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EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF DIFFERENT EXTRACTS OF VIGNA UNGUICULATA SEEDS

***Md. Shahedul Haque Shah, Tahira Foyzun, Kazi Faheema Islam, Md. Saroar Hosan and Tarik Hasan**

Department of Pharmacy, Southeast University, Banani, Dhaka-1213, Bangladesh.

***Corresponding Author: Md. Shahedul Haque Shah**
Department of Pharmacy, Southeast University, Banani, Dhaka-1213, Bangladesh.

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ABSTRACT

Introduction: *Vigna unguiculata* is one of the most important medicinal species. An extensive several of nutritional and medicinal uses have been attributed to its seeds. In the present study an attempt has been made gather the information related to phytochemical compositions and Pharmacological uses. They exhibit antioxidant and free radical scavenging activities. They also exhibit Antibacterial activity against both the Gram positive and Gram negative organisms. So, the aim of the study was to screen its phytochemical and biological activities. **Methodology:** In this study crude methanol, Methanol, Ethyl acetate, Chloroform and N-hexane extracts of *V.unguiculata* seeds were prepared and were screened for their phytochemical properties (Total phenolic and flavonoid determination) and antioxidant properties by employing various established in-vitro methods. Such as reducing power, DPPH scavenging and total antioxidant activity were determined against three gram (+) and three gram (-) bacteria *Bacillus cereus, Micrococcus Luteus, Staphylococcus aureus, E.coli, Pseudomonas and Klebsiella* by disc diffusion method. **Results:** The antibacterial activity of crude Methanolic extract, crude chloroform extract and crude N-hexane extracts seeds of *V.unguiculata* showed that, it is moderately active against both Gram-positive and Gram-negative bacteria. The range of zone of inhibition was found from 6-18mm in disc sensitivity test while 19-29mm showed by standard kanamycin (30 µg/disc**)**. However, the highest activity was found against Bacillus cereus and *E.coli* with 18mm zone of inhibition where concentration per disc was 500µg/disc. The crude extracts of *V.unguiculata* seeds were screened for identification of phytochemical properties by Total phenolic and flavonoid contents where CEE respectively 75.14 ± 0.95 and 73.18 ± 0.95 which were higher than CME, CNE and CCE. Antioxidant properties which were employed by iron reducing power capacity and DPPH Radical Scavenging where the CEE of seeds is higher than that of their extract. **Conclusion:** On the basis of the results obtained in the present study it is observed that the crude ethyl acetate extract of *Vigna unguiculata* which contains phenolic compounds that exhibit a high antioxidant and free radical scavenging activities. Methanolic extract of seeds *Vigna unguiculata* possesses good antibacterial activities. So, traditional medicine can be used as modern medicine to fight against different pharmacological problem as like *Vigna unguiculata*as a medicinal plant.

KEYWORDS: *Vigna unguiculata,* antimicrobial activity, antioxidant activity.

INTRODUCTION

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities. The local people have a long history of traditional plant usage for medicinal purposes. The medicinal use of plants is very old. The writings indicate that therapeutic use of plants is as old as 4000 - 5000 B.C. and Chinese used first the natural herbal preparations as medicines. In India, however, earliest references of use of plants as medicine appear in Rig-Veda, which is said to be written between 1600 - 3500 B.C. Later the properties and therapeutic uses of medicinal plants were studied in detail and recorded empirically by the ancient physicians (an indigenous system of medicine) which are a basic foundation of ancient medical science in India.[1] Medicinal plant is an important element of indigenous medical systems in all over the world. The ethno botany provides a rich resource for natural drug research and development.[2] "Traditional" use of herbal medicines implies substantial historical use, and this is certainly true for many products that are available as "traditional herbal medicines". In many developing countries, a large proportion of the population relies on traditional practitioners and their armamentarium of medicinal plants in order to meet health care needs. Although modern medicine may exist side-by-side with such traditional practice, herbal medicines have often maintained their popularity for historical and cultural reasons.[3] Natural products have played an important

role throughout the world in treating and preventing human diseases. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates and its importance in modern medicine has been discussed in different reviews and reports.[4] In recent years, the use of traditional medicine information on plant research has again received considerable interest. In recent times, there have been increased waves of interest in the field of research in natural products chemistry. This level of interest can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites, the utility of novel bioactive natural compounds as biochemical probes, the development of novel and sensitive techniques to detect biologically active natural products, improved techniques to isolate, purify, and structurally characterize these active constituents, and advances in solving the demand for supply of complex natural products.[5] The World Health Organization (WHO) has also recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines. Proven agro-industrial technologies need to be applied to the cultivation and processing of medicinal plants and the manufacture of herbal medicines.[6] Medicinal plants are resources of new drugs and many of the modern medicines are produced indirectly from plants. It is estimated that there are more than 250,000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. In this review the objective is to consider the past and present value of medicinal plants such as Thymus vulgaris used in traditional and modern medical practices as bioactive natural compounds.

Antimicrobial agents kill or inhibit the growth of microorganisms. The microbial agent may be a chemical compounds and physical agents. These agents interfere with the growth and reproduction of causative organisms like bacteria, fungi, parasites, virus etc. The journal of Antimicrobial Agents stocks up information about antibacterial, antifungal, antiviral, Anti protozoal, anti-algal agents and their methods of detection, different therapies and advanced treatments to overcome diseases. Thus, antimicrobial agents that are used in the treatment of disease include synthetic chemicals as well as chemical substances or metabolic products made by microorganisms and chemical substances derived from plants.[7]

Antioxidants keep free radicals in check.[8] Antioxidants are molecules in cells that prevent free radicals from taking electrons and causing damage. Antioxidants are able to give an electron to a free radical without becoming destabilized them, thus stopping the free radical chain reaction. "Antioxidants are natural substances whose job is to clean up free radicals.[9] Just like fiber cleans up waste products in the intestines, antioxidants clean up the free radical waste in the cells," said Wright. Well-known antioxidants include beta-carotene and other carotenoids, lutein, resveratrol, vitamin C, vitamin E, lycopene and other phytonutrients.

