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Research Article

Variation in the efficacy of organic extrants for the phytochemical validation of Sauropus androgynus – a multivitamin plant

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ABSTRACT

Sauropus androgynus (L.) Merr., known traditionally as a multivitamin plant being cultivated in kitchen garden throughout south Indian region. Different organic solvents such as Ethyl acetate, hexane, and methanol were used to extract the bioactive compounds from the fresh air dried plant leaves. Among which ethyl acetate extracted most of the bioactive compounds efficiently. The compounds like tannins, flavonoids, proteins, terpenoids, coumarins and steroids were extracted at varied concentration remain parallel with their bioactive principles. Test organisms used were *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* showed their sensitiveness with the ethyl acetate extract *in vitro.* The percentage scavenging activity was also observed to be more with ethyl acetate extract, which was found to increase with concentration.

Key words: *Sauropus andogynus* leaves, organic solvents, bacterial species, bioactive compounds.

INTRODUCTION

Sauropus androgynus (L.) Merr. is one of the most important medicinal plants and consumed, as a green leafy vegetable, being preferred as a nutrition rich plant in many places of South India. It is commonly known as 'Chakramani' plant cultivated in kitchen garden, hence a favorite culinary item. In spite of its nutritional importance, it is yet to be validated through rigorous research activities. Its components are rich in antioxidants and also antimicrobials. It is also known for its multivitamin plant in ayurveda for various ailments. Considering these roles in the field of nutrition, the present study has been considered with much emphasis to evaluate the total phytochemicals with special reference to phenols, antioxidants and antimicrobials, in view of its qualitative assessment as of pharmaceutical importance. Hence, based on the knowledge of research lacunae, the present investigations initiated for its scientific validation.

MATERIALS AND METHODS

Plant materials and Sample preparation: Fresh leaves of *Sauropus androgynus* (L.) Merr. belongs to Meliaceae family were collected from the College of Agriculture, Hassan, Karnataka. The leaves were washed in water to remove the debris and rinsed with clean water for surface sterilization. Then they were spread evenly under shade and

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completely dried after 4 days. Then the dried samples were chopped in electric mixer to get fine powder from for further use. 40g of fine powder was obtained from the dried leaves. Soxhlet apparatus was used for the extraction of mutli-solvent extract with temperature in between 60-70ºC for all solvents with their respective boiling point. The samples were evaporated through the Rotary vacuum evaporator at 60ºC for all solvents. Safety cabinet or Laminar Air Flow was used to conduct the antibacterial assay to avoid the contamination. Absorbance spectrophotometer was carried out using a UV-vis spectrophotometer. Wavelength scans and absorbance measurements were in 1ml quartz cells of 1cm path length.

Qualitative Phytochemical Screening: The crude extracts of different solvents system exhibiting broad spectrum of antibacterial and antioxidant activity were subjected to phytochemical constituent analysis according to the standard methods. The presence of several photochemicals like sterols, tannins, proteins, sugars, alkaloids, flavonoids, saponins, terpenoids and cardiac glycosides was evaluated.

Test for Carbohydrates
To 0.5ml of plant extract, 1ml of Molisch's reagent (15g of α-Naphthol in 100ml of chloroform) and few drops of concentrated sulphuric acid were added. Presence of purple and reddish colour indicates the presence of carbohydrates.

Test for Tannins
To 0.5ml of plant extract, 2ml of 5% ferric chloride (5% w/v solution of ferric chloride prepared in 90% alcohol) was added. Appearance of dark green or dark blue colour indicates the presence of tannins (Ugochukwu *et al*., 2013).

Test for Flavonoids – Ferric chloride Test
To 0.5ml of plant extract, a few drops of 10% ferric chloride solution were added. A green-blue or violet coloration indicates the presence of Flavonoids (Auwal *et al*., 2014).

Test for Alkaloids
To 0.5ml of plant extract, 2ml of concentrated HCl was added. The. Few drops of Mayer's reagent (0.136g of Mercuric chloride in 0.5g of Potassium iodide in distilled water mix both solution and make it up to 10ml with distilled water)were added. Presence of green colour or white precipitation indicates the presence of alkaloids.

Test for Quinones
To 0.5ml of plant extract, 1ml of concentrated sulphuric acid was added. Formation of red colour indicates presence of quinones.

Test for Phenols
To 0.5ml of plant extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of bluer or blue or green colour indicates the presence of phenols.

