Detailed Project Report

Project Title: Phytochemical Analysis of some antioxidant plants with special reference to phenolic compounds.

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Introduction:

Plants are the rich source of a variety of chemical compounds which imparts them medicinal properties. Besides other phytochemicals, phenolic compounds and flavonoids are observed to be distributed in plants which have been reported to exert multiple biological effects including antioxidants, free radical scavenging, anti-inflammatory and anticarcinogenic etc. As a crude extracts of herbs, spices and other plant materials, rich in phenolic compounds are of increasing interest in the food industry because they retard the oxidative degradation of lipids and thereby improve the quality and nutritional value of food items. While flavonoids (a group of polyphenolic compounds) with known properties have potential scavenge the free radicles and inhibit the hydrolytic and oxidative enzymes and inflammatory action.

Since the ancient times, the medicinal properties of plants have been investigated. In recent times, the scientific work and development in such investigations highlighted the role of plants with potent antioxidant activities. As antioxidants have been reported to prevent

oxidative damage caused by free radicals, it can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers. The potential reactive derivatives of oxygen attributed as reactive oxygen species (ROS) are continuously generated inside the human body. The generated ROS are detoxified by the antioxidants present in the body. However, overproduction of ROS or inadequate antioxidants, defense can easily persuade oxidative damage to various biomolecules including proteins, lipids, lipoproteins and nucleic acids. This oxidative damage is a critical etiological factor implicated in several chronic human diseases such as Dibetes mellitus, Cancer, Atherosclerosis, Arthritis and Neurodegenerative diseases and also in ageing process. Therefore, the aspect of phytochemical analysis of some antioxidant plants species was planned to investigate from Akola region.

Objectives:

1) Selection of the plants with antioxidant potential from Akola region

2) Qualitative phytochemical analysis with special reference to phenolic compounds.

3) Total phenolic content of the selected plants.

4) Analyzing antioxidant potential of the selected plants.

5) Correlation between phenolic content and antioxidant activity.

Materials and Methods:

Twelve medicinal plants were collected from the Akola region, Maharashtra (India) and identified using flora of Marathwada (Naik, 1998) and flora of Maharashtra State (Singh and Karthikeyan, 2000). The specimen of each collected species is deposited in Department of Botany, Shri Shivaji College of Arts, Commerce and Science, Akola. The samples were cleaned using tap water to remove the soil and then rinsed with distilled water. The plant samples were dried for 10-15 days in the shade at room temperature. These samples were then ground to a fine powder and were stored in polythene bottles at 4° till the time of experiments. All the reagents and chemicals used in the experiments were of analytical grade. All chemicals used were obtained from the local suppliers.

The plant extracts were prepared in methanol by adding 100 ml of methanol to 1 g of plant powder. The infusions were stirred on the magnetic stirrer at room temperature for 5 h. This was then centrifuged at 6000 rpm at 4° for 10 min and the supernatant was stored at -4° for further analysis. Moisture content, total ash, acid insoluble ashes were determined using standard methods. All the spectrophotometric measurements of the following assays were performed by UV/Vis spectrophotometer (BioEra Make, India).

Phytochemical determination

Powdered plant samples (5 g) were extracted with a mixture of methanol and water (150 ml) in the volume ratio 4:1 using Soxhlet for 12 h. The extract was cooled and filtered through Whatman filter paper No. 41. The residue obtained was extracted with 125 ml of ethyl acetate and the percentage of crude fibres was calculated from the residue. The amount of fats and waxes was determined by evaporating the ethyl acetate.

The filtrate (methanol and water) was reduced to approximately 1/10th of its original volume and acidified with 2M H_2SO_4. This filtrate was extracted with 75 ml (3×25 ml) chloroform in a separating funnel. The chloroform layer was separated and evaporated to dryness on a water bath maintained at 45°. This contains phenolics and terpenoids. The aqueous layer obtained after the separation was adjusted to pH 10 with 2M NaOH. It was further extracted with 60 ml chloroform and methanol (3:1) followed by extraction with 40 ml chloroform in a separating funnel.

The separated aqueous basic layer was evaporated to dryness on a water bath. The residue so obtained consists of quaternary alkaloids and N-oxides. The organic layer (chloroform and methanol) was transferred to a beaker and solvent is evaporated to dryness. The residue so obtained was the basic extract consisting of alkaloids.

