Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2015; 7(6); 1072-1079

ISSN: 0975-4873

Research Article

Evaluation of *In-vitro* Antioxidant, Antimicrobial Activities and GC-MS Analysis of *Alternanthera bettizicikiana* Linn. Leaf Extracts

Vidhya T, Suji T, Dhatchayani R, Priya C L, Bhaskara Rao K V*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Science and Technology, VIT University,Vellore, (T.N), India.

Available Online:11th October, 2015

ABSTRACT

The aim of the current study was to perform phytochemical screening, antioxidant and hemolytic activities of *Alternanthera bettizicikiana* leaves. Determination of the antioxidant nature of *A.bettizicikiana* was carried out by DPPH radical scavenging, reducing power and total antioxidant activities. Hemolytic activity was performed by Spectrophotometric method to know the toxicity of *A. bettizicikiana* extract towards human RBC'S. In addition, antimicrobial activity was performed by Kirby-Bauer disc diffusion method using *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhimurium.* Preliminary phytochemical screening was performed to hexane, chloroform, ethyl acetate, methanol and aqueous extracts of *A. bettizicikiana* that resulted in the presence of carbohydrates, oils and fats and absence of proteins and saponins. Among all the extracts, methanol extract shown 92% DPPH radical scavenging activity with IC_{50} value of 293.44µg/ml and also possess good reducing property and total antioxidant activity in the tests performed. Antimicrobial activity of methanol extract resulted in no zone of inhibition for the tested organisms. The quantitative analysis of total phenolic and flavonoids was estimated that resulted in 63mgGAE/g and 38.98mgQE/g of the extract. Further, GC-MS analysis was performed that detected N N-Dimethyltryptamine in methanol extract and FT-IR analysis identified presence of functional groups within the methanol extract of *A. bettizicikiana*. Therefore, the bioactive compound should be isolated in future studies and could be used as a safe and natural antioxidant.

Keywords: *A.bettizicikiana*, Antioxidant, Antimicrobial, Hemolytic, GC-MS analysis, FTIR analysis.

INTRODUCTION

Free radicals are atoms or group of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules[1]. When cells in the body encounter a free radical, the reactive radical may cause destruction in the cell. The primary site of free radical damage is the DNA found in the mitochondria. Hence this free radical generation process disrupts all levels of cell function. Oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses. Oxidative stress seems to play a significant role in many human diseases like cancers, rheumatoid arthritis, cardiovascular diseases and age related diseases[2]. Antioxidants are molecules which can safely react with free radicals and terminate the chain reaction before vital molecules are damaged[3]. Although there are several enzyme systems within the body that scavenge free radicals, the antioxidants protect the enzymes that repair DNA damages, thereby enhancing our body's ability to rejuvenate itself. The aim of the present study was to screen the antioxidant activities of common edible fruits, garden plants and medicinal plants. There are several different types of antioxidants but they can be grouped into two major categories: natural and synthetic antioxidants. Natural antioxidants are those that can be harvested directly from organic sources such as fruits, vegetables, grain and meat[4]. Some of the common natural antioxidants which are found in everyday foods were vitamin C (ascorbic acid), vitamin E (tocopherols), vitamin A (carotenoids), various polyphenols including flavonoids, anthocyanins and ubiquitin. While synthetic antioxidants are those created artificially in laboratories. It is generally used for use in the preservation of foods. Some of the synthetic antioxidants were Butyl hydroxyl anisole (BHA), Butyl hydroxyl toluene (BHT), Tertiary butyl hydroquinone (TBHQ), Propyl gallate (PG), Dodecyl gallate (DG) and Octyl gallate (OG) [5]. Almost all synthetic antioxidants have been found to be carcinogenic in high doses. Medicinal plants are considered as a resource of ingredients which can be used in drug development and synthesis. The use of medicinal plants is not just a custom of the distance past. Perhaps 90% of the world's population still relies completely on raw herbs and unrefined extracts as medicine[6].

A.bettzickiana is a species of flowering plant in the amaranthaceae family. It is commonly used as an ornamental edging plant. It is particularly popular in china where it is cultivated in nearly all the large cities. It is native to South America. Its common name includes calico

**Author for Correspondence*

plant. This genus consists of approximately 80 species and is widespread genus with cosmopolitan distribution[7]. *A.bettzickiana* whole plant is reported to be useful in purifying and nourishing blood and it is claimed to be soft laxative, a galactagogue and an antipyretic, in addition to its wound healing property. Some studies revealed that *A.bettzickiana* improved superoxide dismutase and catalase activities in the liver of ovariectomized mice[8]. The anti-inflammatory and diuretic activities of *A.bettzickiana* were also reported recently[9].

The focus of this study was to investigate leaves extracts of *A.bettzickiana* for the presence of antioxidant activity (DPPH radical scavenging activity), hemolytic, phytochemical and antimicrobial activity.

