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HPLC Analysis Of Polyphenolic -Alkaloid Compounds And Toxicity Determination Of Medicinal Herbal Extracts

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ABSTRACT

Secondary plant metabolites, a large group of bioactive compounds having health-promoting properties containing aromatic structure, hydroxyl groups providing stabilization and protecting free radicals, biological tissues against reactive oxygen and nitrogen species damage. These are an important phytochemical. The recent ethnopharmacological study was aimed at evaluating the effects of ethnobotanical herbs Withania somnifera, Terminalia arjuna, Bacopa monnieri, Ranunculus sceleratus and Acalypha indica using two different solvents on yield extraction estimation, various phytochemical compounds identification, high performance liquid chromatography with diode array detection (HPLC-DAD): quantification of phenols, flavonoids and alkaloids and toxicity cell viability test was performed using compound microscopy by Neubauer chamber. The results showed high yield extraction around 30%-50% percentage, of ethanol and methanol root extracts. These extracts also exhibited high phytochemical i.e., secondary metabolites, mainly polyphenols like flavonoids, tannins, terpenoids, saponins and also alkaloids playing vital roles in biological activities. HPLC method was used to confirm and quantify the presence of polyphenols and alkaloids. Results showed high presence of flavonoids, alkaloids and phenols in extracts. Limit of Detection (LOD) and Limit of Quantification (LOQ) values calculated were 0.1-0.9 ug/ml for respective standard compounds. Obtained values were also compared with other previous literatures, interpreting HPLC-DAD quantification method as the more sensitive and accurate method. Toxicity results revealed non-toxic effects of root extracts on Saccharomyces cerevisiae. 50% inhibition concentration was calculated for all extracts showing ≥ 1mg/ml values. Therefore, these phytochemicals of root extracts can be further used for production of safe, zero side effects, natural medicinal treatment system as economically viable resources.

Keywords: *Phenols, Alkaloids, Ethnobotanicals, High Performance Liquid Chromatography, Ethnopharmacological.*

1. NTRODUCTION

Plant extracts have been used in traditional folk medicinal system in various form as decoctions, tinctures, essential oils, infusions worldwide as a method of treatment and curing of harmful diseases all over the decades. Though these are extensively used for different designs and development of various new drugs in human medicinal treatment plan, as plant antimicrobial, antioxidant compounds, also acting as a great health promise for future as disease controlling agents. An ancient therapy to use plant products as the antimicrobial agents [1], but many ethnopharmacological researches in that area are gaining the attention very lately. As a strict response to the foreign acquired pathogens showing high resistance to the various antibiotics existing commercially, new, natural alternatives must be designed for treatment of such infectious diseases. Hence, it is desirable to know the potential of numerous plant [2-3] as this one could be used as a solution for phytopharmaceutical and medical industry. Plants contain different kinds of natural bioactive compounds known as secondary metabolites and also acts

as antioxidants. The traditional medicinal plants treatment all over the earth are highly rich in these secondary metabolites [4]. The presence of such biologically active molecules in plants especially those molecules which are proven to have an unique therapeutic potentials in respective experimental disease models. These are flavonoids, phenols, tannins, steroids, alkaloids, terpenoids, quinones, saponins etc, responsible for the high medicinal impact of against microbes including with their antioxidant activity [5-6]. The antioxidant effects of a particular compound have been credited to their various mechanisms [7]. One - two pharmacologically markers of active compounds are mainly used to determine the quality and authenticity of herbal preparation.

It provides an overall overview of herbal products as multiple ingredients synergistically work and are mainly responsible for their therapeutic effects. A number of analytical techniques are required to obtain the herbal medicine fingerprints [8]. Chromatography is the golden standard analytical technique having high efficiency in separating various different components of plant extracts [9-10]. Researchers has reported that spectroscopic with spectrophotometric fingerprinting can also be appropriately used as the authentication and identification tool of various herbal medicines. Hence, detecting multiple markers through the extraction of herbal medicines, understanding the formulation of herbal products. Determining the polyphenolic compounds, one of the important, highly valuable part, groups of bioactive compounds are the one used in estimating the pharmacological activity of all medicinal plants [11]. Though many different approaches for the analyzation of polyphenolic compounds have been introduced, identification, quantification and separation of all phenolic compounds still remains very difficult, especially the synchronous determination of different major-minor groups of phenolics in a exclusive analysis. Numerous research work has been published versatile development in HPLC-DAD methods for the analyzation of polyphenolic compound groups from medicinal plants. It allows separation of specifically subgroups of polyphenols, phenolic acids [12].

Polyphenolic (phenols) compounds are the naturally synthesized compounds (phytochemicals) in plants containing aromatic ring attached with a hydroxyl group. This hydroxyl group is majorly attributed for all types of medicinal therapeutic functions. These are universally present in plants and are the primary metabolites products known as secondary metabolites, produced by enzymatic reactions which mainly shields the plants against various ultraviolet radiations as well as resist harmful microbial pathogenic aggression. This class contains, flavonoids, tannins, lignans, stilbenes, terpenoids, lignins, simple phenols, curcuminoids, coumarins, phenolic acids. Phenolic acids having biogenetic precursor i.e., shikimic acid, are found in bound form and are classified into: benzoic acid derivative, depside, hydroxycinnamic acid derivative. These acts as, anti-inflammatory, antipyretic, anti-ulcer, cholagogic etc [13,16]. Phenols greatly benefits the human body as it fights various diseases and keep the human body healthy. Phenolic acids occur chiefly in an ester form of quinic acid or glucose like chlorogenic acid. The derivatives of hydroxyl benzoic acid are vanillic, protocatechuic**,** gallic, tannins, lignins, syringic, p-hydroxy benzoic acid found primarily as glycosides. Phenols are found in all types of foods. Phenolic acids plays a vital, potential, defensive and protective role by neutralizing free radical chain reaction against different oxidative damaged diseases due to reactive oxygen species (ROS) and reactive nitrogen species (RNS), through high consumption of various fruits and vegetables. This amazing antioxidant-nutraceuticals-pharmacological properties of phenols has attracted global attention over the few past decades. Biological activities such as anti-mutagenicity, anti-bacterial function, antiviral action, anti-inflammatory traits, apoptotic operation, etc. are rationalized analysing, detecting and quantitating of compounds [14-15]. Flavonoids belongs from phenols. These are a part of a large family of various polyphenolic compounds naturally synthesized by plants and are structurally derived from the primary substances like flavone. Flavonoids found in foods including vegetables, fruits, cocoa, teas, wines providing high potential and versatile positive health benefits by free radical scavenging and chelating activities.Flavonoids are of C6-C3-C6 with variable phenolic structures [15]. They have 6 major groups, flavones, flavonols, isoflavones, flavanols, anthocyanidins [16]. The maxima spectrum