Our body produces some antioxidants on its own, but an insufficient amount. Oxidative stress occurs when there is an imbalance of free radicals and antioxidants (too many free radicals and too few antioxidants), according to the pharmacognosy review.[10]

Antioxidants can be acquired through diet. "Antioxidants are plentiful in fruits and vegetables, especially colorful fruits and vegetables," said Wright. "Some examples include berries, tomatoes, broccoli, spinach, nuts and green tea".

Antioxidants are able to neutralize free radicals at the levels of prevention, interception as well as repair.[11] Antioxidants can the stop the formation of ROS for e.g. superoxide dismutase (SOD) catalysis the disputation of superoxide to H_2O_2 and catalyze breaks it down to water.[12] Interception of free radicals is mainly by radical scavenging. At the repair and reconstitution level, mainly repair enzymes are involved which neutralize the free radicals.[13]

MATERIALS AND METHODS

Collection of the plant parts

The *V.unguiculata* seeds were selected for the study. The seeds of these plants were collected during month of October, 2017 from the area of Dhaka Bangladesh and were identified by the experts of Bangladesh National Herbarium, Dhaka, where voucher specimen were retained.

Drying, Pulverization and Preservation of plant parts

The leaves were first washed with water to remove the adhering dirt and then cut into small pieces, sun dried for 12-15days. After complete drying, the entire portions were pulverized into coarse powder with the help of grinding machine and were stored in an air tight container for further use.

Extraction of Plant Material

The each ground leaves 100gm were extracted with 3 times methanol of their weight in a round bottom flask container with 1:2 sample and solvent ratio at room temperature through occasional shaking and stirring for 7 days. After 7 days, the extracts were filtered through filter paper. The filtrates were concentrated at 50ºC under reduce pressure in a rotary evaporator to afford a greenish mass of biological investigation. Then the crude extract ready for assaying of antimicrobial, antioxidant, total phenolic content.

Extraction procedure

Chemical constituents from crude plant can be extracted by following two extraction procedures-Cold extraction

& Hot extraction. In our current study we used cold extraction method.

Cold Extraction for the four plant parts
Preparation of ethanolic extracts
For each plant the dried and powdered materials (500 g for) were soaked in 2500 ml of 90% ethanol for about 15 days at room temperature with occasional stirring. After 15 days the solution was filtered using filter cloth and Whatman's filter paper. The filtrates (Ethanolic extract) obtained were evaporated under rotary evaporator and in a water- bath until dried. It rendered a gummy concentrates and were designated as crude extracts of Ethanol.

The disk diffusion susceptibility method is simple and well-standardized. Bacterial inoculums are applied to the surface of a large agar plate. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. Plates are incubated for 16–24hr at 35°C prior to determination of results. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The zones of growth inhibition are measured to the nearest millimeter around each of the antibiotic disks. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Barry, 1976).

Table 1: Apparatus and reagents for antimicrobial test.

Filter paper discs	Screw cap test tubes
Petri dishes	Nose mask and Hand gloves
Inoculating loop	Laminar air flow hood
Sterile cotton	Autoclave
Sterile forceps	Incubator
Spirit burner	Ethanol
Micropipette	Nutrient Agar Medium

Test Sample of *Vigna unguiculata*
Aqueous fraction of methanolic extract, Ethyl acetate extract and N-hexane extract of *Vigna unguiculata* were taken as test sample.

Test Organisms
The bacterial strains used for the experiment were collected as pure cultures from the Southeast University microbiology laboratory. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table.

List of micro-organisms
The following microbes are collected form ICDDR'B
1) *Micrococcus Luteus*
2) *Bacillus cereus*
3) *Staphylococcus aureus*
4) *Escherichia coli*
5) *Pseudomonas aeruginosa*
6) *Klebsiellapneumoniae*

The microbes are cultured in petri dishes in nutrient agar.

Procedure
Preparation of the Medium
To prepare required volume of this medium, 5.6gm of agar medium was taken in a bottle with a cap and distilled water was added to it to make 200ml volume. The contents were then autoclaved to make a clear solution.

Sterilization Procedure
In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.[14]

Preparation of the Test Plate
The test organisms were transferred from the subculture to Petridis containing about 10 ml of melted and sterilized agar medium. The bacterial and fungal suspension was taken by a loop mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial suspension. Then the bacterial sample is applied to the Petridis with the help of this cotton bud.[15]

Preparation of Discs
1. Standard Discs.
2. Blank Discs.
3. Sample Discs.

Preparation of Test Sample
Measured amount of test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical filter paper discs were taken in a blank Petridis under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Application of Test Samples
Standard ciprofloxacin discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Methanol discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Diffusion & Incubation
The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria.[16] The plates were then kept in a refrigerator at 4°C for about 24 hours' upside down to allow sufficient

diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hours.

Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

The antimicrobial potency of the test agents is measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

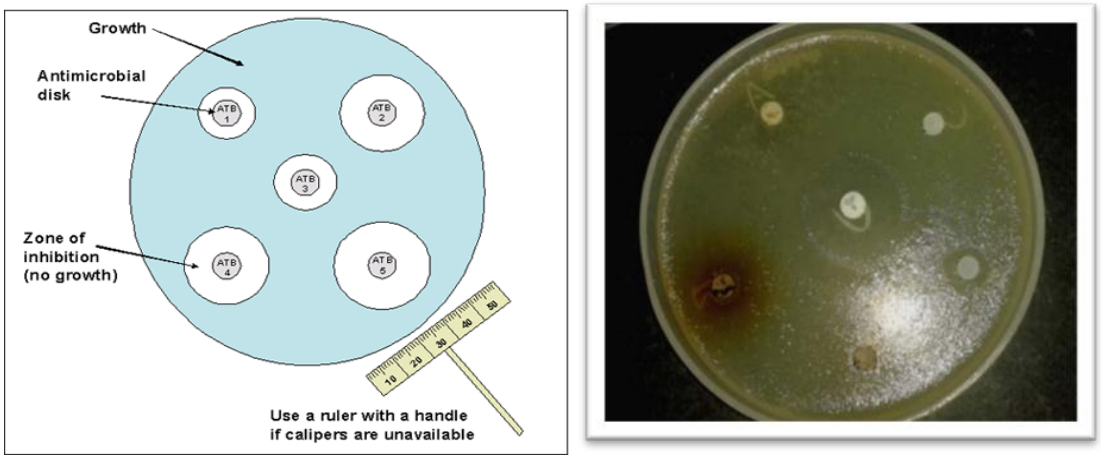

Figure 1: Zone of inhibition.