To determine the total phenolic content in plant extract, gallic acid was used as standard (Dhawan and Gupta, 2017). Total phenolic content was analyzed using the Folin-ciocalteu method along with some modification (Velioglu *et al*., 1998; Cai *et al*., 2004; Chlopicka *et al*., 2012). The stock solution of the gallic acid was made by dissolving 10 mg in 10 mL of distilled water. Five different concentrations of the gallic acid were used as standard – 20, 40, 60, 80 and 100µg/mL were used to make standard curve. Solvent extracts were taken in two concentration 200 and 400µg/mL. About 20µL of each extract was taken and volume adjusted to 2 mL using parent solvent. About 200µL of FCR and 500µL of 20% Na_2CO_3 were added to each tube. The reaction mixture was incubated for 1 h at room temperature and the absorbance was measured at 760nm.The samples were prepared in duplicates for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution Gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenols was read (µg/mL) from the calibration line; then the content of phenolics in extracts was expressed in terms of Gallic acid equivalent (mg of GA/g of extracts).

Test for Terpenoids – Salkowski Test
To 0.5ml of plant extract, 2ml of chloroform was added, followed by a further addition of 3ml of concentrated sulphuric acid to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids (Oladipupo *et al*., 2014).

Test for Cardiac Glycosides – Keller-Killiani Test
To 0.5ml of plant extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was mixed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of cardiac glycoside (Odeja *et al*., 2015).

Test for proteins
To 0.5ml of plant extract, 2ml of water and 0.5ml of concentrated Nitric acid was added to it. Yellow colour is obtained if proteins are present.

Test for Triterpenoids
To 0.5ml of plant extract, 1ml of Libemann-Buchard reagent (1ml of acetic anhydride and 1ml concentrated sulphuric acid in 10ml of absolute ethanol) was added. Formation of blue green colour indicates presence of triterpenoids.

Test for Coumarins
To 0.5ml of plant extract, 1ml of 10% NaOH was added. Formation of yellow colour indicates presence of coumarins.

Test for Saponins
To 0.5ml of plant extract, 2ml of distilled water was added and shaken in a test tube for 15min lengthwise. Formation of 1cm layer of foam indicates the presence of saponins. The persisted froth on warming has considered as an evidence for the presence of saponins.

Test for sugars
To 0.5ml of plant extract was dissolved in distilled water and filtered. The filtrate was heated with 5ml of equal volumes of Fehling's solution A (2.079g of copper sulphate in 30ml of distilled water and Fehling's solution B (7.5g of KOH in 10.38g of sodium-potassium tartrate (Rochelle salt) in 30ml of distilled water). Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars (Auwal *et al*., 2014).

Test for Steroids – Salkowski's Test
To 0.5ml of plant extract, 1ml of chloroform in a test tube, concentrated sulphuric acid was carefully added from the sides of the test tube, to form a layer. A reddish-brown colour at the interphase indicated the presence of steroids.

Antioxidant assay-DPPH radical scavenging assay: The DPPH assay was carried out as described by Hsu *et al*., 2007 with some modifications. A volume of 1.5mL of 0.1mmol/L DPPH solution was mixed with 1.5mL of various concentrations (20 to 100µg/mL) of leaf extract. The mixture was shaken vigorously and incubated at room temperature for 30min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was replicated in three independent assays (Sahoo *et al*., 2013). Inhibition of DPPH free radical in percentage was calculated by the formula:

DPPH radical scavenging activity (%)=(A control-A_1 test)/A control X 100
Where, **A control** is the absorbance of the control and **A_1 test** is the absorbance of samples.
The antioxidant activity of each sample was expressed in bar diagram showing the percentage of scavenging activity.

Hydrogen peroxide scavenging activity: Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method Ruch *et al*. (1989) with little modification. 4mmol/L solution of hydrogen peroxide was prepared in PBS (Phosphate Buffered Saline) (pH 7.4). Plant extract (4 mL), prepared in distilled water at concentration from 40 to 200µg/mL was mixed with 0.6mL of 4mmol/L hydrogen peroxide prepared in PBS solution was taken at 230nm against a blank solution containing the plant extract in PBS without hydrogen peroxide (Priyanka *et al*., 2013) The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equations:

Hydrogen peroxide radical scavenging activity = (A control – A_1 test)/A control X 100
Where A control is the absorbance of hydrogen peroxide radical + methanol; **A_1test** is the absorbance of hydrogen peroxide radical + sample extract.

