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Phytochemical Analysis of Antioxidants From *Abutilon indicum* L. and *Paederiafoetida* L. Supported by TLC, FTIR and NMR Studies

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Abstract: ***Abutilon indicum and Paederiafoetida are two important medicinal plants in traditional healing. Abutilon indicum has proved antibacterial, analgesic, antimalarial property. Studies also show that it has hepato-protective and wound healing capacity. Similarly Paederia foetida, a perennial twinner belonging to family Rubiaceae used for gastric disorder and rheumatic condition. It has also hepato-protective and antidiabetic properties. In present study phytochemical screening of both the plants were done with help of common detection test for antioxidants generally found in plant system. Phytochemical fingerprinting was done with TLC studies where methanolic plant extract were loaded along with standard antioxidant compounds. The result of above studies was backed up by UV, FTIR and NMR studies. The screening confirmed that both the plants have secondary metabolites contributing to their antioxidant potentiality. The DPPH scavenging assay shows lowerIC_{50}forAbutilon indicumi.e. 52µg/ml than 98µg/ml in case of Paederia foetida. Estimation of total flavonoid content shows Abutilon indicumwas having higherphenolics and lower flavonoid content whereas Paederia foetida has almost equal phenolic with high flavonoid content.***

Keywords: Phytochemical screening, TLC fingerprinting, DPPH assay, Total phenolic and flavonoid, FTIR, NMR,UV.

1. Introduction

Traditional medicinal systems of India like Ayurveda, Siddha, Unani are used even today along with modern medicine because of its low cost and minimal side effects [1-2]. The systems rely heavily on medicinal plants. India has a rich source of medicinal plants roughly 8000 in number distributed in 16agro climatic zones. The present study was based on two such medicinally important plants *Abutilon indicum*L. belonging to family Malvaceae and*Paederia foetida* L. belonging to family Rubiaceae[3][4]. The main objective was to evaluate their antioxidant potential. It was clinically proven that oxidative stress generated in aerobic organisms due to ROS lead to cell degeneration which is the main cause of some diseases like arthritis, gastric disorder, cancer, hepatic disorder and neuro degeneration etc. Antioxidants present in plant system can be categorized into two types enzymatic and non-enzymatic. Enzymatic components like super oxide dismutase, peroxidase and catalase act collectively to neutralize oxidative stress generated in living system. In plants, products of primary metabolism such as aminoacids, carbohydrates and proteins are vital for the maintenance of life process while secondary metabolites like alkaloids, phenols, steroids and terpenoids have toxicological, pharmacological and ecological importance. Flavonoids and phenols who come under the secondary metabolites were involved in general antioxidant defense mechanism of plants having similar basic carbon skeleton[5]. In this study rutin andgallic acid are used as standard.Rutinalso called rutoside, quercetin-3-O-rutioside and sophorin, is the glycoside between the flavonolquercetin and the disaccharide rutinose. In the fava d'anta tree, the synthesis is done via a rutin synthaseactivity[6].Gallic acid is a tri-hydroxybenzoic acid, a type of phenolic acid, a type of organic acid, also known as 3,4,5-trihydroxybenzoic acid, found in gallnuts, sumac, with hazel, tea leaves, oak leaves, oak bark, and other plants. The chemical formula is $C_6H_2(OH)_3COOH$. Gallic acid is found both free and as part of hydrolyzable tannins. Gallic acid is commonly used in the pharmaceutical industry. It is used as a standard for determining the phenol content of various analytes by the folin-ciocalteau assay.Identification and quantification of such pharmacologicallyimportant secondary metabolites is necessary to predict the antioxidant potential of medicinal plants. Identification of medicinally potent compounds will help in future use of these plants with accuracy and confidence.

