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Extraction, Purification and *In-vitro* Antibacterial Activity of different crude leaves Extracts of *Sterculia foetida* Linn

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ABSTRACT
Sterculia foetida is a soft handsome woody tree with various pharmacological properties and they are most prevalently found in India, Thailand, Indonesia, Ghana, and Australia. The biochemically active compounds present in the plant possess good medicinal properties which have been already reported in several research papers. The present study was designed to screen the biochemically active compounds present in the leaves of Sterculia foetida. The present work is phytoconstituents of the seed powder of Sterculia foetidaL. extracted with different solvents. The extracted phytochemical compound subjected to qualitative analysis, quantitative analysis, HPLC and GC-MS analysis. There are 8 bioactive compounds were identified through GC-MS analysis of seed powder of S. foetida L. These various bioactive compounds possess a wide range of activities such as disease control, pest control and microbicidal effect.
Keywords: *Sterculia foetidaL., GC-MS analysis, phytoconstituents*

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INTRODUCTION

Plants synthesize numerous natural organic compounds having complex chemical structures. These plant-derived compounds play a crucial role in their ecological functions [1]. Extensive studies have indicated that terpenoids, phenolics, and nitrogen-containing substances are important phytoalexins which provide a defense system and protect plants against attack by harmful microbes and herbivores [2]. The plants that release these active compounds are capable to compete and invade other plant species in their vicinity by suppressing their growth (a natural phenomenon known as allelopathy) [3]. In addition to their physiological functions in plants, numerous phytoalexins have also been reported to possess strong antioxidant, antibacterial, and herbicidal properties. A number of bioactive compounds have been isolated, purified and employed in a wide range of applications including food, pharmaceutical, cosmetic, and agricultural industries [4]. Therefore, the exploration of active medicinal plants and their natural bioactive molecules has become essential to exploit the possible additional values of natural sources.

The pharmaceutical and medicinal properties possessed by the plants are due to the presence of metabolites (chemical substance). They are abundantly seen in all parts of the plant – root, stem, leaves, fruits, flowers, and seeds. There are two types of metabolites; primary metabolites which are responsible for growth and development of plants, secondary metabolites which are responsible for defense mechanism of human [5]. From 20th century onwards, several scientists concentrated their research in isolating secondary metabolites from different parts of the plants since bioactive compounds derived from secondary metabolites possess great therapeutic values and also due to its powerful defense mechanisms in humans [6]. The bioactive compounds present in the secondary metabolites can be derived using phytochemical screening analysis [7-16]. Phytochemical screening was performed to extract the medicinally active substances. Qualitative analysis reveals the presence or absence of secondary metabolites present in the plantsand quantitative analysis reveals the amount of percentage of secondary metabolites present in the plants [17]. *Sterculia foetida* Linn is a tropical evergreen tree; it

belongs to family Sterculiaceae, found in the western and southern part of India. It is commonly called as Java Olive, wild almond Poon tree and Pinari in tamil [18]. The tree is reported to have several useful aspects due its pharmacological properties present in the whole plant and especially in leaves. The phytochemicals present in the plant possess several biological properties like antifungal, antimicrobial, antiviral, antitumor activities [19]. In olden days the leaves of the plant were used as the medicine without the knowledge of their medical properties. Leaves were observed at the ends of the each branch, it was clumped with 7-9 leaflets with length of 10-17 cm with unpleasant smell [20]. The present study was designed to reveal the hidden potentials present in the leaves extract of medicinal plant *Sterculia foetida* Linn and to predict the presence of medicinally important secondary metabolites from various solvents like methanol, ethyl acetate, hexane etc. by using standard protocol for different bioactive molecules.

MATERIAL AND METHODS

The leaves were collected and washed with the running tap water, dried under shades for two weeks. The dried leaves were blended into coarse powder using electric blender and later stored at room temperature. The 100 grams of powdered leaves were subjected to successive extraction with five different solvents Ethanol, methanol, Hexane, Diethyl ether and ethyl acetate using soxhlet apparatus by continuous percolation process. After the extraction the crude extract was filtered through a Whatman number 1 filter paper. Later, the crude extract was subjected to evaporation in a rotary vacuum evaporator for dryness. After the evaporation process completed the concentrated extract was collected and stored at 4°C for further analysis.K_3Fe (CN) $_6$, $FeCl_3$, TCA, DPPH, Nitro blue tetrazolium, Hydrogen peroxide and organic solvents were purchased from Sigma-Aldrich, U.S.A.

