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RESEARCH ARTICLE

EXTRACTION AND PURIFICATION OF PHENOLICS FROM *STAR ANISE*

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ABSTRACT

Star anise (*Illicium verum*) is an aromatic evergreen tree bearing purple-red flowers and anise-scented star-shaped fruit. It grows almost exclusively in Southern China and Vietnam. It's fruit is an important traditional Chinese medicine as well as a commonly used spice. It carry chemical compounds that have known to be antioxidants and have disease preventing and health promoting properties. It's application is in many traditional medicines such as antispasmodic, digestive stimulant and toxic agent. The main objective of the work is to characterize star anise to assess the presence of its vital phenolic components with medicinal properties. Star anise was extracted using different solvents such as aqueous, buffer and organic solvents to estimate the presence of phenols, proteins and carbohydrate. It was observed that the organic solvent extracts of red star anise in acetone and methanol gave a maximum yield when compared to other extracts and also black star anise. Further the phenolic components were purified by adsorption column chromatography and it was found that the methanol extract of red star anise contained higher amounts of phenolics. The presence of phenols was confirmed by thin layer chromatography which showed that the acetone and methanol extract of both the red and black star anise contained higher concentration of phenols compared to other extracts. Further, methanolic extracts were partially purified by column chromatography and the eluents from it were characterized on thin layer chromatography.

Key words: Star Anise, Proteins, Phenolics, Antioxidants, Thin Layer Chromatography.

INTRODUCTION

Star anise (*Illiciumverum*) is an evergreen tree produces unique star-shaped fruits with five to ten boat-shaped sections radiating from the center, tough skinned and are rusty in color. Its fruit is an important traditional Chinese medicine as well as a commonly used spice (Loi, 1970). *I.verum* is a spice that closely resembles anise in flavor, obtained from the star-shaped pericarp. It is native to Southern China and Northern Vietnam and is grown almost exclusively in Southern China, Indochina and Japan. It has similar flavor and taste like that of anise seed (*Pimpinella anisum L)*. It is a tree living in cooler tropics and sub tropics. The spice was first introduced into Europe in the 17th century. The plant belongs to the genus of the family Illiciaceae, order Illiciales, subclass Magnoliidae, class Magnoliopsida. It is a small to medium-sized evergreen tree reaching upto 8m (26 ft.) in height. The trees have evergreen, aromatic leaves and bisexual flowers. The leaves are lanceolate and the axillary flowers are yellow, the female portion of the flower consists of 7–15 carpels (Rosengarten, 1969). The fruits are star-shaped, reddish-brown, consisting of 6–8 carpels arranged in a whorl. Star anise is a plant used in phytotherapy as well as for aromatization of pharmaceutical products, foods and cosmetics, and its usage has a long tradition. The fruits are commonly used as a spice and pharmaceutical treatment for flatulence, spasmodic pain and colics.

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The oil of star anise is employed topically to treat rheumatism and otalgia, and is also used as an antiseptic (De *et al*., 2002). Star anise contains anethole, an ingredient responsible for its characteristic flavour, is the primary precursor for anti influenza drug Tamiflu. Phenolic compounds are ubiquitously distributed throughout the plant kingdom (Naczk *et al*., 2004). In previous study, it has documented that *I. verum*has Phenolic phytochemicals which are known to exhibit several health beneficial activities such as antioxidant, antiinflammatory, antihepatotoxic, antitumor, and antimicrobial (Middleton *et al*., 2000). Thereby, the main objective of the study is to compare and characterize red and black variety star anise and to assess the presence of the vital phenolic components with medicinal properties.

MATERIALS AND METHODS

Chemicals

Sodium hydroxide, HCl, Sodium bicarbonate, Sodium potassium tartarate, methanol, ethanol, Phosphoric acid, Acetone, Anthrone, Bovine serum albumin (BSA), Bromine water, Copper sulphate, phenol, Dinitrosalicylic acid, Folin-Ciocalteu reagent, H_2SO_4 , Lithium sulphate, Phenolphthalein reagent, Sodium carbonate, Sodium molybdate, Sodium sulphite, Sodium tungstate, catechol, glucose, Tris (pH 7.0).