2. Material and Methods

Both the plants*Abutilon indicum*L. and *Paederia foetida* L. were collected from Khurda district of Odisha. The plants were identified in P.G. Dept. of Botany Utkal University and voucher specimen wassubmitted. The plants were acclimatized in the garden of P.G. Dept. of Botany Utkal University. Fresh and healthy leaves were collected, washed;shade dried in room temperature and was converted to coarse powder. 30 g of the powder was extracted with 300ml of methanol in soxhlet apparatus for 72hr. the extract was collected and the solvent was evaporated in rotary evaporator. The semisolid crude extract was weighed and stored at -20˚C for further studies.

2.1 Phytochemicalscreening for antioxidants

The plant extracts were subjected to qualitative tests adopting standard procedure [5][7] foridentification of phytoconstituents known to possess antioxidant property [8-9].

2.1.1 Detection of alkaloids
A few drops of diluted HCl were separately treated with 1ml each of various extracts.Then it was filtered.The filtrates were treated with few drops of Mayer'sreagent.Formation of turbidity confirms the presence of alkaloids.

2.1.2 Detection of flavonoids
Five ml of alcoholic solution of both the plant extract added tofew drops of alcoholic $FeCl_3$ .The formation of blackish red colour indicated the presence of flavonoids.

2.1.3 Detection of phenols and tannins
5ml of alcoholic solution of the plant extracts were treated with few drops of $Fecl_3$solution. The change in colour indicated the presence of phenols.

2.1.4 Detection of phytosterols and terpenoids
Extracts were dissolved in 5ml of chloroform separately. They were subjected to Lieberman-Burchard test. To 1 ml of stock solution a few drops of acetic anhydride and 1ml of concentrated sulphuric acid were added from the sides of the test tubes and allowed to stand for 5 mins. Formation of brown ring at the junction of 2 layers with upper layer turning green indicates the presence of phytosterols and terpenoids.

2.1.5 Detection of sugar
5ml of aqueous extract filtered and subjected to Fehling`s test by adding 1ml of Fehling`s solution A and B and then heated gently. No formation of reddish brown colour indicated the absence of sugar.
2.1.6 Detection of fixed oil and fats
A small quantity of extracts was pressed between the folds of filter paper. Oil stains were seen indicating the presence of fixed oils.

2.2 Estimation of antioxidant potential by DPPH assay:

2.2.1 DPPH Dot Blot assay-
For rapid evaluation antioxidant activity was done by DPPH dot blot assay [10]. The radical scavenging capacity towards DPPH was tested using TLC plate in which 2µl of extract solution was loaded in diluted condition to avoid background colour. The negative control was pure methanol and positive controls were rutintrihydrate and ascorbic acid. The drugs were applied in triplicate and left to dry for 2-3 mins. The TLC sheet was immersed upside down in a 0.4mm DPPH methanolic solution for 10 sec.Stained silica layer revealed a purple background with yellow spots at the location or the drops which indicated radical scavenging capacity.

2.2.2 DPPH radical scavenging assay-
Method first introduced by Blois (1958) [11]. The hydrogen or electron donative ability of the corresponding extracts measured from the bleaching or a purple colouredmethanolic solution or DPPH [12]. 1 ml or 0.1mM DPPH in methanol was mixed with 3ml of the extract solution prepared in methanol with differing concentration (50-250µg). The samples were vortexed and kept in dark for 30 mins at room temperature. The decrease in absorbance was measured at 517nm. Absorbance of DPPH solution in the absence of extract and standards were measured as the control. Pure methanol is used as blank. The IC_{50} value was calculated only for those extract that reduced DPPH in the cuvette by less than 50% when the absorbance reach the plateau. DPPH radical scavenging activity was
Expressed using the formula

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

WhereAo= theabsorbance of the control and $A_{1=}$ the absorbance of the sample. IC $_{50}$ value in µg extract/ml) is the effective concentrations at which DPPH radical, were scavenged by 50%

2.3 TLC study to detect antioxidatively active compounds

Clean glass plates (12x24cm) coated with slurry of silicagel in water in a ratio (1:3 w/v). Thethicknessof slurry was 0.25 mm for qualitative analysis by moving the applicator at a uniform speed from one end to other. The plates so developed left to dry at room temperature. The dried plates were placed in oven at $100\text{-}120^0$c for 1-2 hours to remove the moisture and to activate the adsorbent on the plates. The samples were loaded 1cm above the base line. Acetone: Toluene: formic acid (4.5:4.5:1) used as the mobile phase [13].