Antimicrobial Acvtivity - Agar well diffusion Assay: Antibacterial activity can be determined by many methods, majorly agar well diffusion assay and agar disk diffusion method methodsare using. Antibacterial activity of the extracts was tested by agar well diffusion assay (Zhang *et al*., 2009) with some modifications. 10ml of sterilized nutrient agar media was poured into disposable Petri dishes under aseptic condition and allowed to solidify. Once the media get solidified, 200µl of standardized test microbial inoculum of *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* were spreaded uniformly using 'L' shape sterile spreader. 6mm diameter agar is drawn from plate to form a well using sterile rubber cork. Antibiotic Amphicillin was used as positive control and Methanol solvent used as negative control. 10µl of a volume of the antimicrobial agent or extract solution at desired concentration like 1, 0.75 and 0.5mg/ml is introduced into well. Then, agar plates were incubated at 37ºC for 24h. The diameter of the inhibition zone around the well is measured in millimeter (mm).

RESULTS AND DISCUSSION

Sauropus androgynus is an important medicinal plant, which considered as a source of secondary metabolites with a wide range of pharmaceutical attributes due to its high bio-efficacy. To study the pharmaceutical efficacy of the plant extract, 3 different solvents were used i.e. Hexane, Ethyl acetate and Methanol. There are variations in the yields of crude extracts obtained from 3 different solvents. The yield of extracts using Soxhlet apparatus were 0.76g, 0.49g and 1.26g respectively. The variation in yield may be due to the polarity of the solvents used in the extraction process. It is reported that phenols are responsible for the variation in the antioxidant activity of the plant (Cai *et al*., 2004). They exhibit antioxidant

activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals.

Photochemical Analysis: Qualitative phytochemical analysis was carried out for Hexane, Ethyl acetate and Methanol leaf extracts, which has indicated the presence of carbohydrates, proteins, glycosides, phenols, tannins, flavonoids, alkaloids, cardiac glycosides and saponins. The varied levels of phytochemicals were observed in different solvent extracts of the plant (Table 1). Negative results were recorded for terpenoids, quinones, and sugars in *S. androgynus* leaf extracts. Obtained results were tabulated in Table 1. The results obtained revealed that ethyl acetate extract has highest phytochemicals and hexane extract remain least compared with each other. The presence of the bioactive compounds such as phenols, alkaloids and flavonoids in the leaf extracts prompted to work on antioxidant aspects.

Table 1: Qualitative analysis of phytochemicals in *Sauropus androgynus*

Phytochemical compounds	**Presence of bioactive compounds in different solvents extracts of *S. androgynus***		
	Hexane extract	**Ethyl Acetate extract**	**Methanol extract**
Carbohydrates	+	+	+
Tannins	-	++	-
Flavonoids	-	++	+
Alkaloids	+	++	-
Quinones	-	-	-
Phenols	-	+	-
Terpenoids	-	-	-
Cardiac glycosides	+	+	+
Proteins	+	++	-
Triterpenoids	+	++	-
Coumarins	+	++	+
Saponins	-	+	+
Sugars	-	-	-
Steroids	-	++	+

"--" =absence of the compound.
"+" =presence of the compound.
"++" =indicated the high concentration of compound

The antioxidant assay of different plant extracts from *S. androgynus* was determined using DPPH reagent. DPPH is very stable free radical. Unlike *in-vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelating and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517nm. This purple colour fades when antioxidant molecules quench DPPH free radicals and convert them into a colorless/bleached product i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine, resulting in a decrease in 517nm band (Amarowicz *et al*., 2003). The antioxidant activity of three different extracts was expressed in terms of percentage of inhibition (%). The antioxidant activities of plant extracts showed varied values, ranged from 33.33% to 92.85%. These results indicated the dose dependent antioxidant activity in all the cases. The highest capacity to neutralize DPPH radicals was found in ethyl acetate extract (92.85 %). The minimum capacity to inhibit DPPH radicals were recorded in hexane extract (33.33%), the data revealed the highest antioxidant activity with increase in the concentrations of phenols. The rate of percentage of scavenging activity was represented in bar diagram (Figure 1).

