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SCREENING OF SECONDARY METABOLITES, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF *CEROPEGIA BULBOSA* EXTRACTS'

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ABSTRACT

Phytochemicals are the secondary metabolites produced from all the plants, but some of the secondary metabolites have high medicinal value. To evaluate the medicinal properties from *Ceropegia bulbosa*, phytochemicals extracted from different solvents such as ethyl acetate, acetone, ethanol, hot water and cold water. The qualitative estimation of phytochemical revealed the presence of alkaloids, saponins, tannins, flavonoids, terpenoids, quinines, cardiac glycosides and phenols. Antibacterial activity was analyzed by Resazurin method, results revealed that ethanol extracts showed strong antimicrobial activity. Antifungal activity was carried out using well diffusion method, maximum antifungal activity was observed also in ethanol extract. Antioxdant activity was analysed using DPPH, ABTS and Reducing power, results reveals that, ethanol extract showed for DPPH assay, but hot water showed both ABTS and Reducing power assay. Antidiabetic activity was found in hot water extracts based on IC 50 value of Amylase inhibition assay.

KEYWORDS: Phytochemicals, Antibacterial activity, Resazurin, DPPH, ABTS.

INTRODUCTION

The genus Ceropegia is the largest genus about 200 species distributed worldwide (Bruyns 2003). Among the 200 species Bulbosa is a slander, fleshy, herb popular in India. It has wide uses to cure definers, for treatment of kidney stone. It has many constituents used in ulcer active against, inflammation (Adibatti, 1991) diarrhea, decentry, cold, sneezing and eye diseases (Kirtikar, 1935).

The primary metabolites amino acid, carbohydrates, protein, and chlorophyll are present. Secondary metabolites consist of alkaloid which is active against diarrhoea (Nadkarni, 1976) Flavonoids helps in heart diseases and phenoloids active against the anticancer activity and other compounds saponin, tannins and so on. (Kumar et. al., 2009).

Based on the review, a lot of works done on ceropegia species but less works has been done on *bulbosa* species. In the presence study isolation and characterization of secondary metabolites and biological activity was analyzed.

2. MATERIALS AND METHODS

2.1. Sample collection: Sample was collected from Orissa forest and authenticated from Botanist Regional Ayurveda research institute.

2.2. Extraction of secondary metabolites: Leaves were removed and washed with tap water and kept in hot air oven for drying for 72 hrs at 40℃. After drying, leaves were ground into fine powder using mixer. The dry powder was subjected for successive extraction of phytochemical compounds using soxhlet apparatus. The solvents used are Ethyl acetate, Acetone, Ethanol, and Distilled water (Cold /Hot).

2.3. Qualitative Estimation Secondary metabolites

Test for Alkaloids (Wagner's Reagent): To 1 ml of sample, 3-5 drops of Wagner's reagent added. The solution changes to reddish brown precipitate.

Test for Carbohydrates (Molisch's test): To 1ml of sample, few drops of Molisch's reagent followed by concentrated sulphuric acid along the walls of test tube. There will be the formation of dull violet colour at the interphase of two layers.

Test for Cardiac glycosides: To 1ml of sample, 2ml of glacial acetic acid, drop of ferric chloride, followed by concentrated sulphuric acid was added. Brown ring appears at the interface indicates presence of cardenolides.

Test for Flavonoids: To 1ml of sample, few drops of 20% sodium hydroxide solution is added formation of intense yellow colour which becomes colourless on addition of dilute hydrochloric acid.

Test for Phenols: To 1 ml of sample, 5% aqueous ferric chloride is added. The solution changes to deep bluer black colour.

Test for Phlobatannins: To 1 ml of sample, 1% aqueous hydrochloric acid added and boiled. Formation of red precipitate occurs.

Test for Amino acids and Proteins: To 1ml of sample 2-5 drops of ninhydrin solution is added and placed in boiling water bath for 1-2 minutes. The solution changes to purple colour.