Antioxidant Assays

Determination of Total Phenolic

Principle

The content of total phenolic compounds of different fractions in the plant was determined by Folin–Ciocalteu Reagent (FCR).[17] The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphospho tungstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})_4$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI).

$$\mathbf{Mo\ (VI) + e^- \rightarrow\ Mo\ (V)}$$

Experimental procedure

0.5 ml of plant extract or standard of different concentration solution was taken in a test tube and 2.5 ml of Folin – ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube. Then 2.5 ml of Sodium carbonate (7.5%) solution was added and incubated for 20 minutes at 25^0C to complete the reaction. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution. The total content of phenolic compounds in plant extract and in polyphenolic fractions in Gallic acid equivalents (GAE) was calculated by the following formula

C = (c x V)/m

Where,

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of Gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of different pure plant extracts, gm.

Determination of Total Flavonoids

Principle

The content of total flavonoids in different fractionates of plant extract was determined by the well-known aluminum chloride colorimetric method. In this method aluminum chloride forms complex with hydroxyl groups of flavonoids present in the samples. This complex has the maximum absorbance at 420 nm.[18]

Experimental procedure

One milliliter of aqueous extract containing 0.1 g/ml of dry matter was placed in a 10 ml volumetric flask, then 5ml of distilled water added followed by 0.3ml of 5% $NaNO_2$. After another5min, 0.6 ml of 10% AlCl3 was added and volume made up with distilled water. The solution was mixed and absorbance was measured at 510 nm. Total Flavonoid contents were expressed as catechin equivalents per dry matter, calculated by the following formula.

C = (c x V)/m

Where,

C = total content of flavonoid compounds, mg/g plant extract, in catechin equivalent (CE);

c = the concentration of catechin / extract established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant extracts, gm.

All samples were analyzed trice and results averaged.

Reducing Power Capacity Assessment

Principle

In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compounds. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reducers such as antioxidant substances in the samples causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron.[19] The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Experimental procedure

Various concentrations of standards or plant extract (1.0 ml) were mixed with 2.5 ml of 0.2 M potassium buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixture was incubated for 20 minutes at 50^0C. After 2.5 ml of 10% trichloroacetic acid solution was added, the mixture was centrifuged at 2000 rpm for 10 min. The upper layer solution (2.5 ml) was withdrawn from the mixture and mix with 2.5 ml of deionised water and0.5 ml of fresh ferric chloride ($FeCl_3$), (0.1%) solution. Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution mixture without plant extract or standard and it was incubated under the same conditions as the rest of the samples solution. A higher absorbance of the reaction mixture indicates a higher reducing power.

Determination of Total Antioxidant Capacity

Principle

The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reluctant and Mo (VI) and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm.[20]

$$\mathbf{Mo\ (VI) + e \rightarrow Mo(V)}$$

Experimental procedure

An aliquot of 0.5 ml of sample solution was combined with 3ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95ºc for 10 minutes. After the sample had cooled to room temperature, the absorbance of aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Ascorbic acid, ∞ tochopherol can be used as standard.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Assay

Principle

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds.[21] This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H. A freshly prepared DPPH solution exhibits a deep purple color. The transformation results in color change from purple to yellow, which is measured spectrophotometrically. Thus antioxidant molecule can quench DPPH free radicals (by providing hydrogen atom or by electron transfer, conceivably via a free radical attack on the DPPH molecule) and convert them a colorless products (2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine) resulting in a decrease absorbance at 517 nm. Hence, the more rapidly the absorbance decreases the more potent antioxidant activity of the extract in terms of hydrogen atom donating capacity/electron transfer ability. In this assay, the positive controls can be ascorbic acid, gallic acid, quercetin, BHT, rutin or catechin.[22]

Experimental procedure

2 ml of methanol solution of plant extract or standard at different concentration was taken in a test tube. 3 ml of methanol solution of DPPH was added into the test tube. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution. The percentage (%) inhibition activity was calculated from the following equation.

$\% I = \{(A_o - A_1)/A_o\} \times 100$

Where,

A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract/standard.

Then % inhibitions were plotted against concentration and from the graph IC_{50} was calculated.

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RESULTS AND DISCUSSION

The antibacterial activity of the crude Methanolic extract, crude Ethyl acetate extract, crude N-hexane extract and crude chloroform extract of seeds of *Vigna unguiculata* namely was investigated against a wide range of gram positive and gram negative bacteria using disc sensitivity tests.

Table 2: The gram positive bacteria.

Gram positive bacteria					
Name of microorganism	**Conc.**	**Zone of inhibition (mm)**			
		Methanolic extract	**N-hexane extract**	**Ethyl acetate**	**Kanamycin(30µg\disc)**
Micrococcus Luteus	250ml	15	8	17	27
	500ml	13	9	14	
Bacillus cereus	250ml	8	10	16	25
	500ml	6	12	15	
Staphylococcus aureus	250ml	10	6	14	26
	500ml	9	7	11	

Table 3: The gram negative bacteria.