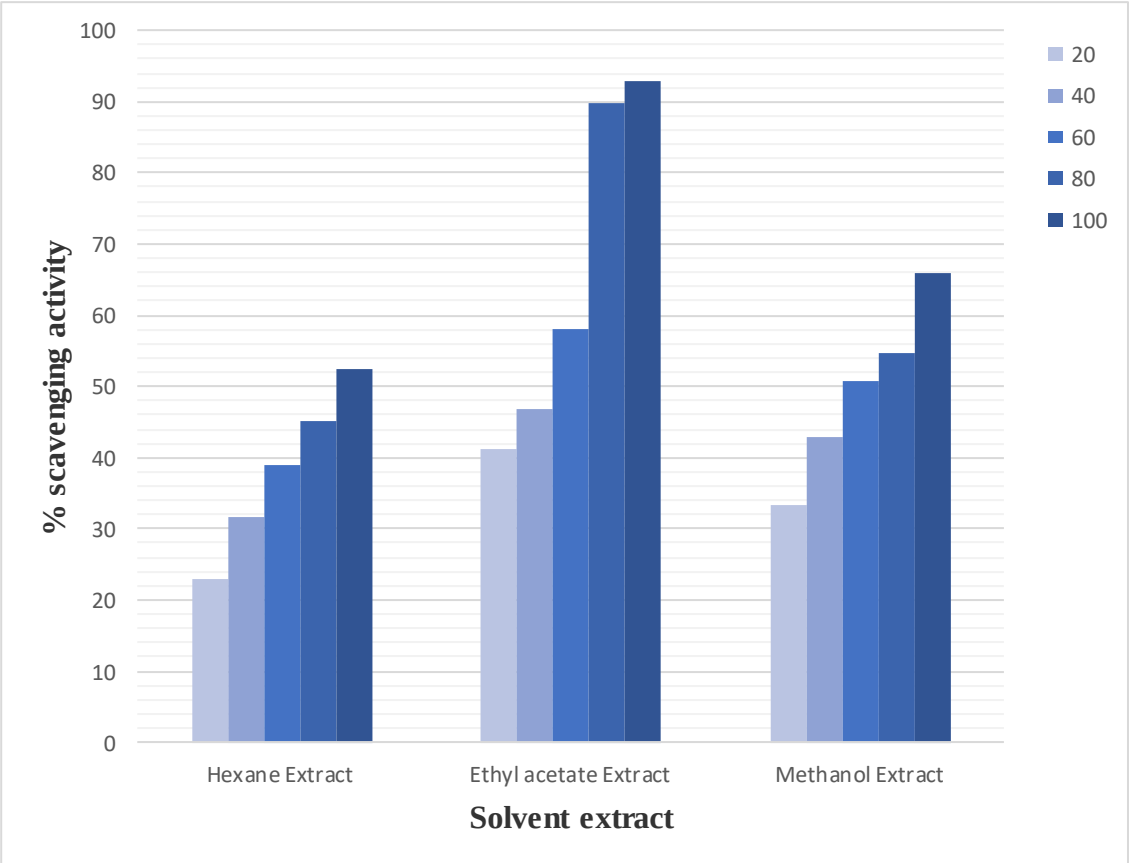

Figure 1: Variation in the DPPH scavenging activity of plant extracts in different organic (Different colors of the bars indicated the concentration of the extract in %)

Hydrogen Peroxide Radical Scavenging Assay

Different solvent extracts of *Sauropus androgynus* leaves was taken in different concentration and were subjected to determine the antioxidant assay employing hydrogen peroxide method. The % scavenging activity was found to increase in all the solvents with little variation. Comparatively, Ethyl acetate extract was found to be highly active. The corresponding rate of scavenging activity of different solvent extracts was represented in Table 2 (Table 2). It was observed that scavenging activity was found to increase with an increase in the concentration of the solvent. Similar to the other antioxidant activity assays, ethyl acetate extract showed highest activity of 96.52 %, followed by methanol (88.64 %) and lowest was observed with hexane (62.93 %).

Table 2: Hydrogen Peroxide scavenging activity of different solvent extracts of *Sauropus androgynous*

Concentration of solvents in (µg/mL)	**Scavenging activity of Hexane extract in (%)**	**Scavenging activity of Ethyl Acetate extract in (%)**	**Scavenging activity of Methanol extract in (%)**
40	36.11	84.54	43.84
80	43.37	86.43	54.73
120	51.73	89.9	69.40
160	56.46	92.9	78.07
200	62.93	96.52	88.64

