Comparative evaluation of Phytochemical and Physicochemical parameters and *in-vitro* antimicrobial activity of flowers of *Ageratum Conyzoides* Linn

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Abstract: The present communication attempts to evaluate the comparative *in-vitro* activity of flowers of *Ageratum conyzoides* Linn.(Asteraceae family). *Ageratum conyzoides* Linn.is a well known medicinal plant in traditional medical systems having various ethnopharmacological uses. As the official source of plant was whole plants and flowers and it had been studied extensively. Previously leaves of *Ageratum conyzoidess* were regarded as useful part of plant, but now a day there is growing interest in flowers of the plant. As there is no detailed work reported on flowers. Antimicrobial activity was also performed using ethanolic extract through cold percolation method against *Staphylococcus aureus*. The extract was found to have positive results against all.

Key Words: *Ageratum Conyzoides* Linn, Antimicrobial, Compoundss,

Introduction:

Ageratum is derived from the Greek words 'a geras' the meaning of the non – aging and *Conyzoides* on the other is derived from 'konyz' the Greek words 'Inula helenium. *Ageratum conyzoides* belongs to the family Asteraceae.[1] The plants in the family are herbaceous while trees and shrubs are comparatively rare. The genus *Ageratum* consists of 30 species but few species contain phytochemically investigated. *Ageratum Conyzoides* is a tropical plant and it is commonly found in west Africa, Asia and south America.[2] The *Ageratum Conyzoides* is an annual branching herb and it is grown to approximately 1m in height. The stems and leaves are covered with fine white hairs. The leaves are ovate and upto 7.5cm long. The flowers are purple to white and it is less than 6 mm across and arranged in closed terminal inflorescences. The fruit are achene and easily dispersed . The seeds are photoblastic.

Ageratum conyzoides is among such medicinal plants that are effective against diseases and may contain such biologically active compounds, which are effective against ill health. It belongs to the family and tribe of Asteraceae and Eupatoriae respectively. It is an erect, annual, branched, slender, hairy and aromatic plant, which grows to approximately 1 m in height. [3]

It is native to Central America, Southeast Asia, South China, India, West Africa, etc. *Ageratum conyzoides* have been known since ancient times for its curative properties and has been utilized for the treatment of various ailments, such as burns and wounds, headaches pneumonia, analgesic, inflammation, asthma, spasmodic and haemostatic effects, stomach ailments, gynaecological diseases, leprosy and other skin diseases. In Nigeria, different tribes have different names for it. For instance, Igedes of the Middle Belt, Yorubas of the Southwest, and Igbos of the Southeast of the country call it "Ufuopioko", "Imiesu" and "Nriewu" respectively. The plant is widely employed in traditional medicine within the above mentioned geopolitical zonesin Nigeria. It is the only plant used in the treatment of HIV/AIDS by Igede people in Nigeria. Sequel to the on-going efforts by researchers to explore the potency of *Ageratum conyzoides* , the present study investigated the chemical profile of *Ageratum conyzoides* using its different parts and as well relating the constituents to their possible pharmacological importance. [4]

Plant profile:

Taple: 1 Taxonomical Classification: [2]

Botanical Name	*Ageratum conyzoides*
Common Name	Jungle pudina
Classification	Kingdom: Plantae Subkingdom: Angiosperm Class: Eudicots Order: Asteroles Family: Asteraceae Genus: Ageratum Species: Conyzoides

Material and methods: The whole plants and flowers were widely collected from the Campus of the Himalayan Institute of Pharmacy and Research, Dehradun, India in the month of January 2016. The plant is proclaimed as to have ethno pharmacological importance. After collection the plant was preserved in 70% ethyl alcohol for various studies.

Screening for Antibacterial activities:

Chemicals requirement: Ciprofloxacin.

Zone of inhibition (Diffusion Method): The ethanolic extract of flowers of *Ageratum Conyzoides* were tested by Cylinder – Plate method. Different concentrations (10mg/ml, 5mg/ml) of the extract were prepared by reconstituting with water. The test microorganisms were seeded into respective medium (Mueller Hinton agar media) by spread plate methods 2ml with the 24hrs cultures of bacteria grown in Mueller Hinton broth and the excess of which was removed by a sterile Pasteur pipette, and plates were again dried at 37° C for 30 to 45 minutes. There were then ready for application of the discs impregnated with the extracts (100µl) and the drug. The discs were placed on the plates with the help of sterile fine pointed forceps at a suitable distance apart so that the respective discs can produce clear zones of inhibition around them. 100µl of ciprofloxacin was used as the minimum concentration of the drug which could inhibit the growth of bacteria. The antibacterial assay plates were incubated at 37°C for 24hrs. The diameters of the inhibition zones were measured in mm by antibiotic zone reader.