Bacterial strains and cultivation

Microorganisms. Bacterial strains utilizing *E.coli* (KF 918342), *Staphylococcus haemolyticus, Aeromonas hydrophila* (KCTC 12487), *E.coli* (ATCC 35150), *Cronobacter sakazakii* (ATCC 29544), *Aeromonas salmonicida* (KACC 15136) were used experiment. These six strains were collected from medicine department of Alagappa University. 50ml of LB broth was prepared in 250ml conical flask and the bacterial strains were grown in this medium at 37C on an orbital shaker. The culture flask was inoculated at 0.1 OD 600nm with freshly prepared LB medium under same culture conditions. The mid log phase bacterial cultures were utilized for the antibacterial studies. *Staphylococcus haemolyticus* are only the gram positive bacteria used in this investigation all other gram negative bacteria.

Antibacterial screening

Disk diffusion method

0.1 OD of overnight various bacterial cultures was swabbed on the 25ml LB agar plates. Then the whatman disk was placed on the plates. About 30ul of different solvent extract of *Sterculia foetida*were add on that whatman disc and incubate for overnight at 37□. For different organ screening the different parts powdered and dissolved in ethyl acetate then add on the disc placed on bacterial cultures inoculated 25ml LB agar plates. Ethanol, Methanol, Hexane, Diethyl ether and Ethyl acetate utilized as a control and streptomycine was used as a standard.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the extracts from *Sterculia foetida* was completed according to the Rajinimenon *et al* reference method using 96 well plates. The extracted powder 1mg/ml was dissolved in water with 2% dimethyl sufoxide (DMSO). The initial extracts concentration was 50 μL crude extract and then serially diluted in two fold. Each well was having 100 μL of LB broth and then extracts were added as mentioned above the concentration. Add 5 μL of suspension containing 10^8 CFU/mL of bacteria. This experiment proceeds for all bacteria in 96 well plates and incubated at 37□ for 17 hours. After incubation time the minimum inhibitor concentration of bacteria was explained by lowest visible growth present in LB broth and the experiment was conducted in triplicate.

DPPH Radical Scavenging Assay

The free radical scavenging activity was performed following the method by Elzaawely *et al*. [21]. A volume of 0.2 to 1 mL extract was mixed with 0.25 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution and 0.1 mL of 0.1 M acetate buffer pH (5.5). The mixture was shaken and kept at room temperature for 30 min in the dark. The absorbance was measured at 517 nm using a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The BHT standard (5–20 ppm) was used as the positive reference. The inhibition concentration (IC50) was the concentration ofthe samples which gave 50% DPPH radical scavenging activity. Thus, a lower IC50 value indicated a higher antioxidant activity. The following formula measured the percentage of DPPH radical scavenging activity. The DPPH radical scavenging activity was evaluated by the following formula.

DPPH radical scavenging activity (%) = $[(A0 - A1/ A0) * 100]$, where $A0$ is the absorbance of the control at 30 min and $A1$ is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

Radical Cation ABTS Decolorization Assay

The ABTS (2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay was carried out as an improved ABTS method of Re *et al.* [22], with some adjustments. Briefly, the ABTS radical solution was prepared by mixing 7 mM ABTS (2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) and 2.45 mM potassium persulfate in water. The solution was incubated in the dark at room temperature for 16 h, and then diluted with methanol to obtain an absorbance of 0.70 ± 0.05 at 734 nm. An aliquot of 120 µL of the ABTS solution was mixed with 20-100 µL of a sample and the mixture was incubated in the dark at room temperature for 30 min. The absorbance of the reaction was recorded at 734 nm using a spectrophotometer (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). BHT standard solutions (0.01–0.25 mg/mL) were prepared and used as a positive control. The percentage inhibition was calculated according to the formula:

ABTSradical scavenging activity (%) = $[(A0 - A1 /A0) * 100]$, where $A0$ is the absorbance of the control at 15 min and $A1$ is the absorbance of the sample at 15 min. All samples were analyzed in triplicate.

