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EXTRACTION AND ISOLATION OF ASCORBIC ACID FROM AUSTRALIAN PINE (*Casuarina equisetifolia*) NEEDLES GROWN IN JOS AND ENVIRONS.

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ABSTRACT

Ascorbic acid was extracted and isolated from the aqueous extract of *Casuarina equisetifolia* (Australian pine needles) harvested from the main campus, university of Jos, Nigeria. The extraction was carried out via cold extraction (maceration) while the isolation was done using gravimetric analysis. The isolated sample was identified using UV-Vis, FTIR, GC-MS and H-NMR spectrophotometric analysis. Fresh needles (leaves) of *Casuarina equiselifolia* which contains 61.00±1.6 mg/100g of ascorbic acid was used for this research when compared with related species within same environment. The proximate composition is moisture content (10.27±0.48) %, crude protein (5.98±0.20) %, Crude fibre (22.89±0.35) %, crude fat (3.72±0.24) %, ash (2.34±0.07) %, carbohydrate (54.81±0.27) % and ascorbic acid (61.00±1.60) mg/100g. The molar concentration of ascorbic acid in the sample was (0.02075±0.001) moldm^{-3} while the weight percent of the lyophilized isolate is (68.04g/500g, 13.608%). The UV-Vis determination gives peaks at 240nm (weak band) and 300nm (λ_{max}). The FTIR spectrophotometric determination gave some distinctive peaks. For this research, only the OH$_s$, the lactone C=O (around 1750 cm^{-1}) and C=C stretch (approximately 1680 cm^{-1}) bands will be considered. 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. 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, with the possible loss of $C_2H_2O_2$ (Acetolactone), a transient species of mass spectrometer. The H-NMR in methanol and deuterated DMSO (Dimethyl sulphoxide) identified the presence of the most deshielded proton and the most acidic proton. There was partial decomposition from the confirmatory titrimetric analysis (56.88% ascorbic acid). The FTIR analysis confirms that the compound isolated is ascorbic acid and further confirmation derived from the UV-Vis. The result of this research should be adopted for the mass production of high quality ascorbic acid in Nigeria.

Keywords: Pine, *Casuarina equisetifolia,* Extraction, Isolation, Optimization, Spectrophotometer, Ascorbic acid, Decomposition, vitamin C, DMSO, H-NMR

INTRODUCTION

Humans are not able to synthesize ascorbic acid (vitamin C). Goats, like many animals make their own ascorbic acid. An adult goat, weighing approximately 70 kg, will manufacture more than 13,300 mg of ascorbic acid per day in normal health, and levels many fold higher when faced with stress. An acute, chronic illness caused by a dietary deficiency of this vitamin is known as scurvy (scorbut), which repeatedly causes morbidity and death [1]. Historically, during the second voyage (1535-1536) of Jacques Cartier the sickness began to spread in all three of his ships, and of 110 crewmembers, 8 were dead and about 50 past all hope of recovery. For the cure of scurvy, they used *Iroquois decoction* from bark and leaves of the "tree of life" known later as an "*Annedda*". They also learned from Indians to cure the scurvy using pine needles and bark [2, 3]. Afterwards, the name of Annedda was narrowed down to different *Pinus* and *Picea* species as well as to *Juniperus communis, Tsuga canadensis, and Thuja occidentalis*, among others. The successful cure for scurvy was very significant for future naval explorations [2]. During Swedish-Russian War in the 18th Century, almost all Swedish soldiers were severely affected by scurvy, after drinking a solution prepared from pine needles their health was restored. During the 19th and 20th Centuries the young shoots of pine and spruce were used in Canada, Norway, and Russia against scurvy [3].

Pine trees are evergreen, coniferous resinous trees (or, rarely, shrubs) growing 3–80 m (10–260 ft) tall, with the majority of species reaching 15–45 m (50–150 ft) tall. The smallest are Siberian dwarf pine and *Potosi pinyon*, and the tallest is an 81.79 m (268.35 ft) tall ponderosa pine located in southern Oregon's Rogue River-Siskiyou National Forest. The parts of a pine tree include bark, foliage, cones etc. The pine tree is used for lumber and construction, ornamental, wildlife, farming and for food and nutrients [4].

