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Original Article

ISOLATION AND PURIFICATION OF APIGENIN FROM *ALLIUM FISTULOSUM*

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ABSTRACT

Objective: The objective of this study is to optimize the extraction of apigenin (4′, 5, 7-trihydroxy-flavone) from *Allium fistulosum.*

Methods: The present study aims at extracting flavonoids from the plant using methanol as a solvent. Phytochemical screening was performed to analyse the phytochemical constituents present in the extract. Apigenin was isolated from the leaves of *A. fistulosum* and further purified and identified by TLC and HPLC.

Results: Phytochemical screening showed the presence of various constituents like terpenoids, tannins, flavonoids, reducing sugars, phenolics, cardiac glycosides and amino acids. TLC results indicated the presence of apigenin in the plant extract with the R_f value of 0.88, which coincided with the standard apigenin of R_f value 0.83. HPLC results showed a similarity in the peak of 17.1 min of the standard with the minor peak of 17.324 min of the sample. This proved the presence of apigenin in low quantities.

Conclusion: This present study suggests that *Allium fistulosum* can be a moderate source of apigenin. This compound was isolated for the first time from this plant.

Keywords: Apigenin, *Allium fistulosum*, Spring onion, Solvent extraction, Methanol, TLC, HPLC

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INTRODUCTION

*Allium fistulosum*is also called the Welsh onion. Commonly known as bunching onion, long green onion, Japanese bunching onion, and spring onion, is a species of evergreen plant growing throughout the year. Its odor and taste is very identical to Allium cepa. But *A. fistulosum*, neither does it shows bulb production nor possesses dense leaves, rather has characteristic hollow leaves and scapes. On the other hand, the Leeks and the Japanese Negi are comparatively bigger in size, while Chives are smaller. A very common vegetable in Asia, where there are many different cultivars, it is usually grown more on a garden scale in Europe and America, mainly for its edible leaves which can be produced throughout the winter if the weather is not too severe [1].

The therapeutic qualities attributed to the welsh onion are many, especially in Chinese medicine. It is used to improve the functioning of internal organs and metabolism and to prolong life. It is further reported to improve eyesight, to aid digestion and perspiration, and to enhance recovery from common colds, headaches, wounds and festering sores. A tea made from the roots is a children's sedative [2]. Use of the bulb in the diet impedes internal parasites. Externally, the bulb can be made into a poultice to drain pus from sores, boils and abscesses [3]. The juice of the plant is used as a moth repellent. The whole plant is said to repel insects and moles [4].

Among the 6000 distinct flavonoids present, quercetin, kaempferol, myricetin, apigenin and luteolin are the five most omnipresent plant flavonoids. Apigenin (4′, 5, 7-trihydroxy-flavone) is one of the prevalent monomeric flavonoids found in an everyday diet. In view of the substance's backbone structure, apigenin is a flavone, one of the subclasses of flavonoids. Apigenin has interested scientists because of its reduced toxicity and different valuable bioactivities. Apigenin is vastly spread in the plant realm, as it has been found in numerous vegetables, herbs, and organic products. Parsley, vine spinach, celery seed, green celery heart, Chinese celery, and dried oregano are dietary sources with high apigenin content. Other plants in which apigenin has been identified include red and white sorghum, rutabagas, oranges, kumquats, onions, wheat sprouts, tea, and cilantro [5]. In nature, apigenin is usually found in a glycosylated form, with the tricyclic core structure linked to a sugar moiety through hydroxyl groups (O-glycosides) or directly to carbon (C-glycosides). The common apigenin glycosides are apiin, apigenin-7-O-glucoside, apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), apigenin-7-O-neohesperidoside (rhoifolin), and apigenin-6-C-glucoside 8-C-arabinoside (schaftoside) [6].

In the colorectal cancer cell lines HCT116 and DLD1, apigenin up regulated BIM expression and down-regulated Mcl-1 expression, thereby synergizing with the Bcl-2 inhibitor ABT-263 to trigger mitochondrial-dependent cell apoptosis [7]. It is reported that apigenin exposure induced G2/M arrest in imatinib-sensitive K562 cells while arresting imatinib-resistant K562/IMA3 cells in S phase, especially at 100µ Mapigenin [8]. Chemotherapy drugs, such as cisplatin and paclitaxel are widely used in the clinic for cancer control. To enhance their antitumor effects and to minimize their limitation, co-administration with other targeted drugs has been widely tested and has achieved great success in clinical applications. Studies have shown that co-administration with apigenin significantly enhances the anti-cancer efficacy of chemo drugs and helps overcome their limitations in various types of cancers by targeting multiple signaling pathways [9].