Ferric Reducing Antioxidant Power Test (FRAP)

The reducing power was measured by the method as reported in Tuyen *et al.*[23]. Briefly, an aliquot of 0.2 to 1mL of the extract was mixed with 2.5 mL potassium ferricyanide (1%) and 2.5 mL of phosphate buffer (0.2 M, pH 6.5). After incubation at 50□ for 30 min, 2.5 mL of trichloroacetic acid (10%) was added to the mixture. The mixture was centrifuged at 4000 rpm for 10 min, and an aliquot of 2.5 mL of the supernatant was subsequently taken and mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (0.1%). The absorbance was measured at 700 nm using a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The BHT standard (5–20 ppm) was used as the positive control. The IC50 value was calculated.

HPLC Analysis

Ethyl acetate Extracts from *Sterculia foetida*extracted by 2.3 was dissolved in methanol and filtered through a filter (0.45 µm pore size) prior to injection. Mixed stock solution including reference a standard was prepared by dissolving weighed accurately samples of each compound in methanol. The calibration curves of six various concentrations were obtain by more diluting the mixed stock solution with methanol, and the six various concentrations of mixed standard solutions were injected (10 µL) and measured. HPLC determinations were showed by utilizing an Agilent HPLC 1200 instrument (Agilent Technologies, USA), equipped with a diode array detector (DAD) detector, an auto sampler, a column heater and a Waters Symmetry® C18 (250 mm × 4.6 mm, 5 µm) column. The mobile phase was composed of acetonitrile (A) and water (B), and gradient elution was as follows: 0-20 min, 20-55% A; 20-40 min, 55-65% A; 40-60 min, 60-100% A; 60-70 min, 100% A. The flow rate was 1.0 mL·min-1 and the injection volume was 10 µL. The column temperature was set at 30 ℃ and the wavelengths were monitored from 190 to 800 nm.

GC-MS analysis

Aim of this analysis is to identify the pharmacologically active biomolecule (anticancer & antioxidant) present in the seed extract. The extract of *S. foetida* leaves powder was subjected to GC-MS analysis on the instrument GC and MS JEOL GC mate equipped with secondary electron multiplier (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was fused silica 50 m×0.25 mm I.D. The study conditions were 20 min. at 100°C, 235°C for column temperature at 3 minutes and 240°C for injector temperature, carrier gas was helium, and split ratio was 5:4. The 1 µl of the sample was evaporated in a split-less injector at 300°C and the run time was 22 min. The phytoconstituents of the extract was identified by Gas Chromatography coupled with Mass Spectrometry. The GC-MS spectrum was analyzed using the NIST08 library which has more than 62,000 patterns,[24].

RESULTS

Antimicrobial screening

The antibacterial activity of five different (Ethanol, methanol, Hexane, Diethyl ether and ethyl acetate) leaf extracts of *Sterculia foetida* were determined using Agar well diffusion technique by measuring the diameter of the zone of inhibition. The negative control (DMSO) does not showed inhibitory activity against all bacterial strains. The ethyl acetate extracts of *Sterculia foetida* showed a significant antibacterial activity against all the five organism *E.coli* (KF 918342), *Staphylococcus haemolyticus, Aeromonas hydrophila* (KCTC 12487), *E.coli* (ATCC 35150), *Cronobacter sakazakii* (ATCC 29544), *Aeromonas salmonicida* (KACC 15136) at the high concentration. On analyzing the above results of antibacterial activity, it was confirmed that ethyl acetate leaf extracts possess best antibacterial activity against all the organisms when compared with other leaf extracts and at the same time growth media is

also responsible for antibacterial activity. Therefore it was revealed that the compound present in the ethyl acetate leaf extract showed good inhibitory activity against the organism of *E.coli* (KF 918342), *Staphylococcus haemolyticus, Aeromonas hydrophila* (KCTC 12487), *E.coli* (ATCC 35150), *Cronobacter sakazakii* (ATCC 29544), *Aeromonas salmonicida* (KACC 15136). The antibacterial effect of crude ethyl acetate extract may be due to the presence of phytochemical like flavonoids, tannins, coumarins, saponin, trepenoids etc. The presence of each bioactive molecules exhibit different mechanism on the microorganism. The most effective bioactive components are present in flavonoid compounds which has the ability to form a complex with cell wall of bacteria and extracellular proteins [25]. Compounds under tannin family has the capacity to arrest the cell wall synthesis of bacteria [26]. The small molecules belonging to terpenoidsgroup, have the ability to weaken the membrane tissue by terminating the cell wall of bacteria [27]. Coumarins have the ability to accumulate in the cell adjacent to infected cell [28]. Saponin compounds act as the inhibitor for the growth of bacteria [29]. Further best antibacterial compound can be identified from the above phytochemicals and can be further used for the treatment of bacterial diseases.