Gram negative bacteria					
Name of microorganism	**Conc.**	**Zone of inhibition (mm)**			
		Methanolic extract	**N-hexane extract**	**Ethyl acetate**	**Kanamycin (30µg\disc)**
E.coli	250ml	10	10	9	28
	500ml	12	11	11	
Pseudomonasvaerugino sa	250ml	10	9	14	24
	5000ml	13	10	16	
Klebsiella*pneumoniae*	250ml	10	6	19	23
	500ml	12	7	17	

The antibacterial activity of crude methanolic extract, crude petroleum ether extract, and crude N-hexane extract of leaves of *vigna uniguculata* showed that it is moderately active against both Gram-positive and Gram-negative bacteria. The range of zone of inhibition was found from 6-18mm in disc sensitivity test while 19-28mm showed by standard kanamycin (30 µg/disc). However, the highest activity was found against Bacillus cereus and *E.coli* with 17mm zone of inhibition where concentration per disc was 500µg/disc.[23] Furthermore zone of inhibition with 13mm and 16mm value was found for Micrococcus Luteus and Pseudomonas as well as and *Staphylococcus aureus* followed behind with 12mm zone of inhibition. The antibacterial activity of the crude Ethyl acetate extract of seeds of *vigna uniguculata* was investigated against a wide range of gram positive and gram negative bacteria using disc sensitivity tests.

Anti-oxidant assay

Determination of Total Phenolic Content

Table 4: Absorbance of Gallic acid at different concentrations after treatment with Folin-Ciocaltue reagent.

Concentration (µg/ml)	Absorbance			Absorbance Mean±STD
31.25	0.127	0.124	0.128	0.126±0.001
62.5	0.253	0.251	0.254	0.253±0.002
125	0.450	0.448	0.452	0.452±0.0023
250	0.860	0.862	0.858	0.861±0.001
500	1.61	1.58	1.62	0.162±0.004

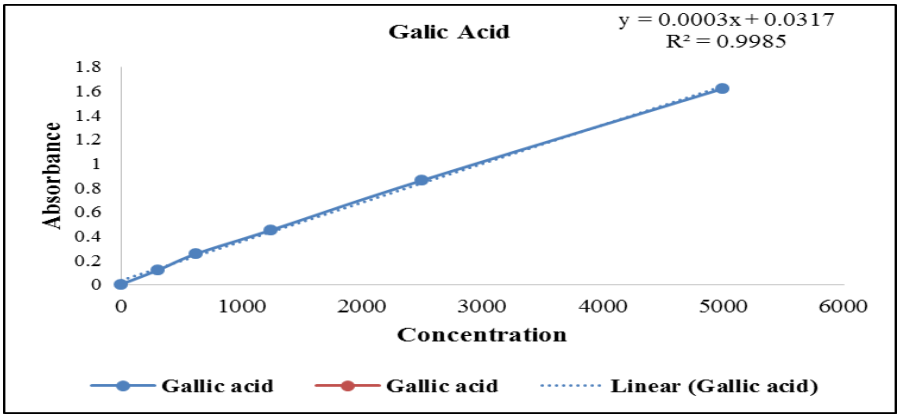

Figure 2: Standard curve of Gallic acid for the determination of total phenolic content.

Table 5: Determination of total phenolic content of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract (CCE) of and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata.*

Sample	No.of sample	Concentration (µg/ml)	Absorbance	GAE/mg of dried sample	GAE/mg of dried sample Mean ± STD
Crude Methanol extract(CME)	1	500	0.936	43.12	42.45 ± 0.19
	2	500	0.943	42.88	
	3	500	0.952	42.23	
Crude Chloroform extract(CCE)	1	500	0.644	32.76	31.08 ± 0.39
	2	500	0.658	33.13	
	3	500	0.663	33.36	
Crude Ethyl Acetate extract(CEE)	1	500	1.172	74.94	75.14 ± 0.95
	2	500	1.174	75.09	
	3	500	1.178	75.40	
Crude N-hexane(CNE)	1	500	0.723	35.23	35.12±0.25
	2	500	0.729	35.56	
	3	500	0.735	35.72	

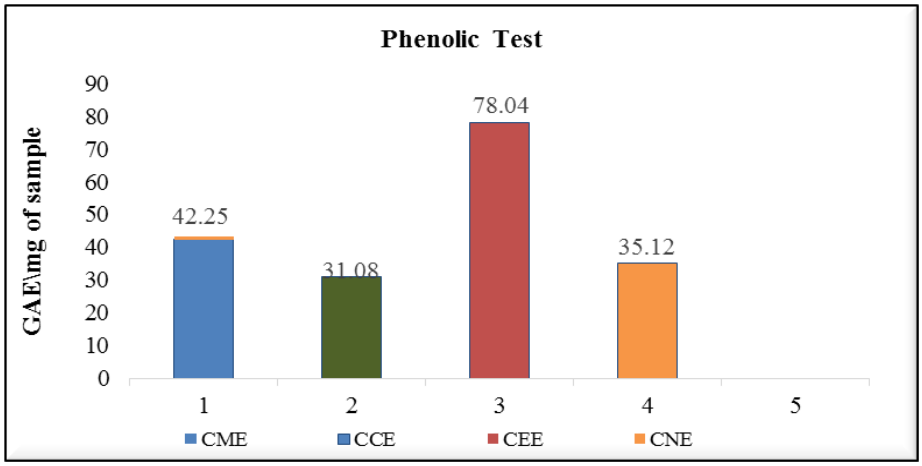

Figure 3: Total Phenolic content (mg/gm plant extract in Gallic acid equivalent) of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude N-hexane extract (CNE) and crude Chloroform extract (CCE) of seeds of *Vigna unguiculata.*

The total phenolic content of crude Methanolic extract (CME), crude Ethyl acetate extract (CEA) and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata* was shown in table which is, crude Ethyle acetate extract (CEE) is higher than crude methanolic extract (CME), Crude N-hexane extract (CNE) and crude chloroform extract (CCE) seeds of *Vigna unguiculata*.

