

ANTIOXIDANT ACTIVITY OF *ACHYRANTHES ASPERA* LINN STEM EXTRACTS

Charles Lekhya Priya, Gaurav Kumar, Loganathan Karthik, Kokati Venkata Bhaskara Rao*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Science and Technology, VIT University, Vellore, Tamil Nadu - 632 014, India

***Corresponding author**

Dr. Kokati Venkata Bhaskara Rao, Assistant Professor (Senior), Environmental Biotechnology Division, School of Bio Science and Technology, VIT University, Vellore, TN - 632 014, India

Telephone: 09894350824

E-mail: kokatibhaskar@yahoo.co.in

Summary

In this study, *Achyranthes aspera* stem was screened for the presence of major phytochemical groups. Stem extracts were screened for antioxidant, antimicrobial and hemolytic activity. Methanol and aqueous extracts were screened for the estimation of total phenolic content by Folin-Ciocalteu assay. Antioxidant potential of both extracts was measured by DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging assay. Antimicrobial activity of both extracts was determined by agar well diffusion method. Hemolytic activity towards human erythrocytes was measured by spectroscopic method. Phytochemical screening showed the presence of carbohydrates, phenolic compounds, saponins, alkaloids, oil and fats and tannins. Methanol extract exhibits high phenolic content than that of aqueous extract. Methanol extract showed high antioxidant potential compare to aqueous extract. Both extracts exhibits very low antimicrobial and hemolytic activity. Further, the presence of –OH functional group of phenolic compounds was confirmed by FT-IR analysis. With all results it concluded that *A. aspera* possess high antioxidant activity and can used for the isolation of antioxidant compounds.

Keywords: Antioxidant; antimicrobial; hemolytic; DPPH radical scavenging assay; phenolic compounds

Introduction

Indian systems of medicine (Ayurveda, Unani, Siddha, Yoga and Naturopathy) have developed over a long period of time. Ayurveda is one of the oldest medication system of disease prevention in the World and is called in its complete form under the name Maharshi Ayurved. ¹ The World Health Organization has approved its efficacy. ² These medication systems usually use plants or plant products for treatment of several diseases. A number of Indian medicinal plants have been used extensively in the traditional medicinal system for the treatment of numerous diseases.

Oxidation is a natural metabolic process in cell, some time resulting in the formation of free radicals such as hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl) and ozone (O₃).³ Free radicals are also generated through cigarette smoke, automobile exhaust, radiation, pesticides and air pollution etc.⁴ Free radicals play a crucial role in normal aging and neurodegenerative disorders.⁵ Free radicals damage the cell membranes, proteins, fats and cause heavy damage to the genetic material in the cell, this oxidative damage increase with age. Damaged protein and enzymes can result in premature wrinkling, aging and even cancerous growths on the damaged skin.^{6,7}

Natural oxidants namely phenols in medicinal and dietary plants can prevent the oxidative damage caused by free radicals. Plants produce a variety of antioxidants compounds to control the oxidative stress caused by sunbeam and oxygen, so they represent a source of compounds with antioxidant activity.^{6,8,9}

A. aspera is a indigenous medicinal plant of Asia, South America and Africa, it throughout the India belonging to the family Amaranthaceae. The plants are known for various medicinal properties and used widely for the treatment of different diseases in humans. In the recent time, *A. aspera* reported to have array of medicinal compounds and medicinal properties. *A. aspera* is used for the treatment of fever, dysentery, asthma, hypertension and diabetics. *A. aspera* seeds were reported to contain emetic and hydrophobic properties. *A. aspera* have application in infantile diarrhea and Cold. The dried leaves of *A. aspera* are reported to cure asthma.^{10, 11, 12, 13} *A. aspera* reported to possess wound healing activity, immunostimulatory properties, larvicidal activity, antibacterial activity and antifungal activity. Roots of *A. aspera* reported to possess antioxidant activity and anti-inflammatory properties.^{14, 15, 16, 17, 18, 19, 20}

Phytochemical analysis of the whole plant of *A. aspera* and particularly roots suggests the presence of various phytochemical compounds such as Saponin, sterols, polysaccharides and alkaloids. The medicinal values of *A. aspera* may be because of the presence of these phytochemicals.

The focus of this study was to investigate stem extracts of *A. aspera* for the presence of phytochemical, antioxidant activity (DPPH radical-scavenging activity), antimicrobial activity and hemolytic activity.