MATERIALS AND METHODS

Chemicals

Petroleum ether, Chloroform, Ethyl acetate, Carbinol, Dimethyl sulfoxide, Nutrient broth, Muller Hinton agar, Ferric chloride, Sodium phosphate monobasic, Sodium phosphate dibasic, DPPH solution and Ammonium molybdate were purchased from HiMedia Laboratories Pvt.Ltd.

Plant Collection and Processing-

The plant was collected from Bharat horticulture nursery, sankarapalayam, Tamilnadu, India. The leaves of *A.bettizicikiana* were collected and chopped into small pieces. The pieces were dried in hot air oven at 50^0c. Then the dried leaves were milled by grinder. The powdered leaf was serially diluted in nonpolar solvents like Hexane, Chloroform and Ethyl acetate. Similarly, polar solvents like Methanol and Aqueous in the ratio of 10g in 100ml solvent [10]. The powder was mixed with the extract and kept in rotating shaker at 45^0c for 24hrs. The extract was filtered, evaporated and vaporized.

Preliminary phytochemical screening-

Phytochemical screening of the leaf of *A. bettizicikiana* was carried out by using the standard protocols as described by JB Harborn [11].

Evaluation of DPPH radical scavenging activity

The DPPH scavenging activity was performed according to the method of Tekao et al with new modification[12].The plant extracts were diluted in distilled water to make 1000, 500,250 and 125µg/ml in triplicates. 1ml of diluted extract solution was mixed with 1ml of DPPH solution. Incubation was carried out for 30 minutes in darkness at room temperature (23^0C). The absorbance was recorded at 517nm. The control samples contained all the reagents. The percentage inhibition was calculated using equations.

$\%DPPH\ Scavenging = [(A_c-A_t)/A_c)] \times 100$

Here, A_c is the absorbance of the control (DPPH); A_t is the absorbance of test sample.

Determination of reducing power

The reducing power of the test samples was determined by the method of yen and duh with few modifications[13]. Different concentrations of leaves extracts (125, 250, 500 and 1000 µg/ml) is mixed with 1ml phosphate buffer (0.2M, pH 6.6) and 1ml of 1% Potassium ferrocyanide. The mixtures were incubated for 20 minutes at 50^0C. At the end of incubation 1ml of the absorbance mixture is mixed with 1ml of Trichloroacetic acid, 1ml of distilled water and 0.5ml of ferric chloride. The blank solution contains 2ml of distilled water. Absorbance was measured at 700nm using calorimeter. The reducing power tests were run in triplicates. Increase in absorbance of the reaction mixture indicated the reducing power of the samples.

Total antioxidant activity-

Total antioxidant capacity of the extract of *A. bettizicikiana* leaf was performed by Ammonium molydbate[14]. 1ml extract of different concentrations (125,250,500 and 1000µg/ml) was mixed with 3ml of the reaction mixture containing 3.3ml of concentrated Sulfuric acid,0.335g of Sodium Phosphate Monobasic and 0.495g of ammonium molybdate which was dissolved in 96.67ml of distilled water. The mixture was incubated in water bath at 95^0C for one hour. The blank contains 3ml of reaction mixture and 1ml of distilled water. The absorbance was measured at 645nm using UV-Visible spectrophotometer.

Antibacterial activity

Test organisms

The bacteria isolates used for this study included *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and clinical isolate of *Salmonella typhi*. All the test organisms were inoculated in nutrient broth (pH7.4) for 8 hours.

Positive and negative control

Cotrimoxazole (23.75µg/disc), Ciproflaxocin (5µg/disc), Chloramphenicol (30µg/disc) and Piperacillin (100µg/disc) were used as a positive control. Sterile distilled water was used as negative control.

Agar well diffusion method

Antibacterial activity of the crude extract was determined by the agar well diffusion method. All test organisms were inoculated in nutrient broth (pH 7.4) for 8 hours[15]. The isolates were seeded on Muller Hinton agar plates using sterilized cotton swabs. Agar surface was bored using sterilized gel borer to make wells. 100µl of the test extract (100mg/ml) and 100µl of sterilized distilled water were poured in separate wells. Plates were incubated at 37^0C for 24hrs. Each experiment was performed in triplicates at each concentration.

Hemolytic activity

Hemolytic activity was performed by spectrophotometer method[16]. 5 milliliters of blood was collected from a healthy individual. The blood was centrifuged at 1500rpm for three minutes. The pellet was washed three times with sterile saline solution (0.89%w/v NaCl by centrifugation at 1500rpm for 5 min. The cells were resuspended in normal saline to 0.86%. A volume of 0.5ml of the cell suspension was mixed with 0.5ml of the plant extracts (125, 250, 500 and 1000µg/ml concentrations in saline). The mixtures were incubated for 30 minutes at 37^0C and centrifuged at 1500rpm for 10 minutes. The free hemoglobin in the supernatants was measured in UV Spectrophotometer at 450nm. Saline and distilled water were used as minimal and maximal hemolytic controls. Each experiment was performed in triplicates at each concentration. The level of percentage hemolysis by the extract was calculated according to the following formula

Percentage hemolysis= $[A_t-A_n/A_c-A_n] \times 100$

Here, A_t is the absorbance of test sample; A_n is absorbance of the control (saline control).