of flavonoids consists of two range 240nm-285 nm, and 300nm-500 nm [17]. Flavonoids exhibits anti-allergic, anti-fungal, anti-ulcer, antibacterial, anti-inflammatory, repellent, anti-viral activities [18]. The *in-vitro* antioxidant activities are due to their capability to minimize the free radical formation and hence exhibit several biological activities. Many research studies have reported that flavonoids including quercetin, rutin, apigenin, kaempferol, catechin shows anti-ulcer, anti-inflammatory, anti-allergic, hepato-protective properties etc [19]. Almost each and every different fruits and vegetables are capable of conducting and displaying various different limits of antioxidant activities referring to the presence of a varied quantities of free phenolic and flavonol contents. The identification and quantification of phenolic acids with flavonoids in plant extracts helps in determining their medicinal, antioxidant, antimicrobial properties [20]. An increasing demand for highly selective and sensitive analytical methods for extraction and analysis of polyphenols analysis has been reported [21]. A very important technique of polyphenols separation is high performance liquid chromatography (HPLC) coupled with photo diode array (PDA), which is also known as diode array detector (DAD) detecting compound/ wavelength in real time. HPLC is a powerful technique for analyzation of natural substances like polyphenols, their identification, quantification and evaluation.

Alkaloid compounds consists of nitrogen bases i.e., large nitrogen containing heterocyclic ring structures belonging to a group of naturally occurring amino-acid derived medicinal compounds in plants that contain one or more atoms of basic nitrogen of low molecular weight [22]. Alkaloids are produced by a large diverse group living organisms including animals, bacteria, plants, fungi [23]. Alkaloids mainly plays a very important role in living organisms, these are in form of pharmacologically active drugs and have protective functions for plants. Some of these toxic alkaloids like swainsonine originally extracted from *Swainsona canescens* prevents insects and animals from eating it. Mostly alkaloids are bitter in taste accounting some other traditional herbal medicines. Highly outstanding and attractive features of alkaloids i.e., have a broad range of pharmacological activities. These have been greatly used in traditional herbal medicinal treatment as well as in modern medicinal treatment to treat malaria, cancer, asthma, malaria, acute pain and other some diseases [24-26].

2. METHODOLOGY

2.1 Plant Material Collection: Five plant roots had been selected depending upon its geographical location, availability and traditional usage. Plants, *Withania somnifera, Terminalia arjuna, Bacopa monnieri*, *Ranunculus sceleratus, Acalypha indica* was collected from agrakhal forest area of Uttarakhand, U.P., India and from forest of surajpur, Greater Noida, U.P., India.

2.2 Plant Material Processing: Five fresh plant roots were properly dried out in sun rays for 2-3 days. Dried roots were packed and preserved in airtight plastic bags and then powdered as per the need.

2.3 Extraction procedure: Dried powdered root was used for extraction with methanol, ethanol by maceration method. The extraction was carried out till the material was defatted. 50 gm root powdered was heated for 20 mins, filtered and dried at oven 50°C-60^{O}C. Root extracts were stored at -20 °C until for further tests were performed [27].

2.4 Determination of percentage yield: The yield percentage of each plant root extract was calculated by using formula [28]:

Percentage Yield % = (Weight of Extract Weight (g)/Root powder Weight (g))*100

2.5 Phytochemical screening: Phytochemical identification and examinations were performed for all five root extracts according to the standard developed protocols [29].

2.6 Yeast Production: The growth of *Saccharomyces cerevisiae* was enhanced using sugar fermentation method. 1g of powdered form of Baker's yeast was dissolved in 50ml of hot sugar solution and incubated overnight and next day the foam was observed [30].

2.7 Growth Kinetics: Growth Kinetic method was method [31]. The absorbance was recorded in spectrophotometer at 600nm. Growth kinetics were calculated by the time taken by the microorganism to acquire the log phase i.e., 1.5×10^8 CFU/ml. In the growth kinetics, a pre-inoculum of microorganism

was incubated for 24-48 hrs at 35°C. The culture was prepared in Potato Dextrose Broth media.

2.8 Trypan Blue Exclusion Assay: Cell viability cytotoxicity test was assessed by Trypan blue dye exclusion assay [32]. It was evaluated in order to determine the *in vitro* cytotoxicity potentials of ethanol and methanol root extracts. Different concentrations (0.25, 0.5, 1, 2, 4 mg/ml) of extracts were prepared. 100ul of root extracts of all sample were added in different test tubes, mixed l ml ($1.5 X10^8$ in 1ml) of *S. cerevisiae* culture was added. Each root plant extract concentration were performed in triplicates. All the samples were incubated at 35°C in B.O.D incubator for 30min. About 100µl of 0.4% tryphan blue dye solution was added to all the test tubes and mixed properly. Within one- two minutes, the cells were transferred in a Neubauer haemocytometry chamber and the total number of viable with non-viable cells per 1 x 1 mm square were counted under compound microscope.

The dead cells, as it loses the membrane semi permeability, blue dye gets retained. Hence, the cells get coloured whereas viable or the live cells remained unstained.

% Cell Cytotoxicity was determined: [(no. of viable cells/ total no. of viable + non-viable cells) x 100].

2.9 HPLC Chemicals: Methyl alcohol anhydrous-32.04 g/mol, HPLC Grade (>99.9%) (Sigma Aldrich, Merck, India), ethyl alcohol-46.07 g/mol, HPLC Grade (>99.9 %) (Sigma Aldrich, Merck, India), gallic acid anhydrous-170.12 g/mol, (>98%),(Sigma Aldrich, Merck, India), vanillic acid-168.15 g/mol, (>98%), (Sigma Aldrich, Merck, India), caffeic acid-180.16 g/mol, (>98%), (Sigma Aldrich, Merck, India), resveratrol-228.24 g/mol, (>99.9%), rutin hydrate-664.6 g/mol, (>98%) (Cayman, Michigan, U.S.A), quercetin hydrate-302.24 g/mol, (≥95%), (Cayman, Michigan, U.S.A), atropine- 289.4 g/mol, (>95%), (Cayman, Michigan, U.S.A), berberine chloride -371.8 g/mol, (>95%), (Cayman, Michigan, U.S.A) were used for HPLC. Deionized water-18.02 g/mol, (18.2 MΩ.cm resistivity) generated using Milli Q Ultrapure water system (Sigma Aldrich, Merck, India).

2.10 Preparation of Standard Solutions: Four standard compounds for phenols namely, gallic acid, resveratrol, vanillic acid and caffeic acid were used. Similarly, two for flavonoids, quercetin, rutin and two for alkaloids, atropine, berberine. The working solution of 30 ug/ml of standard compounds were prepared by using stock solution of 100ug/ml. All the standard compounds were dissolved in 1ml of HPLC-grade methanol followed by centrifugation at 10,000 rpm for 10mins and then sonication for 20 min at 25^OC and the resulting solution volume was made up to 1 ml with the HPLC grade methanol solvent as the mobile phase The same method was followed to prepare the stock standard solutions of the phenolic acids and the flavonoids. The standard working solutions were filtered using 0.22 µm, Nylon membrane hydrophilic syringe filter (Moxcare Labware, India) and the mobile phase was degassed through degasser before the injection of the standard solutions and transferred into 2 mL vials. All the filtered standards were stored at -18°C. All standard solutions were thawed before HPLC analysis [33].