DPPH Radical Scavenging assay

Total free radical scavenging capacity of the extracts from different plant samples were estimated according to (Brand- Williams et al., 1995) with slight modification using the stable DPPH radical, which has an absorption maximum at 515 nm. A solution of the radical is prepared by dissolving 2.4 mg DPPH in 100 ml methanol. A test solution (5 µl) was added to 3.995 ml of methanolic DPPH. The mixture was shaken vigorously and kept at room temperature for 30 min in the dark. Absorbance of the reaction mixture was measured at 515 nm spectrophotometrically. Absorbance of the DPPH radical without antioxidant, i.e. blank was also measured. All the determinations were performed in triplicate. The capability to scavenge the DPPH radical was calculated using the following equation (Yen et al., 1994). DPPH Scavenged (%)= ((AB–AA)/AB)×100…..(1), where, AB is absorbance of blank at t= 0 min; AA is absorbance of the antioxidant at t= 30 min. A calibration curve was plotted with % DPPH scavenged versus concentration of standard antioxidant (Trolox).

Ferric reducing antioxidant power

The antioxidant capacity of the medicinal plants was estimated spectrophotometrically following the procedure of Benzie and Strain (1996). The method is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+}-tripyridyltriazine (blue colored complex) formed by the action of electron donating antioxidants at low pH. This reaction is monitored by measuring the change in absorbance at 593 nm. The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 ml TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37°. Freshly prepared working FRAP reagent was pipetted using 1-5 ml variable micropipette (3.995 ml) and mixed with 5 µl of the appropriately diluted plant sample and mixed thoroughly. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml FRAP reagent+5 µl distilled water) after 30 min incubation at 37°. All the determinations were performed in triplicates. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$. The concentrations of $FeSO_4$ were in turn plotted against concentration of standard antioxidant trolox. The FRAP values were obtained by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mg of Trolox equivalent per gram of sample.

Determination of total phenolic content

The total phenolic contents in medicinal plants were determined spectrophotometrically according to Folin-Ciocalteu method (Singleton et al., 1999). Gallic acid was used to set up the standard curve. The content of phenolic compounds of the samples was expressed as gallic acid equivalents (GAE) in mg per gram dry weight. All the samples were analyzed in triplicates.

Determination of total flavonoid content

The $AlCl_3$ method (Ordon-Ez et al., 2006) was used for quantification of the total flavonoid content of the methanolic plant extracts. 20 µl of the sample extract was added to a solution of 2% $AlCl_3.6H_2O$. The mixture was vigorously shaken and diluted with double distilled water to make the total volume 10 ml. The absorbance of the reaction mixture was measured after 10 min incubation at 440 nm. Flavonoid contents were expressed as quercetin equivalents in mg per gram dry material. All the determinations were performed in triplicates.

