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Study on the *in vitro* antioxidant properties of selected traditional medicinal plants

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Abstract

Oxidative stress is the phenomenon by which the excessive production of free radicals and oxidants that cause detrimental effects to diseases and finally death. Plants are the rich source of antioxidants which have the potential to neutralize these reactive oxygen species, thereby preserving the redox equilibrium of the body. Western Ghats serves as an excellent habitat for many endangered species of flora and a reservoir of edible source of food for many living beings. Some of the commonly available traditional plants like *Ficus benghalensis*, *Psidium guajava*, *Beta vulgaris*, *Syzygium cumini* were known to possess rich pharmacological properties. In the present study, we aim to evaluate and compare the *in vitro* antioxidant properties of the selected traditional medicinal plants using various radical scavenging and reducing capacity assays. Further, the structural elucidation of the presence of phytochemical compounds in the extract was carried out using Infrared (IR) spectra. The above investigation on the free radical scavenging properties and the determination of the functional groups of bioactive compounds may open up new interest on their applications in food, pharmaceutical and cosmetic industries.

Keywords: Antioxidants, free radicals, FTIR, *in vitro*, guava, beetroot, banyan, jamun

Introduction

Free radicals belong to the category of atoms, molecules or ions which own a lone pair of valence electron in the outer most orbit making them highly electronegative. Their instability makes them to snatch electrons from the neighboring atoms within any biological system. They may attack the proteins, enzymes which are present on the cell membranes and DNA leading to the denaturation of the cell and other chronic disorders like mutation which leads to cancer. This unstable and excess production of free radicals and oxidants is termed as oxidative stress. Oxidative stress may lead to a chain of reactions like hardening of the blood vessels which lead to stroke, heart attack and also attack the collagen in the skin thereby causing stiffness in the skin tissues ^[1, 2].

Antioxidants are those substances that inhibit the role of oxidation, in low concentrations. They are majorly classified into two types namely enzymatic and non-enzymatic antioxidants which are both involved in stabilizing the Reactive Oxygen species (ROS). Some of the enzymatic antioxidants include Superoxide Dismutase (SOD), catalase, glutathione, while the non-enzymatic antioxidants include the bioactives which mainly fall under total polyphenols such as flavonoids, anthocyanin, tannins, terpenoids, glyceroids etc ^[3, 5].

Southern peninsular India serves as a home for many diversified species of plants. Most of the plants have been used as a part of the traditional medicinal systems without being identified about its medicinal properties. Western Ghats shelter a wide range of endangered and common plants which are left unused, due to the lack of its study on their pharmacological values ^[6]. Plants with colored fruits, flowers and vegetables are usually rich in antioxidants. A total of 82 Indian medicinal plants traditionally used in medicine were subjected to preliminary screening against several common phytochemical activities. Interestingly, four commonly available plants showed strong and broad spectrum of activities (Table 1). Based on the available information, the active part of the plant was chosen to be the leaves, which were enriched with many bioactive constituents. The identification of the active bioactive compounds in the plant which are responsible for the pharmaceutical properties will serve as an indigenous attribute for their usage in the future. FTIR (Fourier Transfer Infrared) spectroscopy works based on the molecular vibration created by the infrared source. It has emerged as a powerful analytical tool in the pharmaceutical application for its detection in the functional group region and fingerprint region ^[7, 8].

Table 1: Selected medicinal plants with their part of use and their medicinal properties

S. No.	Botanical Name	Common Name	Part Used	Family Name	Properties	Medicinal Uses	Reference
1.	<i>Ficus benghalensis</i>	Banyan	Leaf	Moraceae	Anti-diabetic, anti-inflammatory, anti-microbial, anti-tumor	Biliousness, ulcers, vomiting, vaginal complains, fever, inflammations, leprosy	[9, 11]
2.	<i>Syzygium cumini</i>	Jamun	Leaf	Myrtaceae	Antibacterial, anti-cancer, anti-diuretic	Lowering of blood sugar, liver production, polyuria, piles, diarrhea, dysentery, sore throat	[12, 15]
3.	<i>Beta vulgaris</i>	Beetroot; sugar beet	Leaf	Amaranthaceae	Anti-inflammatory, antioxidant, anti-cancer, anti-hypertensive	Treatment of liver diseases and fatty liver, lower blood pressure	[16, 17]
4.	<i>Psidium guajava</i>	Guava	Leaf	Myrtaceae	Anti-cancer, anti-inflammatory, antioxidant, antidiarrheal, anti-mutagenic	Diabetes mellitus, dyslipidemia, insulin resistance, cancer, metabolic syndrome and cardiovascular diseases	[18, 20]