2.11 HPLC Sample Preparation: 0.1g extract was added in 10 ml volumetric flask and diluted with HPLC grade Methanol. Then the resultant extract solution was filtered using whatmann filter paper No. 1. The extracts were centrifugated at 10,000 rpm for 10mins and then sonication for 20 min at 25^OC and the resulting solution volume was made up to 1 ml with the HPLC grade methanol solvent as the mobile phase finally to obtain the concentration of 1000 µg/ml. The extracts were filtered using 0.22 µm, Nylon membrane syringe filter and the mobile phase was degassed through degasser before the injection of the standard solutions (Acrodisc, Sigma-Aldrich, Bulgaria) and transferred into 2 mL vials. All the filtered samples were stored at -18°C and thawed before hplc analysis [34-35].

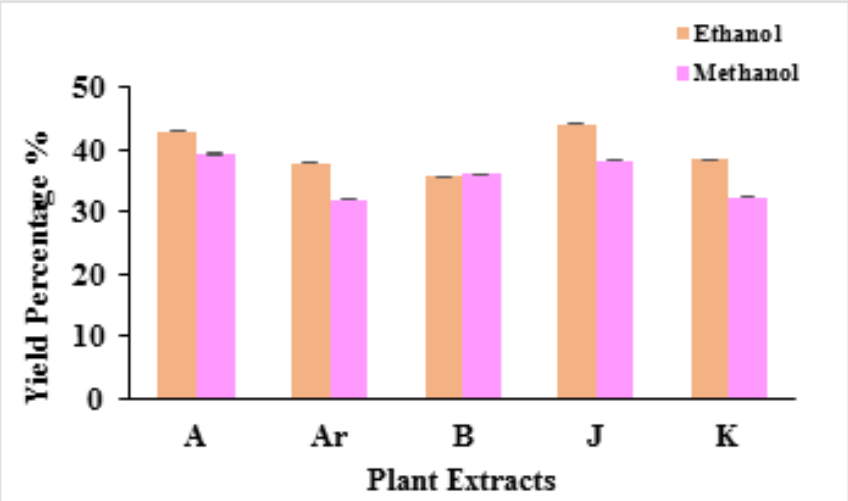
2.12 Reverse Phase HPLC Equipment: The Chromatographic separation and identification was performed with an Agilent 1100 HPLC system (Agilent 1100 HPLC, Agilent Technologies, California, USA) with DAD detector (G1315B, Agilent Technologies, California, USA), and HPLC autosampler G1315A [32-35]. The chromatograms were recorded and integrated by Agilent ChemStation software.Agilent Zorbax SB-C18 column (5 um particle size, 4.6 × 150 mm, CA, USA) was used.

Appropriate standard compounds wavelengths were selected: 278 nm for gallic acid, quercetin 254nm, vanillic acid 254 nm, rutin 256nm, caffeic acid 300nm, resveratrol 305nm, atropine 220nm, berberine 348nm. Chromatographic conditions maintained as each compound separation was performed through linear isocratic elution program with 100 % HPLC methanol (A) for 5 minutes for each standard and sample. The pressure was maintained at 480 bar. The flow rate was 1 ml/min and the column temperature was 25 °C. The injection volume was 20 µl. Diode-array detection was allowed to set to collect data in range of 200-400 nm. Each compound calibration curves of the HPLC-DAD method was obtained by plotting the peak area versus standards concentration (µg\ml). The concentration ranged from 1 to 150 µg/ml for each analytes. Peak purity and absorbance were easily detected by DAD (UV-Vis) detector at different wavelength. Purified fractions of phenols, alkaloids, flavonoids were identified by matching their retention time and spectrum of standards with the unknown compound peaks. External standards were mainly used for the identification and quantification of various compounds. Results are expressed as g/g of dry weight (DW).

2.13 Statistical Analysis: Results were presented as the Mean ± Standard deviation (SD). The data significance was calculated through one-way analysis of variance (ANOVA). All the experiments were performed in triplicates. ANOVA value of $P<0.05$ was considered as a statistically significant.

3. RESULTS & DISCUSSIONS

3.1 Yield and Extraction Analysis- The yield of extracts results o from sample by hydroalcoholic as solvents were shown in graph 1, where A- *Withania somnifera*, Ar- *Terminalia arjuna*, B- *Bacopa monnieri*, J- *Ranunculus sceleratus*, K- *Acalypha indica,* E- ethanol extracts, M- methanol extracts.



Graph 1. Yield percentage of Plant Extracts

The crude extracts of all five plants were obtained after the maceration process, were further concentrated on water bath as well as in hot plate for evaporation of the solvents completely to attain the exact yield of extraction. To calculate the yield percentage of extraction which is very important and necessary phenomenon in bioactive compound extraction process as reported [27] in order to determine the standard extraction efficiency for a single or different plants as well as different parts of the same/different plant or same/different solvents used.

The root extracts with two solvents performed by maceration technique as shown in figure 1, were shown to have different biochemical bioactive compounds. Different wide range of colours had been recorded in each root extracts, therefore, contributing a huge presence of various different phytochemicals including polyphenolics, saponin, alkaloids and flavonoids compounds etc. Also determining the presence of some other phytochemical compounds. These bioactive compounds exhibiting numerous different colours are the mainly due to their structures and were the major cause of potential therapeutic activity against any harmful illness or infections.

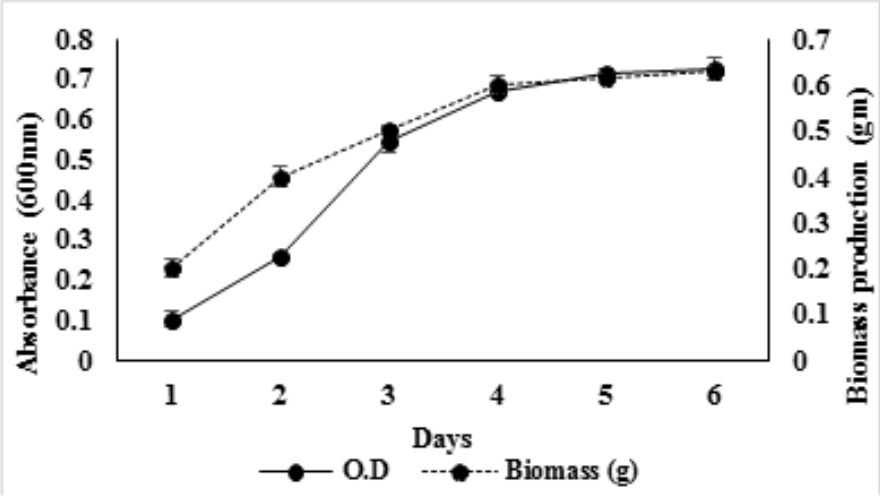
Figure 1. Root Extraction

It also contains a number of different kinds of therapeutic activities. These wide range of colours of root extracts represents high potential medicinal effects. Maceration technique had been termed as the one which is reliable, easy, convenient, less time taking, applicable, safe, less cost as compared to other extraction techniques. It had resulted in acquiring crude extracts consisting of huge mixtures of diverse active compounds, having high purity and efficacy [36]. As per researched, this technique had been highly recommended for limited to small scale molecular compounds as these molecules were able to maintain their potential by being highly stable under temperatures up to 50^{O}C-65^{O}C.

