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Antioxidant Determination And Thin Layer Chromatography Of Extract Withania Somnifera, Terminalia Arjuna, Bacopa Monnieri, Ranunculus Sceleratus And Acalypha Indica.

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Abstract

The main focus of the present paper was to determine Quantitative Analysis, Antioxidant Assays and Thin layer Chromatography Analysis of the roots extracts of Withania somnifera, Bacopa monnieri, Terminalia arjuna, Rannunculus sceleratus and Acalypha indica. Extractions of the plant's roots were carried out by Maceration method using three solvents Ethanol, Methanol and Chloroform. Phytochemical Quantitative analysis, consisting of Total Phenol Content, Total Flavonoid Content and Alkaloid Content were done using the standard phytochemical assays. The extracts were screened for Antioxidant Assays including DPPH (1,1-Diphenyl-2-picrylhydrazyl) Radical Scavenging Assay, ABTS(2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid)) Radical Scavenging Assay, Hydrogen Peroxide Assay. Selected methanol and ethanol extracts were further used for particular compound separation process through Column Chromatography and Thin Layer Chromatography. The results of the Quantitative screening revealed the presence of the three important bioactive compounds i.e. Phenols, Flavonoids, and alkaloids in a high a very high concentration in Ethanol, methanol plant's extracts but highly less concentration in Chloroform extracts. Similarly, the Antioxidant Scavenging Activity Of ethanol and methanol plant's extracts were shown having a very high activity except in Bacopa monnieri and methanol extracts of Rannunculus sceleratus where moderate activity is shown. The Chloroform extracts had shown very less or no scavenging activity. The methanol and

ethanol extracts were estimated having activity of DPPH:45%-65%, ABTS:45%-70%, H_2O_2:90%-97%. In this current investigation, only selected extracts i.e. ethanol and methanol extracts were subjected to column chromatographic separation, around 80 fractions of total extracts were separated. The TLC analysis of the ethanol and methanol extracts showed the presence of the three compounds as spots in specific fractions indicating the extracts as potent antioxidant agents, the existence of phytogenic chemical compounds which could exert several beneficial effects by virtue of their antioxidant activity.

Keywords- *Antioxidant Activity, Column Chromatography, Thin Layer Chromatography, Ethanol, Methanol.*

1.Introduction-

Traditional medicinal agents in nature are the best source for thousands of years and some modern drugs [1]. Medicinal plants have been studied in detailed since ancient time with no practically difference is observed in medicinal herbs values as well as in methods of treatment in ayurvedic, the allopathic and the homeopathic literature.[2] Phytochemicals are in the strictest sense of the word, chemicals produced by plants. The plant's values are because of major phyto-constituents present in them bitter drug, flavonoids, alkaloids, saponins, coumarins, phenol carboxylic acids, terpenes etc. These has specific characteristics and properties in plants. Therefore, these constituents analysis in plants would help in determining many biological activities of plants [3-4]. Herbalism is a folk medicine practice entirely based on use of plants and its extracts. These produces wide array of bioactive molecules, a rich source of medicines. [5]. The World Health Organization (WHO) stated, around 80% of the world's population is being relied on traditional plants for their primary healthcare [6]. Herbal plants usage in Asia has represented a long history of interactions of humans with the environment. These wide range of substances are used to treat chronic as well as infectious diseases [7-8]. Almost whole population of India are using traditional medicinal system since many centuries. Medicinal plants are believed to contain new chemical substances with beneficial potential therapeutic effects [9]. The Infectious diseases has remained as one of the leading cause of deaths worldwide, almost killing 50,000 people per day [10-11]. As defined by World Health Organization (WHO), infectious diseases act as evidence disease caused by pathogenic microorganisms as bacteria, viruses, multi-cellular parasites, fungi, prions. Treatment of infectious disease are done by antimicrobial agents like antibiotics or chemotherapeutic drugs [12]. These are consumed in an appropriate dosage, as prescription of

antimicrobial drugs are vital contributors of drug resistance [13-15]. These are defined as the reservoirs of potential chemical compounds serving as newer leads and clues for modern drug design. Alkaloids, tannins, flavonoids and phenolic compounds are highly important compounds [16-18]. Their medicinal effects produces a definite physiological action on the human body. The most important properties of these active constituents are, these more effective with very less or no side effects when compared to the commonly used synthetic chemotherapeutic agents [19]. Many of the plants contains large amounts of phenolic antioxidants. Phytchemicals are anticarcinogenic, antiatherogenic, antiulcer, anti-thrombotic, anti-inflammatory, immunemodulating, antimicrobial, varodialatory, and analgesic effects. The exploitation of natural antioxidants, mainly of plant origin, has greatly elevated in recent years [20-21]. Flavonoids are the most commonly widely distributed group of plant phenolics [22].