Screening of Antifungal activities:

Chemical requirements: Fluconazole

Zone of inhibition (Diffusion Method): The ethanolic flowers extract of *Ageratum Conyzoides* were tested Cylinder – Plate method. Different concentrations (10mg/ml, 5mg/ml) of the extract were prepared by reconstituting with water. The test microorganisms were seeded into respective medium (fungal agar) by spread plate methods 2ml with the 24hrs cultures of fungus growth in Sabouraud dextrose broth and the excess of which was removed by a sterile Pasteur pipette, and plates were again dried at 37° C for 30 to 45 minutes. There were then ready for application of the discs impregnated with the extracts (100µl) and the drug. The discs were placed on the plates with the help of sterile fine pointed forceps at a suitable distance apart so that the respective discs can produced clear zones of inhibition around them. 100µl of fluconazole was used as the minimum concentration of drug which could inhibit the growth of fungi. The antifungal assay plates were incubated at 37°C for 24hrs. The diameters of the inhibition zones were measured in mm by antibiotic zone reader.

Phytochemical Screening:

Extraction of the plant material using different solvent

The extraction of the powdered plant material was successively performed by following a sequence from non- polar to polar i.e petroleum ether >n- hexane > chloroform > ethylacetate > methanol > water for a period of time through suitable extraction apparatus.

Different phytochemical tests:

Tests for alkaloids:

1. Dragendorff's test: To 2-3 ml filtrate, few drops of Dragendorff's reagent was added. Orange brown precipitate was observed.

2. Mayer's test: To 2-3 ml filtrate, few drops Mayer's reagent gave cream coloured precipitate.

3. Wagner's test: To 2-3 ml filtrate, few drops of Wagner's reagent gave reddish brown precipitate.

4. Hager's test: To 2-3 ml filtrate, few drops Hager's reagent gave yellow precipitate.

5. Tannic acid test: To 2-3 ml filtrate, few drops tannic acid gave buff coloured precipitate.

Tests for Glycosides:

1. Cardiac glycosides

Keller- killiani test: To 2 ml of extract, glacial acetic acid, one drop of 5 % ferric chloride and concentrated sulphuric acid were added. Appearance of reddish brown colour at the junction of the two liquid layers indicated the presence of cardiac glycosides.

2. Anthraquinone glycosides

Borntrager's test : To 3 ml extract, dilute sulphuric acid was added, boiled and filtered. To the cold filtrate equal volume of benzene or chloroform were added. The organic layer was separated and to it was added ammonia. Ammonical layer turned pink.

3. Saponin glycosides

Foam test: The extract and powder were mixed vigorously with water and the formation of foam was observed.

Tests for Flavanoids:

1.With aqueous solution of sodium hydroxide blue to violet colour (anthrocyanins), yellow color (flavones), yellow to orange (flavonones).

2. With concentrated sulphuric acid yellowish orange colour (anthrocyanins), orange to crimson colour (flavonones).

3. Shinoda's test

The extract was dissolved in alcohol, to the solution a piece of magnesium was added followed by concentrated hydrochloric acid drop wise and heated. Appearance of magenta color showed the presence of flavonoids.

Tests for Tannins and Phenolic compounds:

Small quantity of various extracts were taken separately in water tested for the presence of phenolic compounds and tannins with,

Dilute ferric chloride solution (5 %) – violet color

1 % solution of gelatin with 10 % NaCl – white precipitate

10 % lead acetate solution – white precipitate

Tests for Terpenoids:

Noller's test: The substance was warmed with tin and thionyl chloride. The presence of Pink color indicated the presence of triterpenoids**.**

Physico-Chemical Analysis of Plant Material:

Physicochemical analysis of Whole plants and Flowers of *Ageratum Conyzoides* was performed. Various physicochemical parameters like Moisture contents (Loss on drying), Extractive values, Florescence Analysis were determined as per the procedure given in Indian Pharmacopoeia.