Extraction efficiency strongly gets affected by the extraction method, solvents, time, temperature, composition [37-38]. According to some research, under same conditions, solvent is considered as one of the major parameters. The differences of polarity of the solvents could cause a huge variation in the bioactive compounds extraction. A higher extraction yield was observed in methanolic, ethanolic extracts indicating favouring the highly polar solvents because the plant material containing high levels of polar compounds easily soluble in solvents with high polarity i.e., methanol and ethanol [39-40].

Figure 2 had been depicted as the yeast activation i.e., *S. cerevisiae* growth using sugar fermentation. In graph 2 *S. cerevisiae* growth curve had been plotted with days, absorbance (600nm) and yeast biomass formation.

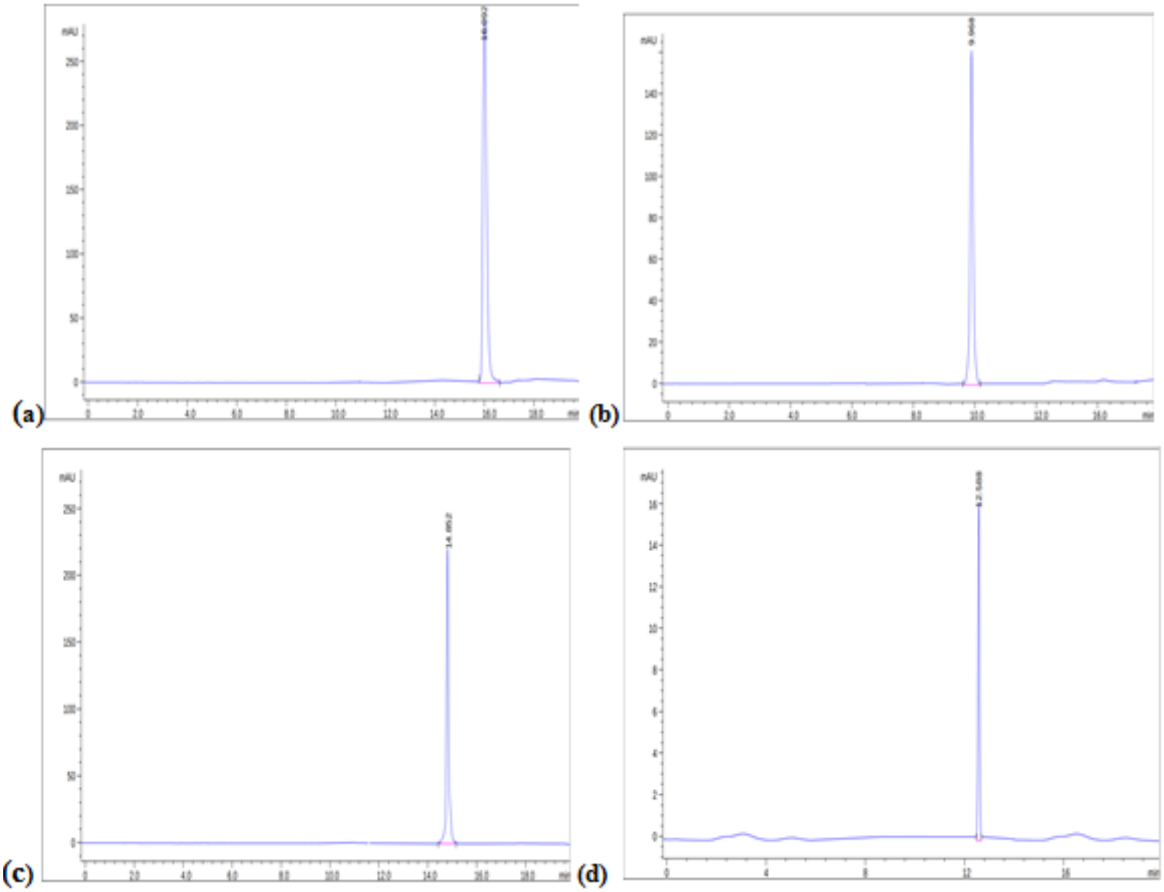
Figure 2. Yeast Activation



Graph 2. Yeast Growth Curve

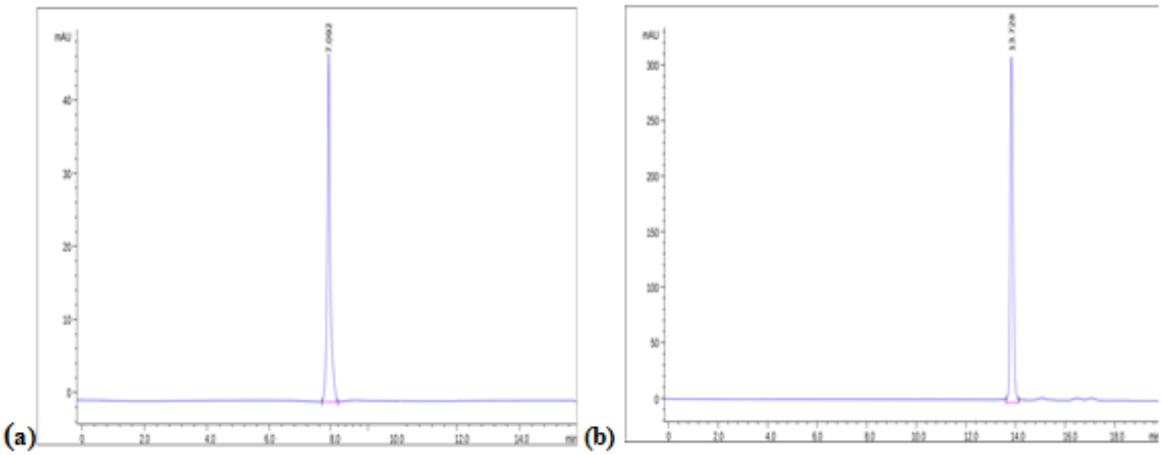
3.2 High Performance Liquid Chromatography Analysis

A reverse phase hplc, C-18 column equilibrated with methanol as the mobile phase was performed. Mobile phase was firstly filtered using whatman filter paper No. 1 and then degassed. It was monitored at 200-400nm for phenols, flavonoids and alkaloids with 10min total run time. The samples were chromatographed and the concentrations were found using the mean of the peak area of the compound as calculated and it was plotted against the drug concentration. The hplc results confirmed the presence of important constituents which are reported to exhibit medicinal, pharmacological, physiological activities. HPLC analysis of standard compounds were summarized in graph 3, 4 and 5 and sample extracts were summarized in graph 6, 7 (ethanol and methanol extracts respectively).