2.4 Qualitative 2,2,diphenyl -11- picrylhydrazyl (DPPH) assay on TLC
The plate developed in the above method till the solvent system is evaporated. 0.5 MM methanolicsolution of DPPH was sprayed on the developed plates. The presence of antioxidant compounds were detected by yellow spots against purple back-ground.

2.5 Estimation of total phenolic contents

Total phenolic content was determined by Folin-Ciocalteu method developed by [15] with little modification Methanolic solution of plant extracts prepared in (1mg/ml)concentration. To 1 ml of the extract solution. 1 ml of folin-ciocalteau reagent was added and after 5 minutes, 10 ml of 7% Na_2CO_3 solution was added,followed by 13 ml of distilled water. The reaction mixture was kept for 2 hours with intermittent shaking. The absorbance was measured at 760 mm. the gallic acid is used as standard to calculate total phenolic content in mg GAE/gm of dry wt.

2.6 Estimation of total flavonoid

The flavonoid was determined using Aluminum Chloride colorimetric method [16]. Extract solution was prepared with methanol in a concentration or 1mg/ml. to 1 ml of the extract solution 1 ml of 2% Aluminum Chloride solution was added. The change of colour of the reaction mixture to yellowish confirmed the presence of flavonoid. The

absorbancy was taken at 415 nm. The rutintrihydrate was taken as standard for flavonoids. Total flavonoid was calculated as mg of RE/gm dry wt.

2.7 TLC study for qualitative analysis for phenol and flavonoid

The activated TLC plates were loaded with methanolic solution of plant extract taken in the concentration of 80 mg/ml along with rutintrihydrate taken in the concentrationof 10 mg/ml as flavonoids and 10mg/ml gallic acid as standard phenol.Sample was allowed to move in the solvent system Acetone: toluene: formic acid (4.5:4.5:1) till 3/4th of the plate. The coincidence of spots were observed under visible light and plates were sprayed with folin–ciocalteu reagent prepared with distilled water in 1:3 for detection of phenol and 2% $AlCl_3$ solution for detection of flavonoid. The R_f value of the corresponding spots were calculated.

2.8 Spectroscopic study toidentify antioxidant compounds (UV, FTIR,NMR)

UV/VIS spectrometric study was done of the diluted methanolic solvent extract with spectrophotometer PerkinElmer model FTIR (Fourier transform infrared spectroscopic) was done in Perkin Elmer spectrumRx_1 model. NMR spectroscopy $^1H_1$ and ^{13}C of both the crude samplewere done with deutereted methanol as the solvent in FT-NMR- Burkeravance -400 models. The peakcharacteristic of the common antioxidants was identified in the data generatedby the analysis of the crude extract.

3. Result and Discussion

Table1: Phytochemical analysis for antioxidants in *Abutilon indicum* and *Paederia foetida* in methanolic extract

Test	*Abutilon indicum*	*Paederiafoetida*
Alkaloids	+	+
Flavonoids	+	+
Phenols	+	+
Phytosterols&Terpenoids	+	+
Sugar	+	+

3.1 Result of quantitative analysis for antioxidant with DPPH

Table 2: DPPH radical scavenging assay

Concentration in µg	% of inhibition AIME	% of inhibition PFME
50	44%	22%
100	82%	56%
150	94%	86%