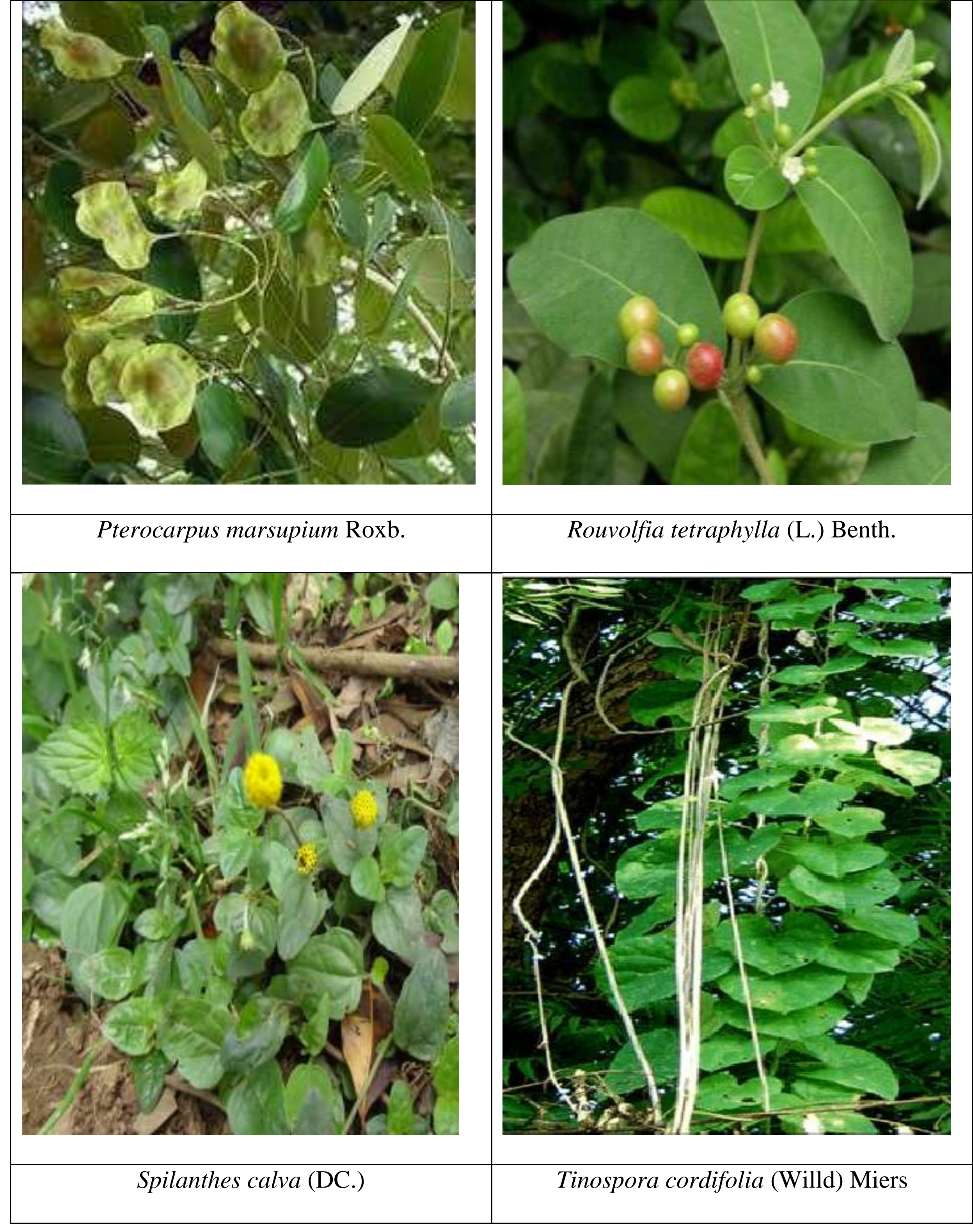
Abrus precatorius L.	*Acacia nilotica* (L.) Willd

Acalypha indica L.