The goal of this project was to identify and isolate the bioactive compound apigenin from the leaves of *Allium fistulosum.*

MATERIALS AND METHODS

Chemicals

Methanol, Fehling's A and Fehling's B solution, α-naphthol, Benedict's reagent, sodium hydroxide, copper sulphate, Nitric acid, Ninhydrin reagent, Dragendroff's reagent, ferric chloride, chloroform, sulphuric acid, glacial acetic acid, ammonia, isopropanol, ethanolic ferric chloride and benzene was procured from Fischer Scientific. TLC silica gel plate was of GF_{254}, which was procured from Merck.

Plant source

The leaves of Spring Onion (*Allium fistulosum*) were collected from Ulsoor market, Bangalore, Karnataka. India.

Extraction

The plant extract was prepared from the leaves of Allium fistulosum. The dust particles were removed by washing under tap water. The leaves of Allium fistulosum was dried at 60 °C in the hot air oven and

made it into fine powder using the mixer grinder. The coarse powder of Allium fistulosum [2.5g (w/v)] was extracted with 80% methanol using magnetic stirrer for 1hr and extract was obtained by squeezing through muslin cloth and used for further experimental analysis.

Phytochemical test

The fresh methanol extract was subjected to various phytochemical screening for the detection of various plant constituents, characterized for their possible bioactive compounds [10].

Test for carbohydrates

Fehling's test

The sample extract was treated with Fehling's A and Fehling's B solution and heated in a boiling water bath for 5-10 min at 60 °C. Appearances of reddish-orange precipitate indicate the presence for carbohydrates [10].

Molisch's test

The filtrate of the extract, which was diluted using water was treated with a few drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates [10].

Benedict's test

The filtrate of the extract was diluted using water and treated with Benedict's reagent and heated in a boiling water bath for 3-5 min. Orange-red precipitate indicates the presence of reducing sugars [10].

Test for proteins

Biuret test

Equal volume of 5% solution of sodium hydroxide and 1% copper sulphate was added to sample extract. Appearance of pink or purple colour indicated the presence of proteins and free amino acids [10].

Xanthoproteic test

The extract was treated with a few drops of conc. Nitric acid. Formation of yellow color indicates the presence of proteins [10].

Ninhydrin test

To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicates the presence of amino acid [10].

Test for alkaloids (Dragendroff's test)

A small portion of the sample extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was carefully tested with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids [10].

Test for tannis

Small quantity of sample extract was taken separately in water and tested for the presence of tannins with 5% ferric chloride solution. Appearance of violet color indicates the presence of tannins.

Test for flavonoids

On the addition of sodium hydroxide solution to the sample extract, blue or violet coloration indicates anthocyanins; yellow coloration indicates flavanones and yellow or orange coloration indicates flavonoids [11].

Test for terpenoids

To the sample extract, chloroform and concentrated sulphuric acid were added and checked for a reddish-brown coloration for the confirmation of terpenoids [11].

Test for steroids

Libemann-burchard reaction

Sample was mixed with chloroform. To this, acetic acid and few drops of concentrated sulphuric acid was added from the side of the test tube. First red, then blue and finally, green color should appear indicating the presence of steroids [10].

Test for glycosides

Cardiac glycosides (Keller Killani test)

To the extract, glacial acetic acid, ferric chloride and concentrated sulphuric acid were added. Appearance of reddish brown color at the junction of the two liquid layers indicates the presence of cardiac glycosides [12].

Anthroquinone glycosides (Borntrager's test)

To the extract, dilute sulphuric acid was added, boiled and filtered. To the cold filtrate, equal volume benzene or chloroform was added. The organic layer was separated and ammonia was added. Ammonical layer should turn pink or red for the presence of anthroquinone glycosides [10].

Test for phenols

Ferric chloride test

Extract was treated with few drops of ferric chloride solution. Formation of bluish-black color indicates the presence of phenols [10].