Material and Methods

Plant material

A. aspera was collected from the natural population growing in the Seshachalam Forest Area, Chittoor district, Andhra Pradesh, India, during December 2009. The plants were carried to the Molecular and Microbiology Research Laboratory, VIT University. Plant was identified in Herbal Garden of VIT University, Vellore. A voucher specimen was maintained in our laboratory (AS/VIT/MMRL/30.12.2009-1).

Processing of plant

The stem of *A. aspera* was collected and washed thoroughly in distilled water and cut in to small pieces. The stem was shade dried at room temperature. Dried stem pieces were uniformly grinded using mechanical grinder to make fine powder. The powder was serially extracted in hexane, chloroform, methanol and distilled water (10% w/v) using a Soxhlet apparatus. These extracts were concentrated at 40°C under reduced pressure (72 mbar) with a Rotary evaporator and dried using lyophilizer. Dried extract was collect in air tight container and stored at 4°C up for further use.

Phytochemical screening

Phytochemical screening of the stem of *A. aspera* was carried out by using the standard protocols as described by JB Harborne.²¹

Estimation of total phenolic content

Total phenolic content of the aqueous and methanol extract of *A. aspera* stem was determined using the Folin-Ciocalteu reagent.²² The crude aqueous and methanol extracts were diluted in methanol to obtain different concentrations (125, 250, 500 and 1000 µg/ml). 50 µl of each extract was mixed with 2.5 ml of Folin- Ciocalteu reagent (1/10 dilution in purified water) and 2 ml of 7.5% Na₂CO₃ (w/v in purified water). The mixture was incubated at 45°C for 15 min. The absorbance was measured at 765 nm. Na₂CO₃ solution (2 ml of 7.5% Na₂CO₃ in 2.55 ml of distilled water) was used as blank. The results were expressed as gallic acid equivalence in µg.

DPPH radical scavenging activity

The DPPH radical scavenging activity was performed according to the method of Gunjan et al. with few modifications.²² The plant extracts were diluted in distilled water to make 10, 20, 40, 60, 80 and 100 µg/ml dilutions. Two millilitres of each dilution was mixed with 1 ml of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20°C for 40 min. Absorbance was measured at 517 nm using UV-Vis spectrophotometer with methanol as blank. Gallic acid was used as positive control. The percentage scavenging of DPPH by the extracts was calculated according to the following formula:

$$\% \text{ DPPH Radical scavenging} = [(A_c - A_t) / A_c] \times 100$$

Here

A_c is the absorbance of the control (DPPH)

A_t is the absorbance of test sample.

Antimicrobial activity**Test microorganism**

The bacterial isolates used in this study included, *Staphylococcus aureus* ATCC 25923, *Klebsella pneumoniae* ATCC 13883, *B. subtilis* ATCC 2063, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922. All isolates were maintained on nutrient agar at 4°C.

Positive and negative control

Vancomycin (30 µg/disc) was used as positive control for *B. cereus*, Cotrimoxazole (23.75 µg/disc) for *K. pneumoniae*, Ciproflaxocin (5 µg/disc) *S. aureus* chloromphenicol (30 µg/disc) for *E. coli* and piperacillin (100 µg/disc) for *P. aeruginosa*, Sterilized distilled water was used as negative control.

Antibacterial assay

Antimicrobial activity of the crude extracts was determined by the agar well diffusion method.²³ All test organisms were inoculated in nutrient broth (pH 7.4.) for 8 hours. The concentration of the suspensions was adjusted to 0.5 McFarland standards. Isolates were seeded on Mueller Hinton agar plates by using sterilize cotton swabs. Agar surface was bored by using sterilized gel borer to make wells. 100 µl of the test extracts (100 mg/ml) and 100 µl of sterilized distilled water were poured in separate wells. The standard antibiotic discs were placed on the agar surface as positive control. Plates were incubated at 37°C for 48 hours. Each experiment was performed in triplicates at each concentration.

Hemolytic activity

Hemolytic activity was performed by spectrophotometer method *in vitro*.²⁴ Five milliliters of blood was collected from a healthy individual. The blood was centrifuged at 1500 rpm for three minutes. The pellet was washed three times with sterile saline solution (0.89% w/v NaCl) by centrifugation at 1500 rpm for 5 min. The cells were resuspended in normal saline to 0.5%. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml of the plant extracts (125, 250, 500 and 1000 µg/ml concentrations in saline). The mixtures were incubated for 30 min at 37°C and centrifuged at 1500 rpm for 10 min. The free hemoglobin in the supernatants was measured in UV-Vis spectrophotometer at 450 nm. Saline and distilled water were used as minimal and maximal hemolytic controls. Each experiment was performed in triplicates at each concentration. The level of percentage hemolysis by the extracts was calculated according to the following formula:

$$\% \text{ hemolysis} = [A_t - A_n / A_c - A_n] \times 100$$

Here: A_t is the absorbance of test sample.