Determination of Total Flavonoids

The total flavonoid content of crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract (CCE) and crude N-hexane extract (CNE) of *Vigna unguiculata* seeds was shown. The results were expressed as mg of Catechin equivalent per gram of dried sample.

Table 6: Absorbance of catechin(standard) at different concentrations for quantitative determination of total flavonoids.

Concentration (µg/ml)	Absorbance			Absorbance Mean ±STD
	a	b	c	
31.25	0.112	0.114	0.113	0.112 ± 0.017
62.5	0.242	0.245	0.243	0.243 ±0.018
125	0.330	0.328	0.333	0.331 ± 0.004
250	0.521	0.524	0.523	0.521± 0.006
500	0.963	0.962	0.965	0.963± 0.040

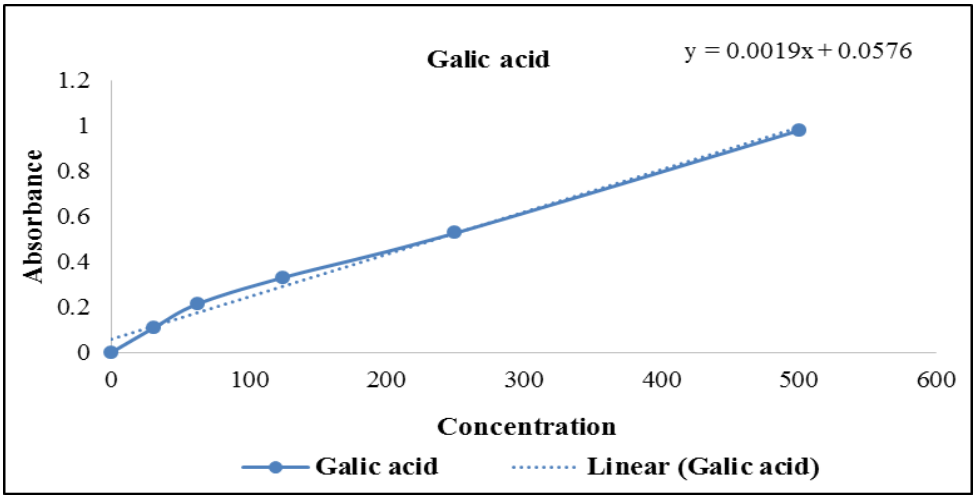

Figure 4: Standard curve of catechin for the determination of total flavonoid content.

Table 7: Determination of total flavonoid content of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract (CCE) and crude N-hexane extract (CNE) of *Vigna unguiculata* seeds.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	GAE/mg of dried sample	GAE/mg of dried sample Mean ± STD
Crude Methanol extract(CME)	1	250	0.684	43.12	54.29± 0.19
	2	250	0.683	42.88	
	3	250	0.685	42.23	
Crude Chloroform extract(CCE)	1	250	0.521	32.76	49.08 ± 0.39
	2	250	0.520	33.13	
	3	250	0.522	33.36	
Crude Ethyl Acetate extract(CEE)	1	250	0.985	74.94	73.14 ± 0.95
	2	250	0.984	75.09	
	3	250	0.986	75.40	
Crude N-hexane(CNE)	1	250	0.489	35.23	39.12±0.25
	2	250	0.488	35.56	
	3	250	0.490	35.72	

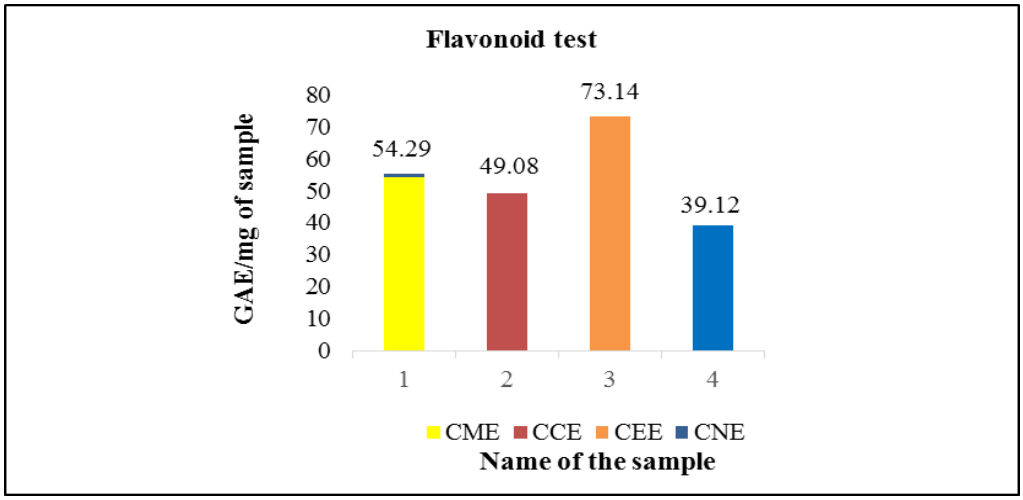

Figure 5: Total flavonoid content (mg/gm plant extract in catechin equivalent) of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE) and crude Chloroform extract (CCE) and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata*.

The results showed that, total flavonoid content (TFC) of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude N-hexane extract (CNE) and crude chloroform extract(CCE)of seeds of *Vigna unguiculata* were 54.29± 0.19, 39.08 ± 0.39, 73.14 ± 0.95 and 49.12±0.25 mg of catechin equivalent /gm of dried

sample respectively. These findings demonstrated that the total flavonoid content of crude Ethyl acetate extract (CEE) was higher than that of crude Methanolic extract (CME), crude N-hexane extract (CNE) and crude Chloroform extract (CCE) of seeds of *Vigna unguiculata*.