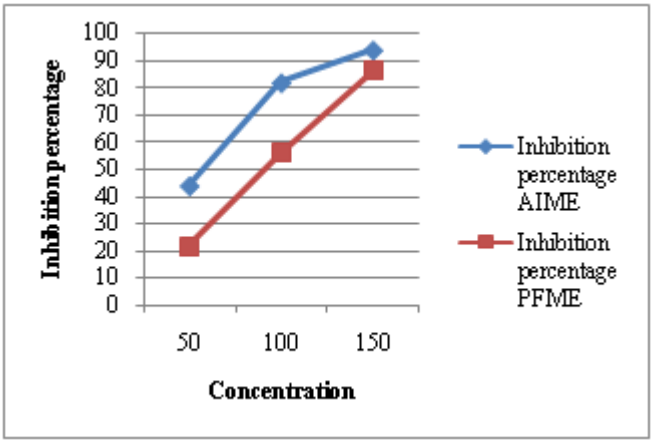

Figure 1: Inhibition percentage of DPPH radical

Table 3: IC_{50} value of AIME and PFME

Name of the extract	IC_{50} value
AIME	52 µg/ml
PFME	98 µg/ml

Abutilon indicum shows lower IC_{50} value than *Paederiafoetida* which indicates higher radical scavengingcapacityin case of*Abutilon indicum*.

Table4: Total phenol and flavonoid content

Name of the Sample	Total phenollic content in mgGAE/gmdry wt	Total flavonoidsmg re/gm dry wt
AIME	4.794+/-0.143	5.291+/-0.442
PFME	3.464+/-O.512	7.854+/-0.019

Abutilon indicum shows slightly higher phenolic content and lower flavonoid content than *Paederia foetida*

3.2 Result of TLC studies for qualitative analysis of antioxidant potentials

3.2.1 DPPH dot blot assay

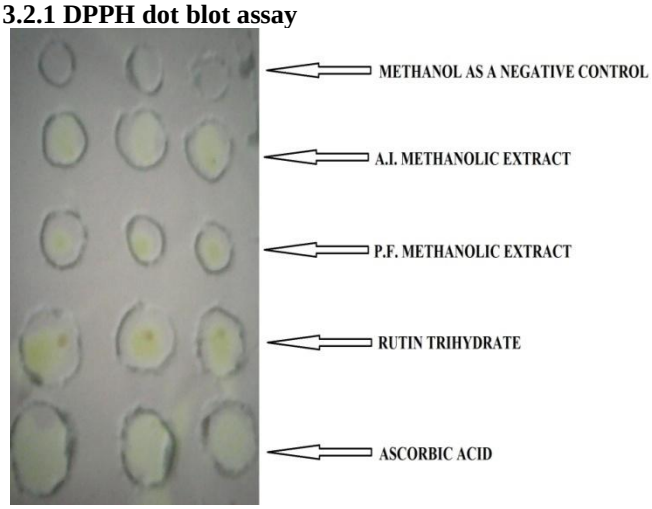

Figure 2: DPPH dot blot assay

Radical Scavenging activity of methanolic extract for *Abutilonindicum* is higher as compared to *Paederia foetida* but much lower than the positive controls ascorbic acid and rutintrihydrate

