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Phytoceuticals in aniseeds: a practical approach for isolation and identification

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Abstract: Nature has provided mankind with a broad and structurally diverse array of pharmacologically active chemical compounds, phytoceuticals, which have proved to be indispensable for the cure of chronic diseases or as lead structures for novel therapeutic agents. Almost, 70% of modern medicines in India have been developed from plants used in the traditional system of medicine. With the technological advances and the development of more sophisticated isolation and analytical techniques, there is great scope for further systematic research to screen and isolate many more phytoceuticals which might be more effective/as effective as synthetic drugs and thereby assess their potential in protecting against chronic diseases. The present investigation is aimed at isolation and identification of phytoceuticals in a spice, aniseed (*Pimpinella anisum* L), therapeutically less-exploited and widely used only for culinary purpose, using analytical techniques viz. column chromatography, high performance thin layer chromatography (HPTLC.) and nuclear magnetic resonance spectrometry. Among all the solvent fractions of methanolic extract tested, methanolic extract and ethyl acetate fraction possessed highest amounts of bioactive compounds viz. phenolics, flavonoids, flavonols which was confirmed by qualitative, quantitative and HPTLC. analyses. Spectral analysis using NMR. of one of the sub-fractions of aniseeds obtained by column chromatography, revealed the presence of a glycosylated flavone, luteolin-6C-glucoside in aniseeds. The present study revealed aniseeds to be a great source of bioactive phytoceuticals which can be novel candidates for development of new therapeutic agents.

Key words: Aniseeds; phytoceuticals; flavonoids; HPTLC.; NMR; luteolin glucoside.

Introduction

Herbal medicines have been main source of primary healthcare in all over the world. From ancient times, plants have been catering as rich source of effective and safe medicines[1]. Plant kingdom represents an important source of sugars, minerals, organic acids, dietary fibre as well as chemical constituents such as polyphenols, alkaloids, flavonoids etc. which are synthesized as secondary metabolites and exist in plants in their biologically active forms. These non-nutritive biologically active chemical constituents are phytoceuticals which exhibit a wide range of pharmaceutical properties viz. antioxidant, antidiabetic, anti-inflammatory, anti-cancer properties[2].

Considering the importance of the healing property of the phytoceuticals, it has become necessary to build up a standard towards an integrated approach to extract, purify and characterize active compounds and ultimately to test their biological activities. Extraction of phytoceuticals is the first step in their utilization as dietary supplements, food ingredients, pharmaceutical and cosmetic products. Extraction of plant materials can be done both by conventional methods viz. maceration, percolation, digestion, hot continuous extraction (Soxhlet) and non-conventional methods viz. ultrasound, enzyme digestion, extrusion, ohmic heating, microwave heating etc.[3]. Successful determination of biologically active compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure and the choice of solvent depends on the targeted compounds to be extracted[4].

Purification of the biomolecules from the crude extract is an integral task as purified compounds are much more effective than the latter. Advances in biotechnology have opened up numerous possibilities for the purification and large-scale production of many biomolecules[5]. Since the plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, different types of chromatographic separations are used viz., paper, thin layer, column and high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC). Practically, most of the phytochemicals have to be purified by the combination of several chromatographic techniques and various other purification methods[6].

Once the compound is purified, it needs to be structurally determined. This involves accumulating data from a wide range of spectroscopic techniques viz. UV-visible spectroscopy, Infra-Red (IR), Nuclear Magnetic Resonance (NMR) which gives some basic clue regarding the structure of the molecule. Nature of the compound can be determined making use of UV-visible spectroscopy[7].

Aniseed (*Pimpinella anisum* L.) is an herb which has been in use in the traditional system of medicine in a variety of preparations. Aniseeds possess expectorant, antispasmodic, carminative, antiseptic, anti-microbial and parasiticidal properties. In traditional medicine, the seeds are used internally for bronchial catarrh, pertussis, spasmodic cough, and flatulent colic, and externally for pediculosis and scabies. Furthermore, it is used as an estrogenic agent. It increases milk secretion, and promotes menstruation[8, 9]. However, scientific information on antioxidant properties of spices like aniseeds, though widely used in culinary and medicine is still fragmentary. Since aniseeds have been used as spice in India since very early times, isolation of phytoceuticals will not only be useful but also helpful in documenting and authenticating the traditional wisdom.