Natural antioxidants are essential to prevent as well as cure the disorders caused by free radicals. These free radicals are highly reactive chemicals species produced in the body, having the potential to damage cells, organelles, DNA, and other biomolecules and resulting in diseases such as cancer, and cardiovascular and neurodegenerative ailments [23-24].These treatment has serious efficacy and safety issues and often are highly expensive, difficult for many people to afford it. Such necessitates efforts are performed in order to discover safe, effective remedies, which can be easily available to all class of people. Nowadays, treatment of harmful diseases has become a challenge because of multi-drug resistance issue, as pathogens rapidly develop resistance to existing antibiotics. Hence, new alternatives are required to treat infectious diseases [25]. It is established that oxidative stress is among the major causative factors of many chronic and degenerative diseases which includes atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immune-suppression, neurodegenerative diseases and others [26]. A huge number of aromatic, medicinal, spice and other plants contain active chemical compounds exhibiting antioxidant properties. Oxidative process is one of the most common routes for producing free radicals in foods, drugs, living systems [27]. The effective path to eliminate and diminish the free radicals activity which cause the oxidative stress is anti-oxidative defence mechanisms. Antioxidants are the substances which possess free radical chain reaction breaking properties. Recently there has been an increase of interest in the therapeutic potential medicinal plants as antioxidants in re-antioxidants in reducing oxidative stress-induced tissue injury [28]. Naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective [29],

inhibits lipid peroxidation (by inactivating lipoxygenase), scavenges free radicals and actives oxygen species by propagating a reaction cycle and chelates heavy metal ions [30]. Medicinal plants studies strongly supports the plant constituents designs with the antioxidant activity which are highly capable of exerting highly protective effects against oxidative stress in the biological systems [31]. Oxidative stress depicts the free radicals and reactive oxygen species existence (ROS), which are produced under normal physiological conditions but are deleterious if not eliminated by the endogenous systems. These results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts initiating oxidation in vivo and in vitro and creating oxidative stress leading to numerous diseases and disorder [27-32] including cancer [33], cardiovascular disease [34], neural disorders [35], Alzheimer's disease [36] mild congnitive impairment [37], Parkinsons disease [38], alcohol induced liver disease [39], ulcerative colitis [40], ageing [41], atherosclerosis [42]. Medicinal plants are the primary source of antioxidants [43]. Natural antioxidants increases the antioxidant capacity of the plasma with reducing the risk of harmful diseases with the secondary metabolites mainly phenolics and flavonoids reported to be most potent free radical scavengers. [44-45]. There is a high need for more effective, less toxic and cost effective antioxidants.

2. Materials And Methods

2.1Plant Materials:-Specimens (roots) of *Withania somnifera, Terminalia arjuna, Bacopa monnieri, Ranunculus sceleratus* and *Acalypha indica* were collected from different locations of Uttarakhand, near Agrakhal and Uttar Pradesh, near surajpur area.

2.2Plants Authentication:-The botanical authentication was performed by Dr. Shailesh Solanki, Head Of The Department of Agriculture, Faculty Of sciences, Noida International University, U.P.

2.3 Plant Extraction- The extraction process was performed by maceration [46]. Three different solvents ethanol, methanol and chloroform were used for the low polarity to high polarity extraction. In 1:10 ratio, the roots and the solvents were mixed, heated and shaken. The mixture was then filtered through Whatman No.1 filter paper and dried. The dried crude extracts were stored in a refrigerator at 4 °C.

2.4Quantitative Assay:-

2.4.1Total Phenol Content (TPC)- In this, Folin-Ciocateu (FCR) reagent method [47] has been used. For standard, Gallic Acid in various concentration with 100ul of extracts, 1ml of Distilled water and 0.2 ml of FCR reagent (0 were mixed and incubated at room temperature

for 15 min. Solution containing 2.5 ml sodium carbonate, 30 mins of incubation at room temperature, measured at 765 nm.