Sample Collection

The fruits of star anise (both red and black variety) were collected using random sampling technique (RST) from local

areas of Bangalore district, Karnataka State, India. The fruits were dried in the sunlight for 24 hrs. After removing immature and damaged fruits, the matured fruits were washed under tap water, dried, powdered and stored in refrigerator until further use.

Sample extraction

A 20% extract of star anise was prepared in distilled water, tris buffer of pH 7.0, 80 % aqueous solutions of methanol and acetone. The sample was extracted for 60 mins on a magnetic stirrer, centrifuged at 27˚C for 15 mins at 6000 rpm and the supernatants were collected and used for the estimation of proteins, carbohydrates and phenols.

Estimation of biomolecules by colorimetric method

Estimation of biomolecules such as protein, carbohydrate and phenols were analyzed by colorimetric method.

Estimation of proteins

The estimation of proteins was carried out by Lowry's method (Lowry *et al*., 1951). Protein is expressed as bovin serum albumin equivalent in gm / 100 gm dry weight of sample.

Estimation of total soluble sugars

The estimation of total soluble sugars was carried out by Yemm E.W and Willis A.J. method (Yemm and Willis, 1954). Total soluble sugars is expressed as glucose equivalent in gm / 100 gm dry weight of sample.

Estimation of reducing sugars

The estimation of reducing sugars was carried out by Nelson Somogoyi method (Nelson, 1944). Reducing sugar is expressed as glucose equivalent in gm / 100 gm dry weight of sample.

Estimation of phenols

The estimation of phenolics was carried out by Bray and Thorpe method (Bray and Thorpe, 1954). The phenolics is expressed as catechol equivalent in gm / 100 gm dry weight of sample.

Purification of Phenols using adsorption gel column chromatography

The partial purification of phenolics was carried out using open glass column (2.5 x 21) cm filled with silica gel G-200 special for column chromatography. The column was washed with 5% methanol solution to pre-equilibrate the column followed by elution buffer consisting of 5% methanol solution. The flow rate of elution buffer was set to 1 ml/ 20 min with the help of flow rate adjuster to force the elute out of the column. 100 µl of the red sample methanol extract was added onto the column and allowed to run down the column with the help of mobile phase. The eleunts were collected in 10 appendoff tube each consisting an eluent volume of 1 ml for each sample extract. These eluents were stored at 4˚C until use.

The eluents collected from gel column were analyzed by UV-Vis spectrophotometer for phenols at absorbance of A_{274} and for proteins at absorbance of A_{280}. Similarly the partial purification of phenolics from black sample methanol extract was carried out as described above. The eluents were collected for each of red methanol and black methanol star anise sample and tested on TLC plates for the presence of phenols.

Determination of maximum absorbance of Phenols

The black and red methanol eluent sample collected from adsorption gel column chromatography were used for estimation of maximum absorbance of phenols and the eluent containing the highest concentration of phenols in both the sample were analyzed between the range of 260 nm to 400 nm for maximum absorbance.

Characterization of phenols using Thin Layer Chromatography

TLC plate of suitable size were chosen and samples loaded were of eluents obtained from column chromatography. Developed TLC plate was placed into a developing chamber containing solvent system of acetone and chloroform in the ratio of 1:5 for development. Plate was observed under UV-transilluminator.

RESULTS AND DISCUSSION

COMPARISON OF PROTEIN CONTENT IN RED AND BLACK SAMPLE

The protein content of the samples were estimated using Lowry method[6]. Figure 1 illustrates that there is a significant increase in the protein content of the red star anise when compared to black star anise. Different aliquots such as 0.1 and 0.4mL of the sample were used for the assay which showed similar results. The highest yield of protein content was observed in acetone extract of *Illicium verum* . The protein concentration in acetone extract was found to be 7.7 mg/g and 6.3 mg/g of the red star anise and black star anise respectively. The lowest yield of protein content was found in aqueous extract of *Illicium verum* when compared to buffer extract and organic solvent extract. We found relevant results when compared to a study where star anise was used to estimate the protein content in proximate analysis of *I.verum*was found to be 4.25mg/g (Madhu *et al*., 2014). We compared our results to study where star anise was subjected to different processing methods of analysis where protein concentrations was found to be 1.34 mg/g in boiling water extract of star anise. Here, we found that the protein concentration is much higher in case of water extract maintained at room temperature than water extract at boiling temperature (Dinesha *et al*., 2014).