After treatment with DPPH

Figure 3: Identification of Antioxidatively active spot by DPPH

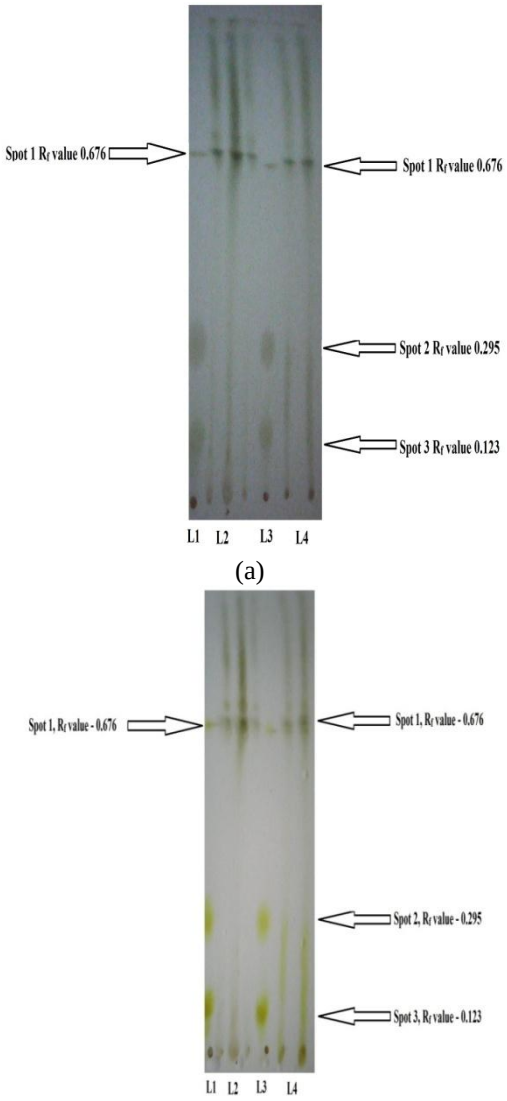

Figure 4: (a): TLC plate with both plant extract loaded with standard Rutin trihydrate

(b): After treatment with aluminum chloride solution

L1: Standard rutin, L2: *Abutilon indicum*, L3: Standard rutin, L4: *Paederia foetida*

Table 5: R_f value of the spots developed for flavonoid

Name of the extract	R_f value of spot 1	R_f value of spot2	R_f value of spot 3
AIME	0.671	-	-
PFME	0.676	0.295	0.123
StandardRutin	0.671	0.285	0.119

3.2.2 TLC Finger printing to locate compounds related to antioxidant property

Antioxidant compounds were visualized as yellow spots on the TLC plates. In case of *Abutilon indicum* three prominent spots were developed whereas in case of *Paederia foetida*four yellow spots developed. This indicates the amount of antioxidatively important compounds present in AIME and PFME.

Spot-1 yellow spotafter spraying with $AlCl_3$ coincide with both the plant extracts with equal $R_f$value.Of 0.671 and 0.676 with standard rutin.

Spot -2 has not developed in case of *Abutilon indicum.* In *Paederiafoetida*lane has equal R_f value of 0.295 & 0.285

Spot -3 Which develop in equivalence with rutin standard in case of *Paederia foetida* has R_f value 0.123 in comparison to 0.119 in standard.

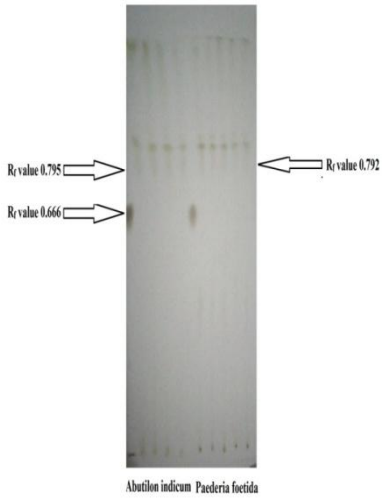

Figure 6: TLC of plant extract with Gallic acid used as standard

Table 7: $R_f$value or spot identical to Gallic acids

Name of the sample	R_f value or spot identical to gallic acids
AIME	0.795
PFME	0.792
Gallic acid to methanol	0.666

Spots developed in comparison to standard gallic acid with equal colour development shows presence of phenol in both the plant extracts, only one spot developed for flavonoid in case of *Abutilon indicum* but two extra spots resolved in *Paederia foetida* in equivalence to standard.

3.2.3 Spectral Analysis (UV, FTIR and NMR)

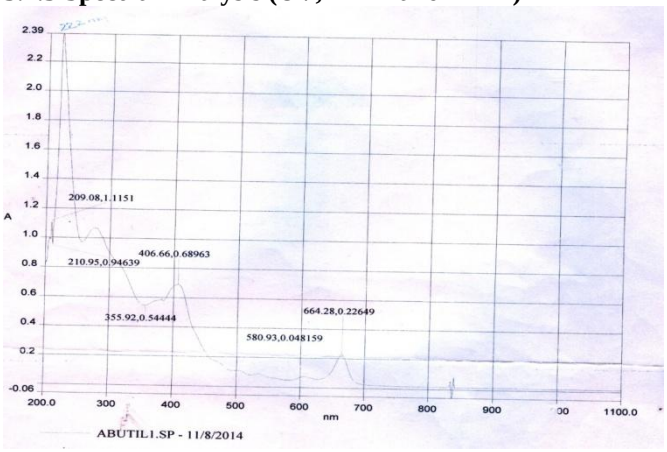

Figure 7: UV Spectral analysis of *Abutilon* species in the range 200nm -1100nm.