Casuarina equisetifolia (Australian/Whistling Pine) is known around the world. Some of the more common names include: Australian pine, beefwood, casuarina, horsetail casuarina, she-oak, beach she-oak and ironwood. *Casuarina equisetifolia* is in the family, *Casuarinacea* (She-oak). *Casuarinaceae* comprises 4 genera and 90 species from tropical, subtropical dry regions, and warm temperate areas. It is assigned to the *Casuarina* genus [5]. The family is native to Southeast Asia, southern Pacific Islands to Tahiti and Samoa, and Australia. The genus *Casuarina* contains 17 species [6]. The botanical name *Casuarina* is derived from the resemblance of the branchlets to the feathers of the Cassowary [7]. The specific name *equisetifolia* is derived from the resemblance of the needles to horse hair.

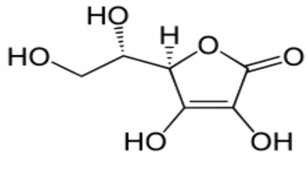


Figure 1: Ascorbic acid

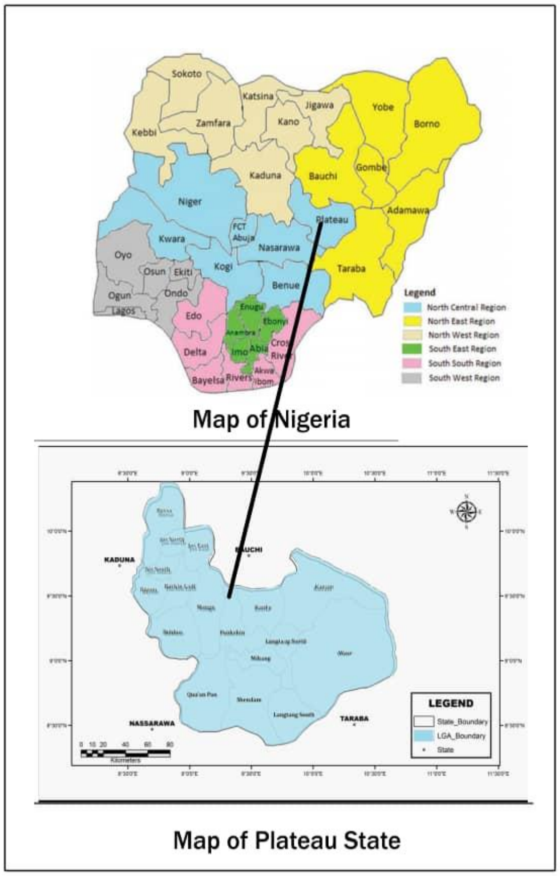
Ascorbic acid (L-ascorbic acid), also known as vitamin C, is a vitamin found in various foods and sold as a dietary supplement. It is used to Ascorbic acid (Vitamin C) was discovered in 1912, isolated in 1928, and in 1933 was the first vitamin to be chemically produced [8]. Prolonged storage or cooking may reduce vitamin C content in foods [9].

The aim of this research is to extract and isolate ascorbic acid (vitamin C) from *Casuarina equisetifolia* (Australian pine needles).

MATERIALS AND METHODS

Study Area

The area studied is the Main Campus of the University of Jos, Plateau state, Nigeria. It is located at 9.9496°31’N and 8.8895°E, Jos North Local Government Area in Plateau state Nigeria.



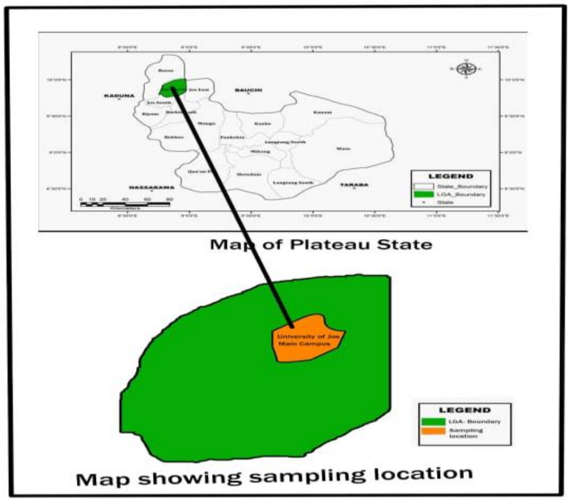


Figure 2**:** Map of Jos Showing Study Areas and Sampling Location.

Materials and Reagents

The materials used for this research were of standard quality and the reagents are of analytical grade (90-98% purity).

