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Preliminary phytochemical screening and quantification of bioactive compounds in the leaves of spinach (*Spinaceae oleraceae* L.)

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ABSTRACT

Plants are the sources for large amount of secondary metabolites. *Spinacia oleraceae* is a green leafy vegetable belonging to Chenopodiaceae family. It is having medicinal properties due to rich nutritional value and is extremely rich in antioxidants. Antioxidant property of the spinach is due to the presence of bioactive compounds. A preliminary phytochemical screening of different plant extracts (aqueous, methanol, ethanol and chloroform extracts) revealed the presence of carbohydrates, phenols, tannins, flavonoids, saponins, alkaloids, terpenoids, cardiac glycosides and steroids. The quantification of bioactive compounds yields 45.24% of phenols, 27.34% of flavanoids, 23.05% of saponins and 4.82% of alkaloids. By this present phytochemical screening and quantification, we suggest that *S.oleraceae* is a good nutrient rich leafy vegetable with antioxidant value that can be used as a therapeutic and curative medicine for many oxidative stress induced diseases.

KEYWORDS: Antioxidant, cardiac glycosides, flavonoids, Phytochemicals.

INTRODUCTION:

Consumption of green is a major source of vitamins and micronutrients. They are amazingly nutritious and a powerhouse of antioxidants. The plants exhibit its curative activity against several human diseases because of the presence of biologically active phytochemicals such as alkaloids, flavonoids, steroids, glycosides, terpenoids, tannins and phenolic compounds). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties[1]. Plants produce these chemicals to proect themselves, but recent research demonstrates that consumption of leafy vegetables is associated with a lowered risk of cancer, heart disease, hypertension and stroke due to antioxidant constituents, which can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidative chain reaction[2].

In view of the importance of phytochemicals of leafy vegetables, *S.oleraceae* L., has been screened for phytochemical presence and their quantification. *S.oleraceae* (commonly called as spinach), belongs to the family Chenopodiaceae and is reported to be a good source of minerals (Iron,copper, phosphorous, zinc, selenium), B complex vitamin (niacin and folic acid), ascorbic acid, carotenoids (ß-carotene, lutein, zeaxanthine), phenols (flavonoids, p-coumaric acid), apocyanin and Omega-3-fatty acids. Recently opioid peptides called rubescolins have also been found in spinach[3]

.

It contains unique flavonoid compound including glucuronides and acylated di and triglycosides of methylated and methylenedioxyderivatives of 6-oxygenated flavonols[4-6] . In this study however, attempts are made to screen the phytochemicals present in different solvent extracts of *S.oleraceae* leaves and to quantify some of these compounds.

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MATERIALS AND METHODS:

a)Collection and Processing of plant samples:

The plant leaves were brought from vegetable market, Anantapur, Andhra Pradesh and washed thoroughly under tap water .They were dried under shade for two weeks. The shade dried leaves were ground into a coarse powder with the help of a blender. The powder was stored in an airtight container and kept in a cool, dark and dry place until further analysis.

b) Plant extraction:

The shade dried plant material (leaves) was soaked in solvents such as water, methanol, ethanol and chloroform with occasional shaking at room temperature (37°C) for aqueous and at 15°C temperature for methanolic, ethanolic and chloroform extracts for 24 hrs. The soluble materials of respective solvents were filtered off and the extracts of plant leaves were used for further qualitative analysis.

c) Phytochemical Screening:

The phytochemical screening was done with four different extracts (water, methanol, ethanol and chloroform) of plant leaves as per the standard methods[7].

1.Test for Carbohydrates:

a. Molisch's Test:

To 2 ml of the plant extract, 2 drops of freshly prepared 20% alcoholic solution of α-napthol was added and then 2ml of concentrated sulfuric acid was added along the sides of the test tube. Formation of the violet ring at the junction of the solutions and its disappearance on the addition of excess alkali solution indicating the presence of carbohydrates.

b. Benedict's test:

To 0.5ml of plant extract taken in a test tube, 5ml of Benedict's solution was added and boiled for 5 minutes and allowed to cool. A red color precipitate of cuprous oxide formed indicating the presence of a reducing sugar.

c. Tollens test:

To the test solution equal volume of hydrochloric acid containing a small amount of phloroglucinol (It is a reagent of the Tollen's test) was added and heated for 10 minutes. Red color produced due to reaction of furfurals with phloroglucinol, indicating the presence of pentoses.

