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COMPARATIVE STUDIES ON PHYTOCHEMICAL, PHYSICO CHEMICAL, ANTI OXIDANT PROPERTIES AND GC-MS ANALYSIS ON LEAVES EXTRACT OF BOERHAAVIA DIFFUSA L. AND BOERHAAVIA REPENS L.

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ABSTRACT

The present study was designed to evaluate the preliminary qualitative phytochemical, physicochemical properties and pharmacological study like antioxidant analysis, synthesis of silver nanoparticles, which are further characterized by GC-MS analysis in the leaf extract of Boerhaavia diffusa L. and Boerhaavia repens L. The results reveal that the leaf extract of Boerhaavia diffusa L. showed maximum phytochemical constituents, good antioxidant and antimicrobial properties compared to the leaf extract of B.repens; B.diffusa contained silver nanoparticles which was characterized by GC-MS analysis. Hence Boerhaavia diffusa L. Was used as medicinal plant for curing various ailments than B. repens.

KEY WORDS

Boerhaavia diffusa L, Boerhaavia repens L., phytochemical properties, antioxidant activity, GC-MS analysis.

1. INTRODUCTION

Medicinal plants play a vital role in the primary health care system of India. Most of the rural people still rely on traditional system to cure their ailments by utilizing the medicinal plants available in their surroundings. In recent years, secondary metabolites or phytochemicals, with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents [1].

Various parts of medicinal plants are used as raw drugs since they possess varied medicinal properties [2]. In India thousands of plant species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times [3].

Plants are the richest source of natural antimicrobial agents that can be used against many diseases [4]. The medicinal values of these plants lie in bioactive phytochemical constituents that produce definite physiological actions of the human body. The bioactive phytochemical constituents in medicinal plants include alkaloids, flavonoids, phenolic compounds, tannins, anthracine derivatives and essential oils [5].

Antioxidant property of plants inhibits the oxidation of other molecules and protects cells against the damaging effects of reactive oxygen species. The antioxidant compounds in food play a vital role as health protecting factors. Today about 300 medicinal species and aromatic plants are used worldwide in the pharmaceutical, food, cosmetics and perfume industries. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization [6].

The Plant Boerhaavia diffusa L. is a herbaceous member of the family Nyctaginaceae which is widely distributed in the tropics and subtropics. It is used to cure jaundice,

hepatitis, edema, oligurea, anemia, eye inflammation, the roots are effectively used for curing several diseases, asthma, urinary disorders, leucorrhea, rheumatism and encephalitis, diabetes [7]. It has many ethno botanical uses; the leaves are used as vegetable and were used in the traditional, Ayurvedic medicine system. Besides, the plant is reported to possess many pharmacological, clinical, and antimicrobial properties. Punarnava is very useful for curing kidney diseases and all type of health problems. B.diffusa L. contains biologically active compounds that may serve as candidates for new drugs in the treatment and prevention of human and livestock diseases.

Boerhaavia repens L. (Family: Nyctaginaceae) is a herbaceous terrestrial annual erect herb. The plant is widely distributed in the trophical areas. B. repens is with rich source of therapeutic constituents [8]. It is found in India which is used as an important medicinal plant having application in jaundice, fever and constipation. It is also reported to have anti-viral property [9]. It has enormous phytochemical constituents and antioxidant properties, cytotoxic and antimicrobial properties.

Thus, the present study aims to comparatively analyze the phytochemical constituents, antioxidant activities (DPPH Scavenging Activity), synthesis of silver nanoparticles and characterization of silver nanoparticles by GCMS analysis on leaves of Boerhaavia diffusa L. and Boerhaavia repens L.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The plants were collected from south rural areas; near Avinashi, Tirupur Dt, Tamil Nadu. The collected plant leaves of both Boerhavia diffusa and Boerhavia repens were made thoroughly free from other foreign organic matter by washing, air dried under shade and grinded to powder by grinder.

2.2 Extraction of plant material

In order to perform a systematic phytochemical screening powdered leaves was extracted with an array of solvents. The usual technique involves extraction of phytochemicals by polar solvent directing towards non-polar solvents. For aqueous extraction cold percolation method was adopted (Harborne, 1998). The plant material was also extracted with Ethanol, methanol, chloroform, petroleum ether, benzene and hexane by using Soxhlet apparatus by successive extraction method.