A_n is absorbance of the control (saline control)

A_c is the absorbance of the control (water control)

Statistical Analysis

All tests were conducted in triplicate. Data are reported as means \pm standard deviation (SD). Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

Results and Discussion

Oxidative damage to the cell results in to several physiological and neurodegenerative disorders. The oxidative damage is because of the generation of free radicals during the oxidation process.^{6,7} Oxidative damage is usually countered in body by antioxidant defiance mechanism. When the amount of free radicals increases extensively high to overcome antioxidant defiance mechanism, it results in to oxidative damage to cell.²⁵ Oxidative damage can be controlled by the therapeutically use of antioxidant compounds. These antioxidants are naturally produced by plants and microbes to protect them from several disorders and stress conditions. The plants are a major source of antioxidant compounds and several antioxidant compounds have already reported from plants. Phenolic or polyphenols are the major phytochemical class of the plant contains the antioxidant properties. Several class of phenolic compounds are reported to be present in plants includes phenols, benzoquinones, phenolic acids, polypropenol, flavonoids, isoflavonoids phenylpropanoids, phenolics quinines, lignins, melanins, tannins etc.²¹ These phenolic compounds can be isolated from plants and can be used as natural antioxidant compounds.

Selection of *A. aspera* L. for the study was based on its tradition use to cure several diseases and therapeutic potential reported earlier. The plant root is already reported to possess antioxidant property, so in this study we screened the antioxidant potential of the stem of *A. aspera*. The plant was collected from Seshachalam Forest Area, Chittoor district, Andhra Pradesh, India. This area is highly rich in biodiversity and very less explored for therapeutic plants.

Plant stem powder was serially extracted in different polarity solvents and the dried extract was measured. The yield of the extracts are expressed as % yield and reported in Figure 1. Methanol extract showed maximum yield (1.5 %), followed by aqueous (1.4 %), hexane (1.2 %) and chloroform extract showed minimum yield (0.9 %). Methanol and aqueous extracts were used for the estimation of total phenolic content, DPPH radical scavenging activity and hemolytic activity, whereas all four extracts were used for the estimation of antimicrobial activity.

Dry powder of *A. aspera* stem was screened for the presence of major phytochemical groups. The results showed the presence of carbohydrates, phenolic compounds, saponins, alkaloids, oil and fats and tannins, whereas, proteins and flavonoids were not found (Table 1).

Total phenolic content of the methanol and aqueous extract was measured. Results are expressed as gallic acid exultance (GAE) in μg . The results are reported in Figure 2. Methanol extract showed slightly high phenolic content than that of aqueous extract. Phenolic content of the plant showed dose dependent increases.

Antioxidant potential of the methanol and aqueous extracts were measured by DPPH radical scavenging activity. The results are expressed as % inhibition of DPPH and reported in Figure 3. Methanol extract showed high antioxidant activity than that of aqueous extract. The DPPH radical scavenging activity was found to be increasing as dose increases. Earlier, *A. aspera* roots and leaves have been reported to possess DPPH radical scavenging activity with IC₅₀ values 241.86 $\mu\text{g}/\text{ml}$ and 129.91 $\mu\text{g}/\text{ml}$ respectively.¹⁹ Presented study reports the high DPPH radical scavenging activity of *A. aspera* stem than that of leaves and roots reported earlier.

Antimicrobial activity of methanol, aqueous, chloroform and hexane extracts was determined by well diffusion method. The results are reported in Table 2. The plant showed very poor antibacterial activity as only hexane extract showed moderate activity against *B. subtilis*, however, earlier studies reported the antifungal activity of the plant.¹⁸

Hemolytic activity was performed to measure the toxic effect of *A. aspera* towards human erythrocytes. Results for hemolytic activity of methanol and aqueous extract are reported in Figure 4. Methanol extract showed low hemolytic activity than that of aqueous extract, however, both extract exhibits very low hemolytic activity and can consider as safe for the human erythrocytes.

Presence of phenolic compounds was further confirmed by FT-IR analysis. FT-IR spectrum of the *A. aspera* is reported in Figure 5. The absorption band at 3451.57 cm^{-1} is representative for -OH group of phenols.