Estimation of total phenolic content

Total phenolic content of the aqueous and methanol extract of *A.bettizicikiana* leaf was determined using the Folin-Ciocalteau reagent[17]. The crude aqueous and methanol extracts were diluted in methanol to obtain different concentrations (125,250,500 and 1000µg/ml). 50µl of each extract was mixed with 2.5ml of Folin-Ciocalteau reagent (1/10 dilution in purified water) and 2ml of 7.5%Na_2CO_3 (w/v in purified water). The mixture was incubated at 45^0C for 15min. The absorbance was measured at 765nm.Na2Co3 solution (2ml of 7.5ml% Na2CO3 in 2.55ml of distilled water) was used as blank. The results were expressed as Gallic acid equivalence in µg.

Estimation of total flavonoids

Total flavanoids content of the extract was performed using the aluminum chloride calorimetric method[18]. 1ml extract at different concentrations (125,250,500 and 1000µg/ml) was mixed with 1ml of $AlCl_3$ (2% in ethanol). The mixture was incubated at room temperature for 60minutes. The absorbance was measured at 420nm with $AlCl_3$ solution (1ml of 2% $AlCl_3$ in 1ml of water) as blank using UV-VIS spectrophotometer. Total flavonoids content was expressed as Quercitin equivalence (QE) in µg.

FT-IR analysis

Kbr pellet procedure for solid samples

The FT-IR analysis was done to test the presence of phenolic compounds present in the extracts of *A.bettickiana*. About 1/8 of the dried leaf extract powder was taken on a micro spatula and mixed with 0.25-0.50 teaspoons of KBr[19]. It should be place in press and pressed at 5000-10000psi. Then place in the FT-IR sample holder. The pressed disc should be nearly clear if properly made.

GC-MS analysis

GC-MS is an analytical method that combines the features of gas liquid Chromatography and Mass Spectrometry to identify different substances within a test sample. For the analysis of volatile compounds, a purge and trap (P&T) concentrator system may be used to introduce samples. The target analytes are extracted and mixed with water and introduced into an airtight chamber. P&T GC-MS is particularly suited to volatile organic compound (VOCS) and TBTEX compounds (aromatic compounds associated with petroleum). In this method, the tallest peak is assigned 100% of the value and the other peaks be assigned proportionate values. All values above 3% are assigned[20]. The total mass of the unknown compound is normally indicated by the parent peak. The value of this parent peak can be used to fit with a chemical formula containing the various elements which are believed to be in the compound.

RESULTS AND DISCUSSION

Selection of *A.bettizicikiana* for the study was based on its traditional use to cure severe diseases and therapeutic potential reported earlier. The plant root is already reported to possess antioxidant property, so in this study we screened the antioxidant potential of the leaf of *A.bettizicikiana.*

Table 1: phytochemical screening of leaves of *A.bettizicikiana.*

Phytochemical screening	Methanol extract	Aqueous extract
Test for Phenolic compound	(+)ve	(+)ve
Test for Oils and fats	(+)ve	(+)ve
Test for Carbohydrate	(+)ve	(+)ve
Test for Tannins	(+)ve	(+)ve

Yield of plant extracts

Plant leaf powder was serially extracted in different polarity solvents and the dried extract was measured. The yields of the extracts are expressed as % yield. Aqueous extract showed maximum yield (1.239%), followed by Hexane (0.54%), Methanol (0.5%), Ethyl acetate (0.3%) and Chloroform (0.25%).

Phytochemical screening

Plants generally produce many secondary metabolites which are biosynthetically derived from primary metabolites. Secondary metabolites are frequently accumulated by plants in smaller quantities[21]. The phytochemical screening in the present study has revealed the presence of phenolic compounds, oils and fats, carbohydrate and tannins. Tannins are phenolic compounds and plant phenolics are a major group of compounds that act as antioxidants or free radical scavengers[22]Since these compounds were found to be present in the extracts, it might be responsible for the potent antioxidant capacity of *A.bettizicikiana.* The presence of phytoconstituents makes the leaves useful for treating different aliments.

DPPH radical scavenging activity-

Antioxidant potential of the methanol and aqueous extracts were measured by DPPH radical scavenging activity. 1, 1-DiPhenyl-2 PicrylHydrazyl (DPPH) is a kind of stable organic radical. The capacity of biological reagents to scavenge DPPH radical can be expressed as its magnitude of antioxidant ability[23]. The results are expressed as % inhibition of DPPH. Methanol extract showed high antioxidant activity than that of aqueous extract. The DPPH radical scavenging activity was found to be increasing as dose increases. The DPPH value for 92% Methanolic extract with IC 50 value of 293.44µg/ml.

Reducing power assay

Reducing power assay measures the electron-donating capacity of an antioxidant. Presence of reducers causes the conversion of the $Fe^{3\dagger}$/Ferricyanide complex to ferrous ions. The color of the reaction mixture changes from yellow to bluish green[24]. Methanolic extract showed high reducing power than that of aqueous extract. Reducing power potential of both extracts increase with the dose.