Hence, in the present study, we aim to evaluate and compare the *in vitro* antioxidant activity of the selected traditional medicinal plants using various antioxidant assays such as Total Antioxidant Capacity (TAC), Ferric Reducing Antioxidant Power (FRAP), Cupric Reducing Antioxidant Capacity (CUPRAC), and ABTS Radical Scavenging Assay. Further, the structural elucidation of the functional groups of compounds responsible for the antioxidant properties was also investigated using the Fourier Transform Infrared (FTIR) spectroscopy.

Materials and Methods

Sample collection and Pretreatment

The leaves of the selected plants such as, *Psidium guajava* (Guava), *Syzygium cumini* (Jamun), *Beta vulgaris* (Beetroot) and *Ficus benghalensis* (Banyan) were freshly procured from places in and around Coimbatore and Nilgiris. The plant parts were authenticated by a Plant pathologist, Botanical Survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore. The voucher specimen of the plant sample was deposited in the herbarium of the department. The leaves were then completely shade dried, ground coarsely and stored in airtight pouches for future experiments.

Hot extraction

Hot extraction of the coarsely ground powder was performed in soxhlet apparatus against a highly polar solvent, ethanol (99%) at a temperature of 70°C. The solvent extract was allowed to cool and collected after completion of 20 cycles. The obtained solvent extract was concentrated using a Flash evaporator and allowed for complete drying. Once drying is over, the powdered extract was scraped and stored in a cool and dry place away from sunlight [21, 22].

In vitro antioxidant assays

Total Antioxidant Capacity assay

100ml of phosphomolybdenum reagent was prepared by adding 0.025M ammonium molybdate and 0.027M disodium hydrogen phosphate successively in 0.6M of sulphuric acid. The leaf samples were prepared in different increasing concentrations (200µg/ml to 1mg/ml) from the prepared stock solution. 2ml of the prepared phosphomolybdenum reagent was added and incubated at 95°C for 90 minutes. The color changes to dark green color on heating and a metallic light blue color is formed on cooling. The absorbance is read at 675 nm and calculated in terms of ascorbic acid equivalents. 0.2ml distilled water in place of the plant sample acts as the blank [23].

Ferric Reducing Antioxidant Power assay

FRAP reagent is prepared using 90ml of acetate buffer (pH 3.6) with 9ml of 20 mM ferric chloride and 9ml of 10mM TPTZ. Varying concentrations of the sample (100µg/ml - 500µg/ml) were prepared and for about 90µl of the extract,

2.7ml of FRAP reagent and 270µl of water was added. The mixture in triplicates was incubated at 37°C for 30 minutes. It was cooled to room temperature and increase in absorbance was measured at 595nm [24].

Cupric Reducing Antioxidant Capacity assay

Varying concentrations of the sample (100µg/ml - 500µg/ml) were prepared in distilled water. 1ml of sample was added with 1ml of 1M ammonium acetate followed by 1ml of 1M cupric chloride and 1ml of neocuproine. The tubes in triplicates were then incubated for 30 minutes and the decreasing absorbance was noted at 450nm [25].

ABTS Radical Scavenging Assay

Equal volumes of 7mM ABTS solution and 2.45mM ammonium per sulfate was mixed well and incubated in dark for 16 hours at room temperature. The initial absorbance of the colored solution was measured at 745 nm and reduce the final absorbance of 0.700(±0.02) by diluting the solution with methanol. Varying concentrations (100-500µg/ml) of the sample in triplicates were prepared. 1ml of ABTS standard solution was mixed with 30µl of sample and incubated for 6 minutes and the decreasing absorbance was read at 745nm. 30µl of ethanol with 1ml of ABTS solution was taken as control and ethanol was used as blank [19]. The percentage inhibition of the ABTS radical by the anti-oxidants in the sample was calculated by the formula,

$$\% \text{ Inhibition} = \left(\frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control}} \right) * 100$$

IR Spectral Analysis

The dry ethanol sample of the leaves were loaded on the sample holder in Shimadzu IRRafinity 1S. The [Measure] tab was clicked followed by the [Sample] button. Samplespectra were displayed and once again the progress in measurement was displayed in the status bar, while the real-time window will display the sample spectrum in transmittance mode. Once the measurement is completed, the main window will switch to the [View] tab. The spectrum was then displayed in two windows, with the upper window being the full view of the spectra, called the [Overview] window, while the lower window being the Zooming window [26].