2.4.2Total Flavonoid Content (TFC)-Aluminum chloride method [48] has performed to determine the flavonoid content. The reaction mixture (3.0 ml) comprised of 100ul of extract, 0.1 ml of aluminum chloride and 0.1 ml of potassium acetate (120 mM), 2.5ml of methanol. Absorbance measured at 415 nm. Quercetin used as standard.

2.4.3Total Alkaloid Content (TAC)- a reaction based method [49] i.e. alkaloid with bromocresol green. 0.1ml extract was dissolved in 1ml of 2 N HCl and filtered.0 .5ml of this was added in 1.6 ml chloroform in a separatory funnel. By using 1 N NaOH, phosphate buffer pH was adjusted to neutral. 1.6 ml of bromocresol solution, 1ml of the solution along 1.6 ml of phosphate buffer were added in separatory funnel. By vigorously shaking, fractions were separated and diluted with 1ml chloroform, O.D. measured at 470 nm.

2.5 Antioxidant Assays: -

2.5.1DPPH (1,1-Diphenyl-2-picrylhydrazyl) Radical Scavenging Assay-The process is based on electron-transfer procedure [50]. The free radical gets reduced in the presence of an antioxidant molecule, giving rise to colourless solution. Plant extracts, antioxidants mainly has an scavenge effect was examined on the DPPH free radical. Ascorbic acid used as control. Solution including 0.5ml sample, 1.33ml methanol with 1ml DPPH reagent, mixed, shaken vigorously, incubated for 5 min and absorbance was measured at 517 nm. A blank sample containing methanol and DPPH was prepared. DPPH reagent solution was freshly prepared and stored in the dark. Radical percentage Scavenging = $((OD_{AC}-OD_{AS})/OD_{AC})*100$, OD is the absorbance, AC is DPPH reagent, AS is DPPH reagent +extract.

2.5.2 ABTS(2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid)) Radical Scavenging Assay-It is a decolorization assay [51]. Ascorbic Acid was used as standard. Methanol was used as blank. 7 mM ABTS in water with 2.45 mM potassium persulfate (1:1) is used to produced ABTS+ cation radical, stored at room temperature in dark for 12-16 h before usage. ABTS diluted with methanol for having an absorbance of 0.700 at 734 nm. 2 µl of extract, 2 ml of diluted ABTS solution and 0.198ml methanol were added. 30 min after mixing, the absorbance were taken. Percent inhibition, ABTS scavenging effect (%) = $((OD_{AB}-OD_{AA})/OD_{AB})*100$, OD is the absorbance, AB is ABTS reagent+methanol, AA is ABTS reagent + extract or standard.

2.5.3 Hydrogen Peroxide Assay-20ml H_2O_2 (40 mM) was prepared with phosphate buffer (0.05 mM, pH 7.4). 0.02ml of extract, 0.6ml H_2O_2 solution with 1.98ml phosphate buffer were mixed and immediately measured O.D at 230 nm [52] was taken. Similarly, for blank containing only phosphate buffer. Ascorbic acid is was used as control. The percentage inhibition of H_2O_2 scavenging: % radical scavenging activity= $((OD_C - ODs)/OD_C)*100$, OD is absorbance, C is Control, S is sample.

2.6 Column Chromatography- Only Selected extracts i.e**.** ethanol and methanol extracts showing higher Quantitative compound content and Antioxidant activity were subjected to Silica gel column chromatography for phyto-constituents isolation. A vertical glass column (40 mm width × 60 mm length of borosilicate) was used with 200 g of silica gel (60–120 mesh size) as the packing material dissolved in hexane poured from the top of the $3/4^{th}$ column by closing the stopcock. Sea sand (50–70 particle size) was added on top to 1 cm height. 10ml 0f extracts were poured down from the top of the column along the sides and was rinsed down with the solvent. Gradient elution method was followed to separate fractions using solvents from low polarity to high polarity (hexane to methanol). The flow rate was adjusted to 2 ml/min and 5-6 extract fractions were collected for each sample extracts.

