RESEARCH ARTICLE

Study of Phytochemical Constituents and Anti-oxidant Activity of *Spinacia oleracea* L. of Bundelkhand Region

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ABSTRACT
Background: *Spinacia oleracea* L. commonly known as palak is an edible flowering plant belongs to *Amaranthaceae* family. The plants exhibit its curative activity against several human diseases because of the presence of biological tannins and phenolic active phytochemicals such as alkaloids, flavonoids, steroids, glycosides, terpenoids. It is used in the treatment of difficulty in breathing, inflammation of liver and lungs and leucorrhoea, useful in urinary concretion, inflammation of the lungs, sore throat, and pain in joints.
Methods*: S. oleracea* L. was collected from local market Jhansi in the month of January 2017. Aqueous and methanolic extraction of *S. oleracea* L. and phytochemical screening of the extracts was done for Saponins, Reducing sugar, Cardiac glycosides, Protein and Amino acid, Glycosides, Alkaloids, Tannins, Flavonoids, Terpenoids, and Steroids.
Results: Phytochemical analysis of leaves of *S. oleracea* had most of the important phytochemicals like Alkaloids, Tannins, Glycosides, Terpenoids, and Flavonoids etc. In which, the aqueous extract of *S. oleracea* showed (in most of the test) positive result for Alkaloids, Phenols, Flavanoids, Saponins, Terpenoids, Reducing sugar, Protein, and Amino acid and showed a negative result for Carbohydrate, Glycosides, and Cardiac glycosides. The methanolic extract of the plant leaves revealed the presence of (in most of the test) Alkaloids, Tannins & Phenolic compounds, Flavanoids, Saponins, Terpenoids and Steroids and negative results for the rest.
Conclusion: The phytochemical analysis of *S. oleraceae* revealed the presence of phytochemicals such as, Tannins, Flavonoids, Alkaloids, Saponins, and Terpenoids etc. in the different extracts. By the presence of these phytochemicals, we were suggested that *S. oleracea* is a good nutrient rich leafy vegetable that can be used as a therapeutic and curative medicine for many oxidative stress- induced diseases.
Key-words- Phytochemical analysis, *Spinacia oleracea*, Flavonoids, Alkaloids, Terpenoids, Methanolic extract

INTRODUCTION
Medicinal plants contain bioactive substances viz. tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids which produce definite physiological action on the human body [1,2]. They are of great importance to the health of individuals and communities. Many of these indigenous medicinal plants are used as spices and food plants. One of the secondary metabolites i.e. phenolics have been known to possess a capacity to scavenge free radicals. The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors. Phenolics are especially common in leaves, flowering tissues and woody parts, such as stems and barks. Fruits and vegetables are important sources of phytochemicals and it is studied that some anti-nutritional content of these vegetables have exhibited potential for reducing the risk of certain diseases in human beings [3]. These diseases include high blood pressure, heart attack, stroke, and other cardiovascular diseases [4]. These anti-nutrients or phytochemicals carry out their healing activities by combining with vitamins or with other nutrients [5]. Information is however scanty on the nutritional and phytochemical contents of the leafy vegetables [6].

S. oleracea (commonly called as spinach) belongs to the family Chenopodiaceae and is reported to be a good source of minerals (iron, copper, phosphorous, zinc, selenium), vitamin B complex (niacin and folic acid), ascorbic acid, carotenoids (ß-carotene, lutein, zeaxanthin), phenols (flavonoids, p-coumaric acid), apocynin and Omega-3-fatty acids. Recently opioid peptides called rubiscolins have also been found in spinach [7]. There are two kinds of spinach (*Amaranthus* sp), the wild spinach and cultivated spinach. Spinach that

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is often consumed is cultivated spinach that comprises of green and red spinach (*Amaranthus tricolor* L.) [8].