Partial purification of rutin and apigenin by thin-layer chromatography

TLC was carried out to segregate apigenin present inthemethanolic concentrate of *Allium fistulosum.* TLC was performed on a silica gel plate (silica gel GF254, Merck). 2-5 µl of different plant extract was deposited to the origins of TLC plates from the 1.5 cm of the origin with the help of capillary tubes.

Development of chromatogram

After the application of the sample on the plates, the plates were kept in TLC glass chamber (solvent saturated) then the mobile phase was allowed to move through the adsorbent phase up to 3/4th of the plate. Solvent system used was-Benzene/Acetic acid/water (125:72:3). TLC plates were visualized under ultraviolet light (λ 254 and 366 nm). Retention values of the different bands were then calculated using the equation:

$$R_f = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

Preparative thin-layer chromatography

Silica gel plate was used. The benzene/acetic acid/water (125:72:3) mobile phase solvent system was used for the separation of apigenin. The methanol extract from *Allium fistulosum* was deposited as a concentrated band 1.5 cm from the edge of its respective TLC plate and allowed to dry. The TLC plates were placed in the chromatographic chamber. Then the respective spots were scraped and further used for characterization using HPLC.

Identification and purification by high-performance liquid chromatography

The partially purified Apigenin from A. fistulosum was subjected to HPLC technique, which was equipped with the binary pump, UV-Vis detector and automatic column temperature control box. Apigenin: 20 µl of the sample was analyzed. The mobile phase was methanol-water-phosphate acid (30:69.3:0.7, v/v/v) with a flow rate of 1 ml min-1. The wavelength used was 360 nm and the run time was 30 min [13].

RESULTS AND DISCUSSION

In this study, apigenin was isolated from the leaves *A. fistulosum*. This plant is easily available in the market and it possesses wide range of phytochemicals and also for its enormous applications in ethnobotanical uses, pharmacological activities and other miscellaneous uses. Especially apigenin have enormous benefits in the medical field.

Phytochemical analysis

Methanoilc extract of *Allium fistulosum* was subjected to phytochemical and pharmacological activities. Their phytochemical

screening was performed which showed the presence of various constituents like terpenoids, tannins, flavonoids, reducing sugars, phenolics, cardiac glycosides and amino acids (table 1). The phytochemicals present in *A. fistulosum,* which revealed that the crude extract of *A. fistulosum* with ethanol indicated the presence of tannins, alkaloids, phenols, saponins, terpenoids and flavonoids whereas aqueous extract showed the presence of tannins, saponins, terpenoids, phenols but not flavonoids [14]. Therefore, the extraction of flavonoids from the plant source using organic solvent is more effective than inorganic solvents.

Table 1: Phytochemical screening of the plant samples

Phytochemical test	*Allium fistulosum*
Fehling's test	++
Molisch's test	-
Benedicts's test	+
Biuret's test	-
Xanthoproteic test	-
Ninhydrin test	++
Dragendroff's test	-
Test for Tannins	+
Teat for Flavonoids	+
Test for Terpenoids	-
Libemann-Burchard's test	-
Keller Killani's test	+
Borntrager's test	-
Test for Phenols	+

Analysis and Partial purification of the plant extracts by TLC

Thin-layer chromatography for the methanolic extract of *A. fistulosum*was performed. TLC profiling of this plant extract denoted an imposing result that guided towards the incidence of a number of phytochemicals (fig. 1). Different solvent systems confirmed the presence of diverse potent biomolecules in these plants. TLC investigation delivers important information about the polarity of the chemical constituents in such a way that compound displaying high R_f value in the less polar solvent system possess lesser polarity and the compound with less R_f value possess higher polarity. These potent biomolecules were further analysed using HPLC. The choice of the good solvent system for mobile phase is important. Combination and polarity of solvents should be used depending on the class of flavonoids targeted [15].

Qualitative analysis of *Allium fistulosum* extract was performed using TLC. Bright orange spot was observed under UV light, whereas yellow spot was observed under visible light was observed with an R_f value of 0.88, which coincided with the standard apigenin of R_f value 0.83, which confirmed the presence of Apigenin. Partial purification was carried out by preparative thin layer chromatography with the respective solvent system using preparative thin-layer chromatography. This method of isolation required less amount of solvent and we obtained apigenin in adequate quantity.