COMPARISON OF TOTAL SOLUBLE SUGARS IN RED AND BLACK SAMPLE

The estimation of total soluble sugars of the samples was carried out by the anthrone method (Yemm, 1954). The figure 2 illustrates that there is a highest yield of the total soluble sugars in the sample having acetone extract as compared to the other extracts.

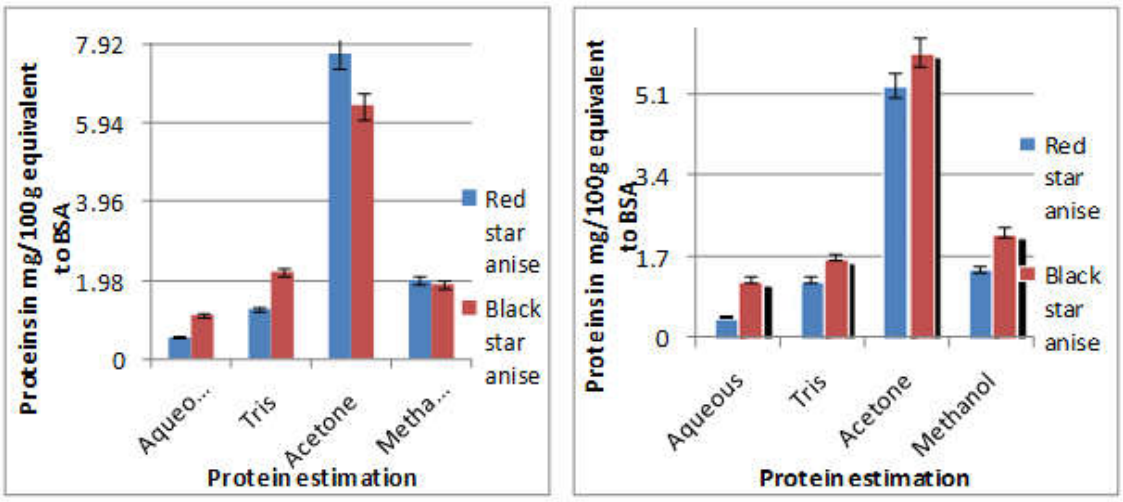

Figure 1. Protein content in red and black sample. Each experiment was performed in duplicates and the resultsare presented as mean ± SE

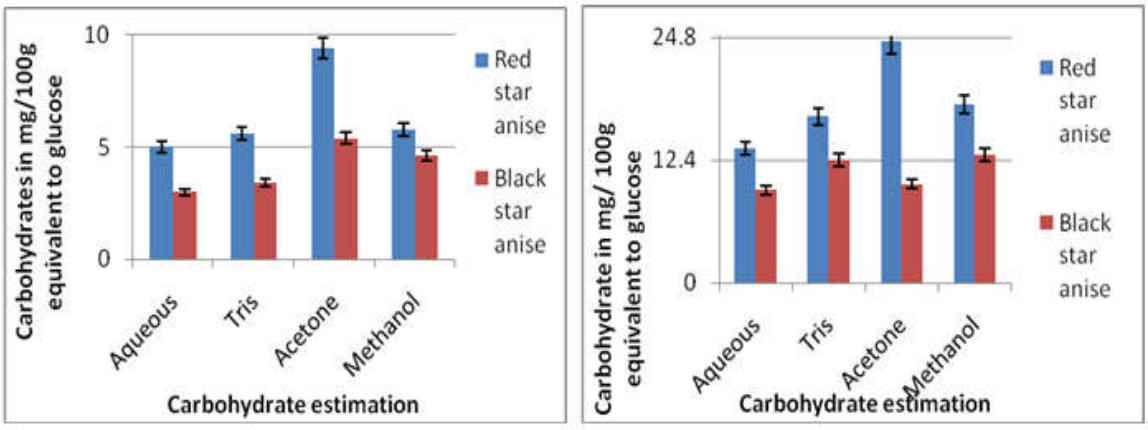

Figure 2: Carbohydrate content in red and black sample
Each experiment was performed in duplicates and data were expressed as mean±SE