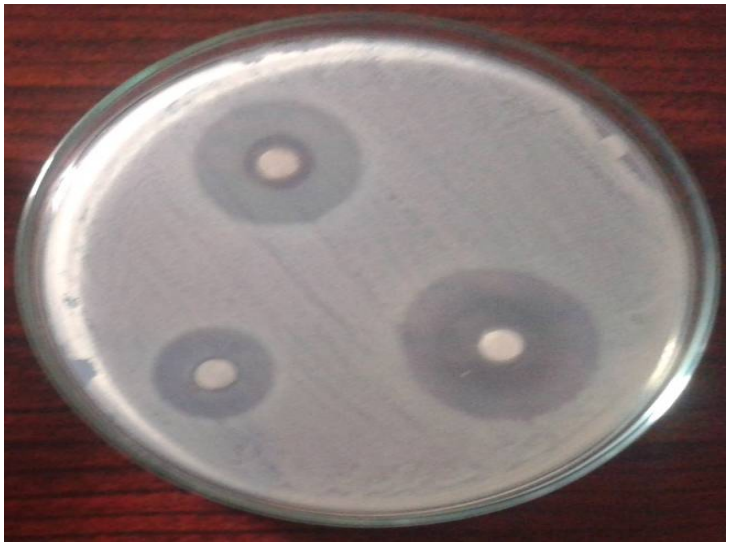


Figure1. Antibacterial activity of ethyl acetate extract of *Sterculia foetida*

Minimum inhibitory concentration

Minimum inhibitory concentration of this crude *Sterculia foetida extract* was investigated by micro broth dilution technique and the outcomes are mentioned in the Table 1. The results performed that the *Sterculia foetida* ethyl acetate extract inhibited all the bacterial growth in broth at various concentrations. Among these bacteria minimum inhibition exposed in *E. coli*. at 6.0μL. Followed by this the growth inhibition demonstrated in *Staphylococcus haemolyticus, Aeromonas hydrophila,and, Aeromonas salmonicida* at 13.5μL. Cronobacter sakazakii inhibitions were showed at 23μL. Streptomycin evaluated the better MIC values in compared the *Sterculia foetida* ethyl acetate extract. Minimum inhibitory concentration results were showed in Table 1.

Table 1 Minimum inhibitory concentration of Ethyl acetate extract from *Sterculia foetida*

Strains	Minimum Inhibitory concentration (MIC)	
	Compound (µl)	Standard (µg)
E.coli (KF 918342)	6.0	25
S. Haemolyticus	13.5	50
Aeromonas hydrophila	13.5	50
E.coli (ATCC 35150)	6.0	25
Cronobacter sakazakii	23	100
Aeromonas salmonicida	13.5	50

DPPH assay

DPPH radical scavenging activity is one of the most efficient techniques for screening the antioxidant activity of plant extract. The results of DPPH free radical scavenging activity of *Sterculia foetida* ethyl acetate extract are demonstrated in Figure 2. The outcomes are demonstrated comparatively active against standard BHT. Ethyl acetate extracts of *Sterculia foetida* having activity depend on concentration manner. *Sterculia foetida* ethyl acetate extract 54.2% DPPH activity revealed at 80μL/ml and 65.88% at 100 μL/ml extract and standard BHT was showed at 60% in 1000μL/ml. DPPH activity showed highest activity 65.88±0.9% at 100µL/ml of ethyl acetate extract.