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Materials and Methods

Procurement and preparation of sample

Aniseeds (*Pimpinella anisum* L.) were purchased in one lot, dried, powdered and were extracted with 80% methanol (Me), thrice (1:1, w/v) at room temperature[11]. The combined extract was concentrated in a vacuum evaporator. A part of methanolic extract was kept aside for investigation and was designated as methanolic extract (Me) and the rest of the extract was dissolved in water and fractionated successively with the solvents in the increasing order of polarity, viz. hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (nBu) and water (Aq) and each extract was evaporated to dryness[12]. The experimental design is depicted in (Figure.2).

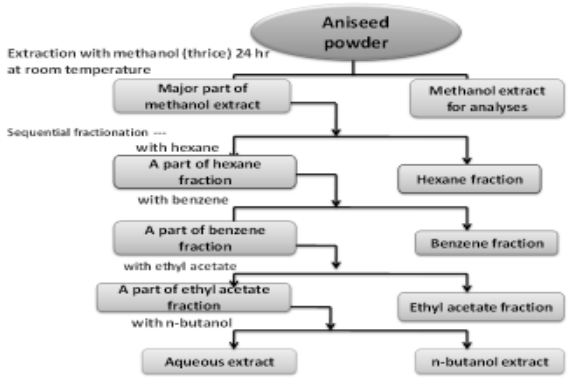

Figure 1: Preparation of fractions of methanolic extract of aniseeds (*Pimpinella anisum* L.)

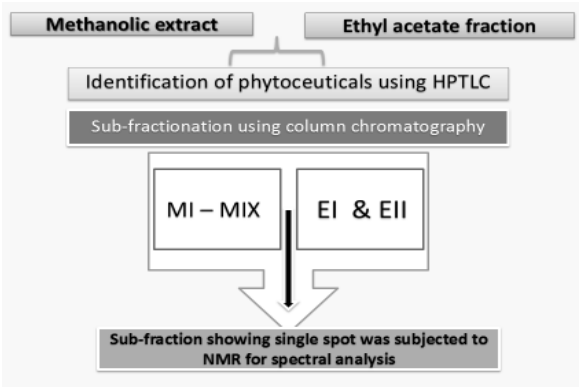

Figure 2: Flow chart depicting the present investigation

Phytoceutical screening

Qualitative analyses: Methanolic extract and the various fractions were qualitatively tested for the presence of carbohydrate derivatives[13], amino acids[14], flavonoids[15,16,17], tannins and phenolic compounds[15], steroids and terpenoids[18] and saponins[16].

Quantitative analyses: Methanolic extract and various fractions were examined quantitatively for total polyphenolics[19], total flavonoids [20] and total flavonols[21].

Identification of phytoceuticals

The multi-component methanolic extract and the ethyl acetate fraction with highest polyphenolic content (phenolics, flavonoids, flavonols) were subjected to high performance thin layer chromatographic analysis for identification of bioactive phytoceuticals.

High performance thin layer chromatographic screening was carried out using "CAMAG® Linomat V" sample applicator, "CAMAG®TLC 3" densitometric scanner and "CAMAG® WinCATs" software (CAMAG, Switzerland, Version 1.2.3). Methanolic extract and ethyl acetate fraction of aniseeds were concentrated by evaporating the solvent, made up to 10ml in a standard flask, 30 µl of samples were applied in a band-shape of 1cm on (E. Merck) aluminium plate pre-coated with silica gel 60 F254 of 0.2 mm thickness, and the TLC. plates were dried. Ethyl acetate: methanol: water (77:15:8) was used as the solvent system and the plates were run up to 8 cm. After air drying the plates were visualized in UV at 254 and 366 nm for the identification of the spots.