With the results obtained in the present study, we conclude that *A. aspera* stem can be used as a good source for the isolation of safe and natural antioxidant compounds.

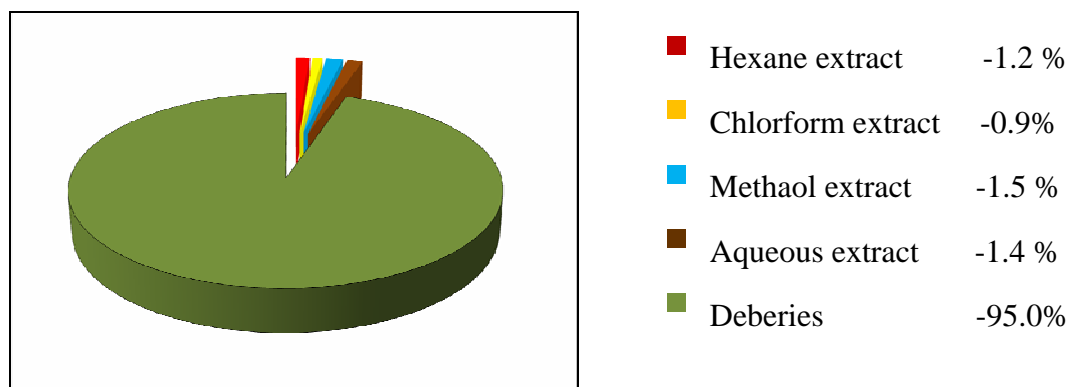


Figure 1: % yield of extracts in different polarity solvents

Table 1: Phytochemical analysis of *A. aspera* leaf

Phytochemicals	<i>A. aspera</i>
Carbohydrates	+
Proteins	-
Phenolic compounds	+
Oil and fats	+
Saponins	+
Flavonoids	-
Alkaloids	+
Tannins	+

Here: +: present, -: not present

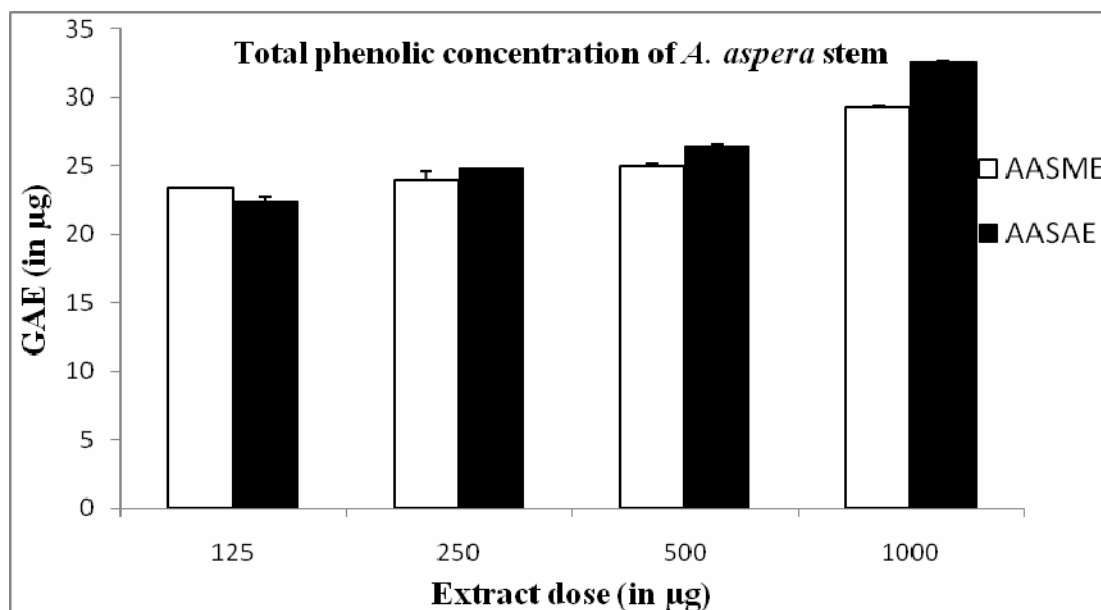


Figure 2: Total phenolic content of *A. aspera*

Here: AASME = *A. aspera* stem methanol extract, AASAE = *A. aspera* stem aqueous extract. All values represent the mean \pm standard deviation ($n = 3$ test).