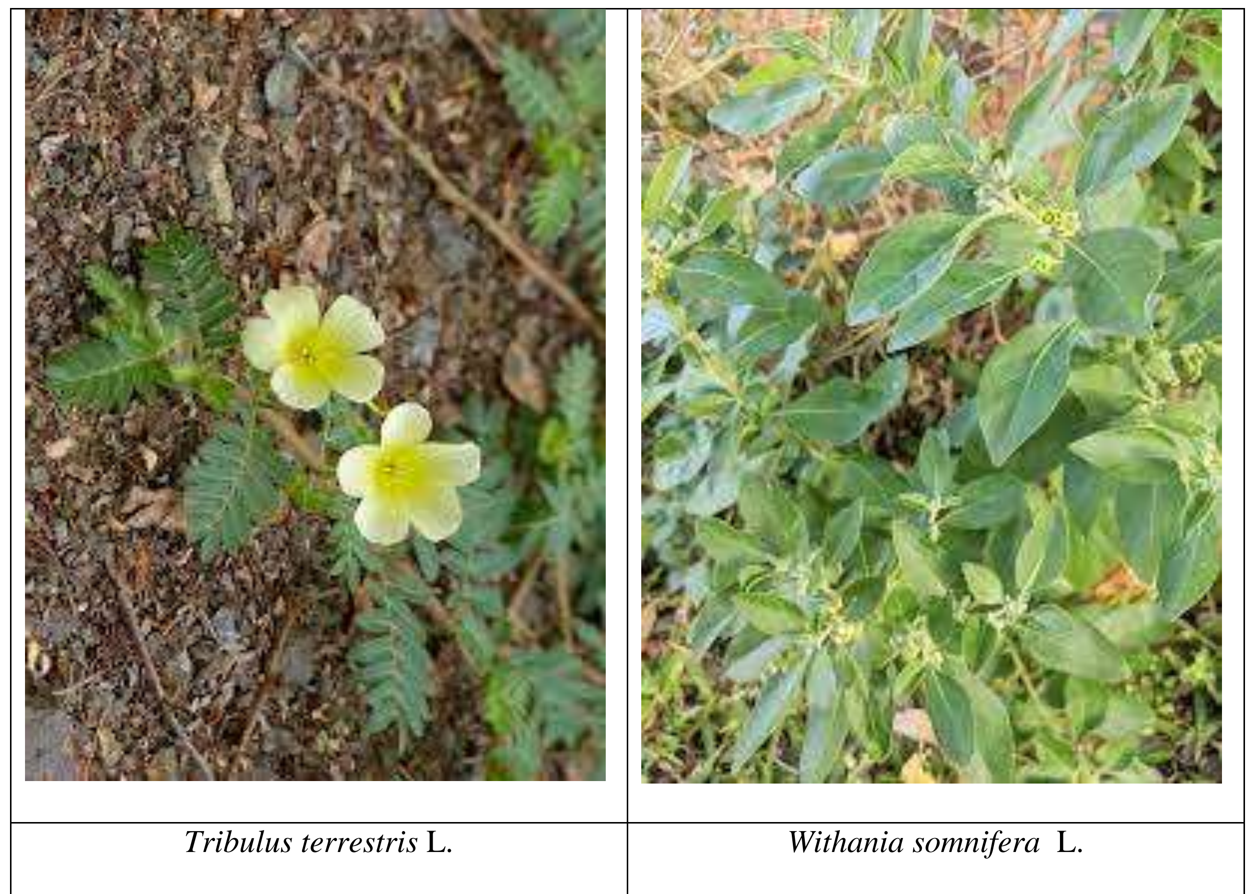
Centella asiatica (L.) Urb

Gymnema Sylvester (Retz.) R. Br.

Khaya Senegalensis (Desr) A. Juss.



Pterocarpus marsupium Roxb.	*Rouvolfia tetraphylla* (L.) Benth.
Spilanthes calva (DC.)	*Tinospora cordifolia* (Willd) Miers



Tribulus terrestris L. | *Withania somnifera* L.

Results

During the present investigation, 12 medicinal plants were analysed for their proximate composition and antioxidant activity. A list of medicinal plants analysed along with parts used is given in **table -1** and medicinal uses are given in separate section (**table-2**). The preliminary phytochemical analysis of selected medicinal plants is presented in **tables -3** and quantitative analysis of alkaloids, phenolics and flavonoids is presented in **table- 4**. **Table 5** given details about the antioxidant activity of given samples.

After surveying the forest area of Akola region and forests around following twelve plants were selected for the phytochemical analysis and antioxidant activity analysis. The table shows the botanical name, vernacular name, family and part used for medicinal purpose. Table 2 showed the reported medicinal properties of selected medicinal plants.

Table-1 Plants selected for the study with their botanical names, vernacular names, family name and parts use in traditional medicine.

Botanical Name	Vernacular Name	Family	Part used
Abrus precatorius L.	Gunj	Fabaceae	Leaves
Acacia nilotica (L.) Willd	Babul	Mimosaceae	Bark
Acalypha indica L.	Kuppi	Euphorbiaceae	Leaves
Centella asiatica (L.) Urb	Mandukparni	Apiaceae	Leaves
Gymnema Sylvester (Retz.) R. Br.	Bedakipala	Asclepiadaceae	Leaves
Khaya Senegalensis (Desr) A. Juss.	Mohogany	Meliaceae	Bark
Pterocarpus marsupium Roxb.	Bija	Fabaceae	Stem
Rouvolfia tetraphylla (L.) Benth.	Sarpangandha	Apocynaceae	Root
Spilanthes calva (DC.)	Akkalkala	Asteraceae	Leaves

Tinospora cordifolia (Willd) Miers	Gulwel	Menispermaceae	Root/ stem
Tribulus terrestris L.	Gokhru	Zygophyllaceae	Fruits
Withania somnifera L.	Ashwagandha	Solanaceae	Root