Sub-fractionation of methanolic extract and the best fraction

Sub-fractionation of methanolic extract and ethyl acetate fraction of aniseeds was carried out by column chromatography using various eluting systems, viz. I) hexane, II) hexane: chloroform (3:1), III) hexane: chloroform (1:1), IV) hexane: chloroform (1:3), V) chloroform VI) chloroform: ethyl acetate (3:1), VII) chloroform: ethyl acetate (1:1), VIII) chloroform: ethyl acetate (1:3), IX) ethyl acetate, X) ethyl acetate: methanol (3:1), XI) ethyl acetate: methanol (1:1), XII) ethyl acetate: methanol (1:3) and XIII) methanol gradient. Separation of bioactive compounds in methanolic extract and ethyl acetate fraction was carried out using column chromatography technique. Sub-fractions were collected in 20 ml portions and monitored on layer chromatography using methanol: chloroform (5%) as the mobile phase and the sub-fractions showing similar spots were combined. The sub-fraction showing single spot was subjected to nuclear magnetic resonance (NMR) spectrometry for spectral analysis.

Identification of phytoceuticals in the sub-fraction using nuclear magnetic resonance (NMR) spectrometry

The sub-fraction was concentrated in a vacuum evaporator, dissolved in dimethyl sulphoxide (DMSO) and used for analysis. The nuclear magnetic resonance (NMR) experiment was performed on a Varian NMR instrument using a Pro Star pump system, a Pro Star UV detector, a Unity INOVA 300 MHz NMR. spectrometer and a micro flow NMR probe. The probe has ^{1}H $\{^{13}C\}$ channels (^{1}H observed with ^{13}C decoupling) with pulsed-field gradient along z axis. For this experiment, 60µL of sample (dissolved in DMSO-d6) was used and the transfer time from the UV cell to the active volume was calibrated to be 21s at a flow rate of 1.0 ml/min. Proton NMR experiments were performed in 'stop-flow' mode, where the HPLC flow was halted after the sample elution fraction was transferred to the NMR. probe which was equilibrated at 25°C (NMR. Research Centre, Indian Institute of Science, Bangalore).

Results and Discussion

Qualitative analyses

Methanolic extract and all the fractions showed poor response to the tests for carbohydrate derivatives and amino acids. Among all the fractions, aqueous fraction showed the presence of moderate amount of carbohydrate derivatives and amino acids followed by ethyl acetate fraction and methanolic extract while hexane, benzene and n-butanol fractions did not show the presence of carbohydrate derivatives and amino acids. All the fractions showed positive response in terms of flavonoids, polyphenols, tannins, saponins and sterols. However, ethyl acetate fraction ranked first in terms of flavonoids, polyphenols and tannins, while hexane and benzene fractions showed highest amounts of sterols as shown in Table 1.

Table 1: Qualitative analysis of methanolic extract and various fractions of aniseeds for phytoceuticals

Test	Me	He	Be	Ea	n-But	Aq
Carbohydrates						
a. Molisch test	+	-	-	+	-	++
b. Fehling's test	-	-	-	-	-	+
c. Barfoed's test	-	-	-	-	-	-
d. Benedict's test	-	-	-	-	-	+
Amino acids						
a. Ninhydrin test	+	-	-	+	-	++
b. Hopkin's-Cole test	-	-	-	-	-	-
c. Erhlich's test	-	-	-	-	-	-
d. Pauly's test	-	-	-	-	-	-
e. Nitroprusside test	-	-	-	-	-	-
Flavonoids						
a. Sodium hydroxide test	++	+	+	+++	+	+
b. Sodium acetate test	++	+	+	+++	+	+
c. Sulphuric acid test	++	+	+	+++	+	+
Polyphenols and tannins						
a. Ferric chloride test	++	+	+	+++	+	+
b. Potassium dichromate test	++	+		+++	+	+
c. Potassium ferricyanide test	++	+		+++	+	+
Saponins						
a. Foam test	+	++		++	+	+
Sterols						
a. Leibermann Buchard's test	+	+++		+	+	+
b. Salkowski test	+	++			-	+

Me- Methanolic extract, He- Hexane, Be- Benzene, Ea- ethyl acetate, n-But- n-Butanol, Aq- Aqueous

Quantitative analyses

Phytoceuticals viz. total phenolics, total flavonoids, total flavonols estimated in the methanolic extract, hexane, benzene, ethyl acetate, n-butanol and aqueous fractions of methanolic extract of aniseeds are presented in Table 2.