Total Antioxidant Activity Determination

The assay was based on the reduction of Mo ((VI) to Mo (V) by the test agents and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The total antioxidant activity was measured and compared among crude Methanolic extract (CME), crude Ethyl acetate extract (CEAE), crude Chloform extract *(CCE)* and crude N-hexane extract (CNE) seeds of *Vigna unguiculata* the reference standard Catechin. The high absorbance values indicated that the sample possessed significant antioxidant activity. The results revealed that all the samples tested had significant antioxidant activities and the effects increased with increasing concentration. The absorbance value of Catechin(Standard), crude Methanolic extract (CME) and crude Ethyl acetate extract (CEE) at 600 µg/ml were 2.042±0.007, 1.842 ±0.009 and 1.182±0.011,2.001±0.0025 respectively, which demonstrated that the total antioxidant activity of Catechin (standard) is higher than that of crude Methanolic extract (CME)crude Ethyl acetate extract (CEE).[24] Between the two extracts, the crude Methanolic extract is more potent than crude Ethyl acetate extract.

Table 8: Total antioxidant activity of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE) and crude Chloroform extract (CCE) and crude N-hexane extract (CNE) of seeds *Vigna unguiculata* and Catechin (standard) at different concentration.

Name of sample	Concentration (µg/ml)	Absorbance			Absorbance Mean ±STD
		A	B	C	
Catechin (Standard)	6.25	0.168	0.169	0.170	0.168±0.001
	12.5	0.320	0.321	0.325	0.320±0.003
	25	0.443	0.446	0.442	0.443±0.001
	50	0.712	0.715	0.714	0.712±0.002
	100	1.202	1.205	1.206	1.202±0.0523
	200	2.104	2.108	2.106	2.104±0.002
	400	2.645	2.644	2.643	2.645±0.001
Crude Methanol extract(CME)	6.25	0.156	0.154	0.155	0.155±0.055
	12.5	0.174	0.173	0.175	0.174±0.001
	25	0.390	0.392	0.391	0.391±0.028
	50	0.625	0.623	0.626	0.623±0.001
	100	0.932	0.930	0.933	0.931±0.025
	200	1.845	1.844	1.843	1.842±0.009
	400	2.202	2.206	2.205	2.203±0.0045
Crude Ethyl acetate extract(CEE)	6.25	0.137	0.139	0.138	0.136±0.012
	12.5	0.295	0.299	0.296	0.296±0.521
	25	0.385	0.386	0.387	0.385±0.001
	50	0.570	0.572	0.571	0.572±0.002
	100	0.992	0.994	0.993	0.991±0.025
	200	1.184	1.186	1.188	1.182±0.001
	400	2.002	2.004	2.006	2.001±0.0025
Crude Chloroform extract(CCE)	6.25	0.157	0.158	0.159	0.156±0.001
	12.5	0.264	0.268	0.265	0.264±0.045
	25	0.380	0.385	0.383	0.385±0.001
	50	0.581	0.582	0.583	0.582±0.235
	100	0.924	0.921	0.925	0.923±0.458
	200	1.346	1.345	1.343	1.345±0.001
	400	2.021	2.022	2.023	2.021±0.478
Crude N –hexane(CNE)	6.25	0.076	0.077	0.078	0.075±0.332
	12.5	0.148	0.147	0.149	0.148±.0012
	25	0.220	0.223	0.222	0.220±0.114
	50	0.398	0.396	0.395	0.396±0.154
	100	0.597	0.598	0.596	0.596±0.001
	200	0.947	0.943	0.949	0.944±0.148
	400	1.524	1.520	1.523	1.524±0.001

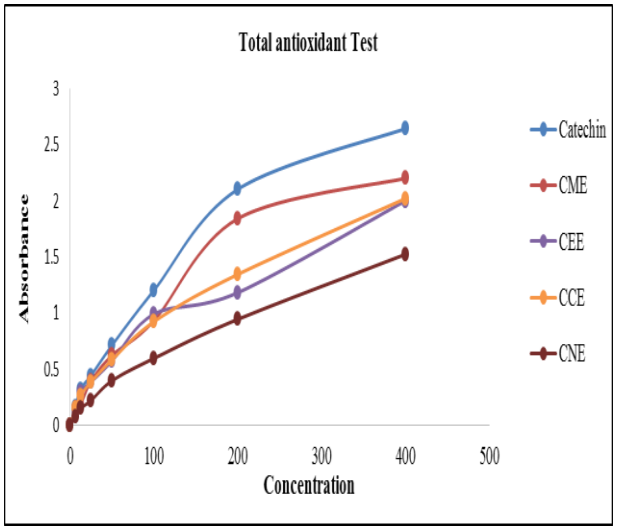

Figure 6: Total antioxidant activity of crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract *(CCE)* and crude N-hexane extract (CNE) of ***Vigna unguiculata*** **seeds and Catechin (Standard) at different concentration**.

From the table it is demonstrated that, total antioxidant activity of crude Methanolic extract of seeds (CME) is higher than that of crude Ethyl acetate extract (CEE), crude Chloroform extract(CCE) and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata* and Catechin (Standard) at different concentrations.[25]

Determination of iron reducing power capacity

The reductive capabilities of crude Methanolic extract and crude Ethyl acetate and standard antioxidant Ascorbic acid were shown in table In this assay the yellow color of the test solutions changes to various shades of green and blue depending on the reducing power of each compound. The presence of reducers causes the reduction of ferric cyanide complex to the ferrous form. Therefore, by measuring the formation of prussian blue at 700 nm.[26] We can monitor the ferrous ion concentration. In assays of the reducing power of the crude extract, significant changes in absorbance at 700 nm were observed with increasing concentration. Reducing power of the crude Ethyl acetate extract was significantly higher than that of crude Methanolic extract.

Table 9: Iron reducing power capacity of crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract(CCE) and crude N-hexane extract (CNE) seeds of *Vigna unguiculata* leave sand Ascorbic acid (standard) at different concentration.