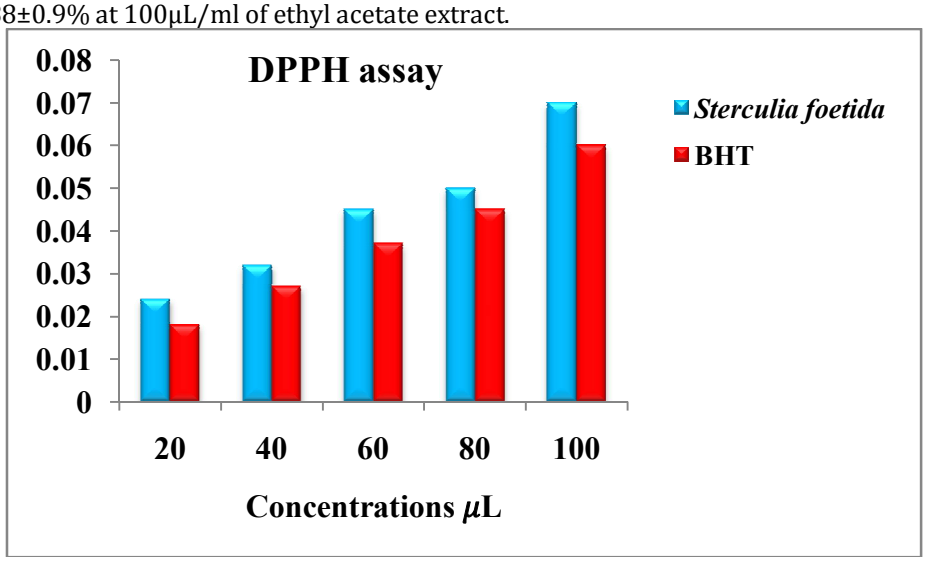


Fig. 2 DPPH Assay of *Sterculia foetida* compared to BHT

ABTS Decolorization Assay

ABTS radical scavenging activity is one of the most efficient techniques for screening the antioxidant activity of plant extract. The results of ABTS radical scavenging activity of *Sterculia foetida* ethyl acetate extract are demonstrated in Figure 3. The outcomes are demonstrated comparatively active against standard ascorbic acid. Ethyl acetate extracts of *Sterculia foetida* having activity depend on concentration manner. *Sterculia foetida* ethyl acetate extract 50.2% ABTS activity revealed at 80μL/ml and 70.4% at 100 μL/ml extract and standard BHT was showed at 60% in 100μL/ml. ABTS activity showed highest activity 70.4±0.9% at 100µL/ml of ethyl acetate extract.

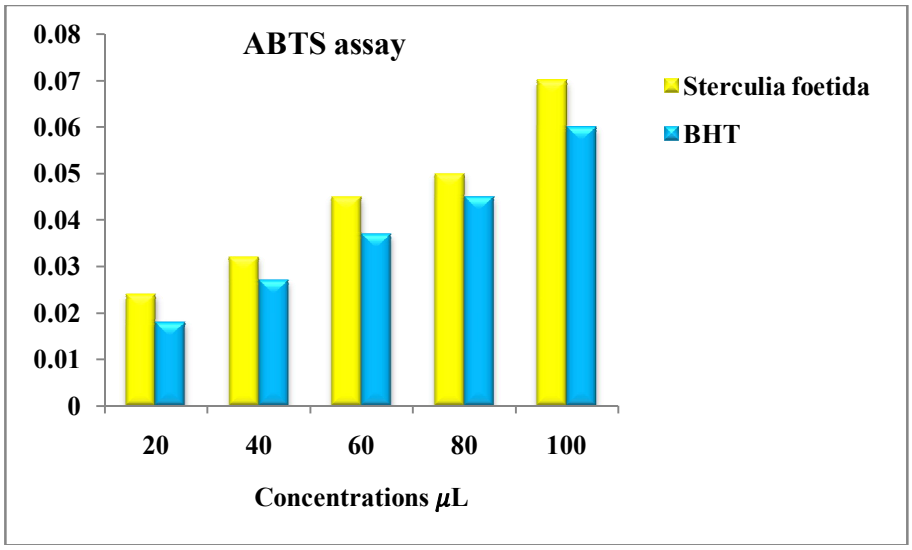


Fig. 3: ABTS assay of *Sterculia foetida* compared to BHT

Reducing power activity

The reducing power activity of the *Sterculia foetida* ethyl acetate crude extract was compared to ascorbic acid. The extract performed comparatively best reducing power activity and increased the absorbance of samples intimated the raised decreasing power activity. Presence of reducers causes the conversion of the Fe3+/ferricyanide complex used in this method to the ferrous form.

The reducing capacity of an extract may serve as a relevant indicator of its prospective antioxidant activity. The reducing power activity of *Sterculia foetida* extract shown in Figure 4.