Statistical Analysis

The obtained values of the antioxidant assays were statistically represented using R² regression analysis to analyze its closeness towards the positive linearity.

Results and Discussion

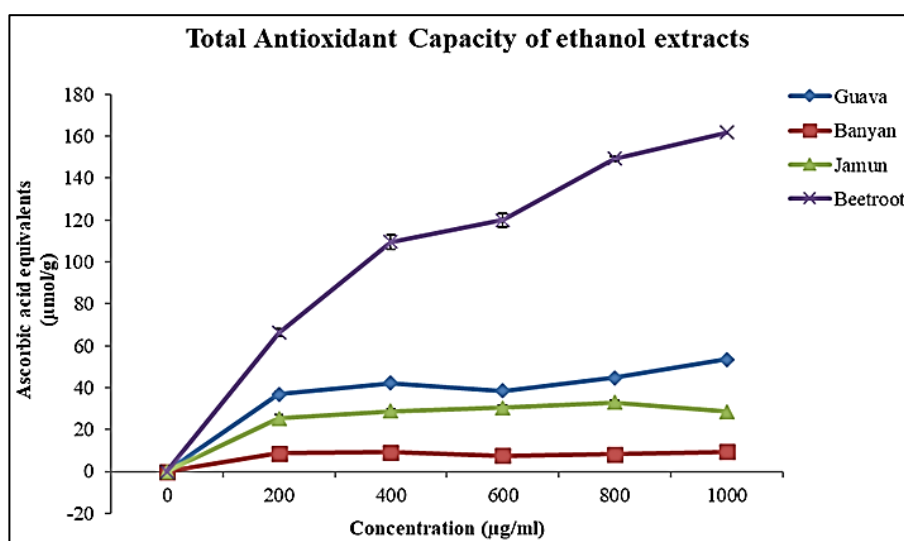
The leaf samples were collected fresh and the pretreatment methods like cleaning, drying and grinding were performed carefully. The hot extracted leaf samples were stored in a dark container at refrigerating temperature.

In vitro Antioxidant activity analysis

Total Antioxidant Capacity

The total antioxidant assay gives the complete estimate of the overall antioxidant action of the different plant samples of the ethanol extract. The formation of phosphomolybdenum complex is seen in all the plant extracts, while the intensity of color was found maximum in beetroot extract (Figure 1). The

intensity recognizes the ability of the extract as a collector of these free radicals. The total antioxidant capacity of the different solvent extracts was expressed as number micromoles of ascorbic acid equivalents ^[23]. Hence the overall effectiveness of antioxidant activity of the extracts can be ordered as,



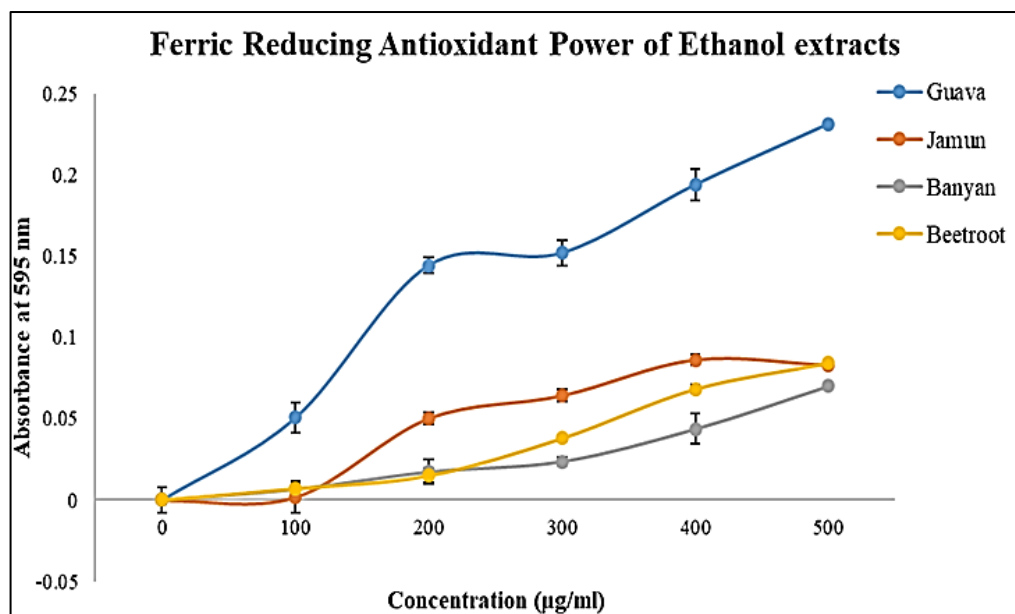
Beetroot > Guava > Jamun > Banyan

Fig 1: Total Antioxidant capacity of the ethanol extracts of the selected medicinal plants