Total antioxidant activity

In the presence of the plant extract, Mo (VI) is reduced to Mo (V) and forms a green colored ammonium

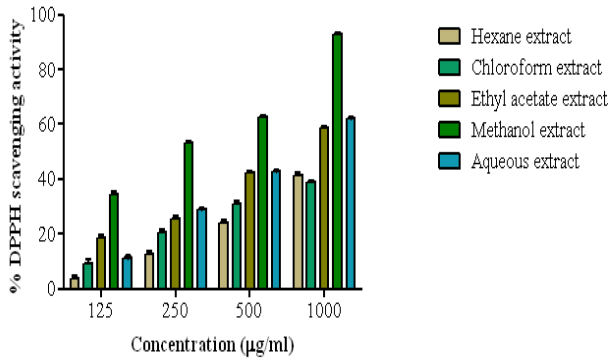


Figure 1: DPPH radical scavenging activity; HE: Hexane extract, CE: Chloroform extract, EE: Ethyl acetate extract, ME: Methanol extract, AE: Aqueous extract: All the values are expressed in mean ±SD (n=3).

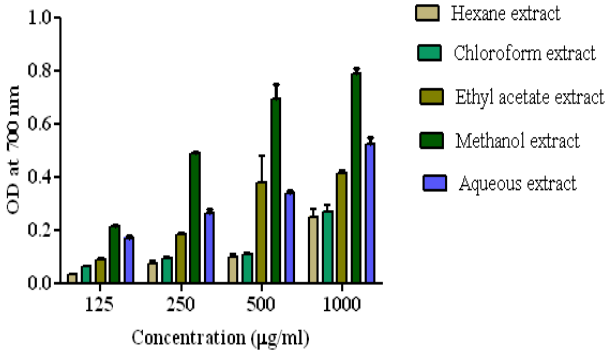


Figure 2: Reducing power assay: All the values are expressed in mean ±SD (n=3).

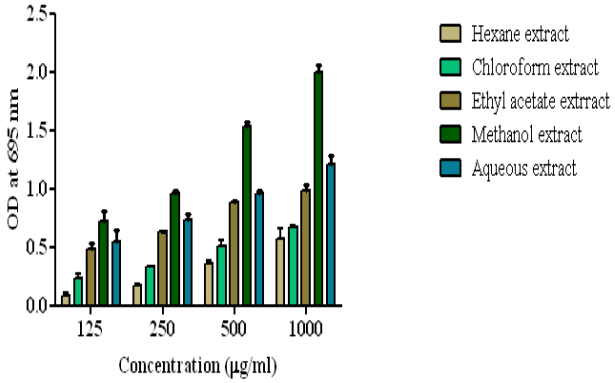


Figure 3: Total antioxidant assay: All the values are expressed in mean ±SD (n=3).

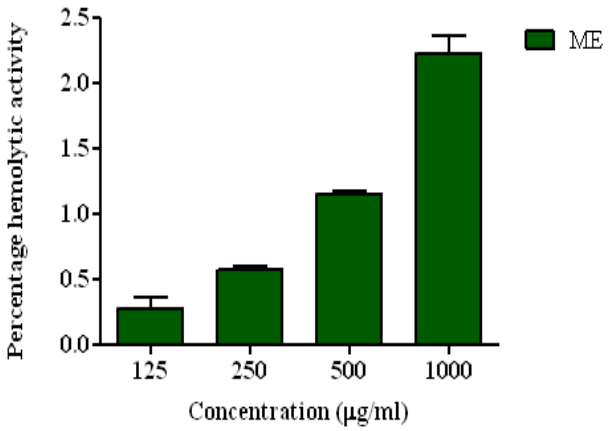


Figure 4: Hemolytic activity: The data was represented as mean ±SD. Experiment was done in triplicates (n=3).

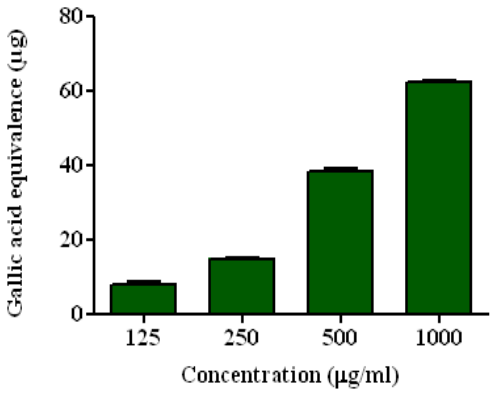


Figure 5: Total phenolic content estimation: The data was represented as mean ±SD. Experiment was done in triplicates (n=3).