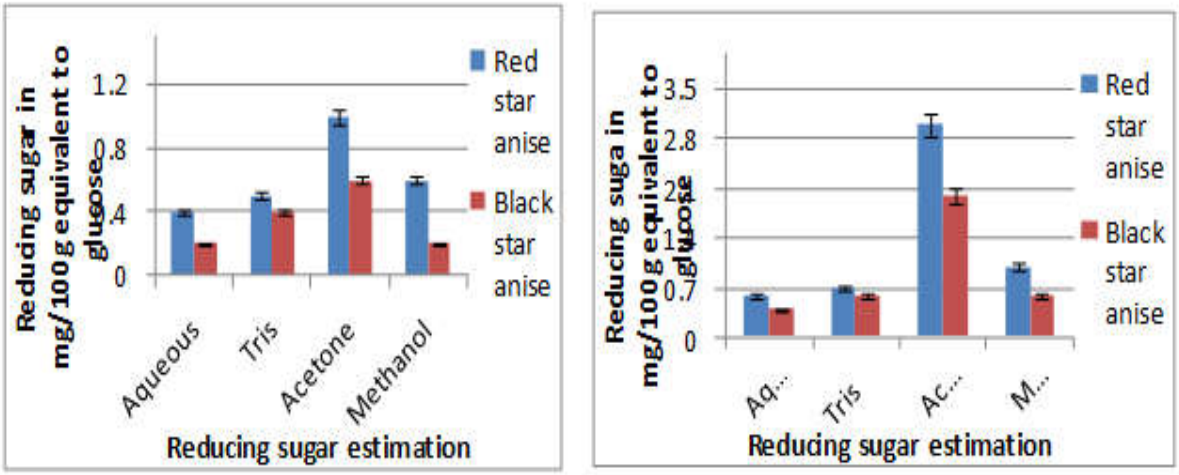

Figure 3: Reducing sugar content in the red and black sample.
Experiment was performed in duplicates and data were expressed as mean±SE

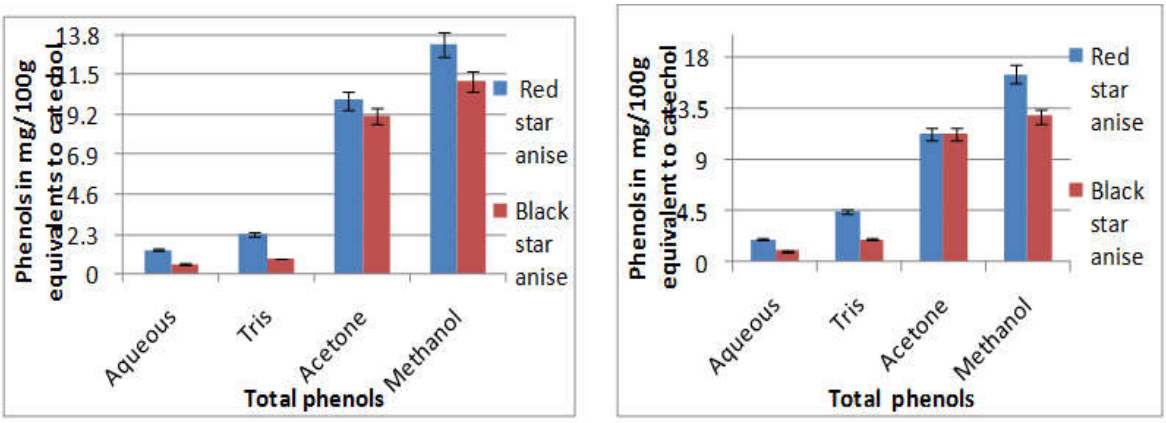

Figure 4: Phenol content in red and black sample. Each experiment was performed in duplicates and the results are presented as mean ± SE