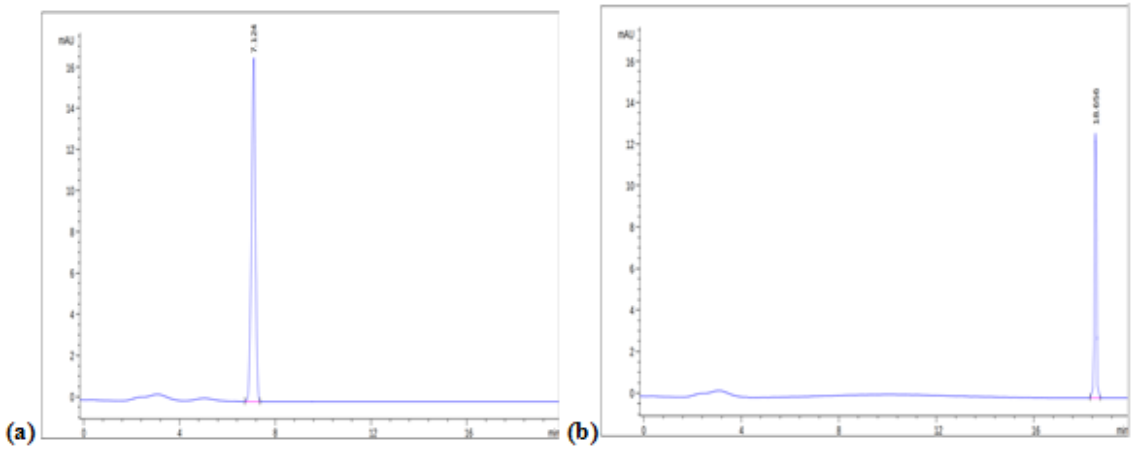


Graph 3: Standard Phenols Chromatogram, **(a)** Gallic Acid, **(b)** Vanillic Acid, **(c)** Caffeic Acid, **(d)**

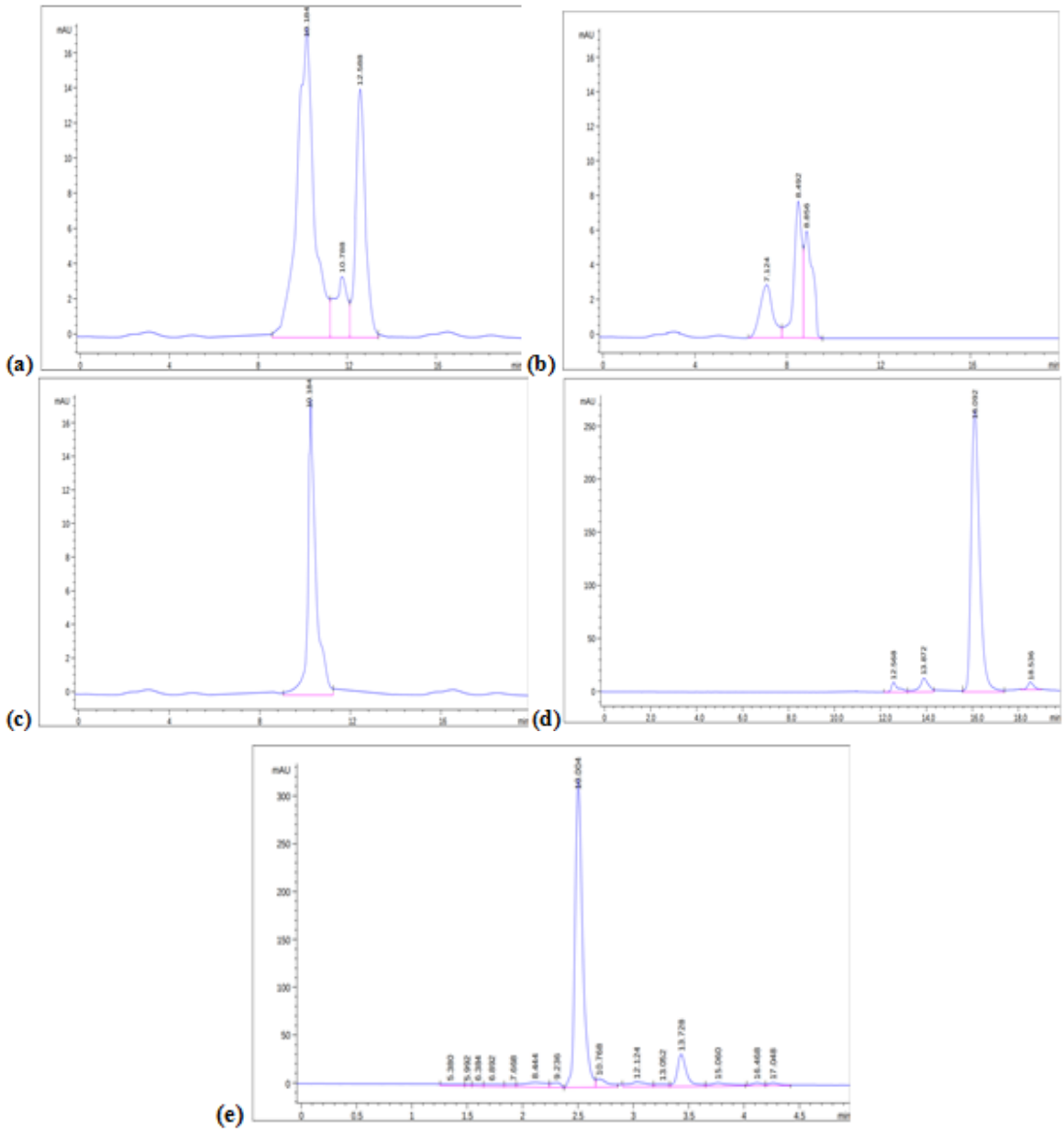
Resveratrol.