Antimicrobial activity-Agar well Diffusion Method: Antimicrobial activity is the method used to check resistance power of the various plant extracts, which are having high medicinal values against various bacteria and fungi (Canadanovic-Brunet *et al*., 2008). In this study, the plant extracts were taken in different concentration to evaluate their efficacy against *Escherichia coli, Bacillus subtilis, and Staphylococcus aureus*, in which positive control Amphicilin and negative control Methanol were used to check the comparative formation of zone of inhibition. The antimicrobial properties of plant extracts against the selected microbes were recorded based on the clear zone formation around the colonies and the data were represented in the tables and figure (Table 3, 4, 5 and Figure 2). Varied levels of antimicrobial activities were observed with different concentrations and microbes used. Ethyl acetate extract was found to be most effective both concentration and microbes wise. Hexane extract was found effective against *B. subtilis* and *S. aureus.* However, methanol extract showed meagre activity against *S. aureus* and it was found negative against *B. subtilis* and *E. coli*.

Table 3: Antimicrobial assay of Hexane extract of *Sauropus androgynus*

Name of the Bacteria	**Zone of inhibition of Hexane extract in mm***			**Positive Control**	**Negative control**
	Concentration of the extract				
	1mg/ml	**0.75mg/ml**	**0.5mg/ml**		
Escherichia coli	—	—	—	14	—
Bacillus subtilis	2	—	—	6	—
Staphylococcus aureus	4	2	1	11	—

*Data based on Agar well – diffusion technique

Table 4: Antimicrobial assay of Ethyl acetate extract of *Sauropus androgynus*

Name of the Bacteria	**Zone of inhibition of Ethyl acetate extract in mm***			**Positive Control**	**Negative control**
	Concentration of the extract				
	1mg/ml	**0.75mg/ml**	**0.5mg/ml**		
Escherichia coli	2	1	—	14	—
Bacillus subtilis	4	2	1	6	—
Staphylococcus aureus	4	2	1	11	—

*Data based on Agar well – diffusion technique

Table 5: Antimicrobial assay of Methanol extract of *Sauropus androgynous*

Name of the Bacteria	**Zone of inhibition of Methanol extract in mm***			**Positive Control**	**Negative control**
	Concentration of the extract				
	1mg/ml	**0.75mg/ml**	**0.5 emg/ml**		
Escherichia coli	—	—	—	14	—
Bacillus subtilis	—	—	—	6	—
Staphylococcus aureus	6	4	4	11	—

*Data based on Agar well – diffusion technique
Positive control = Ampicillin; Negative control = Methanol
— = indicates absence of zone of inhibition**.**

It was inferred that there are variations in the yield of crude extracts of plant obtained using three different solvents i.e. Hexane, Ethyl acetate and Methanol (761.8, 499.3 and 1262mg, respectively). The variation in yield may be due to the polarity of the solvents used in the extraction process. Results also suggested the presence of flavonoids in methanol and ethyl acetate fractions at higher concentration, compared with hexane fractions. It has been reported that the phenols are responsible for the variation in the antioxidant activity of the plant extracts. The exact mechanism of the reaction is complex, but it is essentially a redox reaction occurring between antioxidants with phosphotungstic and phosphomolybdic acids. Since, the reaction is based on redox, the assay would not be specific to just phenolics, but, may confined to any other substances that could be oxidized by the Folin reagent. This is not surprising as numerous workers have reported the poor specificity of this assay. The Ethyl acetate fraction of multivitamin plant exhibited the highest concentration of total phenolic content i.e. 62.09mg of GA/g followed by Methanol extract with 48.49mg of GA/g and Hexane extract with 24.22mg of GA/g, respectively, fetch more interest on the subject.