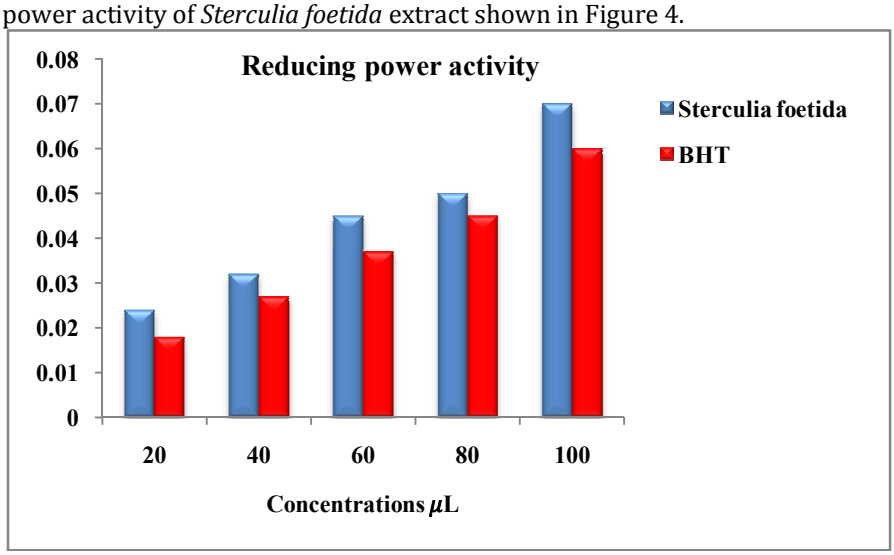
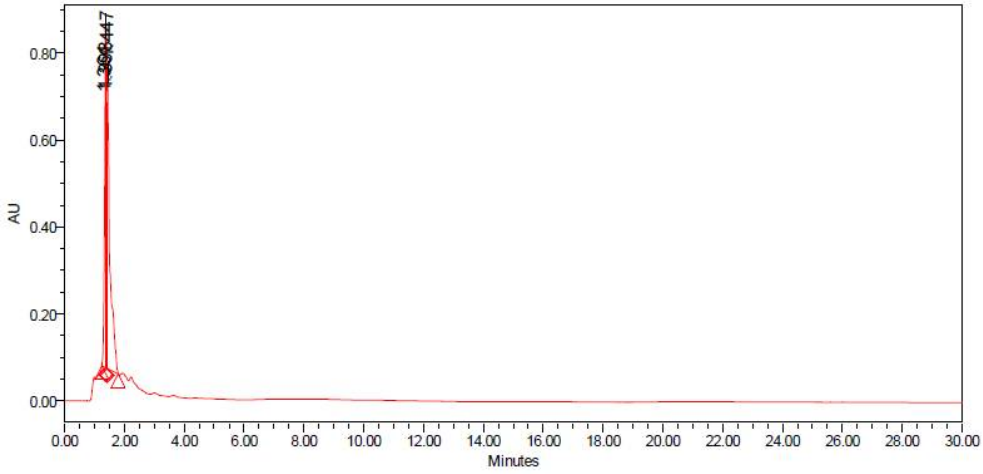


Fig. 4 Reducing Power Assay

HPLC analysis

The phytoconstituents like alkaloids, steroids, phenolic compounds and flavonoids were estimated quantitatively by HPLC method. The results were shown in the figure 5. This study on preliminary screening of leaf extract of *Sterculia foetida* [30] confirmed the presence of metabolites of tannins, 2-deoxysugars, leucoanthocyanin and benzopyrone nucleus. The methanolic leaf extract of *Sterculia foetida* [31] showed the presence of five chemical constituents like alkaloids, proteins, glycosides, phytosterols and saponins whereas result of current study revealed strong presence of secondary metabolites like flavonoids, phenols, carbohydrates, tannins.