2.3 Physico chemical Analysis

The physico chemical analysis was done according to AOAC methods [10].

2.3 a) Moisture Content

One gram of the powdered sample was weighed in a clean crucible/ beaker of known weight. The sample was then dried in oven at 105°C for 8 h. The crucible/beaker was cooled and weighed to determine water loss in powdered sample.

$$\text{Moisture (\%)} = \frac{\text{Difference in weight}}{\text{Weight of sample}} \times 100$$

2.3 b) Estimation of Crude Fiber

Two grams of powdered leaves sample was subjected to acid and subsequent alkali treatment. Oxidative hydrolytic degradation of native cellulose and considerable degradation of lignin occurs. The residue obtain after final filtration was weighed, incinerated, cooled and weighed again. The loss in weight gives the crude fiber contents.

2.3 c) Estimation of Ash percentage

Place about 2-4 gram of the ground air-dried material, accurately weighed, in a previously ignited and tared crucible (usually of platinum or silica). Spread the material in an even layer and ignite it by gradually increasing the heat to 450°C until it is white, indicating the absence of carbon. Cool in a desiccator and weigh.

Ash value can be calculated by using formula

$$\text{Ash value} = \frac{\text{Initial weight of the sample - Final weight of the sample}}{\text{Initial weight of the sample}} \times 100$$

2.3 d) Water soluble Ash

The total ash obtained above was boiled with 25 ml of distilled water for 5 minutes. The insoluble matter was collected on an ash less filter –paper, washed with hot water and ignited to constant weight at low temperature. The weight of the insoluble matter was subtracted from the weight of total ash, represents the water-soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug. The result was calculated with reference to the air-dried drug.

2.3 e) Acid Insoluble Ash

The total ash obtained was boiled with 25 ml of dilute hydrochloric acid for 5 minutes. The insoluble matter was collected on tarred grouch crucible, washed with hot acidulated water, ignited, cooled and weighed. The percentage acid insoluble ash was calculated with reference to the air-dried drug. The same procedure was repeated with other ash obtained.

2.3 f) Estimation of Mineral contents

Acid digestion method was used to digest the dried leaves and root powder for mineral analysis. Organic matter of dry plant powder was well oxidized with the sequential combination of perchloric acid, nitric acid (HNO3) and Sulphuric acid (H2SO4) (1ml: 2.5 ml:1ml) at 125°C temperature. After complete digestion, the sample was cooled, diluted with distilled water up to final volume of 50 ml. The estimation of nutritionally important minerals i.e. Phosphorous, Sodium (Na), Potassium (K), Calcium (Ca) was done by spectrophotometer.

2.3 g) Estimation of Protein percentage [11]

Protein was estimated using micro Kjeldahl method. In this method, 50 mg of powdered leaves sample was digested by boiling with concentrated Sulphuric acid in the presence of catalyst copper sulfate till the formation of the clear solution. The ammonia was released by the addition of excess Sodium hydroxide in presence of compressed water vapors and was removed by steam distillation. The distillate was collected in 4% Boric acid solution and titrated with standard Hydrochloric acid using Methylene blue as an indicator. Total Protein was calculated by multiplying nitrogen percentage by 6.25.

2.4 Phytochemical Screening

Qualitative test for Alkaloids, Flavonoids, Terpenoids, Glycosides, Tannins, Phytosterols, Phenols, Saponins, proteins etc., in the leaves extract of B.diffusa and B.repens was performed according to the procedure [12].

Test for Alkaloids - Mayer's test (Mayer's reagent)

1.36 g of Mercuric chloride was dissolved in 60 ml of distilled water and 5 g of Potassium iodide was dissolved in 10 ml of water. These two solutions were mixed with distilled water which is known as Mayer's reagent. To one ml of the extract, a few drops of reagent were added. Formation of white or pale precipitate showed the presence of alkaloids.

Test for Carbohydrates - Molisch's test

To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 ml of concentrated Sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of Carbohydrates.

Test for Proteins – Ninhydrin Test

Add two drops of freshly prepared 0.2% Ninhydrin reagent to the extract solution and heat. Development of blue colour reveals the presence of proteins, peptides or amino acids.

