrhoea. These effects are attributed to the osmotic effect of unabsorbed vitamin C passing through the intestine. In theory, high vitamin C intake may cause excessive absorption of iron. A summary of reviews of supplementation in healthy subjects did not report this problem but left as untested the possibility that individuals with hereditary hemochromatosis might be adversely affected [8].

The richest natural sources are fruits and vegetables. Ascorbic acid (Vitamin C) is the most widely taken nutritional supplement and is available in a variety of forms, including tablets, drink mixes, and in capsules [9].

Ascorbic acid (Vitamin C) chemically decomposes under certain conditions, many of which may occur during the cooking of food. Vitamin C concentrations in various food substances decrease with time in proportion to the temperature at which they are stored and cooking can reduce the vitamin C content of vegetables by around 60% possibly partly due to increased enzymatic destruction as it may be more significant at sub-boiling temperatures. Longer cooking times also add to this effect, as will copper food vessels, which catalyse the decomposition.

Another cause of vitamin C being lost from food is leaching, where the water-soluble vitamin dissolves into the cooking water, which is later poured away and not consumed. However, vitamin C does not leach in all vegetables at the same rate; research shows broccoli seems to retain more than any other. Research has also shown that freshly cut fruits do not lose significant nutrients when stored in the refrigerator for a few days.

The aim of this research is to investigate the effect of oxalic acid as a stabilizer during the extraction of Ascorbic acid. The main objective of this research is to optimize the production of pure analytical grade Ascorbic acid.

Materials and Methods

Sampling

The *Casuarina equisetifolia* (Australian pine or whistling pine was collected within the Bauchi road campus, University of Jos, within the month of July, 2019. The identification and authentication were carried out by the department of plant science and biotechnology, University of Jos.

The samples were air-dried for a period of three weeks after which fresh ones were obtained again for comparative study. The samples were collected during the flowering period, because it was expected that the vitamin content would be at its highest level at that time [10]. The fresh and dried samples were taken directly to the laboratory and analysis was done immediately upon arrival.

Materials and Reagents

All reagents used were of analytical grade, the apparatus and equipments were in good condition. Before the extraction procedure, all the samples were thoroughly washed with deionized water to remove any adhering contaminants if present. The fresh and dried pine needles were then air dried to remove the water from the washing.

Extraction of Casuarina equisetifolia needles juice

Method 1.0: The fresh pine needles were accurately weighed (500 g) and blended in an electric blender with the addition of 0.5 g oxalic acid and 100 cm^3 of 0.05 M sulphuric acid. The mixture was further ground and strained through four layers of muslin cloth to have a Casuarina equisetifolia juice.

Method 2.0: The fresh pine needles were accurately weighed (500 g) and blended in an electric blender with the addition of 100 cm^3 of 0.05 M sulphuric acid. The mixture was further ground and strained through four layers of muslin cloth to have a *Casuarina equisetifolia* juice.

Note: The extracted juices were analyzed via iodometric analysis to confirm the presence of ascorbic acid before isolation.

Isolation of ascorbic acid from the Casuarina equisetifolia needles juice

Gravimetric analysis was applied for the isolation of the two extracts [11].

Sample analysis

Preparation of 0.07 M Sodium thiosulphate pentahydrate

A 200 cm^3 solution of 0.07 M sodium thiosulphate ($Na_2S_2O_3$) was prepared by weighing out 3.4375 g of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$), adding 100 cm^3 of room temperature deionized water, which had been bubbled with nitrogen gas and contained 100 mg/L Na2CO3, and a single drop of chloroform was added to prevent any bacterial growth [12].

Preparation of 10% starch indicator

A 10 g of the May & Baker, UK starch was added to 100 cm^3 of distilled water. The solution was warmed gently with constant stirring on a heating mantle at a temperature of 500 C. The solution is left to cool after homogenizing [12].

Preparation of 0.01 M Iodine solution

A 0.01 M solution of potassium iodate (KIO_3) was made by weighing out 0.1960 g of dry KIO^3 and then dissolving it with 100 cm^3 of water inside a 100 cm^3 volumetric flask. This solution was then transferred into a clean 100 cm^3 glass bottle and labelled [12].