The nutritional value of spinach indicates it to be a very nutrient-dense food. It is low in calories yet very high in vitamins, minerals, and other phytonutrients. Spinach is also packed with a number of anti-oxidants like polyphenols, flavonoids and carotenoids, which are shown to possess anti-inflammatory effects, anti-mutagenic potential, anti-neoplastic effects as well as chemo-preventive activities [9,10]. It is an important green leafy vegetable. The leaf of this annual plant is used as a major ingredient in Indian cuisine mainly due to its nutritional and therapeutic values. It is low in calories. A cool climate is best for producing spinach.

Phytochemicals are naturally occurring components in fruits, vegetables, legumes and grains. Plants are getting specific color, flavor, and smell and are part of plant's natural defense system i.e. disease resistance. Photochemicals are bioactive, non-nutrient plant compounds in fruits, vegetables, grains and other plant foods that have been linked with reducing the risk of major degenerative diseases [5,11].

Antioxidant compounds in food play an important role as a health protecting factor. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate, and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds such as gallates which have strong antioxidant activity, while others such as the monophenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals [12,13]. In view of the importance of phytochemicals of leafy vegetables, *Spinacia oleraceae* L. has been screened for phytochemical presence, TLC and the antioxidant activity was evaluated.

MATERIALS AND METHODS

Collection of Plant Materials- The vegetables *Spinacia oleraceae* was collected in the month of January 2017 from local market of Jhansi (U.P), India. Firstly the collected plant material was washed with tap water for 3-4 times and then with de-ionized water for two times. After washing, plants were kept in the dark for drying at room temperature and under the constant observation to avoid any contamination. Dried leaves were crushed with the help of electric grinder. Powdered sample was stored for further use.

Extraction Procedure- Extraction was done by three different methods i.e. Aqueous, Quath and Methanolic extraction.

Aqueous Extract- Different concentration of dry powder i.e. 5gm and 10 gm was taken in conical flasks having an equal amount (100ml) of de-ionized water. Both the flasks were heated at 90°C in water bath for 1 hour. After 1 hour flasks were taken out from the water bath and kept at room temperature for cooling purpose. Then the extract was filtered with the help of filter paper and stored at 4°C [14].

Quath Extraction- For quath extraction fresh leaves were used. Firstly leaves washed carefully and then crushed in automatic grinder to make paste. 100 ml of paste was mixed with 300 ml of distilled water in the beaker and heated at 100°C until the final volume remains 100 ml. Cool down the quath extract, filtered with muslin cloth and stored at 4°C [14].

Methanolic Extract- The powdered material was extracted with absolute 80% methanol using Soxhlet apparatus. Different concentration of plant material and solvent were taken. After filling the soxhlet apparatus with plant material and solvent it was run at 60-80°C until it gets colorless and continuously flows of water to cool down the condenser. Finally, the extract was collected in airtight bottles and stored at 4°C [14].

Phytochemical Analysis- The powdered plant material (*Spinacia oleraceae*) was subjected to preliminary phytochemical analysis to test the presence or absence of phytochemical constituents by the method as described elsewhere [15].

Thin layer chromatography- Silica gel 60 F254–TLC aluminium sheets (Merck, Germany) were used for the thin layer chromatographic study and solvent system developed in solvent system Butanol: Acidic acid: Water having a ratio of 2:1:1. The developed TLC plates were air dried followed by hot air oven for 20 minutes. Freshly prepared 0.2 % ninhydrin solution was used to detect the bands on the TLC plates.

The movements of the spots were expressed by its retention factor (Rf).

$$\mathbf{R}f = \frac{\textbf{Distance traveled by solute}}{\textbf{Distance traveled by solvent}}$$

Antioxidant activity- The total antioxidant capacity of the aqueous and methanolic extract of *S. oleraceae* was evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto *et al*. [16]. The assay is based on the reduction of Mo (VI) to Mo (V) by the methanol extract of different part of garlic and subsequent formation of green phosphate/Mo (V) complex at acid pH. The 1.0 mL of various concentrations (3-21 μg/mL) of the extract was combined with 1.0 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. BHT was used as a standard. A typical blank solution contained 3 ml of reaction mixture and the appropriate volume of the same solvent used for the samples/standard. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer.