No. of sample	Concentration (µg/ml)	Absorbance			Absorbance Mean±STD
		A	B	C	
Ascorbic acid (Standard)	5	0.258	0.255	0.257	0.25 ± 0.0015
	10	0.728	0.724	0.725	0.72 ± 0.002
	20	1.318	1.308	1.298	1.30 ± 0.01
	40	1.782	1.765	1.775	1.77 ± 0.008
	80	3.457	3.455	3.442	3.45 ± 0.008
	160	3.825	3.855	3.925	3.86 ± 0.051
Crude Methanol Extract(CME)	5	0.079	0.078	0.076	0.079±0.0015
	10	0.135	0.136	0.134	0.135±0.01
	20	0.225	0.226	0.228	0.225±0.0023
	40	0.659	0.658	0.659	0.659±0.145
	80	0.989	0.988	0.990	0.989±0.012
	160	1.321	1.322	1.332	1.321±0.256
Crude Chloroform extract(CCE)	5	0.221	0.222	0.224	0.221±0.215
	10	0.335	0.339	0.338	0.335±.0002
	20	0.532	0.536	0.535	0.532±0.147
	40	0.782	0.783	0.785	0.782±0.0001
	80	0.885	0.886	0.884	0.885±0.0012
	160	1.002	1.006	1.004	1.002±0.001
Crude N-hexane extract(CNE)	5	0.132	0.134	0.135	0.132±0.005
	10	0.225	0.226	0.227	0.225±0.014
	20	0.420	0.421	0.423	0.420±0.0056
	40	0.683	0.685	0.688	0.683±0.008
	80	0.839	0.835	0.836	0.839±0.0008
	160	0.984	0.985	0.986	0.984±0.001
Crude Ethyl acetate extract(CEE)	5	0.124	0.126	0.127	0.124±0.006
	10	0.219	0.220	0.222	0.219±0.0045
	20	0.453	0.452	0.455	0.453±0.005
	40	0.783	0.786	0.785	0.783±0.0089
	80	1.005	1.006	1.008	1.005±0.0026
	160	2.025	2.024	2.026	2.025±0.008

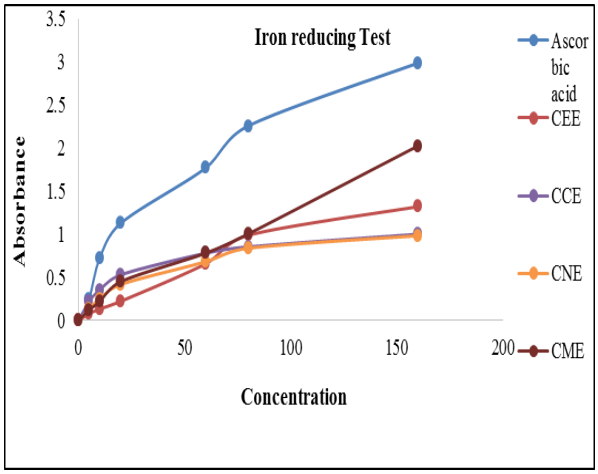

Figure 7: Iron reducing power capacity of Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract *(CCE)* and crude N-hexane extract (CNE) seeds of *Vigna unguiculata* seeds and Ascorbic acid (standard).

From the table it is demonstrated that, Iron reducing ability of crude Ethyl acetate extract (CEE) of seeds is higher than that of crude Methanolic extract (CME), crude Chloroform extract *(CCE)* and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata* compared with the Standard (Ascorbic acid).

Table 10: DPPH radical scavenging activity of the crude Methanolic extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract (CEE) and crude N-hexane extract (CNE) of seeds of *Vigna unguiculata* Ascorbic acid (Standard) at different concentrations.

Sl. no.	Name of sample	Concentration on µg / ml	% of scavenging	IC_{50} µg/ ml
1.	Standard Ascorbic acid	100	96.67	5.8
		50	96.21	
		25	93.97	
		12.5	88.12	
		6.25	85.17	
		3.1	47.55	
		1.6	30.84	
2.	Crude methanolic extract (CME)	100	89.02	24.23
		50	86.40	
		25	82.44	
		12.5	71.16	
		6.25	40.53	
		3.1	34.79	
		1.6	20.46	
3.	Crude N-hexane extract(CNE)	100	81.20	38.45
		50	77.12	
		25	62.12	
		12.5	49.56	
		6.25	41.2	
		3.1	33.54	
		1.6	21.23	
4.	Crude of Chloroform extract(CCE)	100	76.12	52.13
		50	72.89	
		25	62.10	
		12.5	55.12	
		6.25	41.89	
		3.1	33.42	
		1.6	28.42	
5.	Crude Ethyl Acetate extract(CEE)	100	82.56	16.45
		50	72.53	
		25	59.42	
		12.5	48.16	
		6.25	39.26	
		3.1	28.66	
		1.6	22.47	