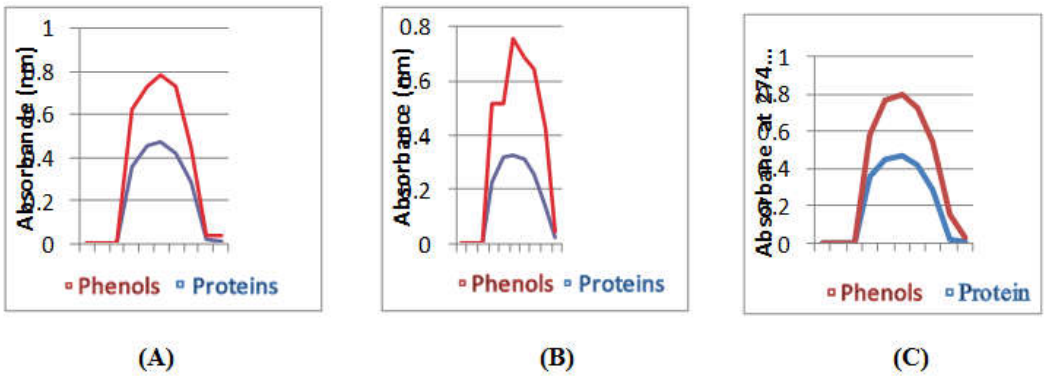

Figure 5: Purification by adsorption chromatography. (A) Amount of phenols and proteins in red methanol at A_{274} and A_{280} respectively (B) amount of phenols and proteins in black methanol at A_{274} and A_{280} respectively (C) comparison of phenols in red and black methanol sample at A_{274}

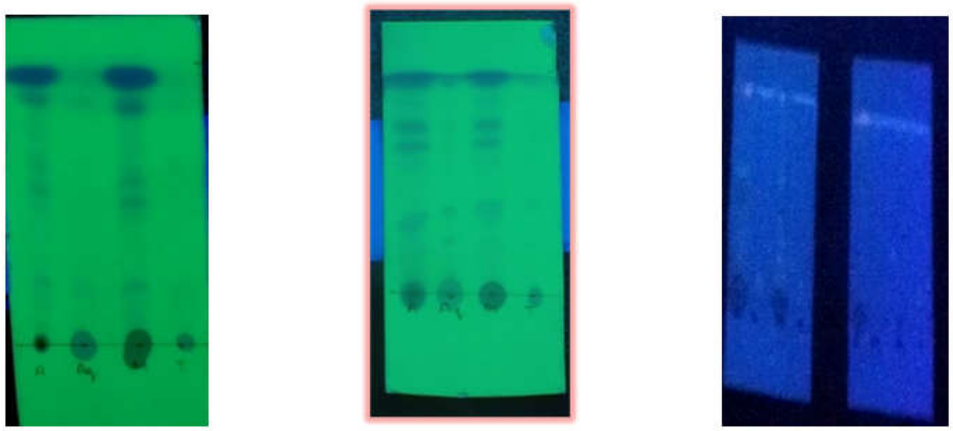

Figure 6. Thin layer chromatography.(A) 20% crude extracts of red sample (B) 20% crude extracts of black sample (C) TLC chromatogram visualised under short UV wavelength

Different aliquots such as 0.1and 0.4 mL of the sample were used for the assay which showed similar results. The total soluble sugar concentration in acetone extract was found to be 7.20 mg/g and 5.60 mg/g of the red star anise and black star anise respectively.

The above histogram depicts that there is significant increase in total soluble sugar content of the red star anise compared to black star anise. The lowest yield of total soluble sugar content was found in aqueous extract of *Illiciumverum* when compared to buffer extract and organic

solvent extract. We found relevant results when compared to a study where star anise was subjected to different processing methods which showed 54.2 mg/g in ambient temperature water extract of star anise and 50.2 mg/g in boiling water extract[11].When compared our results to the study worked on proximate analysis of the *I. verum,* it showed relevant results of carbohydrate content found to be 75.0 mg/g (Madhu *et al*., 2014).