Extractive Value:

Ethanol-Soluble Extractive

Macerated 5 g of the air-dried drug, coarsely powdered, with 50 ml of ethanol of the specified strength in a closed conical flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of ethanol, evaporate 25 ml of the filtrate to dryness in a tarred flat-bottomed (petri disc) shallow dish, dry at 105° and weigh. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Water- Soluble Extractive

Macerated 5 g of the air-dried drug, coarsely powdered, with 50 ml of water of the specified strength in a closed conical flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of water, evaporate 25 ml of the filtrate to dryness in a tarred flat-bottomed (petri disc) shallow dish, dry at 105° and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug.

n-Hexane Soluble Extractive

Macerated 5 g of the air-dried drug, coarsely powdered, with 50 ml of n- hexane of the specified strength in a closed conical flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of n- hexane, evaporate 25 ml of the filtrate to dryness in a tarred flat-bottomed (petri disc) shallow dish, dry at 105° and weigh. Calculate the percentage of n- hexane soluble extractive with reference to the air-dried drug.

Loss on drying (moisture content)

Determination of moisture content can be done by Loss on drying methods. Method includes weighed out 2 gm of powdered drug, placed into weighed thin porcelain dish. After that the porcelain dish was

kept into hot air oven at 105°C, up to two repetitive weights which does not differ by more than 0.5mg. Cooled in desiccators and weighed. The loss in weight is considered as moisture level in powdered drug.

Florescence Analysis:

Many herbs show fluorescence when the cut surface or powder is exposed to UV light and this can be useful in their identification. The fluorescence character of the plant powders (40 mesh) were studied both in daylight and UV light (254 nm and 366 nm) and after treatment with different reagents like sodium hydroxide, hydrochloric acid, nitric acid and ferric chloride etc.

Determination of total Tannins

Estimation of tannin percentage in the plant material was carried out according to the method described in AOAC (Anonymous, 1984).

Regents used:

A. Saturated Sodium carbonate solution: It was prepared by adding 35 g anhydrous sodium carbonate to each 100 ml distilled water, dissolved it at 70-80°c and get cool overnight, filtered through glass wool.

B. Tannic acid standard solution: (0.1 mg/ml) dissolve 10 mg tannic acid in 100 ml of distilled water.

C. Folin & Ciocalteu's phenol reagent.

Preparation of standard curve: Standard curve was prepared using tannic acid as standard (10 mg tannic acid in 100 ml of distilled water).

Procedure: 2 g of powdered plant material was extracted with 100 ml distilled water by boiling on water bath for 6-8 hrs, after filtration the volume was made upto 100 ml in a volumetric flask. We took 1 ml aliquot of it, added 5 ml Folin & Ciocalteu's reagent, 10 ml saturated sodium carbonate and made up the volume up to 100 ml in volumetric flask. The instrument was calibrated through blank and took the corresponding absorbance of different sample.

Determination of total Flavonoides:

Total flavonoids were estimated using the method of Ordon et al. [2006],used to estimate total flavonoid contents of the extract solution based on the formation of a complex flavonoid-aluminium.

Extract preparation: 5 gram of dried powdered leaf and rhizomes were cold percolated with known volume of methanol.

Reagent used:

- **A.** 2% Aluminum chloride
- **B.** Rutin standard: 0.1mg/ml solution
- **C.** Methanol

Procedure: A volume of 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ in methanolic solution was added. After one hour at room temperature, the absorbance was measured at 420 nm, using UV-1 Double beam spectrophotometer. Extract samples were evaluated at a final concentration of 0.01 mg/ml. All the determinations were done in triplicate.

Determination of Phenolic content:

Estimation of total Phenolic content in the plant was carried out according to modified colorimetric Folin-Ciocalteu method.

Extract preparation: 1 gm air dried powdered drug percolated with pure methanol, the extract was filtered three times and lyophilized to drying and then weighed.

Regents used:

- **A.** 7% Sodium carbonate saturated solution: It was prepared by adding 7 g anhydrous sodium carbonate dissolved in 100 ml distilled water.
- **B.** Gallic acid standard solution: (1mg/ml) dissolve 10 mg gallic acid in 10 ml of deionizd water.
- **C.** Folin & Ciocalteu's phenol reagent

A volume of 0.5 ml of deionized water and 0.125 ml of a known dilution of the extract were added to a test tube, Folin-Ciocalteu's reagent (0.125 ml) was added to the solution and allowed to react for 6 min. Then, 1.25 ml of 7% sodium carbonate solution was liquated into the test tubes, and the mixture was diluted to the 3 ml with deionized water. The color developed for 90 min, and the absorbance was read at 760.

Result and Discussion:

Result obtained from the present study shows that the whole plants and flowers of *Ageratum Conyzoides* Linn. Contain alkaloids, Saponins, Terpenoids, Flavonoids, Carbohydrate and Tannin. Results are reported in table below **Tab. 1** and **Tab 2.** Phytochemical screening of successive fraction from soxhlet, (+) shows presence, and (−) shows absence of content.