2. Test for Phenols:

a. Ferric chloride test:

To 1 ml of the leaf extract, 2ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green or violet color indicating the presence of phenols.

b. Libermann's test:

For a small quantity of the leaf extract, 5ml of 20% sulfuric acid followed by the addition of few drops of aqueous sodium nitrate solution was added. A red color obtained, indicating the presence of phenols.

3. Test for Tannins:

a. Lead acetate test:

To 5ml of the extract, a few drops of 1% lead acetate solution was added. A yellow precipitate was formed, indicating the presence of tannins.

b. Chlorogenic acid test:

To the plant extract, aqueous ammonia was added and exposed to air. Gradually, green color developed indicating the presence of tannins.

c. Catechin test: A matchstick was dipped into the plant extract, dried it and moistened with concentrated hydrochloric acid. Then the stick was warmed near the flame. The color of the stick was changed to pink. It showed the presence of tannins.

4. Test for Flavanoids:

Ammonia solution reduction test:

To the 1% Ammonia solution, few drops of leaf extract was added. Yellow coloration was observed, indicating the presence of flavanoid compounds.

5. Test for Saponins:

Froth test:

To 5ml of extract, 2.5ml of distilled water was added and shaken vigorously to obtain a stable persistent froth. To this froth 3 drops of olive oil was added and formation of emulsion was observed, indicating the presence of saponins.

6. Test for Alkaloids:

The extract was stirred with a few ml of concentrated hydrochloric acid and filtered. The filtrate was tested carefully with for the presence of alkaloids by following tests.

a. Mayer's test:

To a few ml of filtrate 2 drops of Mayer's reagent was added along the sides of the test tube. A white creamy precipitate was observed which indicated the presence of alkaloid.

b. Wagner's test:

To a small amount of extract few drops of Wagner's reagent was added along the sides of the test tube. A reddish brown precipitate was observed, confirmed the presence of alkaloid.

c. Dragendorff's test:

To 0.5ml of the extract, 2ml of concentrated hydrochloric acid was added. To this acidic medium, 1 ml of Dragendorff's reagent was added. Orange coloured precipitate was observed, indicating the presence of alkaloids.

7. Test for terpenoids:

Salkowski's test:

To 2ml of the extract, 3ml of chloroform was added and 3ml of concen-

trated sulfuric acid was added carefully along the sides of the test tube to form a layer. A reddish brown coloration was formed at an interface indicating the presence of terpenoids.

.8. Test for Phlobatannins:

10 ml of the plant extract was boiled with 1% hydrochloric acid in a conical flask. There was no deposition of a red precipitate indicating the absence of phlobatannins.

9. Test for Cardiac glycosides:

a) Keller-Killiani test:

To the 5 ml of extract 1ml of concentrated sulfuric acid was added and was mixed with 2 ml of glacial acetic acid containing one drop of ferric chloride. To the above mixture carefully 1ml of concentrated Sulfuric acid was added, it was underneath the mixture. Formation of brown ring at the interface, indicating the presence of cardiac glycoside constituents.

b) Baljet's Test:

A small amount of test solution was mixed with a pinch of picric acid, formation of orange color confirmed the presence of cardiac glycosides.

10) Test for Steroids:

Liberman-burchard's test:

5ml of plant extract with few drops of acetic anhydride, was boiled and cooled. Then concentrated sulphuric acid was added along the sides of the test tube, formation of brown ring at the junction of two layers with green coloured upper layer indicating the presence of steroids.

d) Quantitative analysis of Phytochemicals:

Quantitative analysis of phytochemicals such as flavonoids[8]., alkaloids, saponins[9] and phenols[10] present in the plant powder was carried out using standard methods.

1. Phenols:

To determine the total phenol content of the plant powder, 5gms of the plant powder was taken into a 250ml titration flask and 100ml n-hexane was added, kept for 4h and the filtrate was discarded for fat free residue. The process was repeated for residue and filtrate was collected. 50ml of diethylether was added to the residue, heated in a boiling waterbath for 15 min, cooled to room temperature and filtered into a separating funnel. About 50ml of the 10% sodium hydroxide solution was added to filtrate twice and shake well each time to separate the aqueous layer from the organic layer. The organic layer was kept aside and to the aqueous solution 75ml of de-ionized water was added. The total aqueous layer was acidified to pH 4.0 by adding 10% hydrochloric acid and to this solution 50ml dichloromethane (DCM) was added twice to the aqueous layer in the separating flask. Consequently, the combined organic layer was collected dried and then weighed.