Preparation of 0.03 M Sulphuric acid

17 cm^3 of concentrated sulphuric acid was diluted to 1000 cm^3.

Standardization of the sodium thiosulphate

A 5 cm^3 of KIO_3 was pipette with a 5 cm^3 pipette into three 125 cm^3 Erlenmeyer flasks where each flask was treated separately. Around 0.52 g of solid potassium iodide (KI) was added into each flask along with 2.5 cm^3 of 1.5 M sulfuric acid (H_2SO_4) and then titrated with $Na_2S_2O_3$ solution and 10-100 L and 100-1000 L micropipettes were used to carry this out. When the color of the solution turned pale yellow, the titration was stopped and 1 cm^3 of starch indicator was added to give a dark blue color which was

then titrated away to give a clear solution, which was the end-point. This procedure was repeated for the other two flasks and an average of the concentration was calculated [12].

Determination of ascorbic acid from the plant samples

The analysis employed is iodometric titration. 10 g each of fresh and dried leaves were weighed into separate mortars and 30 cm^3 of 0.03 M sulphuric acid, 20 cm^3 deionized water and 0.5 g oxalic acid were added. The mixtures were stirred for about 20 minutes and rapidly filtered using a suction pump and Buchner funnel. 25 cm^3 of the filtrates were quickly titrated to the endpoint with the standardized 0.01 M iodine solution using 10% starch indicator. The titrations were repeated in triplicates and blank determinations were also carried out following the above procedure but using 25 cm^3 of deionized water instead of the filtrate [13].

Determination of percentage ascorbic acid from the lyophilized samples

The analysis employed is iodometric titration. 0.05 g each of the lyophilized samples was weighed into separate beakers and 30 cm3 of 0.03 M sulphuric acid, 20cm3deionized water and 0.5 g oxalic acid were added. The mixtures were stirred for about 20 minutes. 25 cm^3 of the filtrates were quickly titrated to the endpoint with the standardized 0.01 M iodine solution using 10% starch indicator. The titrations were repeated in triplicates and blank determinations were also carried out following the above procedure but using 25 cm^3 of deionized water instead of the filtrate [13].

UV-VIS Spectrophotometric determination

The UV-Vis absorption spectrum is related to molecular structure; therefore it provides qualitative information as to structure both from ε and λ. In order to take advantage of this it is desirable to have a scanning instrument to measure the entire spectrum (either a double beam, direct reading instrument or a single beam unit with computer interface. By itself an electronic spectrum is not sufficient to identify a compound.

S3000 Fiber Optic Spectrometer (Race-Technology Co., Ltd, Hangzhou, China) equipped with a 3648-element linear silicon CCD array detector (Toshiba TCD 1305), a Y-type optical fiber probe with 100 cm in length and 0.4 mm in diameter, a light source (Oceans Optics Inc., USA) and a home-made sample cell made from dark gray PVC, was used to measure the UV-vis-SWNIR diffuse reflectance spectra within the wavelength region of 200-1100 nm.

Ultraviolet-visible spectra were measured under the following conditions: in the range from 200 to 700 nm, integral time of 397 s and a resolution of 0.29 nm, with a spectralon as background reference. Each ascorbic acid sample of 0.18 g was diluted in distilled water and filled in the sample cell, pressed by free fall impacts of a round rod from a same height. Then the optical probe was placed vertically on the upper surface of the sample [14].

FTIR Spectrophotometric determination

The IR analysis was performed in a Spectrum Two FTIR spectrometer (Perkin Elmer, USA) by using circular KBr cell window, 0.05 mm round Teflon spacers, Spectrum 10 software (Perkin Elmer, USA), Homogenizer (Trumark, India).

An accurately weighed quantity of CPX HCl was dissolved in small quantity of phosphate buffer pH 6.0 and the final volume was made up to 100 cm^3 with methanol as solvent to get 500 µg/cm3 concentration of standard stock solution. Various working concentrations were made by further dilution with same medium.