Test for Phenols - Ferric Chloride Test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

Test for Flavonoids – Alkaline Reagent Test

Extract solution when treated with Sodium hydroxide solution gives yellow or red color immediately.

Test for Saponins - Foam Test

One ml solution of extract was diluted with distilled water to 20 ml and shaken vigorously. Development of stable foam suggests the presence of Saponins.

Test for Tannins – Lead Acetate Test

The test extract was taken in water, warmed and filtered. 5 ml of filtrate was allowed to react with 1ml of 5% Ferric chloride solution. Dark green or deep blue colour indicates the presence of tannin.

Test for Sterols - Liebermann's Test

To the 2 ml of extract add several drops of acetic anhydride and then 2 drops of Conc.H2SO4 and mix carefully. The formation of a green or green-blue colour after a few minutes is positive

Test for cardiac glycosides - keller- killani test

To two ml of extract add with two ml Glacial acetic acid and one drop of FeCl3 was added. Then it is added with one ml of Concentrated H2SO4. A brown ring indicates the presence of cardiac glycosides.

Test for Resins

One ml of various solvent extract was treated with few drops of acetic anhydride solution followed by one ml of Conc. H_2SO_4. Resins give coloration ranging from orange to yellow.

2.5 Determination of Antioxidant Activity

Antioxidant activity was carried to analyze, whether the aqueous leaves extract of both the plants B.diffusa and B.repens contain substance capable to mop up free radicals and prevent them from causing cell damage.

DPPH Free Radical Scavenging Assay

Preparation of standard solution

Required quantity of ascorbic acid was dissolved in methanol to give the concentration 5, 10, 20, 30, 40 and 50µg/ml.

Preparation of test sample

Stock solutions of sample were prepared by dissolving 10mg of dried methanolic extract in 10ml of methanol to give concentration of 1mg/ml.

Preparation of DPPH solution

4.3 mg of DPPH was dissolved in 3.3ml of Methanol and it was protected from light by covering the test tubes with aluminium foil.

Protocol for estimation of DPPH scavenging activity

150 µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different volume levels of test sample (20, 40, 60, 80 and 100 µl) were screened and made 100 µl of each dose level by dilation with 3ml methanol.

Absorbance was taken at 516 nm in visible spectrophotometer after 15 mins using methanol as blank. The reduction % and IC50 were calculated as follows. The free radical scavenging activity (FRSA % antiradical activity) was calculated using the following formula

$$\text{Scavenging (\%)} = \frac{\text{Optical Density of control} - \text{optical density of extract}}{\text{Optical density of control.}} \times 100$$

2.6 Synthesis of Silver nanoparticles

One mM aqueous solution of Silver nitrate (AgNO3) was prepared and used for the synthesis of Silver nanoparticles, 4 ml solution was added to the 4 ml of water extracts of leaves of B.diffusa and B.repens then the colour change was recorded. The prepared solution was taken in elementary flask and covered with aluminum foil air tightly and setup was kept in dark room for 72 hours.

Characterization of silver nanoparticles

a) Colour change

The formation of silver nanoparticles was preliminary confirmed by the colour changes from green to dark brown.

b) Spectrophotometer

The formation and completion of silver nanoparticles was characterized by spectrophotometer. The analysis was carried out in a visible spectrophotometer deep vision 2305 with a resolution of 1nm between 300 and 600 nm.

2.7 GC-MS Analysis: (Gas Chromatography - Mass Spectrometry Analysis).

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the Ethanol leaf extract was performed using a clarus 500 Perkin Elmer gas chromatography equipped with a Elite-5 capillary column (5% Phenyl and 95% methyl Polysaccharides siloxane) and mass detector turbo mass gold of the component which was operated in E1 mode. Elite wax (Polyethylene glycol) (30mmx 0.25mm X0.25umdf) is a polar column used in the estimation. An insert gas such as Hydrogen or Nitrogen or Helium is used as a carrier gas at a flow rate of 1ml/min, split 10:1. The components of test sample is evaporated in the injection part of the GC equipment and segregated in the column by adsorption and desorption technique with suitable temperature programmes of the over controlled by software different components are eluted from based on the boiling point of the individual components. The GC column is heated in the oven between 110ºC to 280ºC. The time at which each component eluted from the GC column is termed as retention time (RT). The total GC running time is 36 min. The eluted component is detected in the mass detector. The spectrum of the known components stored in the NIST library and ascertains the name, molecular weight and structure of the components of the test material in GCMS study. Identification of components was based on comparison of their mass spectra with those of Wiley and NIST Libraries and as well as on comparison of their retention indices with literature.