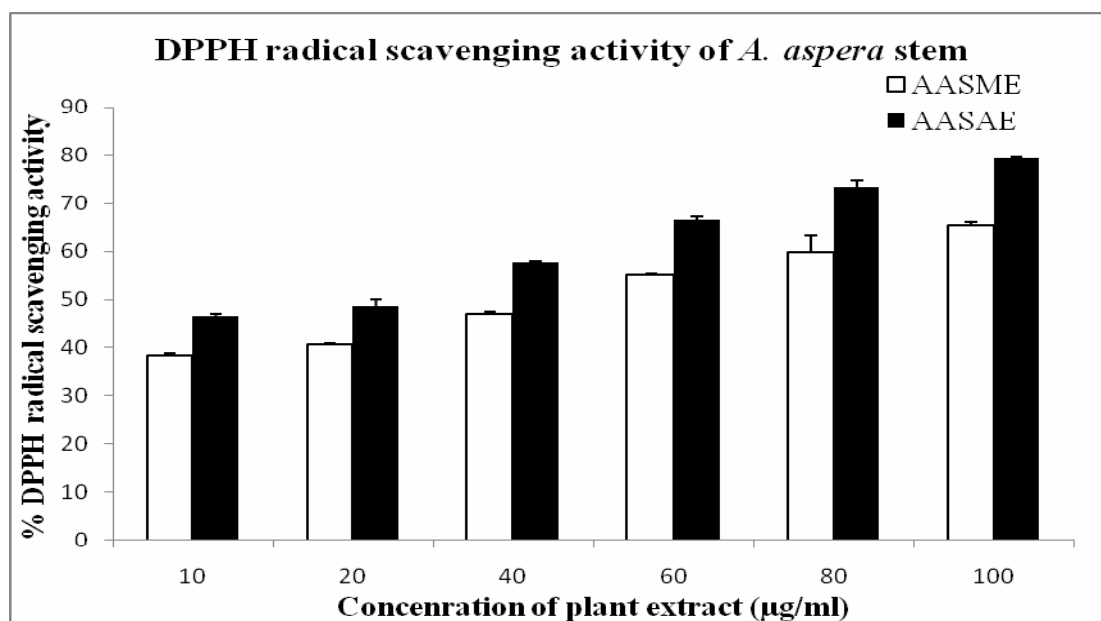


Figure 3: DPPH radical scavenging activity of the *A. aspera*

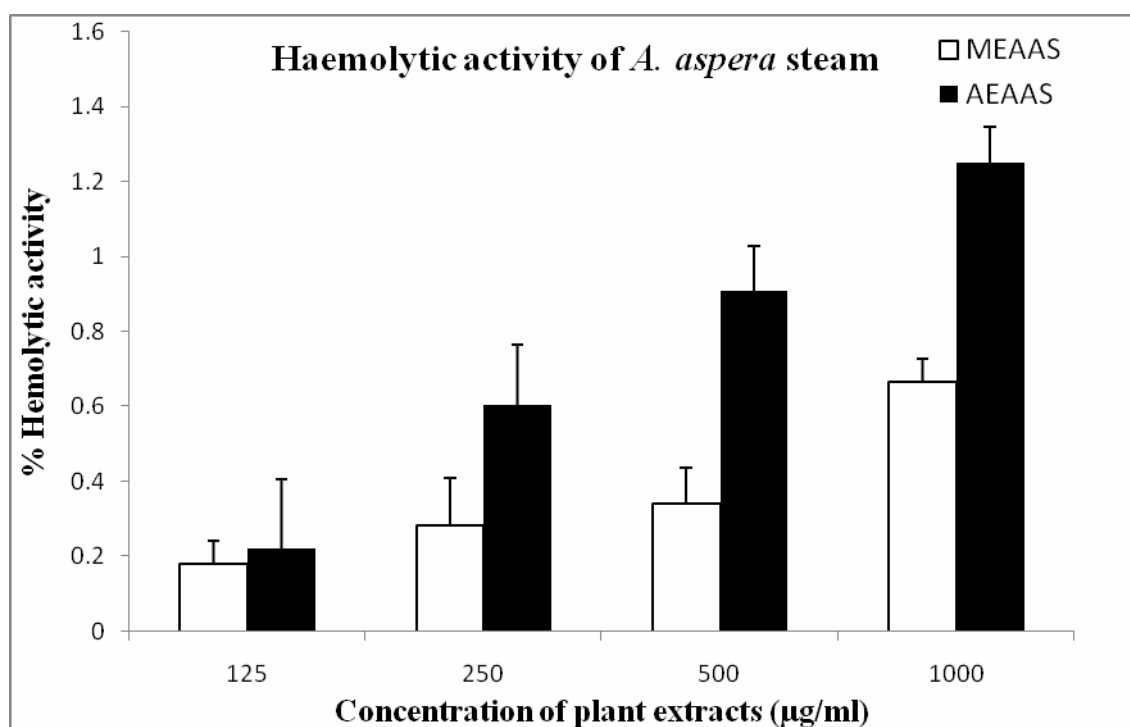
Here: AASME = *A. aspera* stem methanol extract, AASAE = *A. aspera* stem aqueous extract. All values represent the mean \pm standard deviation ($n = 3$ test).