Table: 1 Phytochemicals screening of whole plants extract:

S.NO	Chemical tests	For whole plants extract					
		Pet. ether	n-hexane	Chloroform	Ethyl acetate	Methanol	Water
I.	Test for alkaloids	+	+	_	_	+	+
II.	Tests for flavanoids	+	+	_	+	+	+
III.	Tests for glycosides	+	+	+	+	_	+
IV.	Tests for tannins and phenolic compound	+	_	+	+	+	+
V.	Tests for terpenoids	+	+	+	_	+	+

Table: 2 Phytochemicals screening of flowers extract:

S.No	Chemical tests	For flowers extract					
		Pet. ether	n-hexane	Chloroform	Ethyl acetate	Methanol	Water
I.	Test for alkaloids	+	+	+	_	+	+
II.	Tests for flavanoids	_	+	_	+	+	+

III.	**Tests for glycosides**	+	+	+	_	+	+
IV.	**Tests for tannins and phenolic compound**	+	+	+	+	+	+
V.	**Tests for terpenoids**	+	+	_	+	+	+

Table: 3 Zone of Inhibition to determine antibacterial activity of the extract and standard antibiotic:

Bacterial species	**Zone of Inhibition (mm) *Ageratum Conyzoides***				
	Standard Antibiotic(Ciprofloxacin)	**Flowers extract**			
		10	**5**	**2.5**	**1.5**
Staphylococcus aureus ATCC 12600	16	12	10	6	4

From the table , it con be concluded that the flowers extract of Ageratum Conyzoides has promising antibacterial activity.

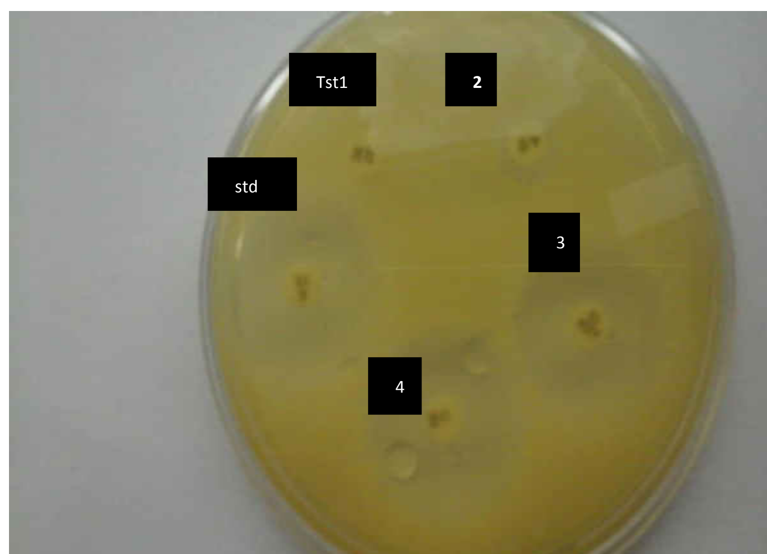


Figure: 1 Zone of inhibition to determine the antibiotic standard drug and different concentration of test sample extract

Table: 4 Zone of Inhibition to determine antifungal activity of the extract and standard antifungal:

Fungi species	**Zone of Inhibition (mm) *Ageratum Conyzoides***				
	Standard Antifungal (Fluconazole)	**Flowers extract**			
		10	**5**	**2.5**	**1.5**
Asparagus recimosus ATCC 16404	18	15	12	6	4

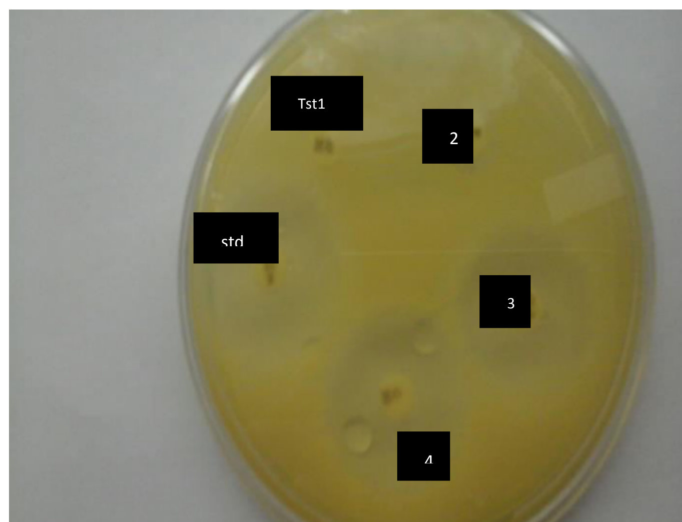


Figure: 2 Zone of inhibition to determine the antifungal standard drug and different concentration of test sample extract

From the table , it can be concluded that the flowers extract of *Ageratum Conyzoides* has promising antifungal activity.