Sampling

The *Casuarina equisetifolia* (Australian pine or whistling pine), *Thuja occidentalis* (Northern white cedar) and *Picea glauca* (White spruce), three conifers were collected within the Bauchi Road Campus, University of Jos, in 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.

Sample preparation

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.

Determination of ascorbic acid from the plant samples

The analysis employed was iodometric titration. Fresh and dried leaves (10 g each) 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].

Proximate analysis

The moisture, ash, crude fats, proteins and carbohydrates of all the samples were determined using [11]. The moisture and ash were determined using weight difference method. Crude fat was extracted by means of the Soxhlet apparatus with petroleum ether (40 to 60° C) for 8 hours. Crude fibers were done by successive digestion of the defatted samples with 1.25% sulphuric acid and 1.25% sodium hydroxide solutions. The nitrogen value, which is the precursor for protein of a substance, were determined by micro Kjeldahl method described by [12], involving digestions, distillation and finally titration of the sample. The nitrogen value was converted to protein by multiplying a factor of 6.25. Carbohydrate was determined by difference method. The carbohydrate was calculated by difference.

Sample extraction

Sample extraction for ascorbic acid content determination

The samples were air-dried for a period of three weeks and fresh ones were also obtained. 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 samples are taken directly to the laboratory and analysis was done immediately upon arrival.

Extraction of *Casuarina equisetifolia* needles juice

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.

The *Casuarina equisetifolia* juice was first centrifuged to produce about 650 cm^3 of the juice; the residue is pressed slowly using a muslin cloth to recover about 150 cm^3 of juice which was then combined with the previous amount. Ethanol (50 cm^3) was added to the juice and left to macerate overnight. After filtration, the solvent was evaporated using a vacuum rotary evaporator at 30^o C. It was made basic by adding equal calcium carbonate and calcium oxide with constant magnetic stirring until a slightly basic pH (8.0-8.5) was reached. The solution becomes progressively milky and a mixture considered to be rich in calcium ascorbate was precipitated. The process was accelerated by adding 600 cm^3 acetone. It was then filtered under a vacuum in a Buchner funnel to yield a yellowish green precipitate. Infrared spectroscopic analysis showed that ascorbic acid of the juice remains in solution as calcium ascorbate, which was then recovered by concentrating the aqueous solution by adding excess calcium chloride which promotes the precipitation of the ascorbate by the common ion effect. The precipitate is stirred in water and 1 N sulphuric acid was dropped in, magnetic stirring being continued to a pH of 2.3-2.8. The mixture was left to settle for a few minutes and the pH was rechecked. The calcium sulphate which has been formed was filtered and the amber filtrate was evaporated using a freeze dryer. The lyophilized sample was obtained and subjected to quantitative and qualitative analysis. This product rich in vitamin C contains other natural organic acids, particularly malic acid, sulphate ion and other impurities. The isolated ascorbic acid was lyophilized (freeze dried) using a lyodry vacuum freeze dryer.

Determination of percentage ascorbic acid from the lyophilized samples

The analysis employed was iodometric titration. The lyophilized samples (0.05 g each) were weighed into separate beakers and 30 cm^3 of 0.03 M sulphuric acid, 20cm^3deionized 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].

RESULTS AND DISCUSSION

Plate 1: The Ascorbic acid before and after lyophilization.

Plate 1 shows the isolated ascorbic acids using method 1.0 and 2.0, before and after lyophilization (freeze drying).

Isolation of ascorbic acid from the *Casuarina equisetifolia* needles juice

Lyophilization is a low temperature dehydration process that involves freezing the product, lowering pressure, and then removing the ice by sublimation. This is in contrast to dehydration by most conventional methods that evaporate water using heat. This is usually carried out on biological samples, pharmaceutical products, foods that other products that decomposes on exposure to heat.

Table 1: Ascorbic acid content of fresh and dried leaves of the *Casuarina equiselifolia* (Australian pine or whistling pine), *Thuja occidentalis* (Northern white cedar) and *Picea glauca* (White spruce) compared with the standard l-ascorbic acid (300mg).