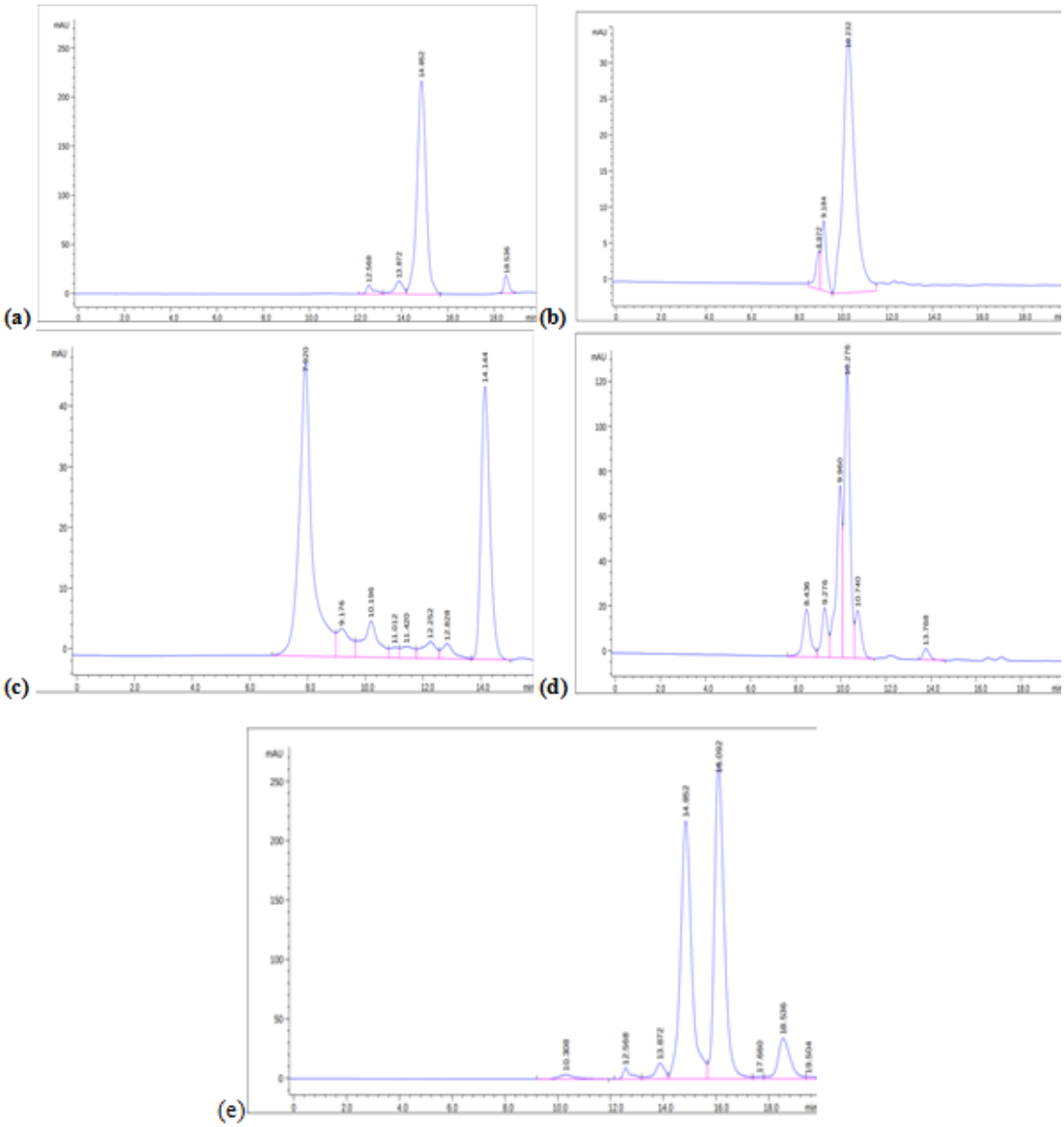
Graph 4: Standard Phenols Chromatogram, **(a)** Quercetin, **(b)** Rutin



Graph 5: Standard Alkaloids Chromatogram, **(a)** Atropine, **(b)** Berberine



Graph 6: Chromatogram of Ethanolic Extracts, **(a)** *Withania somnifera*, **(b)** *Terminalia arjuna*, **(c)** *Bacopa monnieri*, **(d)** *Ranunculus sceleratus*, **(e)** *Acalypha indica*



Graph 7: Chromatogram of Methanolic Extracts, **(a)** *Withania somnifera*, **(b)** *Terminalia arjuna*, **(c)** *Bacopa monnieri*, **(d)** *Ranunculus sceleratus*, **(e)** *Acalypha indica*

The HPLC chromatogram of each standard compounds including phenols- gallic acid (GA), caffeic acid (CA), vanillic acid (VA), resveratrol (Re), flavonoids-quercetin (Q), rutin (R) and alkaloids-atropine (A), berberine (B) with their particular wavelength [41] showed peaks of the significant area with minimal noise and deviation. Similarly of the sample extracts. Some of the compounds in the sample extracts where having extracts retention time as that of standard compounds shown in table 1, 3,4 and 5. Table 1 had depicted the standard compound's retention time, peak area, their wavelength, calculated response factor, concentration. Table 3, 4 and 5 had depicted the retention time, peak area and calculated concentration of each unkown compounds present in each of extract for alkaloids, flavonoids and phenols respectively.

Table 1: HPLC Standard Compounds

S. No	Standard	Wavelength (nm)	Retention Time (min)	Peak Area (mAU)	Response Factor	Concentration (ug/ml)
1.	Gallic Acid	278	16.001 ±0.09	1654.021 ±0.001	55.134	30
2.	Caffeic Acid	300	14.751 ±0.17	778.67 ±0.001	25.956	30
3.	Vanillic Acid	254	9.96 ±0.0015	659.23 ±0001	21.9672	30
4.	Resveratrol	305	12.58 ±0.0005	79.024 ±0.001	2.63415	30
5.	Quercetin	254	7.92 ±0.02	204.012 ±0.0001	6.80041	30
6.	Rutin	256	13.72 ±0.0005	1920.02 ±0.0005	64.0007	30
7.	Atropine	220	7.12 ±0.002	156.001 ±0.0001	5.20004	30
8.	Berberine	348	18.655 ±0.001	62.20 ±0.002	2.07334	30

Table 2: Limit of Detection and Quantification Of HPLC Standard Compounds

S. No	Standard	Limit of Detection (LOD ug/ml)	Limit Of Quantity (LOQ ug/ml)
1.	Gallic Acid	0.256718	0.855726
2.	Caffeic Acid	0.298937	0.996458
3.	Vanillic Acid	0.296549	0.988495
4.	Resveratrol	0.128166	0.427221
5.	Quercetin	0.154254	0.514179
6.	Rutin	0.256674	0.855579
7.	Atropine	0.057616	0.192054
8.	Berberine	0.269815	0.899384