Physico–Chemical Analysis:

Extractive value

The water soluble extractive values indicated the presence of sugar, acids and inorganic compounds [39, 41] . The water soluble extractive values were found to be 22.4 % for whole plant and 17.8 % for flower and ethanol soluble extractive value indicated the presence of polar constituents like phenol, alkaloids, steroids, glycosides, flavonoids [40, 41] .The ethanol extractive valve was found to be 22.2 % for whole plant and 15.42 % for flower. And n- hexane soluble extractive value was found to be 37.4 % for whole plant and 15.6 % for flower.

Table: 5 Extractive values for whole plant:

S. No	Solvent	Extractive Value
1.	Ethanol	22.2%
2.	Water	22.4%
3.	n- hexane	37.4%

Table: 6 Extractive values for Flowers

S. No	Solvent	Extractive Value
1.	Ethanol	15.42 %
2.	Water	17.8 %
3.	n- hexane	15.6 %

Loss on drying

The loss on drying of the powder of *Ageratum Conyzoides* was found to be 11% for whole plant and 11.9 % for flowers.

Table: 7 loss on drying

Loss on drying (105°C)	For whole plant	For flower
	11 %	11.9 %

Fluorescence analysis:

Fluorescence study is an essential parameter for first line standardization of crude drug. In fluorescence the fluorescent light is always of greater wavelength than the exciting light. Light rich in short wavelengths is very active in producing fluorescence and for this reason ultraviolet light produces fluorescence in many substances which do not visibly fluoresce in daylight [42, 43].

Table: 8 Fluorescence Analysis

S. NO.	Solvents Treatment	Visible light	Short UV (254 nm) for whole plants	Long UV (366 nm) for whole plants	Short UV (254 nm) for flowers	Long UV (366 nm) for flowers
1.	Drug + Distilled water	Light yellow	Yellowish brown	Off white	Dark green	Dark black
2.	Drug + Pet.Ether	Reddish brown	Whitish	Yellowish green	Blackish green	Blackish brown
3.	Drug+Chloroform	Light brown	Dark brown	Greenish black	Dark grey	Greenish brown
4.	Drug + Methanol	Yellowish brown	Greenish yellow	Greenish brown	Whitish grey	Dark green
5.	Drug + Conc. HCl	Dark brown	Brownish black	Off white	Greenish black	Dark green
6.	Drug + Conc. HNO_3	Ceramic yellow	Yellow	Greenish yellow	Yellowish green	Dark black
7.	Drug + Conc. H_2SO_4	Greenish brown	grey	Dark green	Black	Dark green
8.	Drug + Picric acid	Yellowish Brown	Yellowish green	Dark brown	Green	Brownish green
9.	Drug + Ammonia solution	Mud brown	Light green	Ceramic green	Dark green	Brownish brown
10.	Drug + 10% Sodium hydroxide	Dark brown	grey	Yellowish green	Dark brown	Blackish green
11.	Drug + Ferric chloride	Greenish black	Yellowish grey	Black	Greenish brown	Dark black

Determination of total Tannins:

Figure: 3 Calibration curve for Tannins content of Whole plant

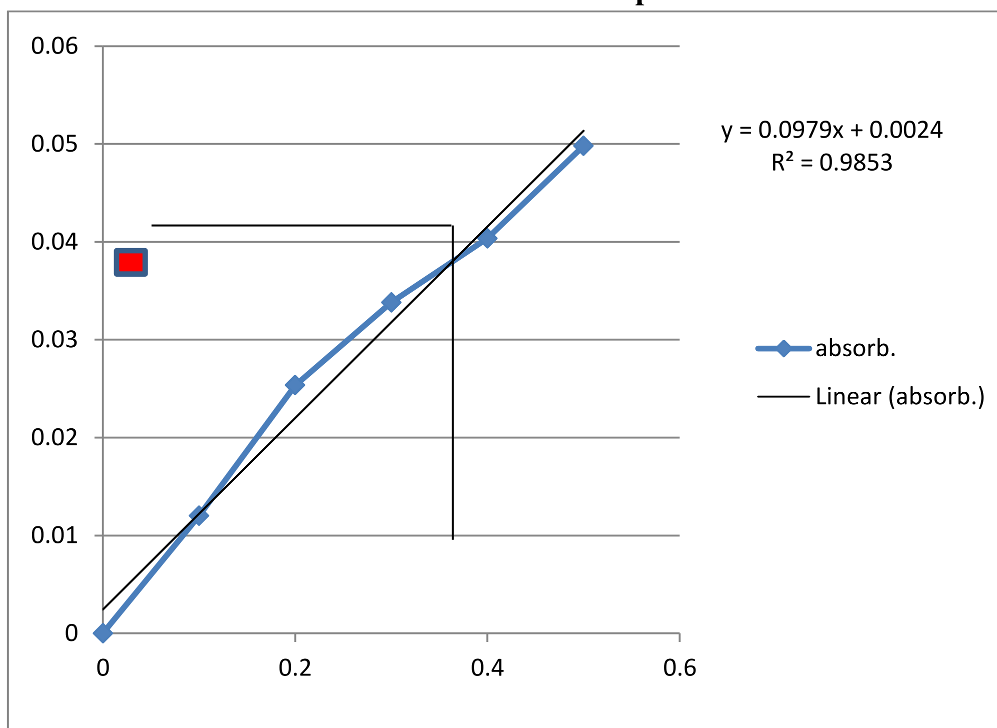
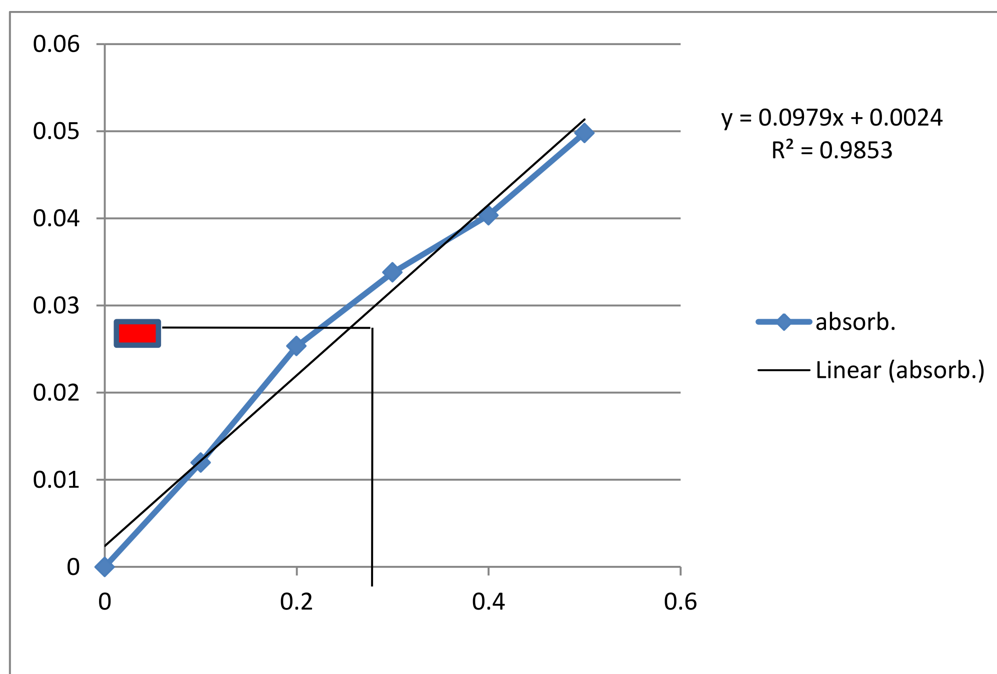


Figure: 4 Calibration curve for Tannins of Flower



Total Tannin content calculated by using y = 0.097x + 0.002, R^2 = 0.985 for whole plant, y = 0.097x + 0.002, R^2 = 0.985 for flower, at 760 nm, using UV-1 Double beam spectrophotometer, where y was the absorbance and x the tannic acid equivalent (mg/ml). The formula for the regression line is y = mx+c. where m is the slope of the line and c is the y-intercept. The methanol extract of whole plant content the highest concentration and flower content lowest concentration. The concentration of Tannins in methanol of whole plant content was found to be 0.373 and flower content 0.280.