Test for Saponins: To 1ml of sample, 3ml of water is added mixture was shaken vigorously and observed for the formation of persistent foam.

Test for Sterols: To 1ml of sample drops of chloroform, acetic anhydride and concentrated sulphuric acid, the solution changes to red colour.

Test for Tannins: To 1ml of sample, 10% of alcoholic ferric chloride is added, the solution changes to greenish or bluish colour.

Test for Terpenoids: To 1ml of sample, 1 ml of chloroform and few drops of concentrated sulphuric acid is added Formation of red precipitate occurs.

Test for Quinones: To 1ml of sample, concentrated HCl is added, the formation of yellow precipitate occurs.

2.4 Antimicrobial activity

2.4.1 Antibacterial activity by Resazurin method

10mg of the extracts were dissolved in 1mL DMSO and 100µL of dissolved samples were used for respective organisms (24hr cultured *Bacillus cereus, E-coli, Salmonella typhi, Pseudomonas, Staphylococcus aureus and Streptococcus mutans).* Luria Bertani (LB) broth (tryptone 10g, sodium chloride 10g, yeast extract 6g and distilled water 1000mL) was prepared and autoclaved at 121°C for 15 mins. 300µL deionized water was added in the wells of micro titer plates (A_1-A_{12}, B_{12}-H_{12}, H_{11}-H_1, and G_1-B_1) to prevent the sample from drying. 100µL sterilized LB broth was added to all the remaining wells. 30µL of 0.1% resazurin in wells B_2- G_2 was added as color blank. In wells B_3- G_3 test organisms and 30µL of 0.1% resazurin was added as culture control. 100µL of the plant extracts was added in wells B_4-G_4

and serially diluted by transferring 100µL of the mixture to subsequent wells upto B_{11}-G_{11} in respective plates and 100µL of the excess sample was discarded from B_{11}-G_{11} respectively.

2.4.2. Anti-fungal activity

Well diffusion method was used to analyze the minimum inhibition concentration. The plant extract were assessed for anti-fungal activity against *Aspergillus niger, Aspergillus flavous and Candida albicans*. 10mg of the extracts was dissolved in 1mL of DMSO (Dimethyl sulfoxide) in sterilized eppendorf, aliquots of sample 100µg, 200µg, 300µg, 400µg of concentration was prepared in sterile eppendrof and the final volume was made upto 50µL by adding DMSO. The required glass wares were washed and autoclaved along Potato Dextrose Agar (Potato-400g, Dextrose-20g, Agar-20g, Distilled water-1000mL) for 15lbs at 121°C and poured into petriplates. After the agar solidified, the fungi culture were spreaded on plate using spreader and wells were made using 8 cms borer. Different concentrations of samples were loaded into the 4 wells. Then the petriplates were incubated for 72 hrs. After incubation the diameter of the zone of inhibition was measured in mm.

2.5 Antioxidant activity

2.5.1 DPPH method

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. The antioxidant activity of the plant extracts were performed on the basis of the scavenging effect on the stable DPPH free radical. The stock solution of 0.1mM DPPH was prepared freshly in methanol and kept in the dark at 4°C. 3mL of different concentrations of plant extracts ranging from 10-100 µg/mL were added to 1mL DPPH stock solution and incubated at ambient temperature in dark for 20 min. After incubation the absorbance was recorded against blank at 517 nm. Gallic acid was used as the standard for antioxidant activity.

2.5.2. ABTS method

ABTS (2, 2-azino-bis-3-ethyl benzthaizoline -6-sulphonic acid) radical cation decolorisation assay: ABTS radical cation were produced by ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark condition at room temperature for 16 hours before use. Various extraction in the plant and standard Gallic acid at various concentration was incubated at 25°C for 30 minutes and were add to 1ml of ABTS solution. The absorbance was read at 713 nm in a spectrophotometer. Gallic acid was used as the standard for antioxidant activity.