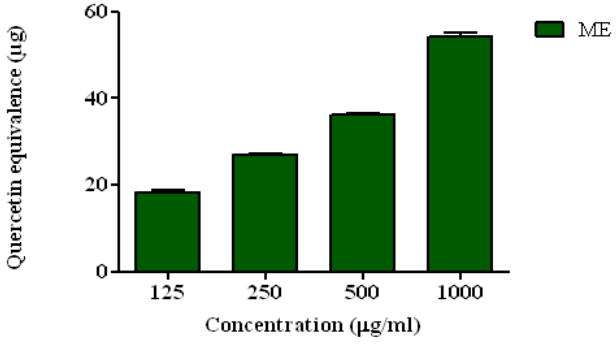


Figure 6: Total Flavanoid content estimation: the data was represented as mean ±SD. Experiment was done in triplicates (n=3).

molybdenum V complex. As the concentration of leaf extract increases, the total antioxidant capacity also goes on increasing continuously[25] The absorbance is measured at 695nm using-Visible spectrometry. As *A.bettizicikiana has* shown the highest absorbance reading, it possesses the highest total antioxidant capacity.

Antibacterial activity

Antibacterial activities of methanol, aqueous, chloroform, ethyl acetate and hexane extracts were determined by well diffusion methods. The plant leaf showed very poor antibacterial activity.

Hemolytic activity

Hemolytic activity of methanol and aqueous extract of *A.bettzicikiana* leaves and its various fractions were screened against normal human erythrocytes. Result indicated that the aqueous extract exhibited minimum hemolytic activity, whereas methanol extract showed the highest hemolytic activity[26]. Lysis of erythrocytes was

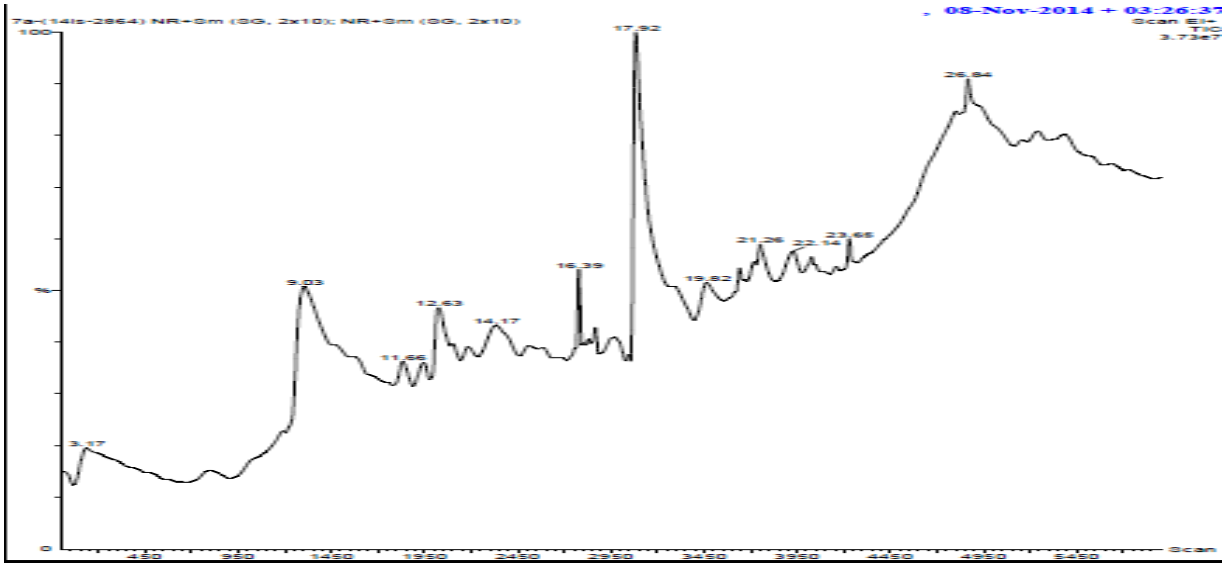
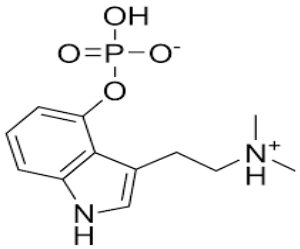
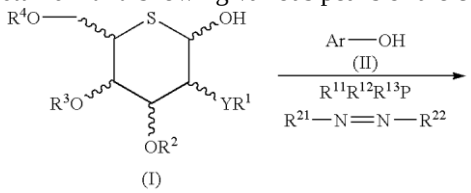


Figure 7: The GC-MS analysis of leaf of *A.bettizicikiana* showing various peaks of the chromatogram





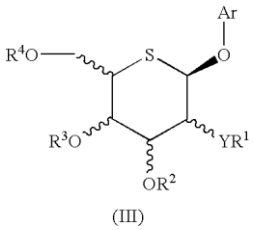
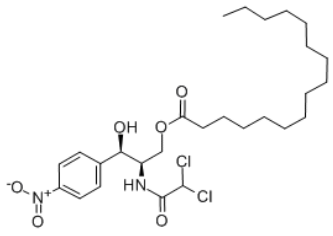


Figure: 8: GC-MS Chromatogram Peak: 9.03 represent N N-Dimethyltryptamine.