Determination of total Flavonoids:

Figure: 5 Calibration curve for Flavonoids of Whole plant

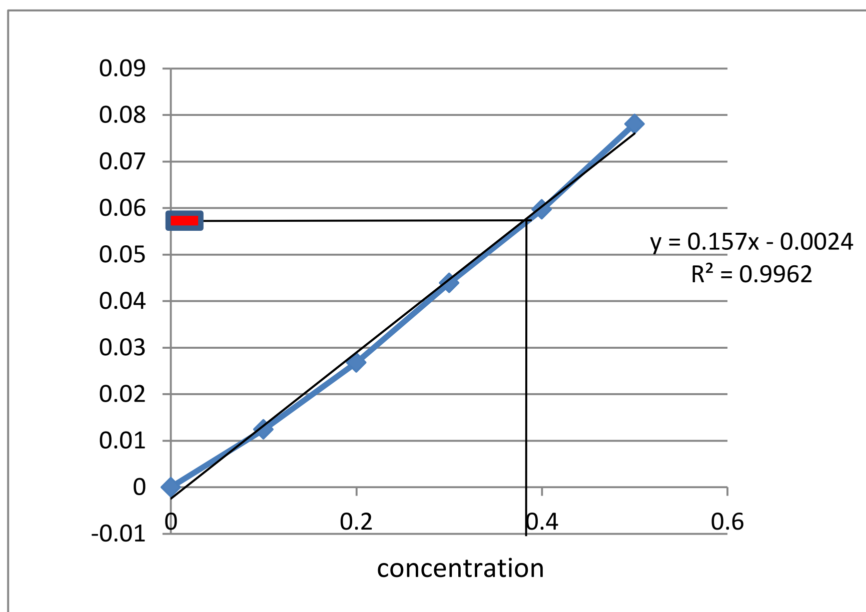
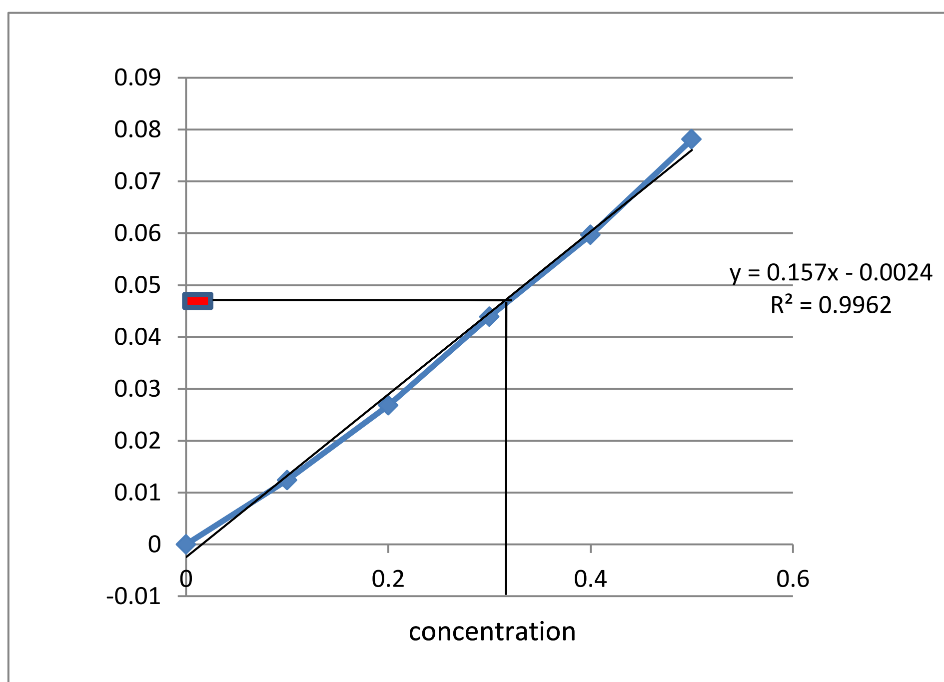


Figure: 6 Calibration curve for Total Flavonoids Content of Flower



Total flavonoids was calculated as Rutin (mg/ml) using y=0.157x - 0.002, R^2 = 0.996 for whole plant and y=0.157x - 0.002, R^2 = 0.996 for flower at 490 nm using UV-1 Double beam spectrophotometer, where y was the absorbance and x the Rutin equivalent (mg/ml). The formula for the regression line is y = mx+c. where m is the slope of the line and c is the y-intercept. The methanol extract of whole plant content the highest concentration and flower content lowest concentration. The concentration of flavonoids in methanol of whole plant content was found to be 0.377 and flower content 0.323.