3 RESULTS

3.1 Qualitative Phytochemical Analysis on Leaves extract of B.diffusa and B.repens.

A systematic study of a crude drug embraces through consideration of primary and secondary metabolites

derived as a result of plant metabolism. The qualitative phytochemical studies of different solvent extracts (water, ethanol, methanol, chloroform, acetone, benzene, petroleum ether and hexane) of leaves of B.diffusa and B.repens was recorded in Table I.

The Ethanol leaves extract of Boerhavia diffusa showed maximum compounds like alkaloids, anthraquinone, cardiac glycosides, flavonoids, tannins, terpenoids, physterols, carbohydrates, proteins, amino acids, resins except saponins and phenols compared to all other extracts. Comparatively, the ethanol leaf extract of B.repens showed maximum compounds except saponins and phytosterols.

3.2 Physio-chemical properties of leaves of Boerhaavia diffusa L. and B.repens, L.

The crude drugs can be identified on the basis of their morphological, histological, chemical, physical and biological studies with the advancement in separation technique and instrumental analysis; it is possible to perform physical evaluation of a crude drug, which could be both of qualitative and quantitative nature.

Leaves powder was screened and compared for physico-chemical analytical values like moisture, total ash, fiber content, protein quantity of Boerhaavia diffusa and B. repens were tabulated in Table II. The results were in accordance to the Ayurvedic Pharmacopoeia of India.

The moisture content (8.33%) was higher in B.diffusa leaves than in B.repens roots. The total ash content was higher in B.diffusa (5.30) leaves than B.repens leaves. The crude fiber content of B.diffusa leaves does not show much difference. The protein content was also higher in B.diffusa leaves (3.78) than in B.repens leaves (3.50).

3.3 Antioxidant property of leaves of Boerhaavia diffusa L. and Boerhaavia repens L.

Antioxidant studies was carried out to find the presence of antioxidants in the ethanol leaf extract of Boerhavia diffusa and B.repens by DPPH free radical scavenging activity. The ethanol leaf extract exhibited higher antioxidant property (74.07%) at the concentration of 100µg/ml than the ethanol leaf extract of B.repens(67.80%). Standard ascorbic acid showed higher value (85.62) than leaf extract and the results were tabulated in Table III.

3.4 Synthesis of Silver nanoparticles in leaves of Boerhaavia diffusa L. and B.repens L.

The presence of silver nanoparticles in the leaf extract was observed by reduction of silver ions into silver nanoparticles. The colour of ethanol extract changed from green to reddish brown in Boerhaavia diffusa indicating high amount of silver nanoparticles than in B.repens which was observed visually.

3.5 GAS CHROMATOGRAPHY-MASS SPECTROPHOTOMETRY (GC-MS) analysis in leaf extract of Boerhaavia diffusa L.

The ethanol leaves extract of Boerhaavia diffusa L. was subjected to GC-MS analysis. The GC-MS analysis of leaf extract of B.diffusa showed ten prominent peaks with retention time ranging from 5.97 to 32.72. Out of ten peaks the peak area percentage was identified to be maximum in Phenol using standard solution under condition.

The GC-MS analysis showed ten compounds Figure 1 they were Phenol, 2-methyoxy-4-(2-propenyl)-(CAS), 2,6,10-Trimethyl-3-oxo-12-(tetrahydropyran-2-yloxy)-dodeca-6,10-dienoicacid, methylester, 3-phenyl- 4 [1 (E) propenyl]3,4,5,6tetrahydro2(1H) pyrimidinone, Hexadecanoic acid, Phytol, Methyl2,3-dimethyl-3-nitrosobutanoate, Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl) ethyl ester at the retention time of 11.72, 15.56, 18.55, 22.70, 25.21, 28.31 and 32.72 respectively. Table IV.

Table I- Qualitative phytochemical analysis of leaves extract of Boerhaavia diffusa L. and Boerhaavia repens L.