Table 2: Phytoceuticals in methanolic extract and various fractions of aniseeds

Sample extract	Total phenolic (mg/100g) GAE	Total flavonoids (mg/100g) RE	Total Flavonols (mg/100g) RE
Methanol	0.63±2.3	0.32±0.9	0.47±1.9
Hexane	0.16±2.9	0.20±0.5	0.24±0.5
Benzene	0.23±0.6	0.27±1.4	0.34±0.3
Ethyl acetate	0.77±0.3	0.45±1.6	0.55±0.1
n-butanol	0.42±1.7	0.15±1.1	0.41±0.9
Aqueous	0.15±2.1	0.07±1.7	0.08±1.4

Values are mean ± SEM of three replicates

Among all the fractions, ethyl acetate fraction of aniseeds possessed highest amounts of polyphenolic compounds (polyphenols, flavonoids and flavonols) followed by methanolic extract, n-butanol, benzene, hexane and aqueous fractions and hence, ethyl acetate fraction was tested for the antioxidant and antidiabetic activities and compared with the activities of multi-component methanolic extract.

High performance thin layer chromatography

High performance thin layer chromatography analysis performed for methanolic extract(multi-component) and ethyl acetate fraction of aniseeds for the identification of individual bioactive phytoceuticals present in them generated useful information by means of development of spots in the chromatograms which were visualized in the UV chamber (Figure. 3).

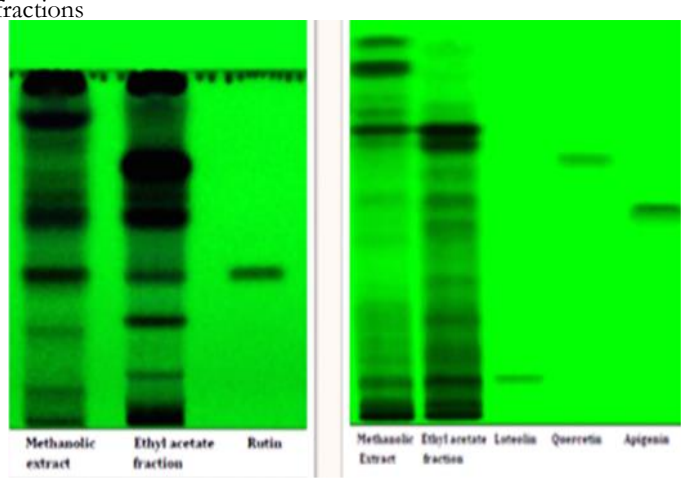

Figure 3: HPTLC fingerprint profile of standards rutin, quercetin, luteolin and apigenin, methanolic extract and ethyl acetate fraction

The chromatograms revealed the presence of many phenolic compounds, out of which apigenin, luteolin, quercetin and rutin could be identified in the methanolic extract and ethyl acetate fraction of aniseeds.

Sub-fractionation of methanolic extract and ethyl acetate fraction

Totally about 50 sub-fractions were collected from methanolic extract and ethyl acetate fractions. Among all the sub-fractions collected from methanolic extract, concentrated sub-fractions were designated as M1-M9 and similarly the sub-fractions of ethyl acetate were designated as E1 and E2. Among the sub-fractions, E1was obtained from ethyl acetate: methanol (3:1) solvent system of column chromatography as a pure compound, detected by TLC using methanol: chloroform (5%) as mobile phase, green in colour and was found to be soluble in chloroform, ethyl acetate and methanol. E1 compound was isolated in workable quantities from the ethyl acetate fraction of methanolic extract of aniseeds and was subjected to NMR for spectral details.

Nuclear magnetic resonance (NMR) spectrometry

^{1}H NMR (400 MHz, DMSO-d6) δ: 7.44 (1H, d, J = 8 Hz, 6'-H), 7.41 (1H, d, J = 2.0 Hz, 2'-H), 6.93 (1H, d, J = 8.6 Hz, 5' -H), 6.54(1H, S, 3-H), 4.79 (1H, d, J = 10.0 Hz, 1"-H).