Figure 9: GC-MS Chromatogram peak: 12.26 represent N-Dimethylaminomethyl-isopropyl Benzyl phosphine.



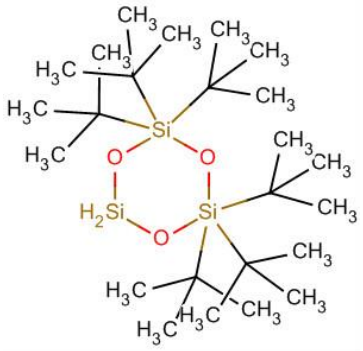


Figure: 10: GC-MS Chromatogram peak 17.94 represent N-Hexadecanoic acid.

Figure 11: GC-MS Chromatogram peak 26.84 represent Cyclotrisiloxane Hexane methyl.

found to be increased with an increase of extract concentration.

Total phenolic content estimation

Quantitative determination of total phenolic contents indicates that methanol extract possess the highest concentration of total phenolic content with 63mgGAE/g of the extract.

Total flavonoids content estimation

Quantitative determination of total flavanoid content indicates that methanol extract possess the highest concentration of total flavanoid with 38.98mgQE/g of the extract. It has been reported that rich phenolic and flavanoid plant leaves could be a vital source of therapeutic potential against the oxidative damages.