2.7 Thin Layer Chromatography

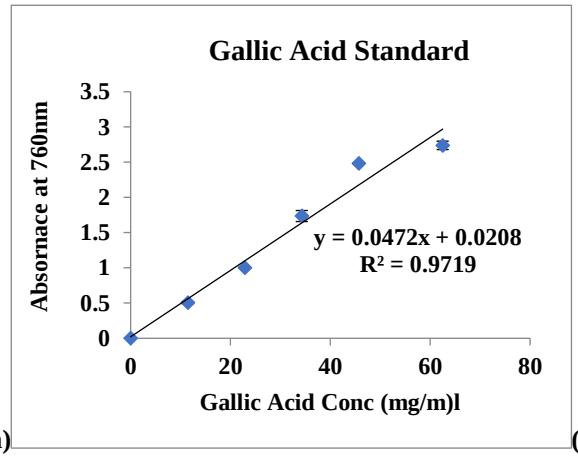
Each fractions of different samples were collected and subjected to TLC (glass plate coated with silica gel G, 0.25mm thickness) to detect the presence of phyto-compounds. Firstly, the plates were completely dried in room temperature and then at 70^oC in hot air oven for 1 hour, for plates activation. The samples extracts were prepared by diluting the each extracts with their respective solvents (50ul) i.e. methanol and ethanol. 1-10 ul volume of each extracts were spotted on the TLC plate by capillary tubes, just 1cm above marked with pencil.The mobile phase and the spraying reagent were added as depicted in Table 1, depending upon the type of compounds and dried at 100 °C in hot air oven for 20-30 min. The plates were analysed in Daylight, Short and Long UV wavelength. The Rf value of each spot was calculated:-

Retention Factor (Rf Value)= Distance moved by the spot / Distance moved by the mobile phase.

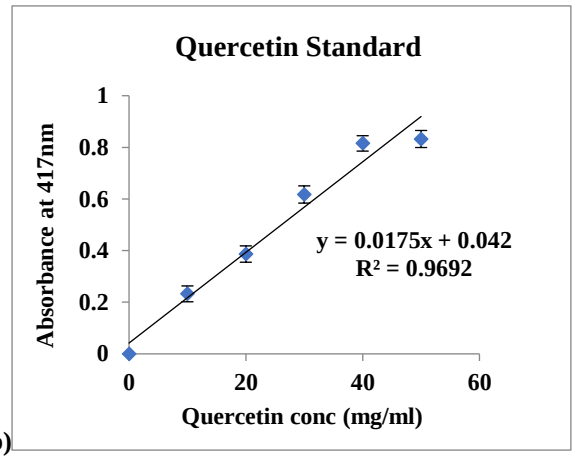
2.8 Statistical Analysis- In triplicates the works were done. The data were recorded as means ± standard deviations (SD).

3. Results And Discussions

3.1 Quantitative Analysis-



a)



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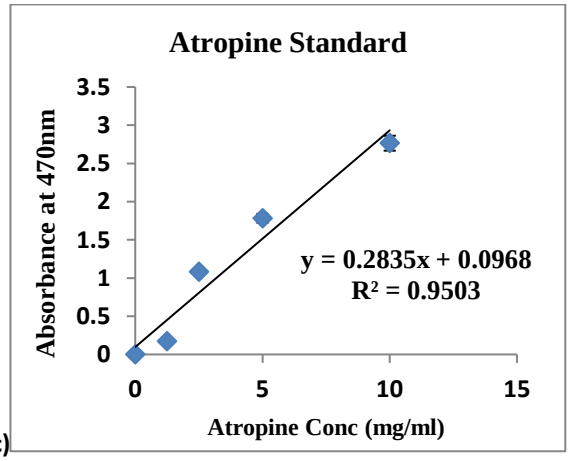
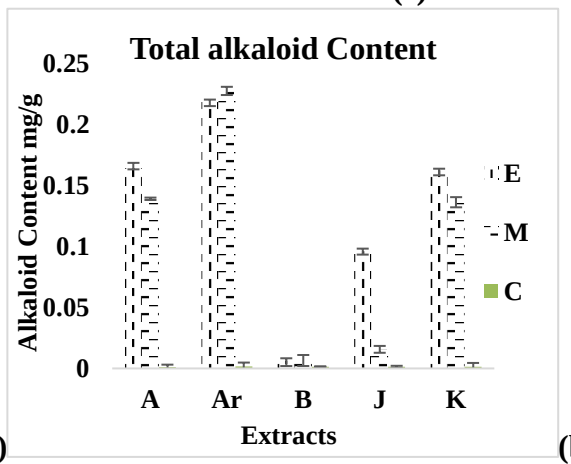
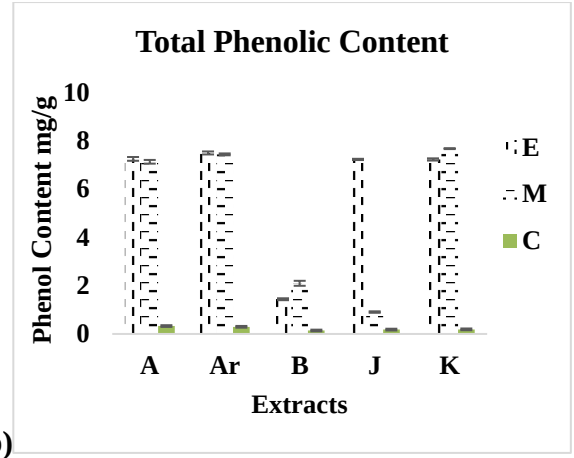