Table-2: Reported medicinal potential of selected medicinal plants

Botanical Name	Medicinal properties
Abrus precatorius L.	The plant is used in some traditional medicine to treat scratches and sores and wounds caused by dogs, cats and mice, and are also used with other ingredients to treat leucoderma. The leaves of the herb are used to cure fever, cough and cold.
Acacia nilotica (L.) Willd	This plant has anti-microbial, anti-plasmodial and antioxidant activity. Also used to treat hepatitis C virus and cancer and treatment of nausea, burns and wounds, stomachache and diarrhea.
Acalypha indica L.	The plant is used to treat inflammation, cancer and wounds.
Centella asiatica (L.) Urb	Plant use as wound healer, to treat various skin problems and rashes, eczema, psoriasis, diarrhea and female urinary tract infections and anxiety.
Gymnema Sylvester (Retz.) R. Br.	The plant is used to treat diabetic conditions, anemic conditions, cough, malaria and antidote against snake bite.
Khaya Senegalensis (Desr) A. Juss.	Traditional use to have Anti-sickling, antimicrobial and anthelmintic properties.
Pterocarpus marsupium Roxb.	The flowers and Bark is anthelmintic and astringent. Also use in treatment of diarrhea and dysentery.
Rouvolfia tetraphylla (L.) Benth.	The leaves and roots are used to treat blood pressure, snake bites, diabetes, piles and malarial conditions.
Spilanthes calva (DC.)	The plant is known to have anti-inflammatory, diuretic and aphrodiastic effect. Flowers effective against tooth problem.
Tinospora cordifolia (Willd) Miers	Plant use traditionally to treat fever, jaundice, cancer, bone fracture, asthma and eye disorders.

Tribulus terrestris L.	Plant fruits use to treat kidney disorder, painful urination, skin diseases and psoriasis.
Withania somnifera L.	The plant use to treat Asthma, diabetes, hypertension, stress, arthritis and cancer.

Preliminary Phytochemical Analysis:

The preliminary phytochemical analysis of the samples prepared from the selected medicinal plant was done. It was noted that all selected plants/ plant samples are rich in phytochemicals. Phenolic compounds and flavonoids are found in every samples. Alkaloids tested negative in *Khaya senegalensis, Spilanthes calva* and *Tribulus terrestris*. Tannins were found absent in the samples of *Abrus precatorius, Khaya senegalensis* and *Spilanthes calva*. While the glycosides were found absent in *Acalypha indica, Acacia nilotica, Khaya senegalensis, Rauvolfia tetraphylla* and *Tribulus terrestris*.

Table-3: Preliminary Phytochemical Analysis of the selected medicinal plant samples in methanolic extracts:

Botanical Name	Alkaloids	Phenolics	Flavonoids	Tannins	Glycosides
Abrus precatorius L.	+	+	+	-	+
Acacia nilotica (L.) Willd	+	+	+	+	-
Acalypha indica L.	+	+	+	-	-
Centella asiatica (L.) Urb	+	+	+	+	+
Gymnema Sylvester (Retz.) R. Br.	+	+	+	+	+
Khaya Senegalensis (Desr) A. Juss.	-	+	+	-	-
Pterocarpus marsupium Roxb.	+	+	+	+	+

Rouvolfia tetraphylla (L.) Benth.	+	+	+	+	-
Spilanthus calva (DC.)	-	+	+	-	+
Tinospora cordifolia (Willd) Miers	+	+	+	+	+
Tribulus terrestris L.	-	+	+	+	-
Withania somnifera L.	+	+	+	+	+

Quantitative Analysis:

The alkaloids, total phenolic compounds and flavonoids were quantified in the given samples. The total phenolics are expressed as gallic acid equivalent (GAE) in g/100 g of sample and flavonoids are presented as quercetin equivalent in g/100 g of sample. It was noted that the *Rouvolfia tetraphylla* has highest content of alkaloids followed by *Centella asiatica* and *Withania somnifera*. The sample recorded phenolic compounds in the range of 0.52 ± 0.01 to 5.08 ± 0.11 g/100 g of samples; the highest content was recorded in the sample of *Acacia nilotica* followed by *Withania somnifera*, its least content was recorded in the sample of *Tribulus terrestris*. The range of flavonoid content in samples was 1.50 ± 0.20 to 6.88 ± 0.35 g/100 g. The highest content was recorded in the sample of *Acacia nilotica* followed by *Tinospora cordifolia* and *Abrus precatorius*. The least amount of flavonoid is recorded in the sample of *Acalypha indica*. The quantitative statement of availability of alkaloids, total phenolics and total flavonoids is presented in table-4.

Table-4: Quantitative analysis of alkaloids, total phenolics and total flavonoids in samples of selected medicinal plants (g/ 100g of sample).