2.5.3. Reducing power method

The reducing power was determined according to the method of Oyaizu (1986). Each extract (0.5–10 mg ml1) in methanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50 C for 20 min. After 2.5 ml of 10% trichloroacetic acid were added, the mixture was centrifuged at 200g for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm against a blank. Gallic acid was used as the standard for antioxidant activity.

2.5. Determination of alpha amylase inhibitor activity

The assay mixture containing 200μL of 0.02M sodium phosphate buffer, 20 μL of enzyme and the plant extracts in concentration range 20-100 μg/mL were incubated for 10 minutes at room temperature followed by addition of 200 μL of starch in all test tubes. The reaction was terminated with the addition of 3 mL DNS (Dinitrosalicylic acid) reagent and placed in boiling water bath for 5 minutes, cooled and diluted with 15 mL of distilled water and absorbance was measured at 540 nm. The control contained all the reagents except the plant extracts. The IC 50 values were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference alpha amylase inhibitor. All tests were performed in triplicates.

3. RESULTS AND DISCUSSION

3.1. Extraction and qualitative analysis of the secondary metabolite.

Extraction of secondary metobolites was done by using different solvent using Soxhlet apparatus. The variation can be also explained by the different polarities of compounds which were selectively more soluble in different solvents (Thanh et al 2017). Flavonoid and alkaloids is present in all extractions. Tannin and phenolic present in ethyl acetate, acetone and ethanol extraction. Steroid is present in ethyl acetate and ethanol extraction. Saponine present in hot, cold and acetone extraction. Glycoside is present only ethyl acetate extraction and Phlobaianine & Anthraquinine absent in all extraction (**Table 1).** The plant extracts were rich in alkaloids, flavonoids, tannins and saponins. They were known to show medicinal activity as well as exhibiting physiological activity (Sofowara, 1993). In very high.

Levels of alkaloids and flavonoids, and are employed in medicinal uses.

Table 1: Presence and absence of the secondary metabolites from different solvent extracts.

Compounds	Ethyl acetate	Acetone	Ethanol	Hot water	Cold water
Tannin	+	+	+	-	-
Saponin	-	+	-	+	+
Flavonoids	+	+	+	+	+
Glycosides	+	-	-	-	-
Alkaloids	+	+	+	+	+
Phenolics	+	+	+	-	-
Phlobaianine	-	-	+	+	-
Anthraquinine	-	-	-	+	-
Steroids	+	-	+	-	-

3.2 Antimicrobial activity

3.2.1 Antibacterial assay: Antibacterial assays, the bacterial concentration is imprecise as they are compared to the Macfarland standard. Resazurin is an oxidation–reduction indicator used for the evaluation of cell growth (McNicholl et al 2007). We use three gram positive organisms such as *Bacillus cereus Staphylococcus aureus and Streptococcus mutans*, three gram negative organisms such as, *E-coli*, *Salmonella typhi*, *Pseudomonas*, Results reveals strong antimicrobial activity showed maximum against gram negative bacteria *E.coli* from all the plant extracts, but for *Pseudomonas* there is no any significant antimicrobial activity. (Table 2). Ethanol extracts showed maximum antimicrobial activity for all the organisms.

Table 2: Antibacterial activity against different organisms concentration showed in µg.

Plant extracts	*E.coli*	*Pseudomonas*	*Salmonella typhi*	*Bacillus cereus*	*Staphylococcus aureus*	*Streptococcus mutans*
Ethyl Acetate	250	-	500	250	125	125
Acetone	62.5	-	250	250	500	500
Ethanol	62.5	500	62.5	500	62.5	250
Hot water	500	-	500	500	500	500
Cold water	500	-	500	500	500	500

3.2.2 Antifungal Activity: Antifungal efficacy was found out using well diffusion method. Fungi used for the analysis were *Aspergillus niger*, *Aspergillus flvous* and *Candida albicans*. Based on the zone of inhibition, MIC was analysed for the plant extracts. Results revealed that Ethanol extracts showed strong anti-fungal activity against all the fungus, but ethyl acetate, acetone are not showed significant antifungal activity (Table 3).