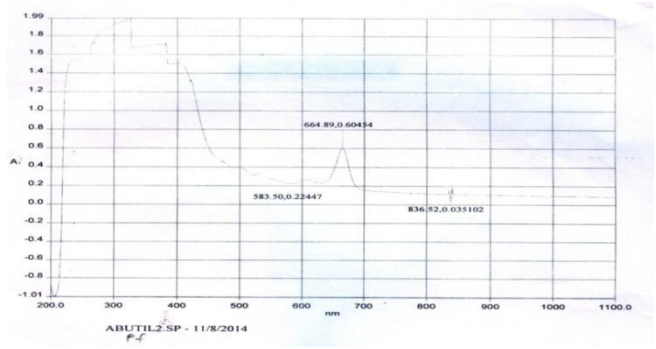

Figure 8: UV spectral analysis of *Paederia* species with 200nm-1100nm.

Fig-6:The UV visible spectra of *Abutilonindicum*, peaks are at 209.08 nm, 210.95 nm, 355.92 nm and 406.66nm in UV region and peak at 580.93 and in 664.28 in visible region.

Fig-7:The UV visible spectra of *Paederiafoetida*, peaks are at 310 nm and 390 nm in UV region where as peaks at 583 and 664 nm in visible region.

The fig. 6 and fig.7 indicates presence of coloured compounds as well as colourless component. Phenolic unsaturated compound shows peak in UV region which indicates presence of phenolic in both the plants [2].