Gas chromatography spectroscopic analysis

Quantification of different pesticides in soils and tuber extracts was accomplished by Perkin Elmer Autosystem Gas Chromatography equipped with ECD under the parameters; Column: 17 m methyl 10 and phenyl silicone 0.32 mm ID, 0.5 µm

film thickness. Injector temperature: 2200°C Detector temp.: 350°C, Detector makeup, Nitrogen: flow 33.3 cm^3/min, Oven: Temp. 80°C, initial time 0 min. Rate 20°C/min. Final temp. 280°C stay for 0 min, final time 10 min. Injection:1µl splitless, integration: peak height was used for quantitation [15].

H-NMR spectroscopic determination

Determination of the protons in isolate 1.0 and 2.0 was accomplished by NMR: 400 MHz, Avance III 400 Bruker. The solvents used are methanol and deuterated DMSO.

Results

TABLE.1**. Molar concentration of ascorbic acid in the pine juice**

	Method 1.0	**Method 2.0**
Ascorbic acid (mol/dm^3)	0.02075±0.001	0.02075±0.00025
Values are means ±standard deviation of triplicate analyses		

TABLE. 2. **Weight and percentage Ascorbic acid of isolate 1.0 and 2.0 obtained after lyophilization (Freeze drying)**

	Weight (g/500g)	**Percentage (%)**
Method 1.0	68.04	13.608
Method 2.0	45.42	9.084

TABLE.3. **Determination of percentage ascorbic acid present in the isolates**

	Method 1.0	**Method 2.0**
Ascorbic acid (%)	56.88±0.48	11.76±0.45
Values are means ±standard deviation of triplicate analyses		