Ferric Reducing Antioxidant Power capacity

The reduction of a colorless complex, ferric tripyridyltriazine complex (ferric (III)) to a blue colored ferrous-(2,4,6-tripyridyl-s-triazine)₂ (ferrous (II)) was carried by the presence of antioxidants which can be monitored by the absorbance at 595nm (Figure 2). The absorption values are in relationship with the reducing power of the electron donating

antioxidants in the sample ^[27]. On the basis of the above said statement, the electron donating antioxidants were found to be significantly maximum in the ethanolic extract of *Psidium guajava* compared to the other extracts. Statistically, the ferric reducing antioxidant potential of plant extract can be ordered as,



Guava > Jamun > Beetroot > Banyan

Fig 2: Ferric Reducing Antioxidant Power (FRAP) of the ethanol extracts of the selected medicinal plants

Cupric Reducing Antioxidant Power capacity

At neutral pH, chromogenic redox reagent bis (neocuprine) copper (II) chelate forms a colored Cu(I)-chelate by the redox reaction with reducing polyphenols in the sample which was measured at 450nm (Figure 3). Here, the ability of the

antioxidants present in the leaves extracts of guava and jamun were found to reduce cupric ions to cuprous ions effectively than that of beetroot and banyan ^[25]. Statistically, the copper reducing capacity of antioxidants can effectively be ordered as,

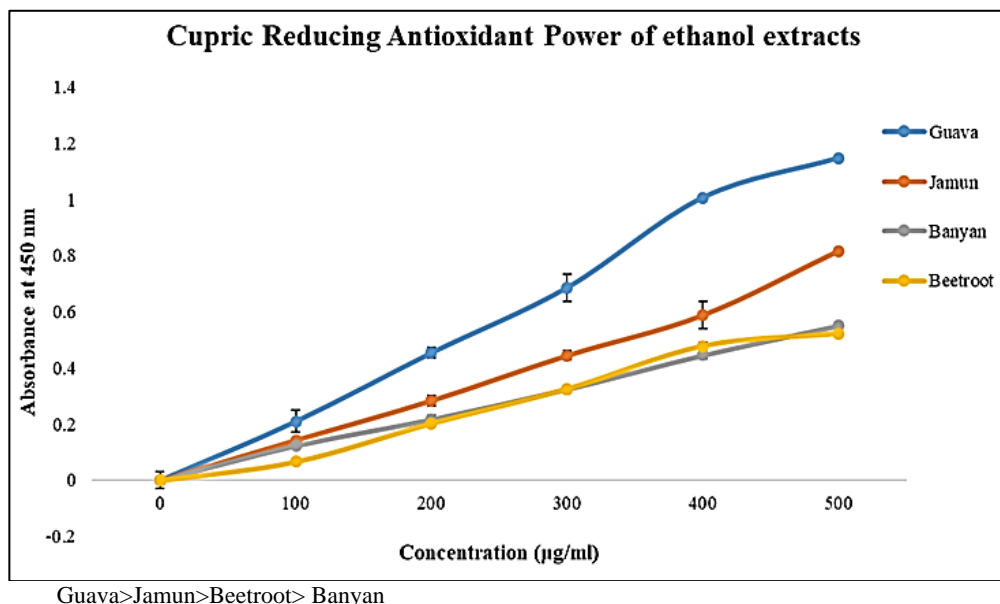


Fig 3: Cupric Reducing Antioxidant Power (CUPRAC) of the ethanol extracts of selected medicinal plants

ABTS Radical Scavenging Activity

The assay is mainly based on the inhibition of the absorbance of the radical cation ABTS⁺ by the plant antioxidants. The radical which has a characteristic long wavelength absorption spectrum is successfully scavenged by all the ethanol leaf extracts. An inverse proportionality can be seen between the absorbance and the percentage inhibition (Figure 4). It was

also found that the maximum IC₅₀ value was 259.82 µg/ml. Collectively the free radical scavenging potential (ABTS) of guava and jamun were more or less similar, while that of beetroot and banyan were in closer relationship [19]. Statistically, the scavenging activity of antioxidants can effectively be ordered as,