Botanical Name	Alkaloids	Total Phenolics	Total Flavonoids
Abrus precatorius L.	0.35 ± 0.01	1.45 ± 0.05	4.20 ± 0.22
Acacia nilotica (L.) Willd	0.50 ± 0.01	5.08 ± 0.11	6.88 ± 0.35
Acalypha indica L.	0.68 ± 0.03	0.85 ± 0.01	1.50 ± 0.20
Centella asiatica (L.) Urb	1.50 ± 0.05	0.95 ± 0.05	6.50 ± 0.50
Gymnema Sylvester (Retz.) R. Br.	0.65 ± 0.05	1.50 ± 0.07	5.85 ± 0.15
Khaya Senegalensis(Desr) A. Juss.	0.85 ± 0.07	1.05 ± 0.05	1.85 ± 0.09
Pterocarpus marsupium Roxb.	0.15 ± 0.02	1.52 ± 0.05	3.50 ± 0.17
Rouvolfia tetraphylla (L.) Benth.	2.45 ± 0.11	0.90 ± 0.07	2.45 ± 0.05
Spilanthus calva (DC.)	0.18 ± 0.01	0.95 ± 0.05	2.12 ± 0.25
Tinospora cordifolia (Willd) Miers	0.70 ± 0.05	0.85 ± 0.15	4.25 ± 0.30
Tribulus terrestris L.	0.30 ± 0.05	0.52 ± 0.01	3.45 ± 0.25
Withania somnifera L.	1.25 ± 0.11	3.05 ± 0.11	3.70 ± 0.45

Antioxidant Activity of Samples of selected Medicinal Plants:

The antioxidant activity of samples of selected medicinal plants was analyzed using standard methods i.e. DPPH assay and FRAP assay and results were expressed as trolox equivalent antioxidant activity in mg/gdw). In case of DPPH assay the highest activity was recorded in *Centella asiatica* (1.85 ± 0.11 TEAA mg/gdw) followed by *Abrus precatorius* and *Withania somnifera*. The lowest activity was recorded in Acalypha indica sample (0.20 ± 0.01 TEAA mg/gdw). In case of FRAP assay more or less similar trend was observed where Cen*tella*

asiatica sample showed highest activity, followed by samples of *Acacia nilotica* and *Withania somnifera*. The least values were recorded in the sample of Khaya senegalensis (2.65 ± 0.21 TEAA mg/gdw).

Table 5: Antioxidant activity of samples of selected medicinal plants (Trolox equivalent antioxidant activity in mg/gdw)

Botanical Name	DPPH	FRAP
Abrus precatorius L.	1.45 ± 0.15	4.36 ± 0.12
Acacia nilotica (L.) Willd	1.15 ± 0.11	10.25 ± 0.25
Acalypha indica L.	0.20 ± 0.01	1.05 ± 0.01
Centella asiatica (L.) Urb	1.85 ± 0.11	10.74 ± 0.41
Gymnema Sylvester (Retz.) R. Br.	0.84 ± 0.04	6.14 ± 0.15
Khaya Senegalensis	0.20 ± 0.01	2.65 ± 0.21
Pterocarpus marsupium Roxb.	1.05 ± 0.05	4.22 ± 0.15
Rouvolfia tetraphylla (L.) Benth.	0.95 ± 0.11	5.65 ± 0.25
Spilanthus calva (DC.)	0.90 ± 0.07	3.03 ± 0.11
Tinospora cordifolia (Willd) Miers	1.15 ± 0.11	6.89 ± 0.45
Tribulus terrestris L.	0.34 ± 0.05	3.25 ± 0.21
Withania somnifera L.	1.35 ± 0.15	7.62 ± 0.24

Correlation of Phenolic compounds, flavonoids and Antioxidant activity:

It was observed that the phenolic contents correlates well with its FRAP and DPPH assays. It confirming that phenolic compounds are likely to contribute to radical scavenging activity of these plant extracts. Further it could be stated that flavonoids content also directly correlated

with antioxidant potential. Thus there found a strong correlation between total phenolic content, flavonoid content and radical scavenging activity.

Conclusion:

From the current study, it is concluded that the plants selected for the study are having significantly rich in phytochemical composition. The quantitative analysis showed that most of them are having significant level of phenolic compounds and flavonoids and it was also reveal in the antioxidant activities of respective samples.

It was observed that the phenolic content in the extracts showed a much higher correlation with reducing power than with the radical scavenging activity. It could be estimated that the phenolic compounds present in the extracts act as an antioxidants directly through the mechanism of the reduction of oxidized intermediate in the chain reaction.

The present study provides the useful information about Preliminary phytochemistry, antioxidant properties and polyphenolic contents of some Indian medicinal plants, which are used for the therapeutic purposes. The findings of this study support the fact that some medicinal plants commonly consumed in India are promising sources of potential antioxidants.

Signature of Principal Investigator

Signature of Principal