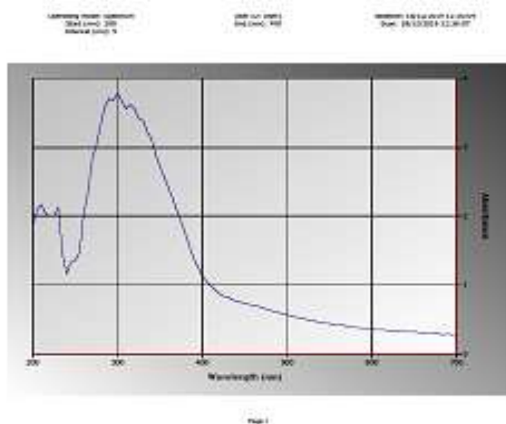

FIG. 1. **The UV-Visible spectrum of Isolate 1.0**

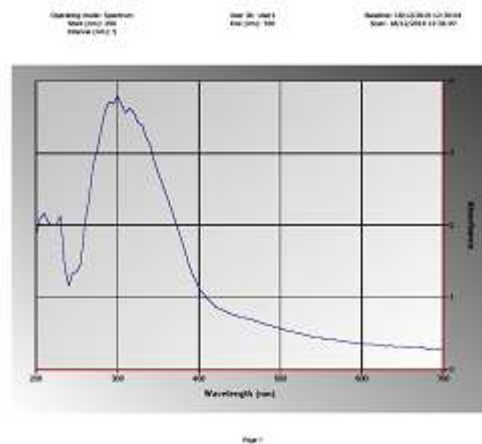

FIG. 2. **The UV-Visible spectrum of isolate 2.0**

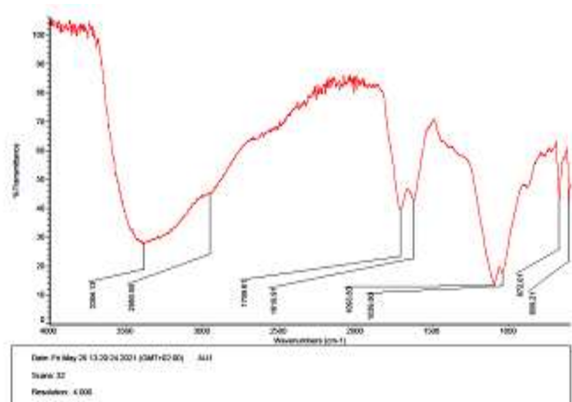

FIG. 3. **The FT-IR spectrum of Isolate 1.0**

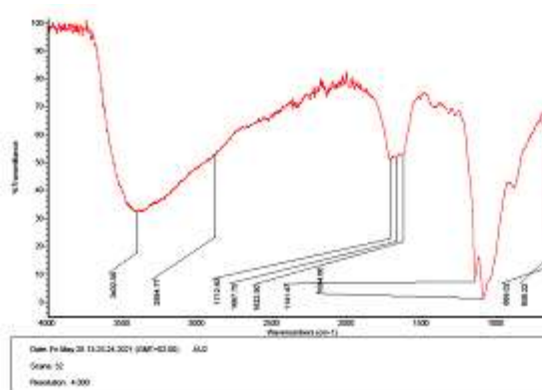

FIG. 4. **The FT-IR spectrum of isolate 2.0**

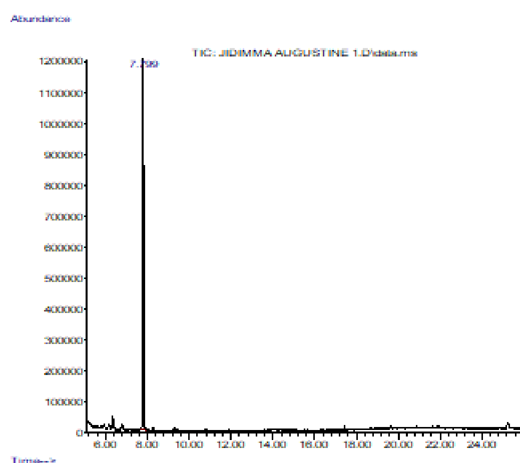

FIG. 5. **The Gas chromatogram of Isolate 1.0**

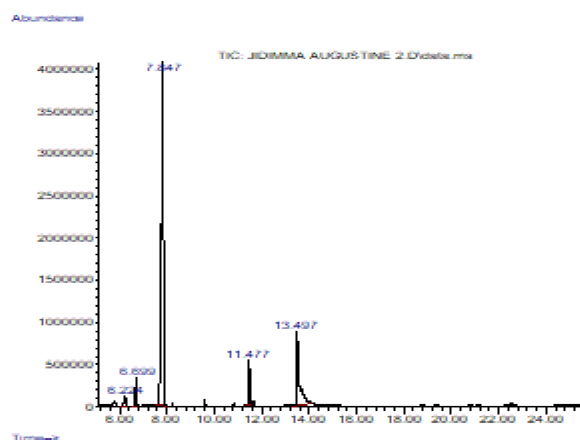

FIG. 6. **The Gas chromatogram spectrum of isolate 2.0**

TABLE. 4. **H-NMR fractions of isolate 1.0 in methanol and deuterated DMSO (Dimethyl sulphoxide)**

Protons	Methanol				Deuterated DMSO			
	peak (ppm)	scale	shift	ppm (Hz)	peak (ppm)	scale	shift	ppm(Hz)
1	3.4	0.25	-0.06	-22.97	4.99	1.42	0.0014	0.56
2	2.5				3.4			

TABLE. 5. **H-NMR fractions of isolate 2.0 in deuterated methanol and deuterated DMSO (Dimethyl sulphoxide)**

Protons	Methanol				Deuterated DMSO			
	peak (ppm)	scale	shift	ppm (Hz)	peak (ppm)	scale	shift	ppm(Hz)
1	3.4	0.35	-0.06	-30.29	4.99	0.35	0.0014	0.56
2	2.5				3.4			

Discussion

TABLE 1 is the molar concentration of ascorbic acid in the pine juice prepared using method 1.0 and method 2.0 respectively. Method 1.0 requires the addition of 0.5g oxalic acid to the *Casuarina equiselifolia* needles juice, while method 2.0 does not require the addition of oxalic acid to the needles juice, all other procedure remains the same. This is carried out to optimize the necessary conditions for maximum productivity. From this table, the molar concentration of the ascorbic acid (0.02075±0.001, 0.02075±0.00025) mol/dm^3 is the same for the method 1.0 and 2.0 respectively. This shows that the oxalic acid has no reaction with the ascorbic acid; it creates no interference to the titration process.