Peak Results

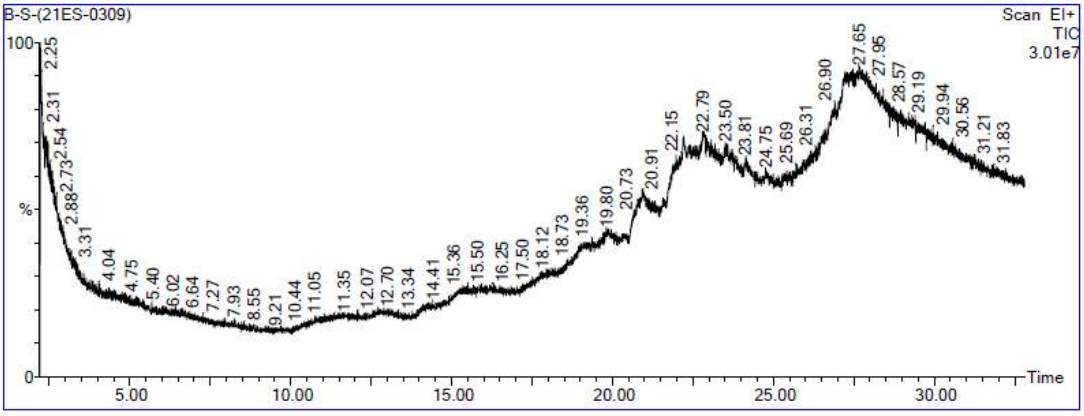
	Name	RT	Area	Height	Amount	Units
1		1.361	2019444	784212		
2		1.398	2145401	792490		
3		1.447	4148358	684110		

Figure 5 Shows that the HPLC analysis

GC-MS analysis

The GC-MS analysis of methanolic extract of *S. foetida* seed powder divulge the presence of thirteen phytochemical compounds that could possess the pharmacological and microbicidal activity. The identification of the biomolecule was confirmed based on the retention time and molecular formula. The biologically active compounds with their Retention time (RT), Molecular formula, Molecular weight, Molecular structure and their Biological activity are presented in figure 6. The pharmacologically active major compounds present in the seeds were Flavanthrone & Flavone (Anticancer activity), 5-Undecyne & Methyl abietate (Antioxidant), 2- cyclohexen-3-ol-1-one,2 (11-phenylundecanoyl) (Antidiuretic), Androstan-6-0l-17-one,3-acetoxy-5Achloro (Vasodilator) and Testosterone Cypionate(Antiinflammatory)

apart from these compounds other major and minor compounds were also present. The GC-MS graph showing the compounds which showing anticancer and antioxidant activities are presented. Similar to this study, twenty seven major phytochemical compounds were characterized through GC-MS analysis of the methanolic leaves extract of *S.urens* Roxb[32]. Asif Jafri *et al*.[33] reported that there were thirty five bioactive compounds were characterized via GC-MS analysis of the ethanolic extract of *S. foetida* seed and they were confirmed further that among 8 bioactive compounds many of them possess pharmacological activity and these findings are similar to the present work.



#	RT	Scan	Height	Area	Area %	Norm %
1	20.951	3749	3,281,818	1,323,498.2	5.606	11.48
2	22.211	4001	5,999,526	3,400,409.8	14.402	29.49
3	22.841	4127	5,599,344	2,986,827.8	12.650	25.90
4	23.497	4258	3,803,805	1,318,705.0	5.585	11.44
5	27.648	5088	8,435,124	11,532,088.0	48.843	100.00
6	28.619	5282	4,359,665	879,951.3	3.727	7.63
7	28.954	5349	3,871,186	1,538,490.0	6.516	13.34
8	29.429	5444	2,769,590	630,572.7	2.671	5.47