2. Flavanoids:

To determine flavonoid content of the plant powder, 100ml of 80% aqueous methanol was added to10gms of plant sample in a 250 ml titration flask, at room temperature and shaken for 4 hrs in an electric shaker. The entire solution was filtered through whatmann filter paper no. 42 and again, this process was repeated to residue. The residue was evaporated to dryness over a water bath and weighed.

3. Saponins:

To 20gms of plant powder in a conical flask, 100 ml of 20% ethanol was added. The sample was heated in a hot water bath for 4 hrs with **continuous stirring at about 55**°c. The solution was then filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40ml over a water bath at about 90^0c . The concentrate was then transferred into 250ml separating funnel and 20ml of diethylether was added and shaken vigorously. The aqueous layer was recovered while diethylether layer was discarded and the purification process was repeated. To this 60ml n-butanol was added and n-butanol extract was washed twice with 10ml of 5% sodium chloride. Finally the solution was heated in a water bath and evaporated. After evaporation the sample was dried in a oven to a constant weight.

4. Alkaloids:

To 5gms of plant powder in a 250 ml beaker, 200 ml of 20% acetic acid in ethanol was added. This was covered and allowed to stand for 4h. The solution was then filtered and the extract was allowed to become concentrate in a water bath until it reached $1/4^{th}$ volume of the original volume. To this concentrate ammonium hydroxide was added until the precipitation was completed. The whole solution was left to settle down and precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The precipitated residue was dried and weighed.

RESULTS:

The different plant extracts of *S.oleraceae* leaves (aqueous, methanolic, ethanolic and chloroform) were prepared for the preliminary phytochemical analysis to identify the bioactive constituents such as carbohydrates, phenols, tannins, flavanoids, saponins, alka-

loids, terpenoids, phlobatannins, cardiac glycosides and steroids. The results of various phytochemical tests performed on the extracts are presented in **Table 1**.

The aqueous extract of *S.oleraceae* showed positive result for carbohydrates, phenols, flavanoids, saponins, terpenoids, cardiac glycosides, and showed negative result for alkaloids, tannins, phlobatannins and steroids. The methanolic extract of the plant leaves revealed the presence of carbohydrates, phenols, tannins, flavanoids, saponins, alkaloids, cardiac glycosides and absence of terpenoids, phlobatannins and steroids. The ethanolic extract of the plant leaves showed the presence of carbohydrates, flavanoids, phenols, alkaloids, tannins, saponins, cardiac glycosides and absence of terpenoids, phlobatannins and steroids. The chloroform extract of plant leaves showed only the presence of carbohydrates, cardiac glycosides, steroids and absence of all other phytochemicals.

The quantitative analysis of phytochemicals such as flavonoids, alkaloids, saponins and phenols present in the plant powder is summarized in **Table 2**. *S.oleraceae* contains about 45.24g of total phenols, 27.34g of flavanoids, 23.05g of saponins and 4.822g of alkaloids 100g of plant powder.

Table 1: Preliminary Phytochemical Analysis of Extracts of *S. oleraceae* Leaves:

Constituents	Test Name	Different extracts of *S.oleraceae* leaves			
		Aqueous	Methanol	Ethanol	Chloroform
Carbohydrates	Molisch's test	+	+	+	+
	Benedict's test	+	+	+	+
	Tollen's test	+	+	+	+
Phenols	Ferric chloride test	+	+	+	-
	Libermann's test	+	+	+	-
Tannins	Lead acetate test	-	+	+	-
	Chlorogenic acid test	-	+	+	-
	Catechin test	-	+	+	-
Flavanoids	Ammonia solution reduction test	+	+	+	-
Alkaloids	Mayer's test	-	+	+	-
	Wagner's test	-	+	+	-
	Dragendroff's test	-	+	+	-
Saponins	Froth test	+	+	+	-
Terpenoids	Salkowski's test	+	-	-	-
Phlobatannins	Hydrochloric acid test	-	-	-	-
Cardiacglycosides	Keller-Killiani test	+	+	+	+
	Baljet's test	+	+	+	+
Steroids	Liberman-Burchard's test	-	-	-	+

Table 2: Percentage yield of different phytochemicals of *S.Oleraceae*:

Phytochemical Names	Weight (gms/100gms dry weight)
Phenols	45.24g
Flavanoids	27.34g
Saponins	23.05g
Alkaloids	4.822g

The table showed the dry weight obtained from the sequential steps of dry weight extraction methods.