Table 3: Antifungal activity of different fungus MIC showed in µg.

Plant Extracts	*Aspergillus niger*	*Aspergillus flavus*	*Candida albicans*
Ethyl Acetate	-	-	-
Acetone	-	-	-
Ethanol	200	200	100
Hot water	400	400	200
Cold water	400	400	400

3.3 Antioxidant activity

3.3.1 DPPH Method: DPPH method is commonly used for the analysis scavenging activity for organic compounds. Results revealed the maximum scavenging activity in ethanol extract compared to other extracts such as ethyl acetate, Acetone, Cold water and Hot water. Each Solvent extracts contributed with a different antioxidant activity as these groups were found to have differing correlation with antioxidant capacity (Fig 1). Phenolic compounds, flavonoids and saponins were mainly responsible for antioxidant activity for these tested materials (Chavan et al 2013).

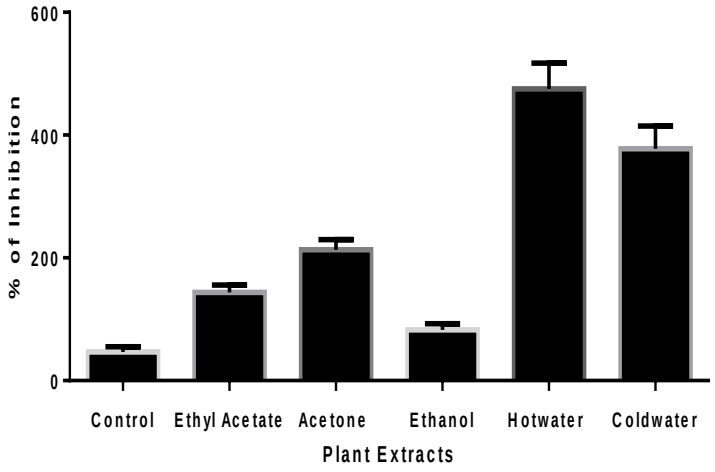

Fig 1: Comparison of scavenging activity by DPPH of Different solvent extraction.

3.3.2 ABTS Method: ABTS method is extensively used for the analysis scavenging activity for inorganic compounds. Results reveals the same maximum scavenging activity obtained from hot water compare other extract (Fig 2). $ABTS^{\bullet+}$ can be solubilized in both aqueous and organic solvents, and thus multiple media can be used to determine both hydrophilic and lipophilic antioxidants. Most applications of this assay on samples are carried out to test the antioxidant activity of the phenolic extracts, and so ABTS is solubilized in polar solvents.

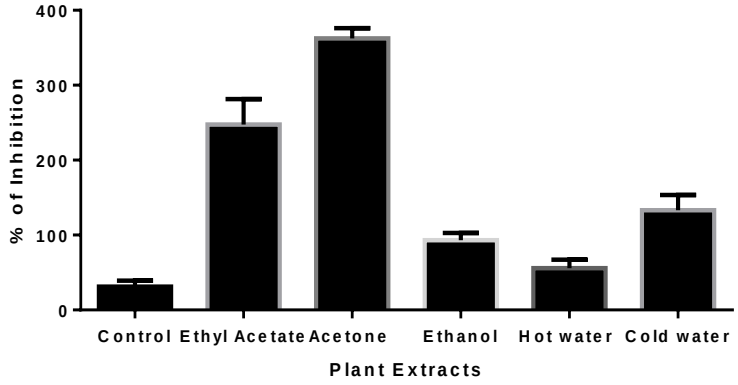

Fig 2: Comparison of scavenging activity by ABTS of Different solvent extraction.