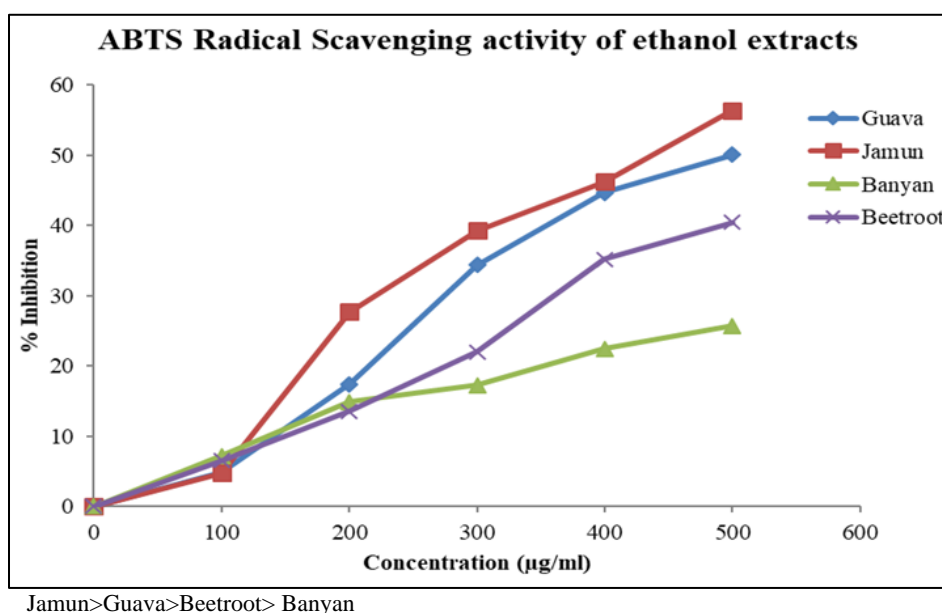


Fig 4: ABTS Radical Scavenging Activity of the ethanol extracts of selected medicinal plants

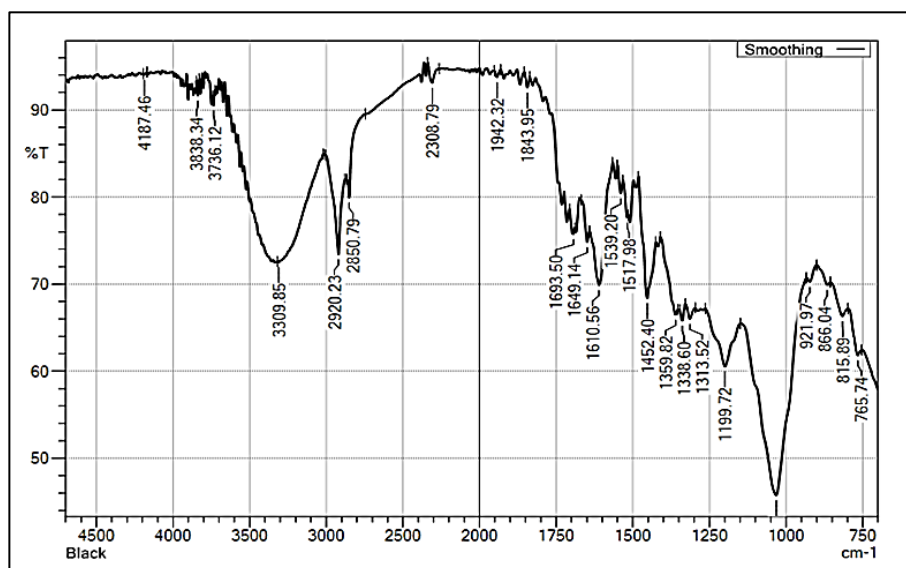
FTIR Spectral Analysis

FTIR (Fourier Transfer Infra-Red Spectrum) spectroscopy works based on the molecular vibration created by the infrared source. It has emerged as a powerful analytical tool in the pharmaceutical application for its detection in the