TABLE 2 is weight of isolated Ascorbic acid obtained after Lyophilization (Freeze drying). The result shows that method one's weight to percentage of ascorbic acid obtained via iodometric titration is (68.04g/500g, 13.608%) compared with the method 2.0 (45.42g/500g, 9.084%). Ascorbic acid decomposes on exposure to heat, light and other external conditions. Oxalic acid helps in maintaining the structure of the ascorbic acid during the decomposition [16]. This may be the reason behind method 1.0 higher weight to percentage isolated ascorbic acid in comparison to method 2.0.

Table 3 shows the percentage of the ascorbic acid present in the dried isolate. From the result, method 1.0 was 56.88±0.48% while method 2.0 was 11.76±0.45%. Therefore, method 1.0 produced more ascorbic acid than method 2.0.

FIG 1 and 2 shows the electronic transitions of isolates in water using a UV-Visible spectrophotometer (Start:200, End:700, Interval:5). The determination gives peaks at 240 nm and 300 nm (λ max) for the two methods respectively. The differences are on the peak and trough absorbance. Method 1.0, (240nm, 1.260; 300nm, 3.675) and method 2.0, (240nm, 1.164; 300 nm, 3.794) respectively. When UV spectra of ascorbic acid in distilled water have been recorded, all of the compounds exhibit weak absorption band at about 245nm and a strong absorption band at 300nm, the strong absorption band is due to $\Pi - \Pi^*$ excitation of the C = C double bond. Again, this indicates the existence of $C_{(2)} = C_{(3)}$ double bonds.

The Infrared spectra, frequencies (cm^{-1}) and tentative assignment of isolated ascorbic acid 1.0 and 2.0 are shown in **FIG 3** and 4. The assignments proposed for the four O-H stretching bonds above 3000cm-1 are contradiction [17]. This is because of the importance and relevance of the O-H groups. For the present discussion, only the OHs and the lactone C=O (around 1750 cm^{-1}) and C=C stretch (approximately 1680 cm^{-1}) bands

will be considered. Based on the results, these are observed only in the isolated ascorbic acid 1.0, this may be due to the decomposition of the ascorbic acid isolated at method 2.0. The four O-H bands observed in the isolated ascorbic acid 1.0 can be assigned as follows: $C_{(1)}OH^-$ (3524.13, O-H stretch, free hydroxyl (alcohol)), $C_{(4)}OH^-$ (3404.48,O-H stretch, H bonded (alcohol)), $C_{(3)}OH^-$ (3303.21, O-H stretch, H bonded (alcohol)), $C_{(2)}OH^-$ (3022.28, OH stretch (carboxylic acid)) and the lactone C=O (1751.98 cm-1) and C=C stretch (1654.63 cm-1) bands. It is now clear that FT-IR spectrometry is capable of direct determination

of vitamin C in several formulations. The method is simple, precise and not consuming compared to the other chromatographic methods [16].

FIG 5 shows the Gas chromatogram and fractions of isolate 1.0. The isolated ascorbic acid showed only one peak from the chromatogram of the isolate. The presence of fatty acids and their derivative in an isolate shows the pharmaceutical properties of the isolate. Fatty acid and alcohols in the isolate undergo esterification to form esters [17]. This compound was identified as Ethane dioic, dimethyl ester and has a molecular formula $C_4H_6O_4$ with a retention time of 7.799 min, which was due to a possible loss of $C_2H_2O_2$ (Acetolactone), a transient specie of gas chromatography, it comprises 100% of the extract. Many fatty acids are known to have antibacterial and antifungal properties [18]. Ethanedioic, dimethyl ester is used as an additive and intermediate for plastics, pigments, pharmaceuticals and agricultural products. It is also an intermediate for the production of paints, adhesives and copolymers [18].