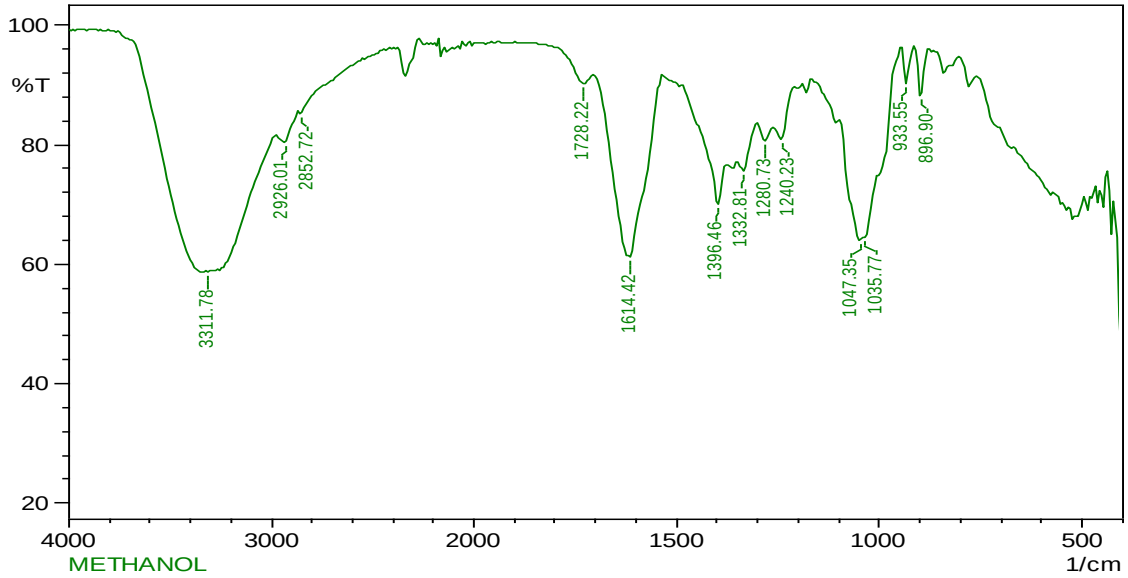


Figure 12: FT-IR analysis of leaf Methanolic extract of *A.bettzicikiana.*

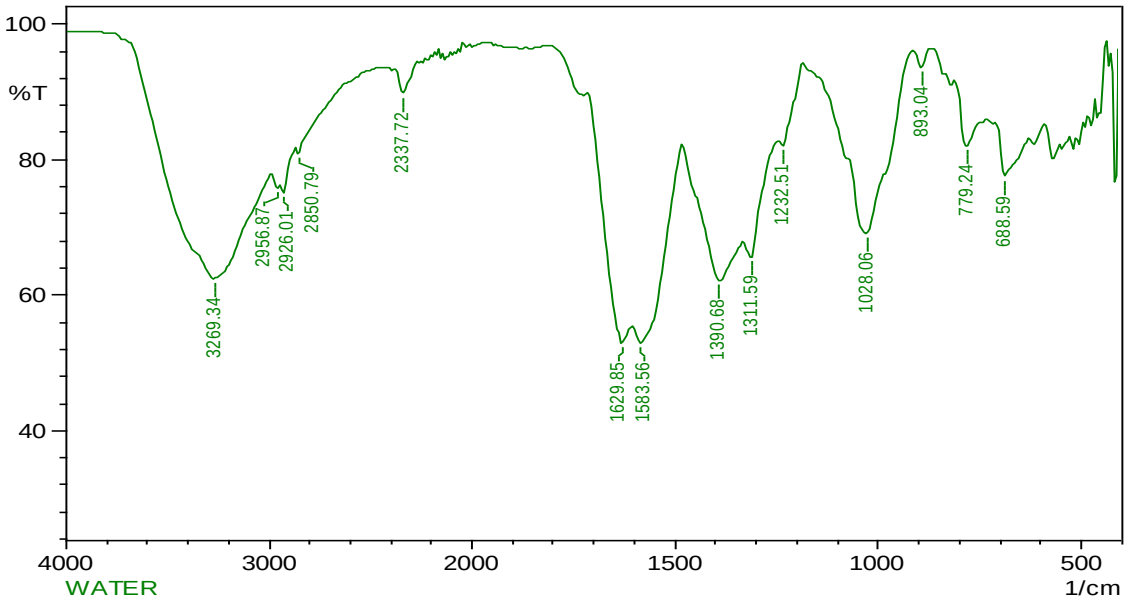


Figure 13: FT-IR analysis of leaf aqueous extract of *A.bettzicikiana.*

GC-MS analysis

The Various chemical compounds present in leaf extracts of *A.bettizicikiana* determined by GC-MS method were as follows

FT-IR analysis

FT-IR Analysis was carried out to examine the some organic and inorganic compounds present in the extracts. The absorption band at 3311.78cm^{-1} is representative of –OH group of phenols. It has also revealed the presence of various functional groups such as alcohols, carbonyls, aromatic amines, ether, alkynes, alkenes, carboxylic acids, amines, esters, primary and secondary amines

DISCUSSION

Use of plants as a source of medicine has been an ancient practice. Medicinal plants are consider as a rich sources of pharmalogical properties and can be used in drug synthesis. Use of medicinal plants as a drug would be beneficial rather than using drugs from synthetic chemicals having worst side effects[27]. Polyphenols represent the aromatic compounds formed during secondary metabolism in plants. Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Polyphenols show natural antioxidant properties. Consumption of diets rich in plant polyphenols offered some protective against development of cancers, cardiovascular diseases, osteoporosis and neurodegenerative disorders[28]. In the present study phytochemical screening showed the presence of phenolic compounds, oils and fats, carbohydrate and tannins. Free radical generation is directly related with oxidation in biological systems. Hence DPPH radical scavenging activity was performed that has given high radical scavenging activity of methanolic extract followed by aqueous extract. Therefore methanolic and aqueous extract

were selected for further antioxidant studies like Total antioxidant and reducing power assays. Hemolytic activity is found to be high in methanolic extract[29]. The highest concentration of total phenol and flavanoid compounds could be vital source of therapeutic potential for different allilies[30].Further GC-MS analysis was carried out to identify the polyphenols present within the extracts which is responsible for its antioxidant properties[18]. GC-MS analysis identified the presence of N N-Dimethyltryptamine, N-Dimethylaminomethyl-isopropyl benzyl phosphine, N-Hexadecanoic acid and Cyclotrisiloxane Hexane methyl. FT-IR analysis was also carried out to identify some organic and inorganic molecules present in the extracts[17]. Hence it is evident that the polyphenols that has been detected in the present study are good antioxidants and their presence within the methanolic extract of A.betzicikiana leaves has contributed to the antioxidant activities.

CONCLUSION

This study concluded that the methanolic and aqueous extract of A.bettzicikiana leaves exhibited high antioxidant potential for the tests performed. GC-MS analysis further confirmed the presence of many polyphenols. The total phenolic and flavanoid compound estimation was found to be higher which serves as a source of therapeutic potential for treating different diseases. In future this bioactive compound to be isolated and can be used as a natural remedy for various kinds of damages caused by oxidative stress.