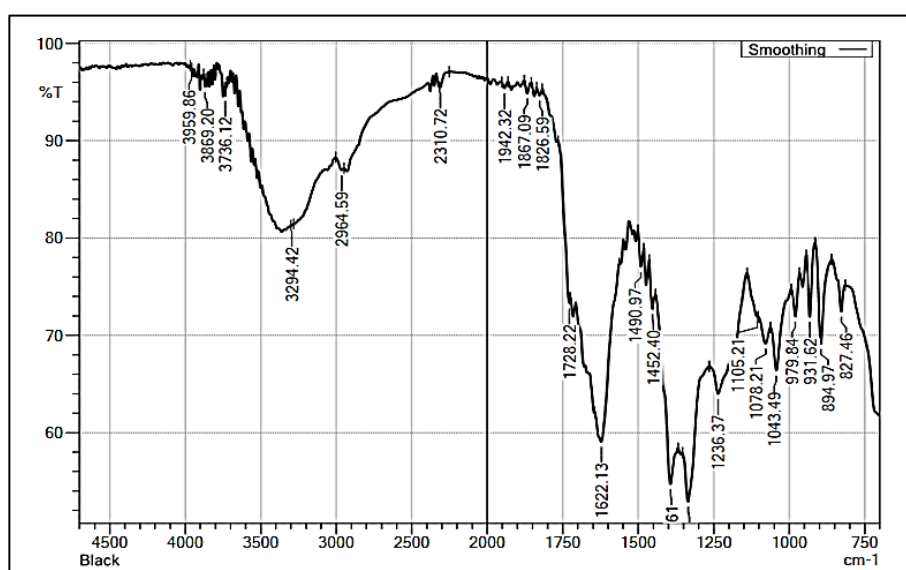
functional group region and fingerprint region [7]. Each spectral range corresponds to the unique features in a molecular structure and hence they can be called as fingerprint regions (Figure 5). Spectral analysis of the selected medicinal plants is tabulated as in Table 2.

Table 2: FTIR Spectrum of the ethanolic extract of selected medicinal plants with their corresponding molecular assignment

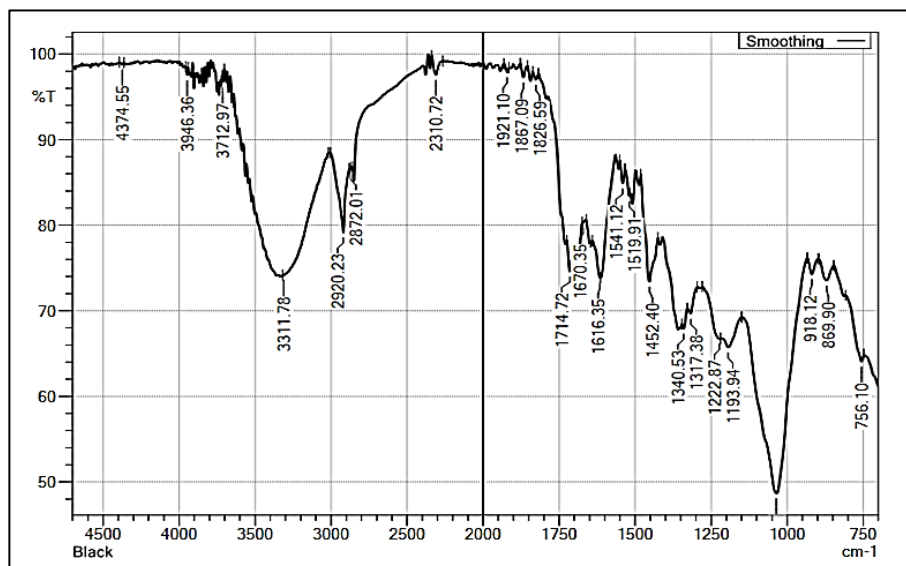
Sample	Spectral range (cm ⁻¹)	Molecular assignment
<i>Psidium guajava</i> (Guava)	3309	Phenolic O-H stretch (H Bonded)
	2920	-CH ₃ (C-H stretch)
	1610	-NH ₃ (N-H bending)
	1452	-CH in plane bend
	1334	Carbonyl (C=O Stretch)
	1199	C-N stretch of amines
	1031	Phenolic C-O stretch
<i>Beta vulgaris</i> (Beetroot)	3294	Phenolic O-H stretch (H Bonded)
	2964	-O-CH ₃ aliphatic ether
	1622	-NH ₃ (N-H bending)
	1392	C-N stretch
	1043	Phenolic C-O stretch
<i>Syzygium cumini</i> (Jamun)	3311	Phenolic O-H stretch (H Bonded)
	2920	-CH ₃ (C-H stretch)
	1616	-NH ₃ (N-H bending)
	1452	C=N stretch of aromatic compounds
	1340	Carbonyl (C=O Stretch)
	1193	C-N stretch of amines
<i>Ficus bengalensis</i> (Banyan)	3379	Phenolic O-H stretch (H Bonded)
	2918	-CH ₃ (C-H stretch)
	1635	-NH ₃ (N-H bending)
	1373	C-H mode in CH ₂ and CH ₃
	815	Triazine ring stretch