RESULTS

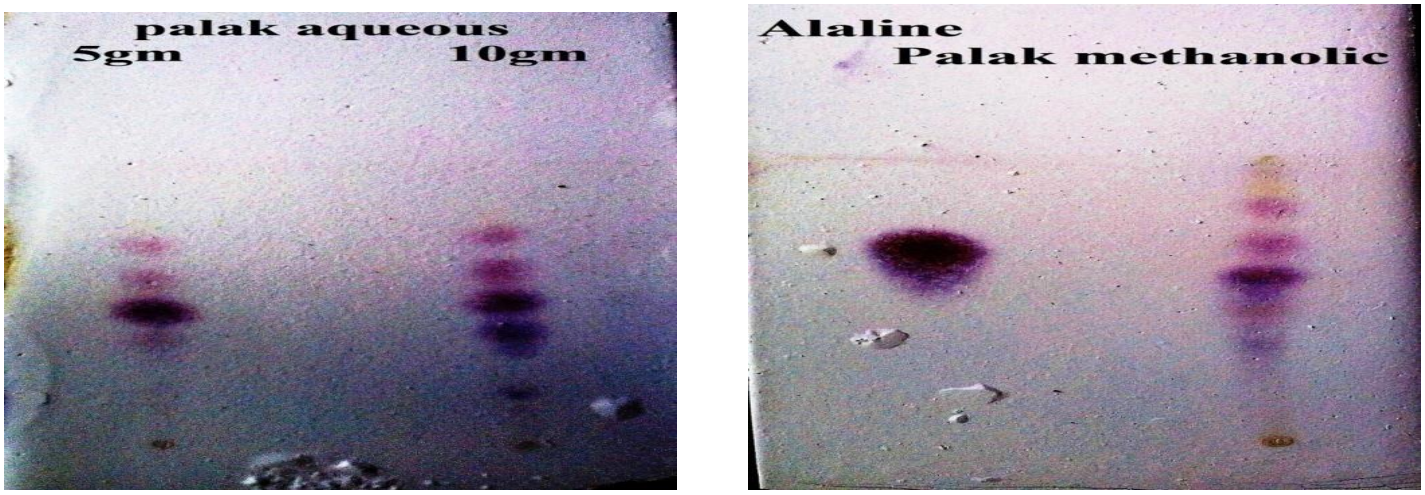
Phytochemical analysis of the leaves of *S. oleracea* L. had most of the important phytoconstituents like Alkaloids, Reducing sugar, Flavonoids, Glycosides, Cardiac glycosides, Tannins, Saponin, Protein, Amino acid, Terpenoids, and steroids. Alkaloids are a class of nitrogenous organic compounds of plant origin which have diverse and important physiological effects on humans and other animals. All the tests for alkaloids i.e. Mayer's test, Wagner' test, Hager's test were shown positive in all the extract. Mayer's test was shown negative with aqueous extraction. Carbohydrate is absent by all the tests used for aquous, quath and methanolic extraction. While reducing sugar was detected in all the extracts (except methanolic extraction by Fehling's test). Flavonoids are hydroxylated polyphenolic compounds that carry out important functions in plants, including attracting pollinating insects; combating environmental stresses, such as microbial infection; and regulating cell growth. Six major subclasses of flavonoids, namely anthocyanidins, flavan-3-ols, flavonols, flavanones, flavones, and isoflavones; flavonols are the most widespread in the human diet. Aqueous and quath extraction shows presence of flavonoids by all the tests used. Methanolic extraction was shown presence of flavonoids only with lead acetate test. Tests for glycosides shows variable results and varies from test to test as well as extraction procedure and solvents. Cardiac glycosides are class of organic compounds that increase the output force of the heart and decrease its rate of contraction by acting on the cellular Na-K ATPase pump. Cardiac glycosides were present only in quath but absent in aqueous and methanolic extraction. Phenolic compounds are any compounds derived from the phenol group and contribute to the colour, structure, astringency, etc. Tannins are large molecular weight compounds resulting from polymerization reaction of smaller phenolic compounds. Tannins and phenolics were mostly positive with tests we applied. Saponin is present in all the extracts. Amino acids and proteins are absent in quath extraction. Ninhydrin tests show positive while Biuret test showed negative results with aquas and methanolic extraction. Terpenoids are present in aqueous and methanolic extraction. Further, steroids are present in all the extracts. Further, the antioxidant activities were observed in all the extract i.e. aqueous, quath, and methanolic extraction.