Table 2: Antimicrobial activity of *A. aspera*

Test organisms	Inhibition zone diameter (mm)					
	Stem extracts				Control	
	HE	CE	ME	AE	PC	NC
<i>B. subtilis</i>	12.3±0.57	n.a.	n.a.	n.a.	14.0±1.0	n.a.
<i>E. coli</i>	n.a.	n.a.	n.a.	n.a.	25.3±1.5	n.a.
<i>K. pneumoniae</i>	n.a.	n.a.	n.a.	n.a.	20.3±0.57	n.a.
<i>P. aeruginosa</i>	n.a.	n.a.	n.a.	n.a.	25.3±0.57	n.a.
<i>S. aureus</i>	n.a.	n.a.	n.a.	n.a.	29.3±0.57	n.a.

Here, HE: hexane extract, CE: chloroform extract, ME: methanol extract, AE: aqueous extract, PC: positive control, NC: negative control, n.a.: no activity

All values represent the mean±standard deviation (n = 3 test).

Figure 4: Hemolytic activity of *A. aspera*

MEAAS = methanol extract of *A. aspera* stem, AEAAS= aqueous extract of *A. aspera* stem.

All values represent the mean±standard deviation (n = 3 test)

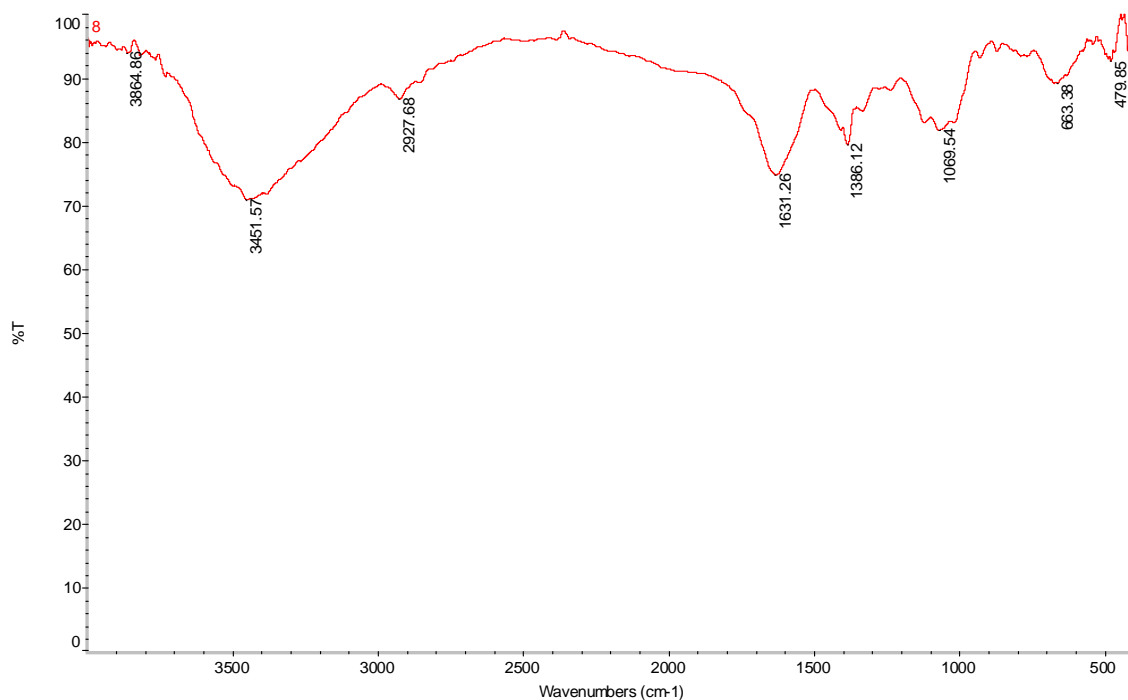


Figure 5: FTIR spectrum of *A. aspera* stem

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