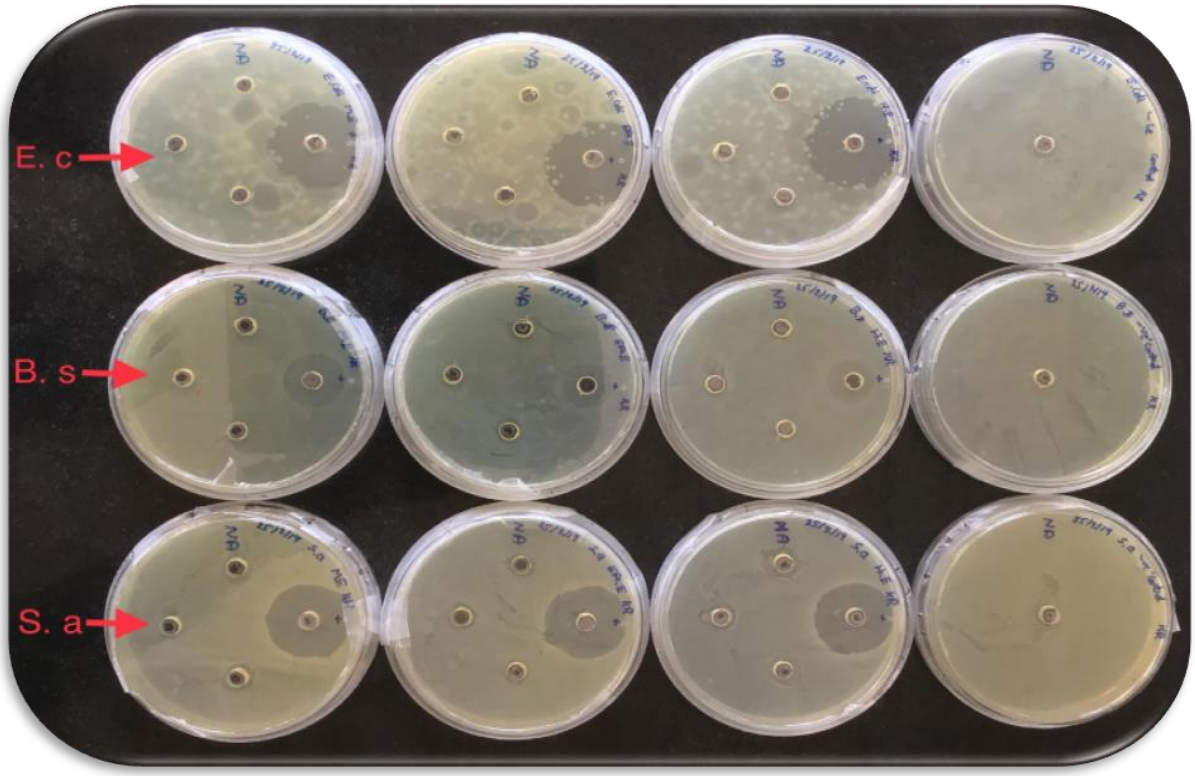

Figure 2: Relative efficicacy of solvent extracts on the growth inhibition of bacterial species
(E.c–*Escherichia coli*, B.s – *Bacillus subtilis*, S.a – S*taphylococcus aureus* are the bacterial species, which were used to assess the antimicrobial effects based on the range of neutralization or the formation of zone of inhibition).

The antioxidant activity of multi-solvent extracts of multi-vitamin plant was measured by two different spectrophotometric methods, DPPH assay and Hydrogen peroxide radical assay. In DPPH assay, DPPH is a stable free radical with purple color. These antioxidants scavenge DPPH radical by donating hydrogen atoms leading to a non-radical with yellow color. DPPH radical scavenging activity of *Sauropus androgynus* (L.) Merr. obtained from three different solvents i.e. Hexane, Ethyl acetate and Methanol, revealed that the ethyl acetate fractions of *Sauropus androgynus* in both DPPH and Hydrogen peroxide radical methods which are of great use in exhibited the highest free radical scavenging activity i.e. 92.85% and 96.52%, followed by methanol extracts with i.e. 65.87% and 88.64% and hexane extracts with 52.38% and 62.93%, respectively. Methanol and ethyl acetate has been proved as effective solvents to extract phenol compounds in the plant. It can be claimed that the ethyl acetate as an organic solvent can be preferred for the extraction of antioxidant compounds mainly because of its lower toxicity.

On comparison, the activity of hexane, ethyl acetate and methanol extract against *E. coli*, *B. subtilis* and *S. aureus* indicated that the dried ethyl acetate extract is useful helping in more inhibitory activity. It was observed that scavenging activity was found to increase with an increase in the concentration in all the cases. In all the antioxidant activity assays, ethyl acetate extract was found to have highest activity followed by methanol and lowest activity was observed with hexane. Hence, the comparative efficacy of the solvents indicated their usefulness in the extraction of beneficial compounds needed for the life.

CONCLUSION

Based on this information, it could be concluded that the plants are natural sources of antioxidant substances of high importance. The highest concentration of phenol compounds was obtained in the plant extracts using solvents of high polarity; the ethyl acetate extract manifested greater power of extraction from *S. androgynus*. The study provides justification for the therapeutic use of these plants as natural antioxidants and antimicrobials in folklore medicine after clinical trails through suitable formulation as designer food or nutraceuticals.

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