REFERENCES

1. Rattan SI. Theories of biological aging, genes, proteins and free radicals. Free Radical Research 2006; 40(12):1230-1238.
2. Saiket S, Raja C, Sridhar C, Reddy YSR, Biplab D. Free radicals, antioxidants diseases and phytomedicines: current status and future prospect. Journal of Pharmaceutical Sciences 2010; 3(1):91-100.
3. Mayne ST. Antioxidant nutrients and chronic disease: Use of biomarkers of exposure and oxidative stress in epidemiologic research. The Journal of Nutrition 2003; 133 (3):9385-9405.
4. Tavasalkar SU, Mishra HN, Madhavan S. Evaluation of Antioxidant Efficacy of Natural Plant Extracts against Synthetic Antioxidants in Sunflower Oil. Open Access Scientific Reports 2012; 1(11): 504-509.
5. Saura CF, Serrano J, Coni I. Intake and bioaccessibility of total polyphenols in a whole diet. Food Chemistry 2007; 101(2): 492-501.
6. Steinar D, Haruki S, Kenjiro W, Kari H, Rune B. Several culinary and medicinal plants are important sources of dietary antioxidants. The Journal of Nutrition 2003; 133 (5): 1286-1290.
7. Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A. and Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. Molecular Nutrition & Food Research 2007; 51(6): 675-583.
8. Suphanthip P, Waranya C, Orawan M, Pradit P, Kanokwan J. Alternanthera sessilis and Alternanthera bettzickiana Improved Superoxide Dismutase and Catalase Activities in the Livers of Ovariectomized Mice. Journal of Applied Biopharmaceutics and Pharmacokinetic. 2013; 1(2): 64-71.
9. Chandana CB,Shameem AB, Archana T,Jayanti DR, Bhaben B, Debesh CP, Dilip KS, Rumi SB, Asheesh G.Influence of Alternanthera brasiliana (L.) Kuntze on Altered Antioxidant Enzyme Profile during CutaneousWound Healing in Immunocompromised Rats. ISRN Pharmacology 2012; 2012: 948792.
10. Priya CL, Gaurav K, Karthick L, Bhaskara Rao KV. Antioxidant activity of *Achryanthes aspera* Linn stem extracts, 2010, Pharmacologyonline, 2: 228-237.
11. Olayinka AA, Anthony IO. Preliminary phytochemical screening and *In vitro* antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. BMC Complementary and Alternative Medicine 2010; 10: 21-24.
12. Kriengsak T, Unaroj B, Kevin C, Luis CZ, David H. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis 2006; 19(7): 669-675.
13. Mustafa O,–Neil Reese R,–Artemio ZT,–Joseph CS, Raymond Miller A. Modified 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Method to Measure Antioxidant Capacity of Selected Small Fruits and Comparison to Ferric Reducing Antioxidant Power (FRAP) and 2,2‘-Diphenyl-1-picrylhydrazyl (DPPH) Methods. Journal of Agricultural Food Chemistry 2006; 54(4): 1151-1157.
14. Chun, O. K., Kim, D.-O., Smith, N., Schroeder, D., Han, J. T. and Lee, C. Y. (2005), Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet. Journal of the science of Food and Agriculture 2005; 85: 1715-1724.
15. Iqbal A, Farrukh A. In vitro efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. Microbiological Research,2007; 162(3): 264-275.
16. Guarav K, Karthick L, Bhaskara Rao KV. Hemolytic activity of Indian medicinal plants towards human erythrocytes: an invitro study. Elixir Applied Botany 2011; 40: 5534-5537.
17. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin – Ciocalteu reagent, Nature protocols 2007; 2(4): 875-877.
18. Aline M, Charles EL, Marco R, Jeanne M, Odile GN. Determination of the total phenolic, flavanoid and proline contents in Burkina fasan honey as well as their radical scavenging activity. Food Chemistry 2005; 91(3): 571-577.
19. Michael B,Peter L, Dieter N. Analytical applications of Fourier transform-infrared (FT-IR) spectroscopy in microbiology and prion research. Veterinary Microbiology 2007; 123(4): 305-319.

20. Zhisheng X, Qundi L, Zhikun L, Mingqian Z, Xiaoxue Y, Depo Y, Xinjun X.The GC-MS Analysis of volatile components extracted by different methods from Exocarpium Citri Grandis. Journal of Analytical Methods in Chemistry 2013; 2013: Article ID 918406,
21. Jacobs DI, van der Heijden R, Verpoorte, R. Proteomics in plant biotechnology and secondary metabolism research. Phytochemical Analysis 2000; 11(5): 277-287.
22. Hollman PC. Evidence for health benefits of plant phenols: local or systemic effects. Journal of the Science of Food and Agriculture 2001; 81(9): 842-852.
23. Mishra K, Himanshu O, Nabo Kumar C. Analytical methods Estimation of antiradical properties of antioxidants using DPPH assay. Food Chemistry 2012; 130 (4): 1036-1043.
24. Mohammad AE, Somayyeh E, Bahman E. *Saamucus eblus elburensis* fruits: A good source of antioxidants. Pharmacognosy Magazine 2009; 5(19): 213-218
25. Nicoletta P, Mauro S, Barbara C, Daniele DR, Sara S, Marta B, Furio B. Total Antioxidant Capacity of Plant Foods, Beverages and Oils Consumed in Italy Assessed by Three Different In Vitro Assays. Journal of Nutrition 2003; 133(9): 2812-2819.
26. Ken-Hsuan L, Yu-Shen L, Christopher WM, Christy LH. Cytotoxicity of Graphene oxide and Graphene in Human Erythrocytes. ASC Applied Material and Interfaces 2011; 3(7): 2607-2615
27. Mohammed I, Shuid A, Borhanuddin B, Fozi N.The application of phytomedicine in modern drug development, The Internet Journal of Herbal and Plant Medicine 2012; 1(2): 1-5.
28. Augustin S, Ian TJ, Mike S. Polyphenols: antioxidants and beyond. The American Journal of Clinical Nutrition 2005; 81(1): 215-217 .
29. Krishana Kumar HN, Navyashree SN, Rakshita HR, Jyothi BC. Studies on the free radical scavenging activity of *Syagrus romanzoftiana*, International Journal of Pharmaceutical and Biomedical Research 2012; 3(2): 81-84.
30. Bahorun, T., Luximon-Ramma, A., Crozier A, Aruoma OI. Total phenol, flavonoid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. Journal of Science of Food and Agriculture 2004; 84(12): 1553–1561.