Determination of total Phenolic:

Figure: 7 Calibration curve for Total Phenol content of Whole plant

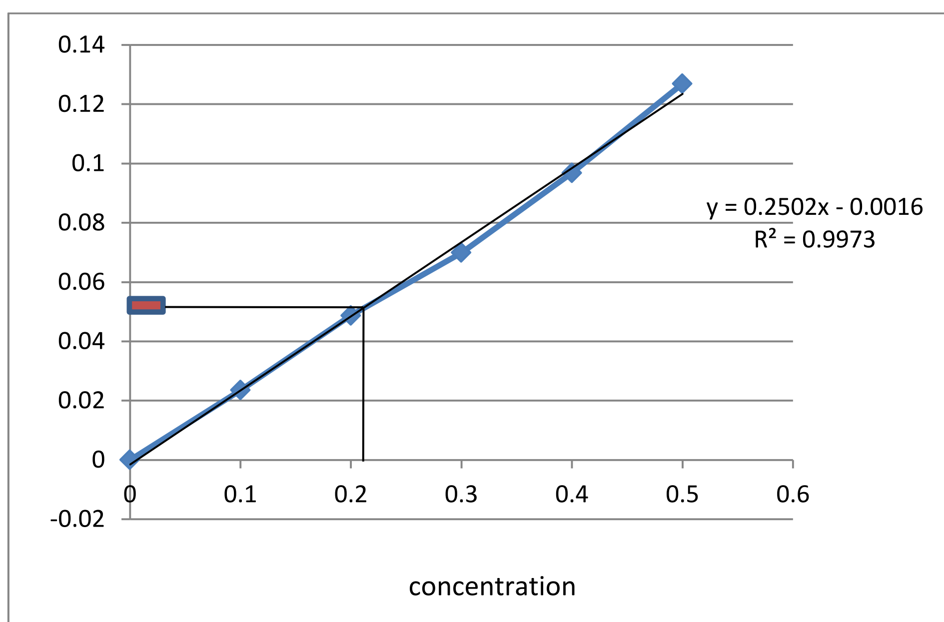
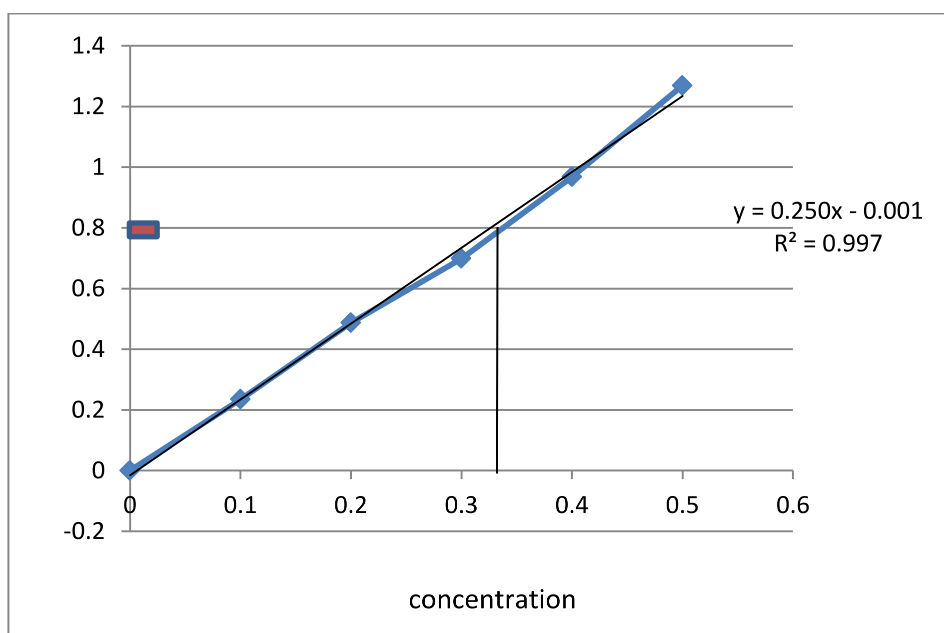


Figure: 8 Calibration curve for Total Phenol Content of Flower



Total phenol was calculated as Gallic acid (mg/ml) using y=0250x - 0.001, R^2 = 0.997 for whole plant and y=0.250x - 0.001, R^2 = 0.997 for flower at 760 nm using UV-1 Double beam spectrophotometer, where y was the absorbance and x the Gallic acid equivalent (mg/ml). The formula for the regression line is y = mx+c. where m is the slope of the line and c is the y-intercept. The methanol extract of whole plant content the lowest concentration and flower content highest concentration. The concentration of phenol in methanol of whole plant content was found to be 0.238 and flower content 0.354.

Conclusion:

Results of the proposed study suggest that there is possible use of whole plants and flowers of *Ageratum Conyzoides* Linn. as a natural antimicrobial agent. Thus, it may be concluded that the plant can be effectively used further in the treatment of above mentioned ailments. It may also be concluded that the present property of the plant is due to the presence of high level of polyphenolic compounds including flavonoids, flavonols etc. Plant also showed positive and considerable anti-microbial activity against all micro-organisms used.

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