S/No	Samples	Molar conc. (mol/dm^3)	Ascorbic acid content(mg /10g) of sample
1	l-ascorbic acid (Merck, Germany) - Standard	0.00163	287.43±0.85
2	Fresh leaves of *Casuarina equiselifolia*	0.0000352	6.10±0.16
3	Dried leaves of *Casuarina equiselifolia*	0.0000172	3.09±0.30
4	Fresh leaves of *Picea glauca*	0.000180	3.33±0.14
5	Dried leaves of *Picea glauca*	0.0000155	2.74±0.18
6	Fresh leaves of *Thuja occidentalis*	0.0000155	2.67±0.28
7	Dried leaves of *Thuja occidentalis*	0.0000155	2.57±0.23

Values are means ±standard deviation of triplicate analyses

Table 2 shows the results obtained from the study of the ascorbic acid content of conifers around university of Jos, Main campus, Jos. This is the ascorbic acid content of fresh and dried leaves of the *Casuarina equiselifolia* (Australian pine or whistling pine), *Thuja occidentalis* (Northern white cedar) and *Picea glauca* (White spruce) compared with the standard l-ascorbic acid (300mg).The arrangement of the six samples under study based on decreasing ascorbic acid concentration is:

Fresh *Casuarina equiselifolia*> Fresh *Picea glauca*> Dried *Casuarina equiselifolia*> Dried *Picea glauca*> Fresh *Thuja occidentalis*> Dried *Thuja occidentalis*

Based on the arrangement above, Fresh needles (leaves) of *Casuarina equiselifolia* which contains 61.00±1.6 mg/100g of ascorbic acid will be used for this research.

Table 2: Proximate composition and ascorbic acid content of *Casuarina equisetifolia* needles

S/No	Parameters	Values (Mean±SD)	Bello *et al.*, (2015)
1	Moisture content (%)	10.27±0.48	8.24±0.16
2	Crude protein (%)	5.98±0.20	6.61±0.67
3	Crude fiber (%)	22.89±0.35	21.85±0.2
4	Crude fat (%)	3.72±0.24	4.42±0.20
5	Ash (%)	2.34±0.07	2.25±0.28
6	Carbohydrate (%)	54.81±0.27	56.64±0.35
7	Ascorbic acid (mg/100g)	61.00±1.60	34.51±0.11

mean±SD for triplicate determination

Table 2 is the proximate composition of the *Casuarina equiselifolia* needles in comparison with the work of [14]. The values for the moisture content (10.27±0.48, 8.24±0.16) %, crude protein (5.98±0.20, 6.61±0.67) %, Crude fiber (22.89±0.35, 21.85±0.2) %, crude fat (3.72±0.24, 4.42±0.20) %, ash (2.34±0.07, 2.25±0.28) %, carbohydrate (54.81±0.27, 56.64±0.35) % and ascorbic acid (61.00±1.60, 34.51±0.11) mg/100g for this research and [14] respectively. There is no variation in the results, with the exception of the ascorbic acid content which is almost twice the content analyzed by [14]. This variation may be due to the time of sampling. Ascorbic acid is said to decompose on exposure to heat and light, the sampling for this research was carried out in the early hours of morning, 06:55 am to be precise.

Table 3: Molar concentration, weight percentage of ascorbic acid in the pine juice after lyophilisation and percentage ascorbic acid in the isolated ascorbic acid

	Molar concentration	Weight percent	% AA in isolated
Ascorbic acid (mol/dm^3)	0.02075±0.001	68.04 (13.608%)	56.88±0.48%

Values are means ±standard deviation of triplicate analyses

Table 3 is the molar concentration, weight percent of ascorbic acid in the pine juice and percentage of ascorbic acid in the isolated ascorbic acid. From this table, the molar concentration of the ascorbic acid is (0.02075±0.001, 0.02075±0.00025), this shows that the oxalic acid has no reaction with the ascorbic acid; it creates no interference to the titration process. The result also shows that the weight percent ascorbic acid in the juice after lyophilisation is (68.04g/500g, 13.608%). The percentage ascorbic acid in the isolated ascorbic acid was analysed titrimetrically via iodometric titration, this shows that the method is very efficient for the extraction and isolation of ascorbic acid.

Table 4: Electronic transitions of isolated ascorbic acid in water, using a UV-Visible spectrophotometer (Start: 200, End: 7.0, Interval: 5)

Turning points

	Wavelength (nm)	Peak (absorbance)	Trough (absorbance)
Method 1.0	240		1.260
	300 (λ_{max})	3.675	

Table 4 shows the electronic transitions of isolated ascorbic acid in water using a UV-Visible spectrophotometer (Start: 200, End: 700, Interval: 5). the determination gives peaks at 240nm. 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.