DISCUSSION:

Plant derived substances are nutritionally, medicinally or physiologically highly active compounds. The chemical constituents present in a plant are responsible for therapeutic and other pharmacological properties. These are plant secondary metabolite compounds which are important sources of many food ingredients[11]. These compounds include phenolics, tannins, flavonoids, saponins, alkaloids, terpenoids, cardiac glycosides, steroids etc., to protect themselves from the continuous attack by naturally occurring pathogens, insect pests and environmental stresses[12,13] . For the discovery of novel drugs, the essential information regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts. These components are well known for their curative activity against several human diseases [14,15].

Plant phenolics include phenolic acids, tannins, flavonoids etc., despite of their wide distribution, the health effects of dietery polyphenols have come to the attention of nutritionists only in recent years. Researchers and food manufacturers have become more interested in polyphenols due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases.

Recently, Phenolic compounds have been considered as powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C, E and carotenoids[16,17] . The phenolics have biological and pharmacological properties such as antimicrobial activity[18] , antiviral[19], anti-inflammatory[20]antimutagenic (Anpin et al., 2011) and anticarcinogenic activities[21] .

Spinach ranks high among vegetables in terms of antioxidant capacity because of an abundance of phenolic compounds[22,23], suggesting that spinach consumption may afford protection against oxidative stress mitigated by free-radical species. Spinach phenolic compounds exhibit a wide range of biological effects including antioxidative[24],anti-inflammatory[25],antiproliferative[26], anticarcinogenic[27] and antimutagenic[6].

Flavonoids are the most abundant polyphenols in our diet. The free radicals produced in the body are neutralized by flavanoids, which are well known for their antioxidant properties. Thus flavanoids decrease the damage caused by free radicals due to the presence of hydroxyl functional groups, which are responsible for their antioxidant and chelating properties, In addition to antioxidant function, flavonoids may also modulate cell signaling pathways and could have marked effects on cellular function by altering protein and lipid phoshorylation and modulating gene exression[28] . Many potential health benefits associated with spinach are thought to be related to unique flavonoids present in the leaves. Tannins are astringent in nature and useful in treating intestinal disorders such as diarrhoea and dysentery[29] . It also aids in wound healing[30].

The presence of saponins will reduce the blood pressure and cholesterol levels in blood. This is due to the binding of saponins to cholesterol to form insoluble complexes that are excreted via bile. This prevents cholesterol reabsorption and results in the reduction of serum cholesterol. Saponins have been found to be potentially useful in the treatment of hypercholesterolemia which suggests that saponins might interfere with the intestinal absorption of cholesterol[31,32].

Alkaloids being bitter substances exert notable antimicrobial actions. So, it is quite reasonable that the plant containing alkaloids exerts beneficial therapeutic effects against infectious diseases for which it is used.

Terpenoids reduce complications associated with diabetes and lowers the sugar level in blood[33] . Terpenoids have been found to be very useful in anti-aging and overall beauty enhancement[34] . Hawkins and Ehrlich (2006) reported that terpenoids have capacity to improve lung function in respiratory treatment. Cardiac glycosides shown to aid in treatment for congestive heart failure and cardiac arrhythmia.

In the present study the qualitative analysis of different extracts of *S.Oleraceae* leaves showed the presence phenolic compounds, tannins, flavanoids, saponins, alkaloids, cardiac glycosides, and absence of phlobatannins.

The quantification of phytochemicals of *S.oleraceae* showed the presence of highest amount of phenols and flavonoids justified the potent antioxidant nature of the plant. The study also revealed that flavonoids represent the main group of phenolic compounds in *S.oleraceae*. In addition to phenolic compounds the plant also contains saponins and alkaloids are also present in considerable quantities, which increases its medicinal properties of plant.

CONCLUSION:

The phytochemical analysis of *S.oleraceae* revealed the presence of phytochemicals such as phenolics, tannins, flavonoids, alkaloids, saponins, alkaloids, terpenoids, cardiac glycosides and steroids along with carbohydrates in different extracts. Furthermore, the quantification of phytochemicals showed the leaves of *S.Oleraceae* contains 45.24g of phenolic compounds , 27.34g of flavonoids, (23.05g) of saponins, 4.82g of alkaloids per 100g of plant powder. Hence the presence of these beneficial secondary metabolites imparts high nutritive and antioxidant potential to *S.oleraceae*. By this present phytochemical screening and quantification, we suggest that *S.oleraceae* is a good nutrient rich leafy vegetable with antioxidant value that can be used as a therapeutic and curative medicine for many oxidative stress induced diseases.

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