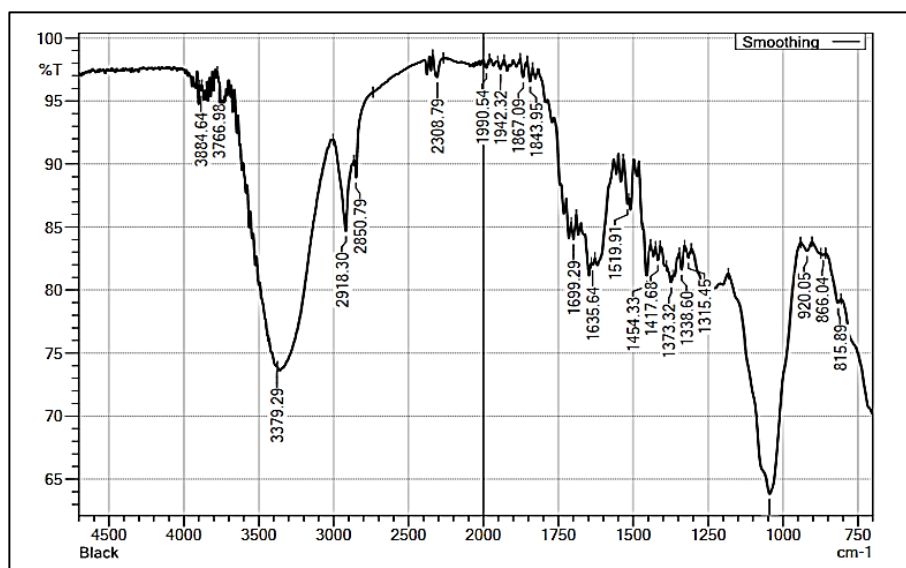
(I)



(II)



(III)



(IV)

Fig 5: FTIR spectra represented in % Transmittance of the ethanol leaf extracts of (i) Guava, (ii) Beetroot, (iii) Jamun and (iv) Banyan

In herbal medicine application, the FTIR spectroscopy owns an indigenous part in the preliminary detection of the compounds, which is essential for the quantification and purification of the compounds present [28]. From Table 2, the presence of phenolic C–O stretch, –CH₃ and phenolic O–H stretch could be seen majorly in all the leaf samples. They mainly denote the possibility of the phenol family and the presence of flavone backbone structure in all the leaf extracts. The broad and wide spectrum at around ~3300 cm⁻¹ represents the O–H bonds, while the presence of carbonyl group is confirmed using the spectrum at ~1330 cm⁻¹. The presence of a spectrum ranging between 1600 cm⁻¹ and 1640 cm⁻¹ show the major possibility of the amide groups showing the presence of another possible bioactive constituent, tannin. It is also visualized that the guava and beetroot extracts shows minimal variation in the wave number indicating the presence of more or less similar nature compounds in both the extracts.

Conclusion

Plants are one of the great sources of phytochemicals and antioxidants that are known for treating a wide range of medical disorders. The collected leaf samples were collected,

dried, ground and subjected to hot extraction using ethanol in soxhlet apparatus. The *in vitro* antioxidant potential of the four plant samples were analyzed using phosphomolybdenum based total antioxidant activity, ferric and cupric ion reducing power activity and free radical scavenging activity using ABTS radical. The total antioxidant capacity showed the effectiveness of the beetroot leaf extract, while both the reducing power assays showed the maximum effectiveness of the guava leaf extracts. This was supported by the FTIR spectra showing the presence of phenolic –OH, C–O stretch and –NH₃ bends showing the presence of maximum phytochemicals such as phenols, flavones, tannins and terpenoids. The estimation of the *in vitro* antioxidant potential and the preliminary investigation of the phytochemicals present has opened avenue for research interest towards various applications.

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