^{13}C NMR (400 MHz, DMSO-d6**)** δ: 161.57 (C-2), 102.97 (C-3), 159.6 (C-5), 127.29(C-6), 156.5 (C-9), 103.6(C-10), 121.4 (C-1'), 112.90 (C-2'), 116.29 (C-5'), 72.37(C-1"), 70.16 (C-2"), 78.90 (C-3"), 70.10 (C-4"), 61.17 (C-6").

Structural analysis of E1 sub-fraction was done using nuclear magnetic resonance spectrometry.^{1}H chemical shifts for the A, B and C rings are around 7.6ppm for flavones, one of the classes of flavonoids. For glycoside flavones additional peaks are observed between 3-5ppm. Both these features were observed in the ^{1}H NMR spectrum in the present analysis (Figure. 4). Presence of a glycoside flavone was confirmed. ^{13}C spectrum of flavones show peaks at 107, 162 and 177ppm for the C ring. The peaks for the B ring falls between 126 - 132ppm. Peaks at 156 ppm and 134 ppm are characteristic to A ring along with peaks in the range of 118 -125 ppm. All these peaks and the peaks between 60-70 ppm for the glycoside linkage in the ^{13}C spectrum (Figure. 5) further confirmed the presence of glycoside flavone. Further analysis of the ^{1}H NMR revealed characteristic peaks at 6.8ppm and 7.2ppm for luteolin. This is also confirmed by ^{13}C spectrum where characteristic peaks for luteolin at 161ppm, 164ppm and 112ppm are seen.

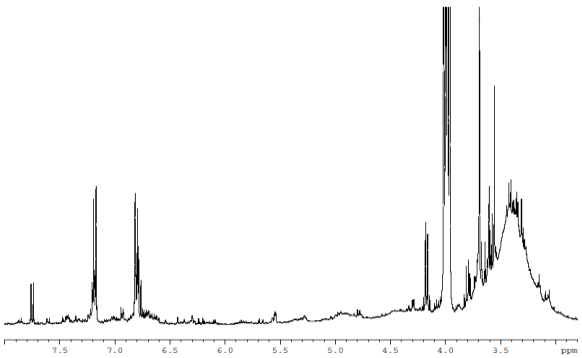

Figure 4: ^{1}H spectrum of E1 compound

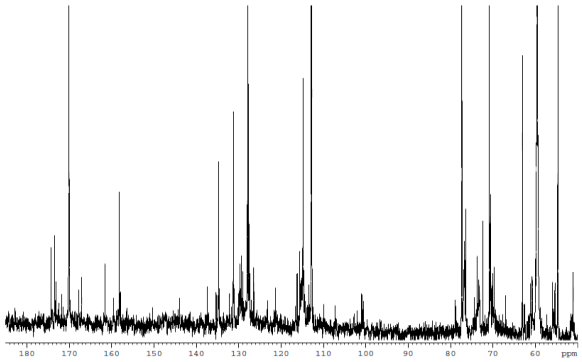

Figure 5: ^{13}C spectrum of E1 compound

Therefore, E1 sub-fraction of ethyl acetate fraction is identified to be a glycosylated flavone, luteolin-6-C-glucoside also called as isoorientin. The chemical structure of luteolin-6-C-glucoside is depicted in Figure. 6.

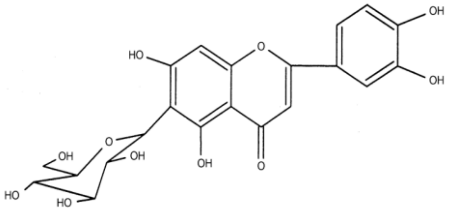

Figure 6: Chemical structure of luteolin-6-C-glucoside

Conclusion

Methanolic extract of aniseeds and the fractions possessed phenolic compounds, flavonoids and flavonols. Phenolic compounds viz. apigenin, luteolin, rutin and quercetin were identified by high performance thin layer chromatography in both methanolic extract and ethyl acetate fraction. One of the sub-fractions of ethyl acetate fraction is identified as glycosylated flavone, luteolin glucoside. Aniseeds possessing varied phytoceuticals can be useful in developing new products in pharmaceutical, cosmetic and food industries.

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