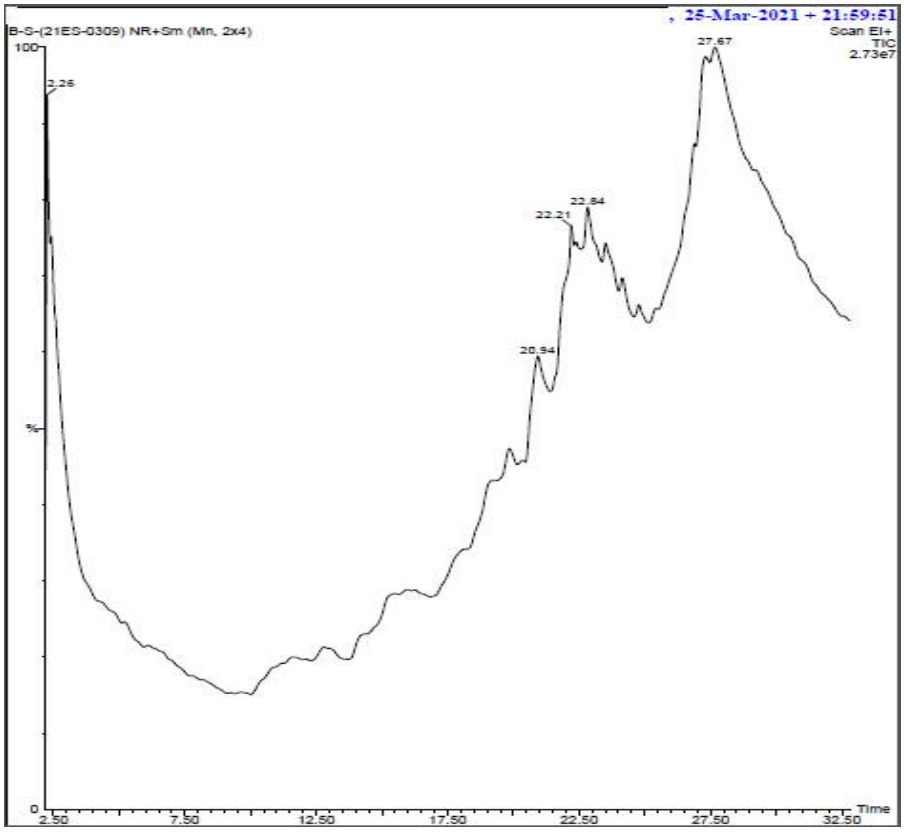


Figure 6: GC chromatograms of *Sterculia foetida* extract

Oxidants are involved in many human diseases and the aging processes. Chronic damage associated with the development of aging can generate destructive oxidants and oxygen free radicals, which are very toxic to tissues and may result in further tissue necrosis and the cellular damage. Cellular mechanisms and external factors involved in the production of oxidative stress include the inflammatory response, free radical leak from mitochondria, auto-oxidation of catecholamines, xanthine oxidase activation, prooxidant activities of toxins such as CCl4 and exposure to ionizing radiation [34]. Aerobic cells are endowed with extensive antioxidant -tocopherol,αdefense mechanisms including both low molecular weight scavengers, such as -carotene, reduced glutathione, ascorbic acid and enzymatic systems, such asβcysteine, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red) and glucose-6-phosphate dehydrogenase (G6PD), which counteract the damaging effects of reactive oxygen species [35]. However, when the balance between these reactive species and antioxidants are altered, a state of oxidative stress results, possibly leading to permanent cellular damage. Our results demonstrate that MSF exhibited an interesting antioxidant activity in cell-free systems. It was able to quench the synthetic DPPH radical and exhibited a SOD-like effect, inhibiting O2 •− formation in a dose-dependent manner. It is reported that green tea tannins are able to protect renal cells against ischemia reperfusion injury [36], characterized by an overproduction of O2 •− due to both an electron leak in the mitochondrial respiratory chain and the conversion of xanthine dehydrogenase to xanthine oxidase [37], which produces O2 •− when it oxidizes xanthine into uric acid. This study, therefore, also considered a possible inhibitory action of the extract on the primary function of this enzyme. The results obtained showed that MSF may determine a dose-dependent inhibition of xanthine oxidase activity. The IC50 values observed were found to be slightly lower than that of standard rutin in nitric oxide radical inhibition assay. The results clearly indicated that the extract was found to be more effective in scavenging the DPPH free radical when compared to the nitric oxide g/mLμ-carotene oxidation at 100 βradical. We also observed the extract inhibited the concentration. The first step in investigating the antioxidant potential of MSF involved defining the relationship between concentration and effect by evaluating its ability to inhibit oxidative processes. Initially, we used a single concentration of extract against all the methods. Having demonstrated that the extract was effective at inhibiting this oxidative process, we then proceeded to confirm this finding with various concentrations of the extract that inhibited the oxidation. On the basis of these experiments, it could be concluded that the methanolic extract of *Sterculia foetida* Linn. can act as primary and secondary antioxidant. It scavenges free radicals and therefore inhibits the lipid peroxidation and may have beneficial effect on prevention of diseases, where reactive oxygen species are involved. Antioxidant properties of its flavonoid compounds can be at the origin of these effects, but further in vivo experiments are planned to verify the relationship between chemical composition and antioxidant activity.

CONCLUSION

Nowadays the use of traditional method as an alternative medicine has been increased. The researchers turn their attention towards the medicinal plants for treating various infections. The present study clearly proved that *Sterculia foetida* Linn showed better antibacterial activity in crude methanol extract. Further isolation, purification and identification procedure is going on for the identification of the particular bioactive compound which is responsible for the antibacterial activity. Later, the isolated bioactive natural compound may serve as leads for the development of new pharmaceuticals against bacterial diseases.

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