(c)

Graph.1 Standard Graphs of (a) Total Phenolic Content, (b) Total Flavonoid Content, (c) Total Alkaloid Content



(a)



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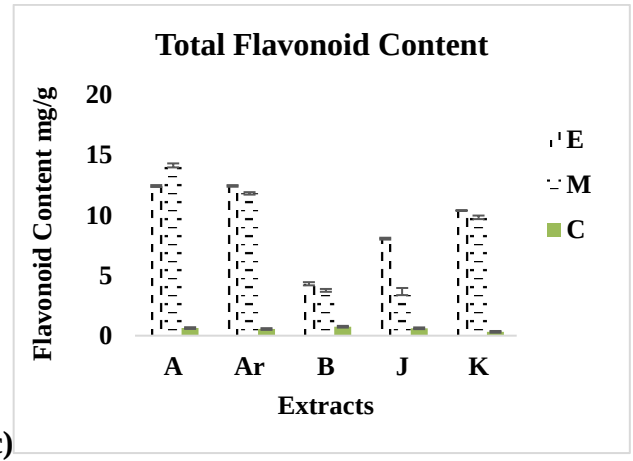
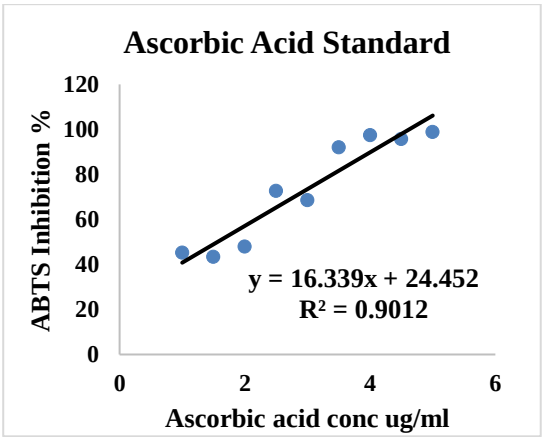


(c)