COMPARISON OF REDUCING SUGAR IN RED AND BLACK SAMPLE

Estimation of reducing sugars of the samples was done by the dinitrosalicylic acid method (Nelson, 1944). Figure 3 illustrates that there is a significant high yield in reducing sugars in the samples having acetone extract as compared to the other extracts. Different aliquots such as 0.1 and 0.4 mL of the sample were used for the assay which showed similar results. The reducing sugar concentration in acetone extract was found to be 3.40 mg/g and 1.80 mg/g of the red star anise and black star anise respectively. The highest concentration of reducing sugar was observed in the red sample than black sample. The lowest yield of reducing sugar content was found in aqueous extract of *Illiciumverum* when compared to buffer extract and organic solvent extract data were expressed as mean±SE. We compared our results to the study conducted on star anise showed increase in reducing sugar content after being subjected to various processing methods. It was found to that reducing sugar content is higher in case of water extract of star anise maintained at room temperature 3.63 mg/g then 1.43mg/g boiling water extract of star anise (Dinesha *et al*., 2014).

COMPARISON OF PHENOLS IN RED AND BLACK SAMPLE

Phenolics are the most abundant secondary metabolites of plants with more than 8000 known structures ranging from simple compounds such as phenolic acids to complex structures such as tannins (Antolovich *et al*., 2002; Dai *et al*., 2010) . In plants accumulation of phenolics varied from one part to another part and also depended on the age and developmental stage of the concerned plant parts. It is well known that the phenolics extracts are always a mixture of different classes of phenols, which are selectively soluble in the solvents. A phenolic –OH group is very reactive and can easily form hydrogen bonds with the active sites of enzymes (Rasooli *et al*., 2009). The phenol content in the red and black sample of *Illicium verum* were analysed by Folin- Ciocalteu method (Bray, 1954). The figure 4 illustrates that there is a significant increase in the phenolic content of red star anise when compared to black star anise. The highest yield of phenol content was observed in methanol extract of *Illiciumverum*. The phenolic concentration in methanol extract was found to be 20.40 mg/g and 12.80 mg/g of red star anise and black star anise respectively. In a study of star anise it was found that the total phenolic content of methanol extracts was 96.3 mg gallic acid equivalent/g (Soher *et al*., 2016). When we compared our results to above study, there is a reduction of phenolic concentration.

Recent studies have also shown that many polyphenols contribute significantly to the total antioxidant activity in fruits and vegetables (Luo *et al*., 2002). It was reported that higher phenolics compounds in star anise is important for building blocks for cell wall structures, serving as a defence against pathogens[15]. The content of phenolic compounds (mg/g) in methanol extract of microwave extract of star anise (MSA) and soxhlet extract of star anise (SSA) was found to be 271 mg/g and 289 mg/g plant extract and expressed in gallic acid equivalents. These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components (Kareti Srinivasa *et al*., 2012). Thereby we can conclude that Illicium verum has high antioxidant properties. in duplicates and the results are presented as mean ± SE.

PARTIAL PURIFICATION OF PHENOLICS

ADSORPTION GEL COLUMN CHROMATOGRAPHY

Partial purification was carried out by adsorption gel column chromatography. The figure 5 depicts that phenols are present in higher amount than proteins. The above histogram also illustrates that red methanol extract has high concentration of phenols than black methanol extract. Thus the phenols were eluted immediately after the void volume in 4th eluent.

CHARACTERISATION OF PHENOLICS

THIN LAYER CHROMATOGRAPHY

TLC chromatogram visualised under short UV wavelength Characterisation of phenolics was carried out by thin layer chromatography. The above figure 6 indicates the presence of phenols in both 20% of red and black crude extracts. The presence of polyphenol compounds in the extract was evidenced by the intense fluoresecene produced under short UV light (Figure 6 C). The results of the TLC showed the extracts of acetone and methanol contained higher concentration of phenol compound compared to other extracts. In a study where star anise was subjected to different methods for TLC, the results showed that the fractions of ethyl ether and ethyl acetate contained higher concentration of flavonoid compound compared to other extracts while our present study, methanol extract contained higher concentration of phenolics (Cheng-Hong Yang et al., 2012). The pictures of the results obtained were fluorescent bands and were difficult to picturise.

Conclusion

Star anise is a rich source of phenolic compounds which are known to exhibit several health beneficial activities. The highest yield of phenolics in star anise was exhibited by organic solvent such as methanol. The phenolic components were purified by adsorption column chromatography and it was confirmed by thin layer chromatography where we found the phenolic presence in red star anise is higher than that of the black variety of star anise. As a result, *Illicium* plants should be explored further as an alternative source of medicine.

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