Table 3: HPLC Concentration Of sample extracts for Alkaloids

S. No	Extracts	Retention Time (min)	Peak Area (mAU)	Concentration (ug/ml)	Dry weight Concentration (ug/g)
Atropine (220nm)					
1.	ArE	7.12 ±0.0008	20.40 ±0.0008	32.92343	329.2343
2.	KM	7.12 ±0.0009	29.46 ±0.0001	59.66591	596.6591
Berberine (348nm)					
3.	JE	18.53 ±0.0008	277.95 ±0.0008	134.0592	1340.592
4.	AM	18.53 ±0.0008	277.95 ±0.0008	134.0596	1340.596
5.	KM	18.65 ±0.0001	79.056 ±0.0008	38.1302	381.302

Table 4: HPLC Concentration Of sample extracts for Flavonoids

S. No	Extracts	Retention Time (min)	Peak Area (mAU)	Concentration (ug/ml)	Dry weight Concentration (ug/g)
Quercetin (254nm)					
1.	KE	7.66 ±0.001	17.21 ±0.0008	20.53163	205.3163
2.	BM	7.91 ±0.008	383.77 ±0.0008	56.43384	564.3384
Rutin (256nm)					
3.	KE	13.72 ±0.0008	201.61 ±0.0004	31.15021	311.5021
4.	JE	13.87 ±0.0009	85.94 ±0.0001	12.34295	123.4295
5.	AM	13.87 ±0.0009	85.94 ±0.0001	19.34294	193.4294
6.	KM	13.76 ±0.0008	24.63 ±0.0004	40.38495	403.8495

Table 5: HPLC Concentration Of sample extracts for Phenols

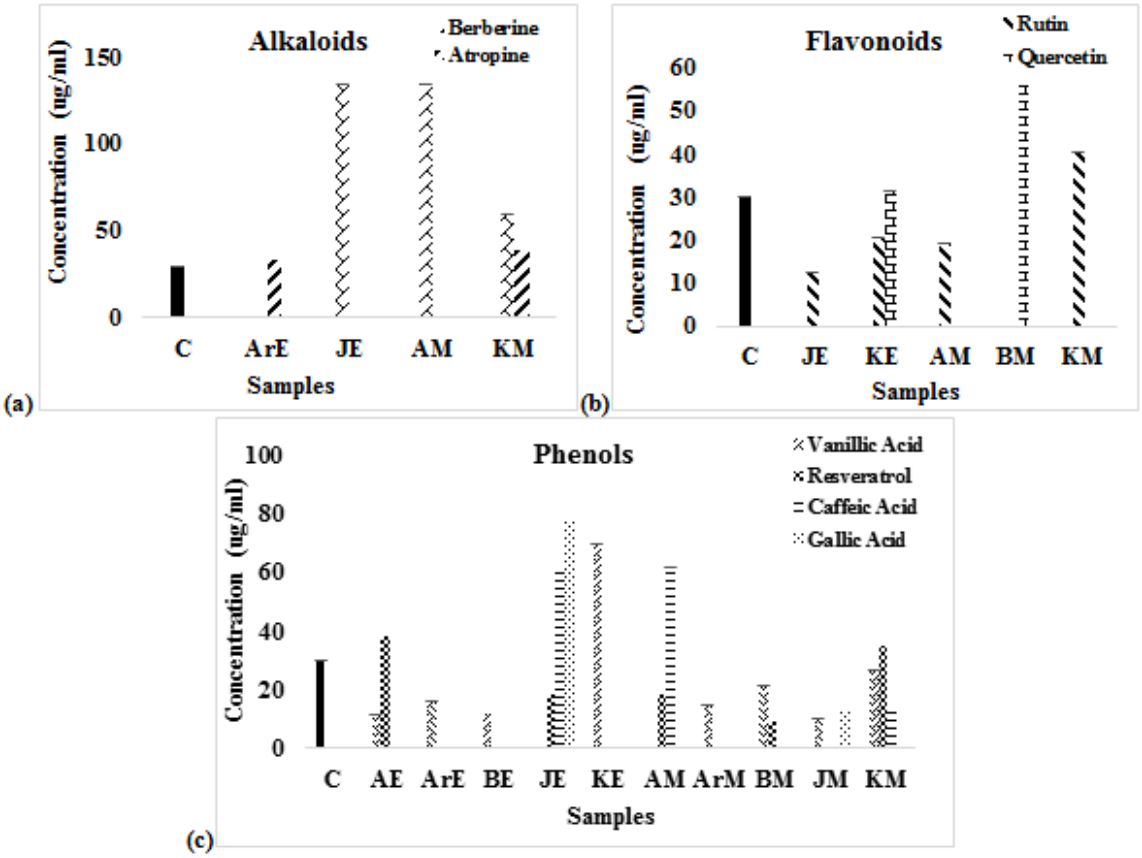
S. No	Extracts	Retention Time (min)	Peak Area (mAU)	Concentration (ug/ml)	Dry weight concentration (ug/g)
Vanillic Acid (254m)					
1.	AE	10.18±0.0008	256.12±00004	11.65938	116.5938
2.	ArE	9.97±0.012	353.34±0.0008	16.08505	160.8505
3.	BE	10.18±0.001	258.25±0.0004	11.75622	117.5622
4.	KE	10±0.0008	1531.045±0.0001	69.69684	696.9684
5.	ArM	10.23±0.0008	328.0305±0.0001	14.93273	149.3273
6.	BM	10.19±0.0009	54.84±0.0001	21.49641	214.9641
7.	JM	10.18±0.0012	218.52±0.0001	9.94753	99.4753
8.	KM	10.28±0.0008	571.93±0.0008	26.03545	260.3545
Gallic Acid (278nm)					
9.	JE	16.092±0.0012	1709.85±0.0001	77.83663	778.3663
10.	JM	16.28±0.001	19.36±0.0001	10.35117	103.5117
Resveratrol (305nm)					
11.	AE	12.59±0.0008	99.13±0.0001	37.63088	376.3088
12.	JE	12.56±0.0012	47.23±0.32	17.9312	179.312
13.	AM	12.56±0.001	47.701±0.0004	18.1088	181.088
14.	BM	12.25±0.0008	24.16±0.0009	9.172932	91.72932
15.	KM	12.59±0.0008	92.073	34.95371	349.5371
Caffeic Acid (300nm)					
16.	JE	14.85±0.0009	1592.039±0.0008	61.33617	613.3617
17.	AM	14.85±0.001	1592.038±0.0004	61.33616	613.3616
18.	KM	14.804±0.0032	41.36±0.0002	12.59333	125.9333

Table 6: 50% Inhibition Concentration Activity of Plant Extracts

S. No	Extracts	IC50 (ug/ml)
1.	AE	1879.84
2.	ArE	19231.5
3.	BE	1826.42
4.	JE	1597.30
5.	KE	1123.34
6.	AM	1198.50

7.	ArM	1387.23
8.	BM	1112.9
9.	JM	1591.74
10.	KM	1694.53

Graph 8, bar graph for each unkown sample compound concentration in each extract had been plotted along with the control as the standard compound. The results obtained in this work mainly suggest that the identified phytochemical bioactive compounds present were the one bioactive constituents majorly responsible for the therapeutic activities and efficacy of the five medicinal plants. The presence of some of major compounds like quercetin, rutin, berberine, resveratrol, vanillic acid, caffeic acid had also been confirmed to possess various pharmacological effects [42]. The work had also established quality control parameters including analytical and phytochemical standardization. Optimum baseline separation of each target analytes were achieved, thus enabling the quantification in the sample extract. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as shown in table 2, using (signal-to-noise ratio 3:1) for LOD and (signal-to-noise ratio 10:1) for LOQ for each compound [43].