Table 1: Phytochemicals analysis of aqueous, quath and methanolic extracts of *S. oleracea* L. leaves

S. No.	Phytochemical Test	Quath	Aquoes (5 gm)	Aquoes (10 gm)	Methanolic
1.	Test for alkaloids				
	(a) Mayer's test	+ve	-ve	-ve	+ve
	(b) Wagner' test	+ve	+ve	+ve	+ve
	(c) Hager's test	+ve	+ve	+ve	+ve
2.	Test for carbohydrate				
	(a) Molisch test	-ve	-ve	-ve	-ve
	(b) Barfoed's test	-ve	-ve	-ve	-ve
3.	TEST FOR REDUCING SUGAR				
	(a) Fehling's test	+ve	+ve	+ve	-ve
	(b) Benedict's test	+ve	+ve	+ve	+ve
4.	TEST FOR FLAVONOIDS				
	(a) Alkaline reagent	+ve	+ve	+ve	-ve
	(b) Lead acetate	+ve	+ve	+ve	+ve
	(c) Ammonia test	+ve	+ve	+ve	-ve
5.	TEST OF GLYCOSIDES				
	(a) Borntrager test	+ve	-ve	-ve	+ve
	(b) Legal's test	-ve	-ve	-ve	-ve
	(c) 10% NaOH test	+ve	+ve	+ve	+ve
6.	TEST OF CARDIAC GLYCOSIDES				
	(a) Keller killani test	+ve	-ve	-ve	-ve
7.	TANNIN AND PHENOLIC TEST				
	(a) Ferric chloride test	+ve	-ve	-ve	+ve
	(b) Lead acetate test	+ve	+ve	+ve	+ve
	(c) Dilute iodine test	+ve	+ve	+ve	+ve
	(d) Ferric chloride 10%	-ve	-ve	-ve	+ve
	(e) Hydrolysable tannin	-ve	+ve	+ve	+ve
8.	TEST FOR SAPONIN				
	(a) Saponin test	+ve	+ve	+ve	+ve
9.	AMINO AND PROTEIN				
	(a) Ninhydrin test	-ve	+ve	+ve	+ve
	(b) Biuret test	-ve	-ve	-ve	-ve
10.	TERPENOIDS & STEROID	-ve	+ve	+ve	+ve
	(a) Test for terpenoids	+ve	+ve	+ve	+ve
	(b) Test for steroid				

"+" = Positive (Present); "-" = Negative (Absent)

Thin layer chromatography- The thin layer chromatography of sample showed different spots for methanolic, quath and auous extraction of *Spinacia oleracea*. In the methanolic and aqueous (10 gm) extraction of palak shows 6 spots having R_f 0.33, 0.44, 0.55, 0.66, 0.77, 0.83 and 0.16, 0.33, 0.35, 0.44, 0.55, 0.66 respectively. But in 5 gm aquous extraction only 4 spots revealed having R_f 0.38, 0.44, 0.55, 0.66 respectively (Fig. 1).