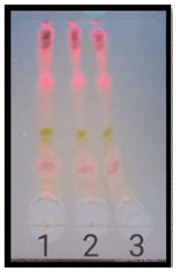

Fig. 1: TLC profiling of Apigenin under long UV (365 nm), Lane 1, lane 2 and lane 3–crude extracts of *A. fistulosum*

Purification and identification by HPLC

High-Performance Liquid chromatography was performed to identify the presence of the desired flavonoid compound apigenin. This can be done by comparing the retention time of the peaks with that of the standard compounds (fig. 2). Partially purified sample was subjected to HPLC for further purification and identification of Apigenin in *Allium fistulosum* extract (table 2). The HPLC system consisted of a binary HPLC pump and the separation was based on C-18 Dynamic column and the column was maintained at 25 °C.

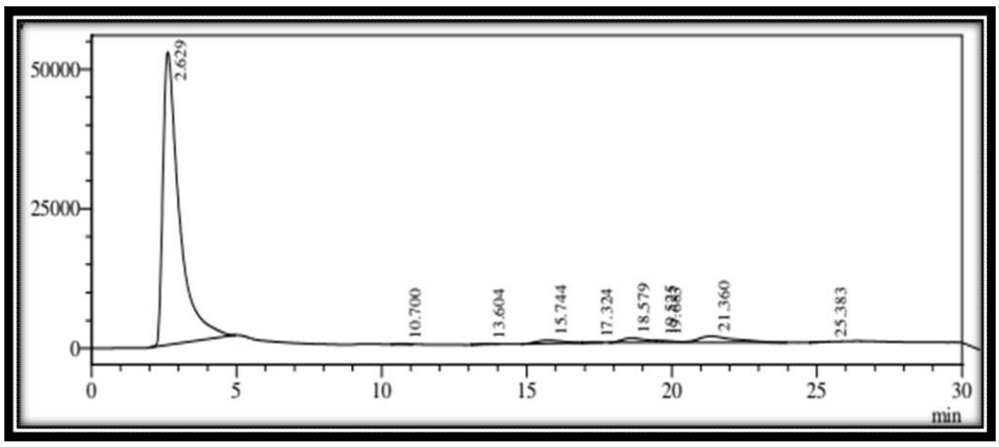

Fig. 2: HPLC Chromatogram of partially purified A. fistulosum

Table 2: HPLC profile of apigenin isolated from *A. fistulosum*

Detector A Ch 1 360 nm					
Peak#	**Ret. Time**	**Area**	**Height**	**Area %**	**Height %**
1	2.629	2065728	52510	90.845	93.612
2	10.700	4542	143	0.200	0.255
3	13.604	3511	110	0.154	0.196
4	15.744	36522	587	1.606	1.047
5	17.324	1369	55	0.060	0.097
6	18.579	46965	799	2.065	1.425
7	19.525	4219	377	0.186	0.672
8	19.683	9342	354	0.411	0.632
9	21.360	100532	1126	4.421	2.007
10	25.383	1172	32	0.052	0.058
Total		2273902	56094	100.000	100.000

The demonstrated HPLC chromatogram of the partially purified methanolic extracts of *A. fistulosum* determined the presence of apigenin. It showed a similarity in the peak of 17.1 min of standard with the minor peak of 17.324 min of the sample [13]. The samples also showed one major peak at 2.629 and 9 minor peaks at 10.700 min, 13.604 min, 15.744 min, 17.324 min, 18.579 min, 19.525 min, 19.683 min, 21.360 min and 25.383 min. This proved the presence of apigenin in low quantities along with other flavanoids in the extract [13].

CONCLUSION

The current study was focused at the isolation of apigenin from the leaves of *Allium fistuloum.* This study showed that their leaves contained different phytochemical constituents. Phytochemical screening of the methanolic extract showed the presence of various constituents like terpenoids, tannins, flavonoids, reducing sugars, phenolics, cardiac glycosides and amino acids, Apigenin was analysed and partially purified from the extract using TLC and preparative TLC method. This investigation using TLC showed the presence of apigenin and it was purified using HPLC technique. Apigenin is considered as an antioxidant compound and it is also commercially available but it is expensive. Allium fistulosum belong to the family Amaryllidaceae, identified as a moderate source of apinenin. Allium fistulosum is cheap and easily available in the market and the method of isolation required less amount of solvent. From this plant, we can obtain apigenin in low quantities but it is not very expensive.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

None

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