FIG 6 shows the Gas chromatogram and fractions of isolate 2.0. The isolate showed five peaks from the chromatogram of the isolate. The presence of fatty acids and their derivative in an isolate shows the pharmacological properties of the isolate. Fatty acid and alcohols in the isolate undergo esterification to form esters (Akinmoladum et al., 2007). Compound 1 was identified as Isopropyl alcohol and has a molecular formula C_3H_8O with a retention time of 6.224 min, it comprises of 2.29% of the isolate. Compound 2 was identified as Tetramethyl silicate and has a molecular formula $SiC_4H_{12}O_4$ with a retention time of 6.699 min, it comprises of 3.90% of the isolate. Compound 3 was identified as Ethanedioic, dimethyl ester and has a molecular formula $C_6H_6O_4$ with a retention time of 7.347 min, it comprises of 67.48% of the isolate. Compound 4 was identified as Dimethyl fumarate and has a molecular formula $C_6H_8O_4$ with a retention time of 11.477 min, it comprises of 4.60% of the isolate and Compound 5 was identified as Dimethyl dimalate and has a molecular formula $C_8H_{18}O_5$ with a retention time of 13.497 min, it comprises of 21.74% of the isolate. Therefore from the result, the most abundant compound present in the isolate is Ethanedioic, dimethyl ester with percentage area of 67.48% followed by Dimethyl dimalate with a percentage area of 21.74%. Dimethyl fumarate is an anti-inflammatory; it is indicated for multiple sclerosis patients with relapsing forms and is also being investigated for the treatment of psoriasis. Dimethyl dimalate has inhibitory role of neutrophils on influenza virus multiplication in the lungs of mice. Ethanedioic, dimethyl ester is used as an additive and intermediate for plastics, pigments, pharmaceuticals and agricultural products. It is also an intermediate for the production of paints, adhesives and copolymers [18]. Ascorbic acid may protect against the oxidative damage of light in the eye and may also play an important role in sperm maturation [19]. It helps in stabilizing plasma components and has been shown to be an effective scavenger of super-oxide radical anion (H_2O_2), the hydroxyl radical (OH), singlet oxygen (O) and reactive nitrogen oxide (NO) [19].

TABLE 4,5 revealed the presence of the two essential protons (the most deshielded proton and the most acidic proton) in the structure of ascorbic acid when dissolved in deuterated methanol and deuterated DMSO and concentrated by sonication. Isolate 1.0 in methanol at a chemical shift of -0.0574 shows distinct peaks at 3.40 for the most deshielded proton and 2.50 for the most acidic proton while isolate 2.0 in deuterated methanol at a chemical shift of 0.0574 shows similar distinct peaks at 3.40 for the most deshielded proton and 2.50 for the most acidic proton. In deuterated DMSO (Dimethyl sulphoxide), at a chemical shift of 0.0014, isolate 1.0 shows distinct peaks at 4.99 for the most deshielded proton and 3.40 for the most acidic proton while isolate 2.0 in deuterated DMSO at a chemical shift of -0.0765 shows similar distinct peaks at 4.99 for the most deshielded proton and 3.40 for the most acidic proton. Ascorbic acid is slightly soluble in alcohol (20 g/cm3). This may be the reason why the peaks in deuterated DMSO are more deshielded while a higher chemical shifts than the peaks in deuterated methanol. The solubility of ascorbic acid in water is 330 g/cm3.

Conclusion

This research revealed that oxalic acid protected the isolated ascorbic acid from total decomposition. There was partial

decomposition from the confirmatory titrimetric analysis (56.88% ascorbic acid) and the GC-MS analysis of 100% ester of oxalic acid, a major decomposition product of ascorbic acid. The FTIR analysis confirms that the compound isolated is ascorbic acid and further confirmation derived from the UV-Vis. The proximate analysis of the *Casuarina equisetifolia* needles was carried out to confirm some level of nutritional composition of the leaves. Although, method 2.0 produced some decomposition products with high pharmaceutical importance (Dimethyl fumarate is an anti-inflammatory agent and it is indicated for multiple sclerosis patients with relapsing forms and is also being investigated for the treatment of psoriasis and dimethyl dimalate has inhibitory role of neutrophils on influenza virus multiplication in the lungs of mice [20]. The application of method 1.0 and 2.0 was carried out to optimize the necessary conditions for maximum productivity.

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