A. 5 gm & 10 gm Aqueous Extract **B.** Methanolic Extract

Fig. 1: TLC Plate showing different solvent extract of leaves of *S. oleracea* L.

DISCUSSION

The preliminary phytochemical analysis of *S. oleracea* revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolic compounds [16] but in our study alkaloids, reducing sugar, flavonoids, glycosides, tannins & phenolic compounds, saponin, amino acids, terpenoids and steroids were present because we applied more than one methods for phytochemical screening. Medicinal values of plants have assumed an important dimension in the past few decades. Plants produce a very diverse group of secondary metabolites with antioxidant potential. Antioxidants block the action of free radicals which have been implicated in the pathogenesis of many diseases and in the aging process [17-19]. An important role is being played by free radicals in governing the various biological processes which are necessary for the body. They have their role in implicating cell-signaling mechanism occurring in our body.

As prevention is a more effective strategy than treatment for chronic diseases, a constant supply of phytochemical containing plants with desirable health benefits beyond basic nutrition is essential in reducing the risk of diseases in humans. The importance of these phytochemicals is their presumed ability to inhibit carcinogenesis. They play a variety of roles such as antioxidants, inhibitor of tumor growth, anti-mutagens, enzyme modulators, chemical inactivators, and free radical scavengers [20]. Terpenoids reduce complications associated with diabetes and lowers the sugar level in blood [21]. Further, terpenoids have been found to be very useful in anti-aging and overall beauty enhancement. Hawkins and Ehrlich [21] reported that terpenoids have capacity to improve lung function in respiratory treatment. Cardiac glycosides showed to aid in treatment of congestive heart failure and cardiac arrhythmia.

Free radicals are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function but they are also involved in various diseases such as diabetes [22], rheumatoid arthritis [23-24], high blood pressure [25], urinary tract disorders [26], bronchial asthma [27,28] and non-healing wounds [29]. Free radicals can initiate the oxidation of bio molecules, such as protein, lipid, amino acids and DNA which will lead to cell injury and can induce numerous diseases [30]. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage and oxidative stress is the main cause of several diseases: cancer, cataracts, age related diseases and Parkinson's disease. Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases. This activity is due to the ability of antioxidants to reduce oxidative stress by neutralizing or scavenging of reactive species by hydrogen donation [31,32].

The natural drugs were always a better substitute for synthetic drugs. Thus numerous drugs have entered the I.P through ethno botany and traditional medicine. The medicinal value of a plant lies on bioactive phytochemical constituents that produce a definite physiological action on the human body. These phytoconstituents work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions. The most important of these bioactive constituents of plants are tannins, flavonoids, carbohydrates, glycosides, steroids, terpenoids, lignin's, and fats [33]. Phenolic compounds in general and flavonoids in particular have the ability to

provide protection against oxidative stress. Thus in this study, the presence of flavonoids and phenolic compounds in the extract could be considered responsible for conferring antioxidant ability.

CONCLUSIONS

Spinacia oleracea L. is a leafy vegetable that belongs to the goosefoot family. It has various pharmacological activities such as anti-oxidant, antiproliferative, anti-infammatory, antihistaminic, CNS depressant, protection against gamma radiation, hepatoprotective, etc. Phytochemical investigation of *S. oleracea* L. had shown the presence of Alkaloids, Reducing sugar, Flavonoids, Glycosides, Cardiac glycosides, Tannins, Saponin, Protein, Amino acid, Terpenoids and steroids etc. There was no effect of concentration on the phytochemical constituents. Mostly results are same in aqueous and quath extract so we concluded that there is no need to dry the green leaves. Result depends upon the solvent as well as the method of test we apply. Further, we observe the antioxidant activities in the extracts. Thus, *S. oleracea* merits further phytochemical, pharmacological and clinical investigations for the development of an effective natural remedy to provide therapeutically effective lead compounds or extracts. This vegetable can be used as a therapeutic and curative medicine for many oxidative stress-induced diseases.

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