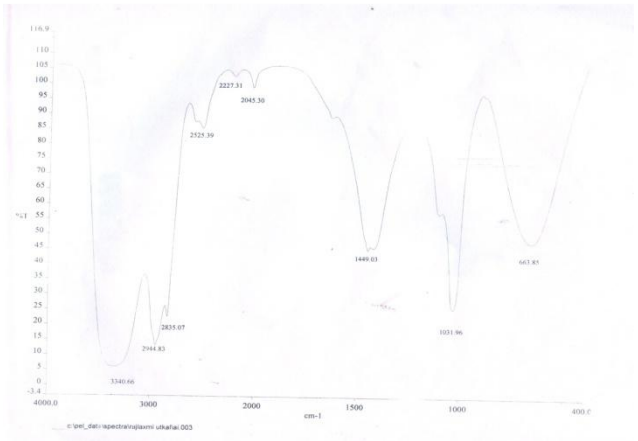

Figure 9: FTIR analysis of *Abutilonindicum*

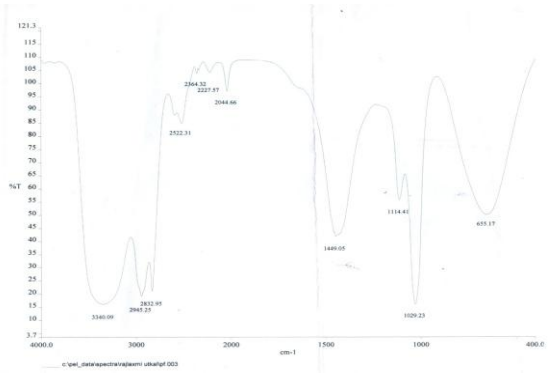

Figure 10: FTIR spectral analysis of *Paederia foetida*

FTIR of AIME has absorption regions at 3340, 2944, 2835, 2525, 2227, 2045, 1449, 1031 and 663 in cm-1FTIRofPFME shows absorption peaks at 3340,2945,2835,2522,2364,2229,2044,1449,1114,1029 and 655 in cm-^{1}IR spectroscopy is one of the more powerful analyticaltool, in identification of functional groups.The broad peak at 3340.09 and 3340.66 both the plants coincides with band 3550-3200 typical to phenolic -OH group. Peaks of PFME at 2945, 2832, 2522 and peaks of AIME at 2944, 2835 and 2525 corresponding to carboxylic acid stretch with characteristic band of 3000-2500 cm-1. Nitrile group C≡N has its characteristic band at 2260-2220. AIME has a peak at 2227.31cm-1 and PFME at 2227.57 showing the presence of this functional group.Alkyl C-H with band 2950-2880 coincides with 2444.83, 2835.07 to peaks in AIME and 2945.25, 2832.95 of PFME.Alcohol, Ether, carboxylic acid and ester having functional group O-H has a peak at the range 1260-1000 which coincides with 1114-1029 of PFME and AIME.

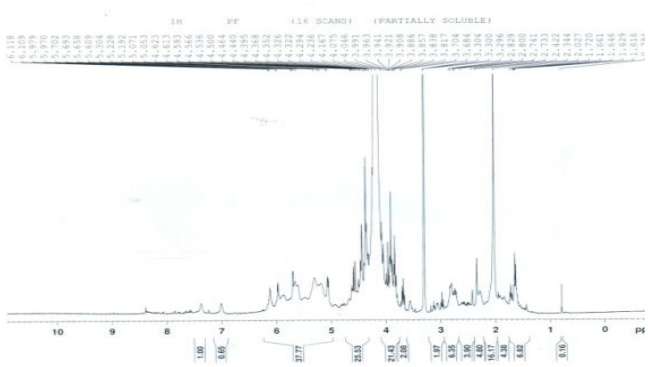

Figure 11: $^{1}H_{1}$ NMR analysis of *Paederia foetida*

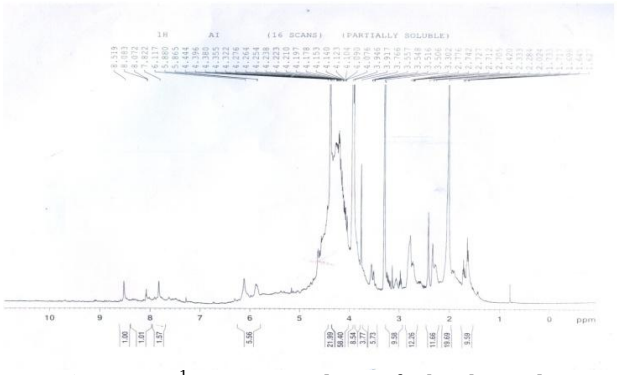

Figure 12: $^{1}H_{1}$ NMR analysis of *Abutilon indicum*

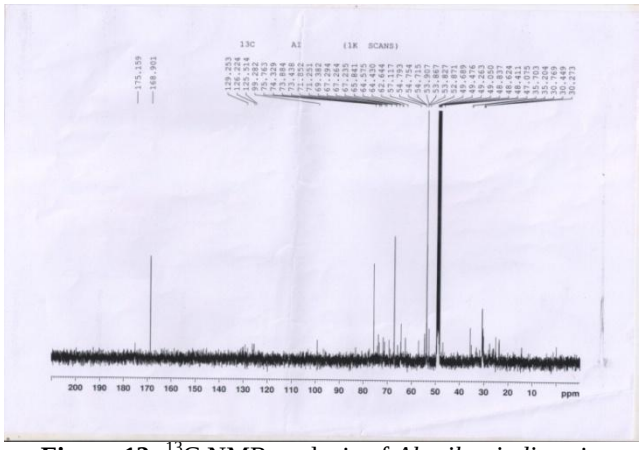

Figure 13: ^{13}C NMR analysis of *Abutilon indicum*in methanol.