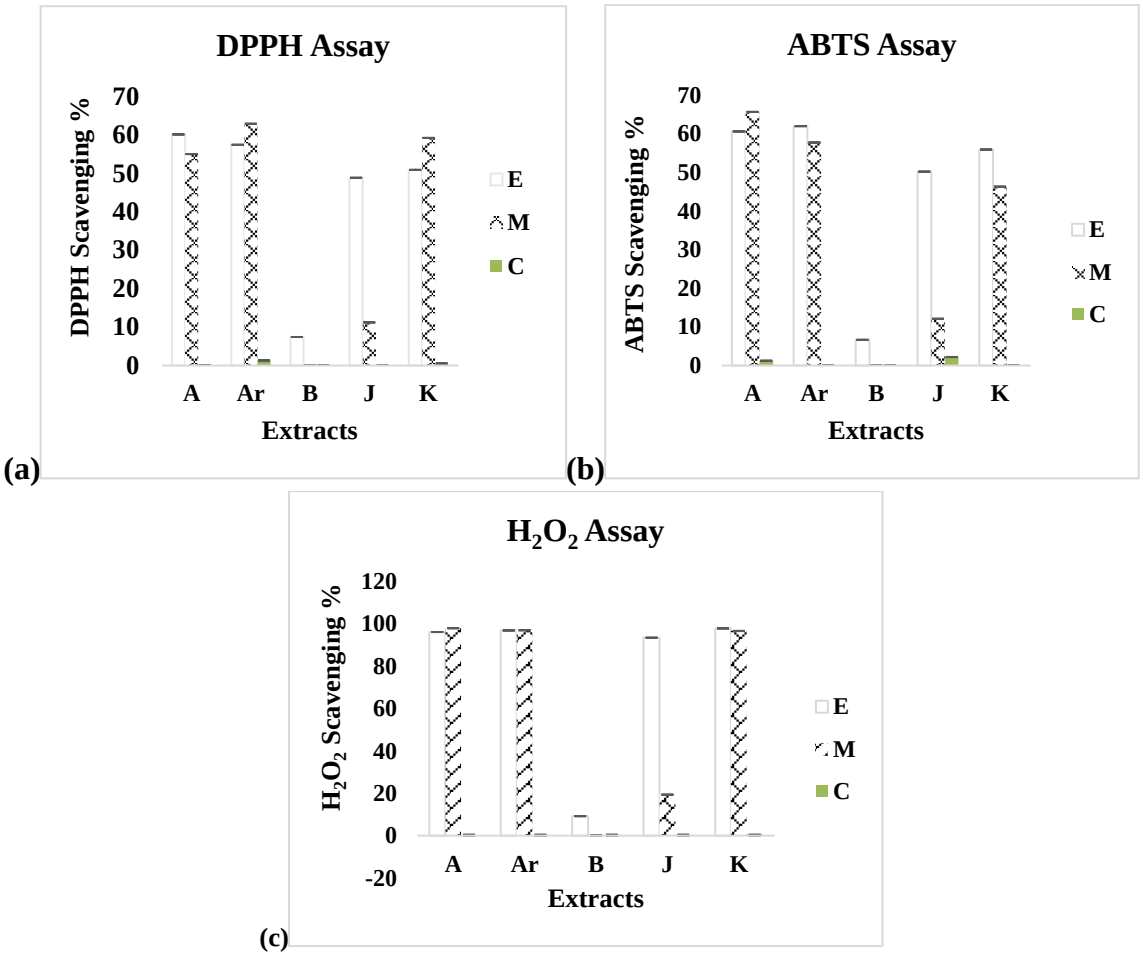
Graph. 2 Quantitative Analysis of Root Extracts (a) Total Alkaloid Content, (b) Total Phenolic Content, (c) Total Flavonoid Content, where A- *Withania somnifera*, Ar- *Terminaliya arjuna*, B- *Bacopa monnieri*, J- *Rannunculus sceleratus*, K-*Acalypha indica*, E- Ethanol, M-Methanol, C- Chloroform.

The Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Alkaloid Content (TAC) in each extract were measured, using equation of standard curves of Gallic acid (GAE), Quercetin (QE) and Atropine (AE) respectively as shown in Graph.1. Graph. 2, *Bacopa monnieri*, ethanol and methanol extracts has shown less concentration1.43±0.03 mg GAE/g, 2.09±.1 mg GAE/g and 4.31±.13 mg QE/g, 3.76±.11 mg QE/g and .006±.001 mg AE/g , .007±.004 mg AE/g respectively. In *Ranunculus sceleratus*, ethanol extract showed good concentration 7.22±.02 mg GAE/g, 8.06±.07 mg QE/g, 0.09±.007 mg AE/g as compared to methanol extracts 0.9±.02 mg GAE/g, 3.66±0.2 mg QE/g, 0.01±.002 mg AE/g. Chloroform extracts of five plants has shown a very low concentration. The data were shown as Mean±Standard Deviation.

3.2 Antioxidant Activity



Graph.4 Ascorbic Acid, ABTS Standard Curve



Graph.5 Antioxidant Assays of Root Extracts (a) DPPH, (b) ABTS, (c) H_2O_2

The Antioxidant activity is calculated by using the standard graph equation as in Graph. 4. Ascorbic acid used as control in DPPH and H_2O_2 Assays. All the three ethanol and methanol plant's extracts, *Withania somnifera*, *Terminaliya arjuna* and *Acalypha indica* showed increase in response curve i.e. maximum scavenging activity against DPPH, ABTS and H_2O_2 in Graph. 5. Ethanolic and methanolic extract of *Bacopa monnieri* showed least activity for all the antioxidant assays. Ethanolic extract of *Ranunculus sceleratus* was having maximum H_2O_2 scavenging activity and optimum ABTS and DPPH radical scavenging activity. Chloroform extracts has shown negligible activity. It is noted that the phytochemicals present in these extracts mainly phenols, flavonoids functions as in donating hydrogen to free radical in order to scavenge the potential damage [53]. It can be stated as the high contents of phenolic and flavanoid compounds of Ethanol and Methanol extracts indicates compounds contribution to the antioxidant activity and can be regarded as a highly promising plant species for natural sources of radical scavenging activity with potential value for treatment of many life threatening diseases.