Phytochemical studies	Water		Ethanol		Methanol		Chloroform		Acetone		Benzene		Petroleum ether		Hexane	
	B.diffusa	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*	*B.diffusa*	*B.repens*
Alkaloids (mayers reagent)	_	+	+	+	+	_	+	_	+	_	+	_	+	+	_	+
Anthraquinone (ammonia test)	+	_	+	+	_	+	_	+	+	_	_	_	_	_	+	_
Cardiac glycosides (keller-killani test)	+	+	+	+	+	_	+	_	_	+	+	+	_	_	_	+
Flavanoids (alkaline reagent test)	_	_	+	+	_	+	_	+	_	_	_	_	_	_	+	_
Tannins (lead acetate test)	+	+	+	+	+	_	+	_	+	_	+	+	_	+	_	+
Terpenoids (salkowski test)	+	+	+	+	_	+	_	_	+	_	_	_	+	_	+	_
Phenols (ferric chloride test)	_	_	_	+	+	_	+	_	_	+	_	+	_	+	_	_
Phytosterols (liebermans test)	+	+	+	_	_	+	_	+	+	_	+	_	_	_	+	_
Saponins (froth test)	_	_	_	_	+	_	+	_	_	_	_	_	+	_	_	_
Carbohydrates (molischs test)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteins (ninhydrin test)	+	+	+	+	+	+	+	_	+	_	_	_	_	+	_	_
Amino acids (biuret test)	+	+	+	+	_	_	+	+	_	+	_	+	_	_	+	+
Resins (sulphuric acid test)	+	+	+	+	_	_	_	_	_	+	_	_	_	_	_	_

'+' indicates presence, '—'indicates absence

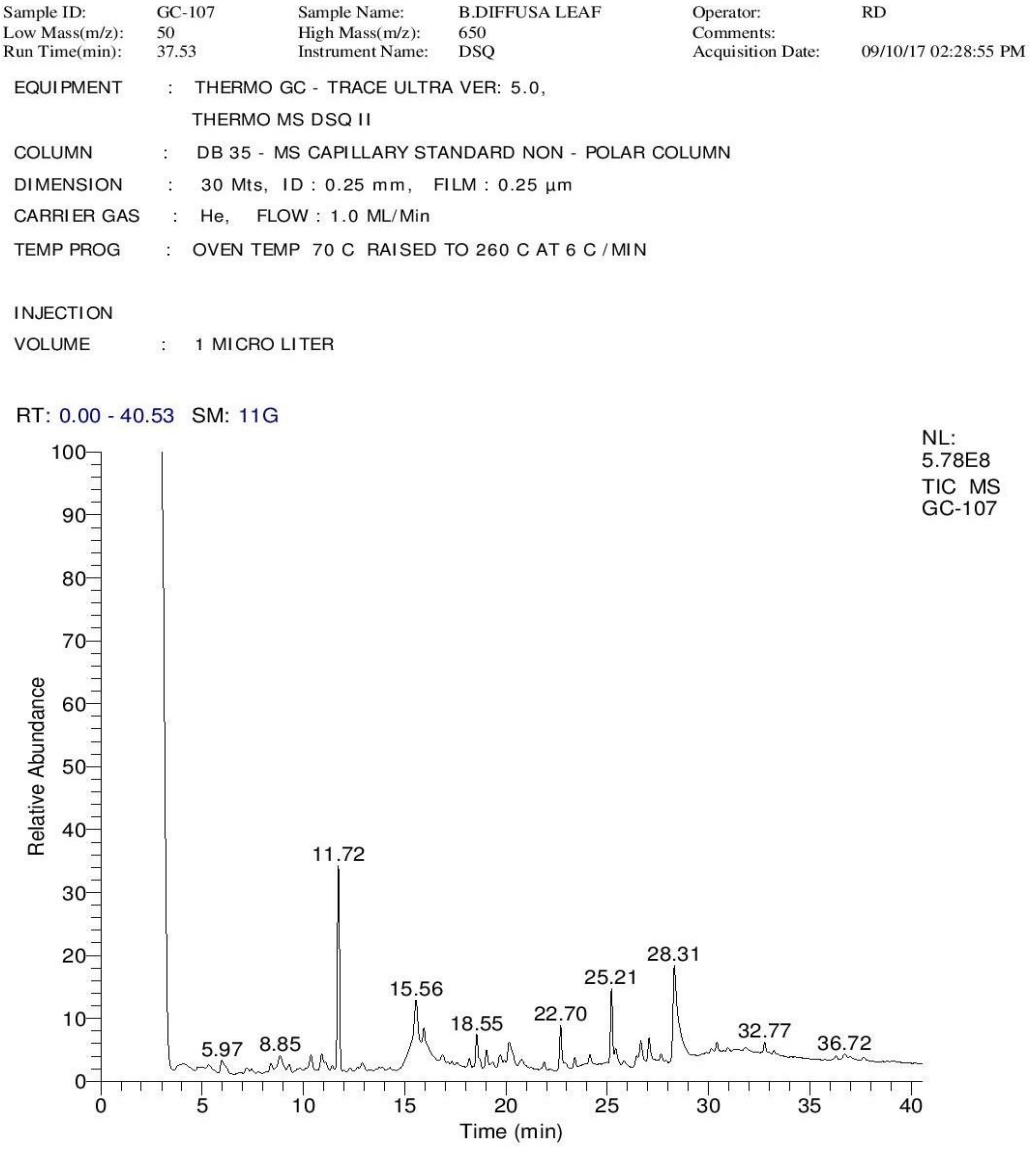
Table II- Physico-chemical properties of leaves of *B.diffusa* L. and *B.repens* L.