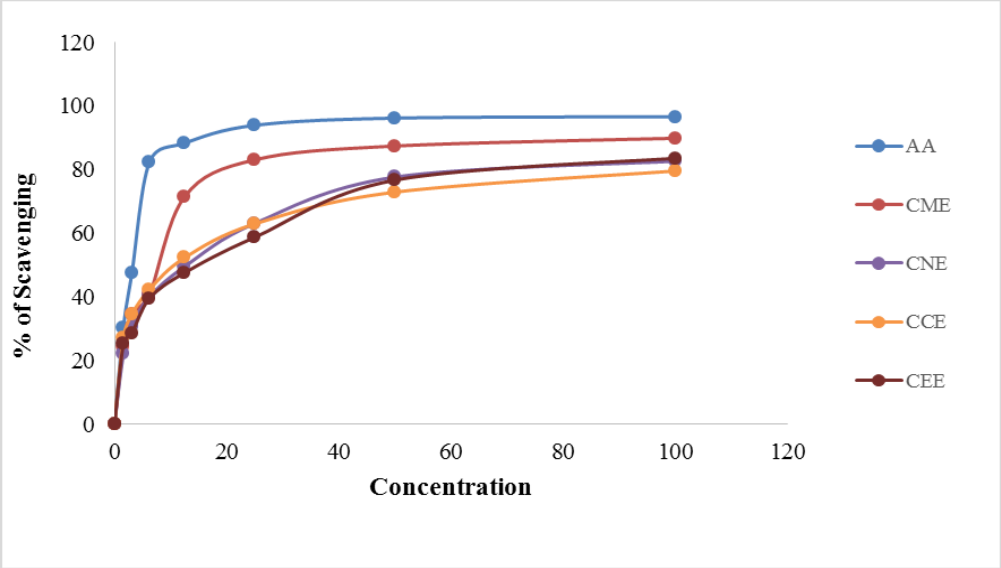

Figure 8: DPPH radical scavenging activity of crude Methanol extract, crude Ethyl acetate extract, crude Chloroform extract, crude N-hexane extract of *Vigna unguiculata* seeds and Ascorbic acid(Standard).

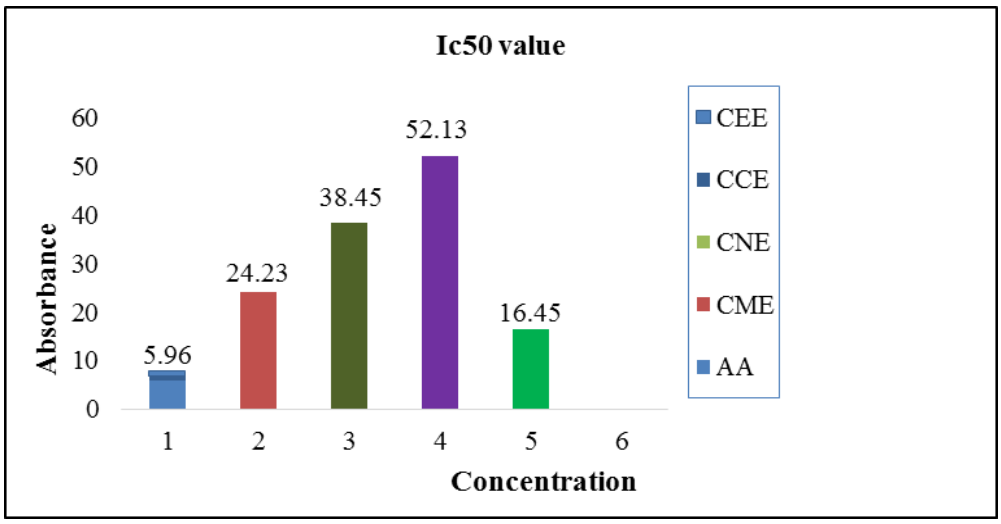

Figure 9: IC_{50} (µg/ml) values of crude Methanol extract (CME), crude Ethyl acetate extract (CEE), crude Chloroform extract (CCE), crude N-hexane extract (CNE) and Ascorbic acid (Standard) for DPPH radical scavenging activity.

In 1' – 1' diphenylpicryl-hydrazyl radical scavenging assay, anti-free radical activity of Crude Ethyle acetate extract (CEE) of leaves is higher than that of other extract. Fig shows the dose response curve at DPPH radial scavenging activity of crude N-hexane (CNE), crude Ethyl acetate (CEE) and crude Chloroform extract (CCE), extract of seeds of *Vigna unguiculata* compared with a standard antioxidant BHT. At a concentration of 100 (µg/ml) the scavenging activities of crude Methanolic (CME), crude Ethyl acetate and crude N-hexane (CNE), extract of seeds were 52.12%, 38.45%, 24.23%, 16.45% respectively while at crude N-hexane extract (CNE) and crude Ethyl acetate (CEE) extract of leaves were 5.96 (µg/ml) respectively compared with the standard BHT with a IC_{50} value.[27]

CONCLUSION

Vigna unguiculata seeds have exhibited high phenol content, rich polyphenol profile and strong antioxidant capacity. The antioxidant potential and radical scavenging activities vary considerably among different scavenging assays, and the results showed a promising source of natural antioxidant, which can prevent and protect from various diseases. Phytochemicals are a rich source of phenols and medicinally important for curing and treating diseases. It has been suggested that the *Vigna unguiculata* is a wonderful plant with antioxidative properties and, hence, for radical scavenging activity and the phenols contribute maximum to the antioxidant activity. The plant part of *Vigna unguiculata* significant amount of polyphenols (CME, CEE, CNE, CCE).The crude Methanolic extract and crude Ethyl acetate extract of seeds showed potent antioxidant activity in most antioxidant test and the IC50

value of crude Methanolic extract and crude Ethyl acetate extract of seeds was 23.63 and 16.45 (µg /ml) respectively where the IC50 value of the Ascorbic acid (standard) was 5.8(µg /ml). This result indicate that the IC50 value of crude Methanolic extract and crude Ethyl acetate extract of seeds almost similar with the IC50 value of the Ascorbic acid (Standard). On the other hand, the antioxidant activity of crude N-hexane extract and crude Chloroform extract of leaves are very low. The powerful antimicrobial properties of *Vigna unguiculata* against both the Gram-positive and Gram-negative bacteria also suggests a promising natural alternative for the synthetic drugs that are also not immunized from the blame for their negative health impacts on human and animals. The range of zone of inhibition was found from 6-18mm in disc sensitivity test while 19-29mm showed by standard kanamycin (30 µg/disc**)**. However, the highest activity of CEE was found against Bacillus cereus and *E.coli* with 18mm zone of inhibition where concentration per disc was 500 µg/disc. The antibacterial activity of crude Ethyl acetate extract of leaves (CEE) was active against *Micrococcus Luteus* and *Bacillus cereus* 16mm and 18mm zone of inhibition.

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