3.3 Thin Layer Chromatography

Table 1. Rf Values Of the Standards and Extracts

S.NO	Active Compounds	Solvent Systems	Confirmatory Test	Fractions	Rf Values
1.	Phenol	EA:FA:AA: M 7:1.1:1.1:2.6	$FeCl_3$ Solution	**GA** AE3 JE2 KE3 AM4 KM3	**0.96** 0.95 0.96 0.89 0.94 0.86
2.	Flavonoid	M: C: H 7:2:1	$AlCl_3$ Solution	**Q** AE3 JE2 JE4 KE3	**0.89** 0.87 0.91 0.89 0.9
3.	Alkaloid	N-Butanol: AA: M 2:2:6	Dragendorff's Reagent	**At** JE2	**0.83** 0.8

EA- Ethyl Acetate, FA- Formic Acid, AA- Acetic Acid, A- *Withania somnifera*, Ar- *Terminaliya arjuna*, B- *Bacopa monnieri*, J- *Rannunculus sceleratus*, K-*Acalypha indica*, M- Methanol, C- Chloroform, H- Hexane, GA- Gallic Acid, Q- Quercetin ,At-Atropine.

The results of TLC profiling are summarized in Table 1, has shown good sensitivity and separation of compound. It depicts the presence of major three beneficial important bioactive compounds in the column fractions. For identification of different compounds different solvent systems and different spraying reagents has been used which has revealed homogenous spots with different Rf values. Each compound has been visualized as a spot in Visible Day light, Short and Long UV wavelength. For Phenols, confirmatory test used was Ferric chloride solution, for flavonoids, Aluminium Chloride solution and Dragendroff Reagent used for alkaloids detection. Flavonoids have shown fluorescence spots in long UV Wavelength, Phenols have shown black spots in Visible light range and Alkaloids has shown orange spot in visible light and in short wavelength UV light. Only fraction of ethanolic extracts of *Ranunculus sceleratus* shown an appropriate spot indicating presence of alkaloid compound in a good concentration. Gallic Acid, Quercetin and Atropine has been used as Reference

compounds. Wavelength ranging from 200-750nm, the spots were detected using Optima Tokyo Japan (SP/3000PLUS) spectrophotometer.

It was observed that ethanol and methanol was found to be effective in extracting three beneficial secondary metabolites. Different Rf values of the compounds gives an idea about their polarity which helps in selecting a particular solvent system [54]. Compound having high Rf value in less polar solvent system have low polarity while with a low Rf value have high polarity. For pharmacological study of novel drugs, the information's regarding the chemical constituents are mainly provided by this method. In this present study analysis of extracts showed significant indication about the presence of potentially active metabolites [55-56].

Accordingly, WHO has stated medicinal plants acts as the best source for obtaining a variety of drugs[57]. Bioactive compounds exerts different Rf values in different solvent system. These Rf values variation provides helps in understanding their polarity and in selection of proper solvent system for separation of pure compounds present in different fractions by column chromatography. Variable polarity in mixture of solvents in different ratios are used in separation of pure compound from plant extract. Hence, the selection of appropriate solvent system for a particular plant extract is mainly achieved by analyzing the Rf values of compounds in different solvent system [58].

4. Conclusion

The Quantitative Assays revealed the presence of potentially important bioactive compounds phenols, flavonoids and alkaloids in a high concentration in ethanol and methanol root extracts. Ethanolic and methanolic extracts of *Withania somnifera, Terminalia arjuna* and *Acalypha indica* showed more antioxidant scavenging activity, as compared with the chloroform extracts showing very less activity. The same was resulted in ethanolic extract of *Ranunculus sceleratus*. The separation and purification of plant constituents is mainly carried through Column Chromatography and Thin Layer Chromatography showing the presence of compounds in fractions. Therefore, these are a potential source of natural antioxidant having great importance as therapeutic agent and preventing oxidative diseases.

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