Graph 8: HPLC concentration of samples **(a)** Alkaloids **(b)** Flavonoids **(c)** Phenols

The selection of the exact solvent and the conditions of extraction wan an important analytical step in the development of the technique for the hplc qualitative and quantitative estimation of the biologically active compounds present in raw plant material. The solvent of the extraction is number one factor enhancing the prognosis step of the qualitative and quantitative level of the isolated compounds with their concentrations [44].

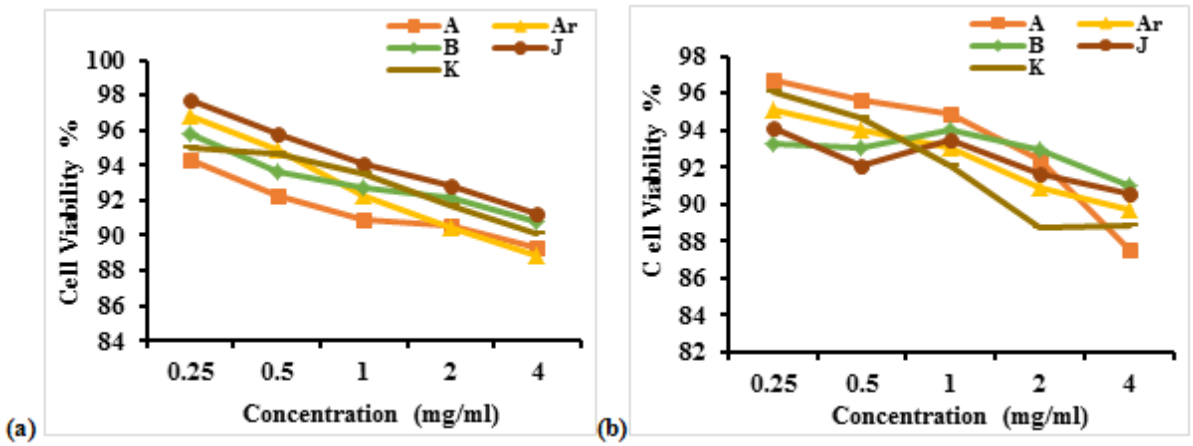
HPLC/DAD should be importantly considered for determining the quality of herbal medicines considering the fact that numerous constituents were present in herbal plants and its product [43]. The extract chromatogram indicated the various peaks of the polyphenols that were detected in extracts. HPLC is highly sensitive, accurate and very specific method for the separation and identification of number of phenolic acids, simple phenols, alkaloids, flavonoids in plants. [44].

Many research studies had indicated better correlations between phenolics content and their antioxidant capacity determined by different methods including HPLC [45] and it had also been asserted that these plants exhibited such medicinal properties due to the presence of polyphenols. Hence, plant polyphenols had contributed to the medicinal values as these lies in those chemical substances that produces a definite physiological action on human body system.

Such distribution of minerals in medicinal plants were an important application for health maintenance in developing countries. Therefore. there is a high need to verify the local herbs for mineral and nutrient composition establishing their potential, indigenous source of natural medicines [46].

3.3 Trypan Blue Exclusion Cell Viability Test Analysis

By using trypan blue dye cell viability method, the toxicity of each plant extracts were determined. Graph 9 shows each ethanolic and methanolic extracts showing cell viability percentage at five different concentration of plant extracts i.e., 0.25, 0.5, 1, 2, 4 mg/ml. It was seen that at a very low concentration 0.25 mg/ml-1 mg/ml the extracts showed high cell viability activity i.e., the amount of dead yeast cells was very low while treating with trypan blue dye indicating plants extracts inhibiting yeast cells at a very low rate. Whereas, as the concentration increases the cell viability activity of plant extracts in yeast cells decreases i.e., plants extracts were able to inhibit the yeast cells at an intermediate amount showing toxic effects but in the lower range. This was due to the presence of phytochemicals in these plant species [47].



Graph 9: Cell Viability Percentage of plant extracts **(a)** Ethanol, **(b)** Methanol

The extracts yeast cell viability ranged from of 98%-85% which is widely acceptable, proving as the non-toxic plant extracts. Morphological changes had been reported in S. cerevisiae cells grown overnight stained with trypan blue dye except cell size or wall volume [48]. The IC50 was calculated as shown in table 6 and the extracts had shown values between 1000ug/ml-2000ug/ml. Therefore, phytochemicals were considered as the active medicinal part as the chemical constituents of the plants [49]. The medicinal plants had been seen as the natural substitutes as the source of chemotherapeutic agents as well. Natural products had reported to received high increasing attention over the past 30-35 years [50].

4. CONCLUSION

The present work reports on the root extraction process of *Withania somnifera, Terminalia arjuna, Bacopa monnieri, Ranunculus sceleratus* and *Acalypha indica* using two different solvents. Ethanol and methanol extracts were seen to be the best solvents for extracting various bioactive compounds as it had resulted in a huge amount of yield extraction percentage and highest quality contents of phenols, flavonoids and alkaloids. The phytochemical screening reveals presence of major important compounds. The confirmation of various biologically active and pharmacologically important alkaloids, phenols and flavonoids compounds in an accurate quantitable amounts in all the five plants using high performance liquid chromatography has been reported. The results of the toxicity test, IC50 of all five

plants were recorded to be ≥ 1mg/ml. Therefore, these five root extracts acts as a promising medicinal agents for the nutraceutical, medical, pharmacological and pharmaceutical industries further for determining the quality control profile by various industries. The investigation initiated for the need for different kinds of biological agents from different plant sources with potent activity and no side effects.

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6. AUTHORS' CONTRIBUTIONS

Author has conducted all the experiments, performed all the sample analysis and prepared first draft of the manuscript, analysis and prediction of experimental data and preparation of figures/tables; developed the concept of the present study and performed the final editing of the manuscript. Author have reviewed and approved the final version of the manuscript for publication and agreed to be accountable for all aspects of the work.

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8. CONFLICTS OF INTEREST

The author reports no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve any experiments on animals or human subjects.

10. DATA AVAILABILITY

All data represented in this research article are available from the author on reasonable request.

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