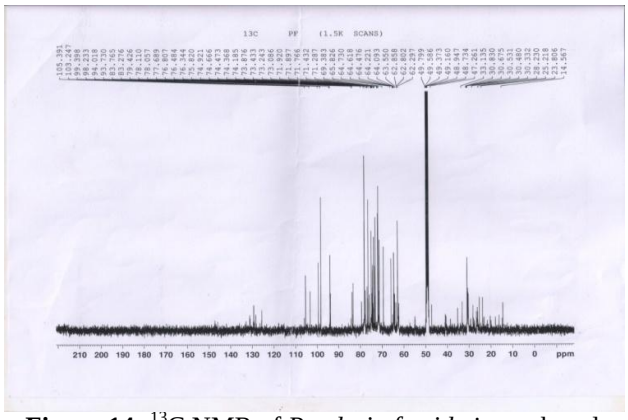

Figure 14: ^{13}C NMR of *Paederia foetida* in methanol

1H NMR spectroscopy provides information about the nature, number and chemical frame structure of different protons in the compound 13 c NMR shows chemical shift, 13c -1H coupling constant and no. of attached hydrogen atoms [17][18].In case of alkaloids UV-Vis spectra lies in 250-303 nm heterocyclic protons appear in the regimes 5.5-7 ppm methyl group attached to C appear in 20-30 ppm, methyl group attached to 0-atom appeared around 50-60 ppm. Methyl group attached to N-atom appeared in the region 40-50 ppm [17]IR spectrum shows band in 3200-3600 and 1047-1934 corresponding to methylene dioxyphenyl grouping Double bond indicates by peak near 1600 cm-1. So all these indicates presence of alkaloids, in AIME, PFME. Flavonoids show absorption in UV-Vis region. IR shows band at 3470-3200 cm-1 for chelaters non-chelaters [19] group and at 1600-1653 for sulphate moiety at 1260(s=0), 1050 (-c-o-s). In aromatic region it has peaks at 7.0,7.8, 8.08 and 8.5. in aliphatic region it has peaks at 3.3,3.7, and 3.9 with J range 8.5 confirms flavone moiety.Absorption at 3.7-4.1 ppm shows methoxyl protons and absorption in the range 1.65 – 2.5 ppm shows acetoxyl proton in flavonoid. 13C NMR spectrum confirmed the presence of p-hydroxyl phenol moiety [17].

3.3 Discussion

Phytochemical screening shows the presence of alkaloids, flavonoids, phenols, and terpenoids in both *Abutilon Indicum* and *Paederia foetida*.This shows that methanolic extract of both the plants contain most of the antioxidants found in dry leaf [8].The DPPH radical assay Is based on ET (Electron Transfer) mechanism of antioxidant estimation. The capacity of a compound to transfer hydrogen atom from its active site to the radical to deactivate it indicates its antioxidant nature. In DPPH assay this process changes the characteristic purple colour of the DPPH solution to yellowish. The colorimatic method involves decrease in absorbancy by increasing activity of the plant extract to neutralizing free radicals generated by the DPPH. *Abutilon indicum* and *Paederia foetida* both shows decrease in absorbancy but *Abutilon indicum*has higher power of inhibition compared to *Paederia foetida* as former shows IC_{50}value 52µg/ml and later shows IC_{50}value 98µg/ml.

Phenol content of *Abutilon indicum* found to be little higher with value of 4.79mg/gm dry wt. than *Paederia foetida* 3.46 mg/gm dry wt. but flavonoid content of *Paederia foetida* is higher as compared to *Abutilon indicum*, former shows the value of 7.854mg/gm dry wt. and later 5.291 mg/gm dry wt. The –OH group present in the active site of phenolic compounds are responsible for the antioxidant nature of the compound. TLC study carried out to identify the phenol and flavonoid with standard Gallic acid and rutin confirms the presence of more flavonoid in *Paederia foetida*. Spectral analysis of samples with UV, IR, $^{1}H_{1}$NMR and ^{13}C NMR as explained above indicates presence of –OH groups associated with carboxylic stretching, ether and ester which corresponds with characteristic functional group of phenolic compounds [20][21].Presence of phenolic compound in appreciable amount confers the antioxidant property to the above studied medicinal plants.

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