Table 5: Infrared frequencies (cm^{-1}) and tentative assignments of isolated ascorbic acid

S/No	Frequencies (cm^{-1})	Tentative assignments
1	3524.13	O-H stretch, free hydroxyl (alcohol)
2	3404.48	O-H stretch, H bonded (alcohol)
3	3303.21	O-H stretch, H bonded (alcohol)
4	3022.28	O-H stretch (carboxylic acid)
5	1751.98	C-O stretch (esters)
6	1654.63	-C=C- stretch
7	1497.15	C-H bend
8	1444.65	C-C stretch
9	1316.94	C-O stretch
10	1104.04	C-O stretch (carboxylic acid)
11	1023.24	C-O stretch (carboxylic acid)
12	988.05	=C-H bend; -OH bend (carboxylic acid)
13	819.85, 869.12	C-H aromatics
14	720.47, 754.34	C-Cl stretch

15	667.14	-C=C-H; C-H bend
16	534.89, 592.35	C-Br stretch

The Infrared spectra, frequencies (cm^{-1}) and tentative assignment of isolated ascorbic acid is presented in table 5. The assignments proposed for the four O-H stretching bonds above 3000cm^{-1} are contradiction [15, 16]. This is because of the importance and relevance of the O-H groups. For the present discussion, only the OH_s and the lactone C=O (around 1750 cm^{-1}) and C=C stretch (approximately 1680 cm^{-1}) bands will be considered. The four O-H bands observed 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 [15, 16].

Table 6: GC-MS fractions of isolated ascorbic acid

Peak (#)	Retention time (min)	Area (%)	Library ID (NIST 14.L)	Molecular formular	Molecular weight (g/mol)	Class of compound
1	7.799	100.00	Ethane dioic, dimethyl ester	$C_4H_6O_4$	118.0880	Acidic ester

NIST: National institute of standards and technology.

Table 6 shows the GC-MS chromatogram and fractions of isolated ascorbic acid. 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 pharmacological 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 $C_4H_6O_4$ (m/z118.0880) with base peak at m/z 8.0, which was due to the loss of $C_2H_2O_2$ (Acetolactone), 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 [19].

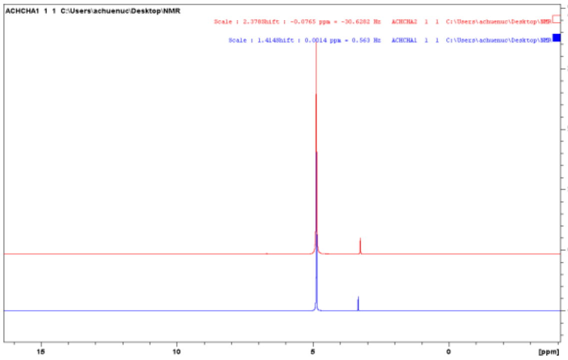


Figure 3: H-NMR spectrum of isolate in methanol and deuterated DMSO

Figure 3 identified the presence of the most deshielded proton and most acidic proton in the structure of the isolate in further confirmatory test that it is ascorbic acid. The spectrum confirms that the isolate is clearly, unequivocally ascorbic acid.

CONCLUSIONS

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.

From the results of this research the following recommendation are made:

i. The extraction and isolation should be carried out on food that contains higher concentration of ascorbic acid. For example, Kakada plums (*Terminalia ferdinandiana*), an Australian nature food containing 100 times more vitamin C than orange, it has the highest known concentration of vitamin C, containing up to 5300mg/100g. Other foods such as guava (228mg/100g), sweet yellow pepper (183mg/100g), blackcurrent (181mg/100g), parsley (133mg/100g), thyme (160mg/100g), mustard spinach (130mg/100g), broccoli (89mg/100g), lemons (77mg/100g), papaya (62mg/100g) and orange (53mg/100g) [20].

ii. The recommended daily amount of ascorbic acid for adult men (90mg), adult women (75mg), teenage boys (75mg) and teenage girls (65mg) for the prevention and treatment of scurvy. Higher dosage is known to cause discomfort and diarrhoea [21]. *Casuarina equisetifolia* needles can be used to prepare supplement of this acid after anti-nutritional and toxicological investigation of this needle.

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