3.3.3 Reducing Power assay: The reducing property is commonly associated with the presence of reluctants. The antioxidant action of reductants is based on the breaking of free radical chain by donation of a hydrogen atom. Reductants also react with certain precursors of peroxide, thus preventing peroxide formation. The data presented here indicated that the marked reducing activity of the extracts seems to be due to the presence of polyphenols, (Singh et al, 2002) which may act as reductants by donating the electrons and reacting with free radicals to convert them to more stable products and terminate radical chain reaction. Fig 1 showed the reducing power (as indicated by absorbance at 700 nm) of extracts which increased with increasing concentration to certain extent and then leveled off with further increase in concentrations. Results obtained indicate that the strong reducing power showed in Hot water and ethanol compared to other extracts. The investigation reveals that both the sample extracts had good hydroxyl scavenging activity and can be used as a good hydroxyl radical scavenger.

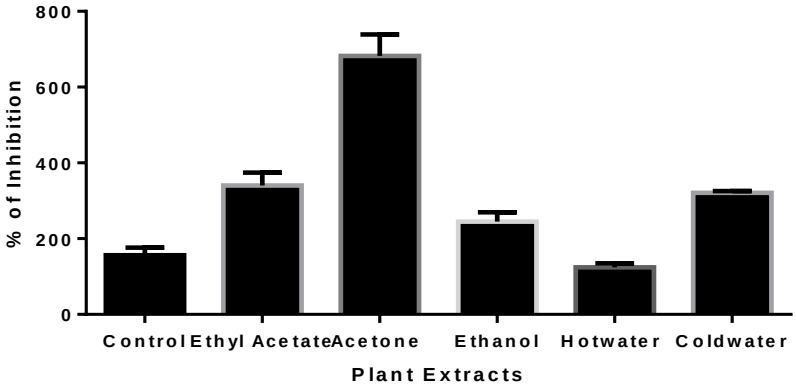

Fig 3: Comparison of scavenging activity by Reducing power assay of Different solvent extraction.

3.4 Antidiabetic activity

Inhibition of α-amylase can lead to reduction in post prandial hyperglycemia in diabetic condition. Based on the IC 50 value hot water showed strong inhibition of amylase activity compare to others plant extracts. It may be due to the presence of more chemical constituents such as lignans (phyllanthin and hypophyllanthin), terpenes, triterpenes, flavonoids (quercetin, quercetrin, rutin), and alkaloids in the ethanol and hexane extracts. The plant-based α-amylase inhibitor offers a prospective therapeutic approach for the management of diabetes.

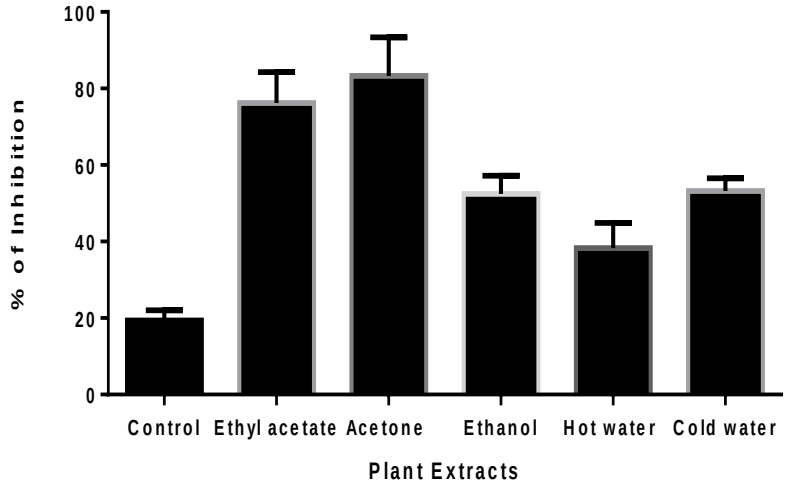

Fig 4: Comparison of anti-diabetic activity by α-amylase inhibitor assay of Different solvent extraction.

CONCLUSION

The medicinal plants appear to be rich in secondary metabolites, widely used in traditional medicine to combat and cure various ailments. The antimicrobial, antioxidant and antidiabetic can be attributed to their high alkaloids, phenols, tannins and flavonoids. As we could extract valuable compounds that could be used for human health care, second need is to standardize and evaluate its diagnostic features for its proper use.

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