Parameters studied	Value of Leaves expressed in % (W/W)	
	***Boerhaavia diffusa* L.**	***Boerhaavia repens* L.**
Moisture	8.33	6.33
Total Ash	5.30	4.33
Crude fiber	23.82	20.03
Protein	3.78	3.50

Table III- Antioxidant property of leaves of Boerhaavia diffusa L. and B.repens L.

S.No	Concentration of plant extract (µg/ml)	DPPH Scavenging activity (%)		
		Ascorbic acid (standard)	Leaf extract *B.diffusa*	*B.repens*
1.	20	58.59	29.13	23.19
2.	40	68.59	38.65	34.24
3.	60	74.37	48.76	36.64
4.	80	78.22	64.53	40.75
5.	100	85.62	74.07	67.80

Figure 3- GAS CHROMATOGRAPHY-MASS SPECTROPHOTOMETRY (GC-MS) analysis in leaf extract of Boerhaavia diffusa L.



4.DISCUSSION

Phytochemical analysis is of paramount importance in identifying new source of therapeutically and industrially valuable compounds. Certain studies were carried out in the leaves extract of Boerhaavia diffusa L. and Boerhaavia repens L. In which B. diffusa showed phytochemical constituents such as tannins, cardiac glycosides, alkaloids, saponins and steroids [13].

The Antioxidant activity was carried out to analyze, whether the ethanol leaf extract; chloroform and methanol extract of both the plants B. diffusa and B. repens, contain substance which was capable to mop up free radicals and prevent them from causing cell damage using DPPH scavenging method with Ascorbic acid as standard. The ethanol leaf extract of Boerhaavia diffusa L. showed dose dependent DPPH radical scavenging activity due to its ability for donating hydrogen molecule [14]. The antioxidant activity in B.repens ethanol leaf extract was due to the presence of high saponins and vitamins [15]. Silver nanoparticles are of great importance because of its high medicinal value. The silver nitrate was added to the ethanol leaf extract of B.diffusa and B.repens. After 72 hours the colour of the extract was changed into reddish brown

colour [16]. The colour change confirmed the presence of silver nanoparticles in the plant extract [17]. In the GC-MS analysis, ten bio active phytochemical compounds were identified in the ethanol leaf extract of Boerhaavia diffusa L. The identification of phytochemical compounds was based on the peak area, molecular weight and molecular formula.

5.CONCLUSION

This study has revealed the presence of many secondary metabolites and bioactive compounds in the leaf extract of Boerhaavia diffusa L., with important medicinal value. It was observed that the leaf extract of Boerhaavia diffusa L. showed maximum phytochemical constituents, good antioxidant properties compared to the leaf extract of B.repens; B.diffusa contained silver nanoparticles which was characterized by GC-MS analysis. Hence Boerhaavia diffusa L. was used as medicinal plant for curing various ailments than B.repens which is being naturally and widely present along with cost effectiveness can prove as an advantage of using B.diffusa as therapeutic drug.

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