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Research Article

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Phytoconstituents and lipoxidase and xanthine oxidase inhibitory effects of methanolic extract of aniseeds (*Pimpinella anisum* L.)

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ABSTRACT

Epidemiological evidences suggest that high intake of plant foods is associated with lower risk of chronic diseases. In recent years, secondary metabolites such as polyphenols, flavonoids, flavonols etc. extensively present in a wide range of plant foods are identified to have different biological roles. The molecular mechanisms involved in the anti-inflammatory activity of flavonoids include inhibition of pro-inflammatory enzymes viz. cycloxygenase (COX), lipoxidase (LOX), nitric oxide synthase (NO synthase), xanthine oxidase(XO) etc. In the present study, influence of the methanolic extract and solvent fractions (hexane, benzene, ethyl acetate, n-butanol and water) obtained by sequential fractionation of methanolic extract of aniseeds (Pimpinella anisum L.) on the activities of inflammatory enzymes viz. lipoxidase and xanthine oxidase were evaluated at 100-500 µg/ml. Ethyl acetate fraction exerted maximum inhibition on the activities of soyabean lipoxidase (IC_{50}-95µg/ml) and xanthine oxidase (IC_{50}- 260 µg/ml) as compared with methanolic extract and the other fractions indicating very effective anti-inflammatory activity attributable to the phenolic compounds viz. flavonoids, flavonols extracted in to ethyl acetate fraction from the methanolic extract of aniseeds. Hence, aniseeds containing a large amount of phenolic compounds can be useful as therapeutic agents.

Keywords: *Aniseeds (Pimpinella anisum L.), anti-inflammatory activities, ethyl acetate fraction, benzene fraction, flavonoids.*

INTRODUCTION

Reactive oxygen species (ROS) are generated in living organisms through many pathways[1]. Accumulation of ROS in aerobic organisms is known as an exacerbating factor in cellular injury and in the ageing process[2]. An efficient way to combat health risks is to balance these ROS by the consumption of foods rich in polyphenolic compounds which delay or prevent the oxidation of cellular oxidizable substrates by direct radical scavenging action or indirect antioxidant action, such as inhibition of ROS-producing enzymes (xanthine oxidase, lipoxygenase etc.)[3]. Phenolic compounds such as flavonoids and phenolic acids, reported to be the most effective are found in many plants, particularly in leaves, fruits, seeds and spices. On account of high content of phenolic compounds, fruits, vegetables, spices etc. have been recognized to have medicinal properties e.g. digestive stimulation action, antioxidant, anti-inflammatory, antimicrobial, hypolipidemic and antimutagenic effects and thereby possess beneficial impact on health[4]. Aniseed *(Pimpinella anisum* L), commonly known as 'saunf', is one of the oldest spice plants with great potential to accrue health benefits due to the presence of considerable amounts of phenolic compounds[5] that possess varying degrees of antioxidant activity[6]. However, very few reports are available on the medicinal properties of aniseeds. In order to study the potential of the phytochemical compounds present in the herbs and spices, it is necessary to extract them from the source prior to the analysis. Extracts of plant materials are always a mixture of different classes of phenolics that are soluble

in the solvent system used[7]. The main objective of this work was to investigate the anti-inflammatory activity of methanolic extract and various solvent fractions of methanolic extract of aniseeds.

MATERIALS AND METHODS

Preparation of aniseed extract

Aniseeds (*Pimpinella anisum* L.) purchased in one lot from local market were shade dried, powdered and extracted with 80% methanol (Me), thrice (1:1, w/v) at room temperature[8] (Petra et al., 1999). The combined extract was concentrated in a vacuum evaporator and the residue was dissolved in water and fractionated successively with hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (Nb) and water (Aq) and each fraction was evaporated to dryness and before use, each fraction was dissolved in various solvents as required to obtain concentration of 1mg/ml[9].

Phytochemical screening

Total Polyphenolics

The total phenolic content was determined by the Folin–Ciocalteu (FC) method[10]. To 0.5ml of various fractions and methanolic extract of aniseeds, 3.16 ml of distilled water, 0.2ml of FC reagent were added, after 5 min., 0.6ml of 20% sodium carbonate solution was added, mixed and left at room temperature for 2hrs. and absorbance was read at 765 nm using Cyberlab double beam spectrophotometer. Gallic acid (50-500µg/ml) was used as a standard and treated in a similar way as the test. Polyphenolic content was expressed as grams of gallic acid equivalents per 100 g extract from the calibration graph.

Total flavonoids

The flavonoid content of various fractions of methanolic extract of aniseeds was estimated using aluminium chloride ($AlCl_3$)[11]. To 0.5ml of various fractions and methanolic extract of aniseeds, 1.5ml of methanol, 0.1ml of aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water were added, kept at room temperature for 30 min and the absorbance was measured at 415nm. Rutin used as standard (100-500µg/ml) was treated in a similar way as the test and sample concentration was intercepted from the calibration curve.

Total flavonols

The total flavonol content was determined using aluminium chloride[12] with some modifications taking rutin as a standard. This method was also based on the formation of complex with maximum absorbance at 440 nm. To 1 ml of various fractions and methanolic extract of aniseeds, 1ml of $AlCl_3$ (20mg/ml) and 3ml of sodium acetate (50 mg/ml) were added and absorbance was read at 440nm after 2.5 hrs. The absorbance of standard rutin (0.5 mg/ml) in methanol was measured under similar conditions and sample concentration was intercepted from the calibration curve. All the determinations were carried out in triplicates.

Anti-inflammatory effects

Inhibition of lipoxidase (LOX) activity

Inhibition of the activity of lipoxidase by the extract was studied using linoleic acid as substrate using the method given by Shinde et al., [13]. To various fractions and methanolic extract of aniseeds at different concentrations (100-500µg/ml) made up to 0.5ml with 2M borate buffer (boric acid and NaOH, pH 9.0), 0.25 ml of soyabean lipoxidase enzyme solution (20,000U/ml) was added, incubated for 5 min. at 25°C, 1.0 ml of linoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. Reaction mixture without extract was used as control. The per cent inhibition was calculated:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control}) - (\text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Inhibition of xanthine oxidase activity

Inhibition of the activity of xanthine oxidase was determined by the method given by Bondet et al., [14]. To 1ml of various fractions and methanolic extracts of aniseeds at different concentrations (100-500 µg/ml), 2.9ml of 50mM phosphate buffer (pH 7.0), 0.1 ml of xanthine oxidase solution (0.4U/ml in phosphate buffer) and 0.1mM xanthine in phosphate buffer were added, incubated at room temperature (24°C) for 3 min and uric acid produced was determined by measuring the absorbance at 295nm. Buffer was used as

blank and reaction mixture without extract was used as control. Allopurinol was used as a positive control. The per cent inhibition of the activity of xanthine oxidase was calculated according to the formula given above.

Statistical analysis

The results obtained were subjected to two-way analysis of variance (ANOVA) using SPSS software and the significant difference between means was calculated. Values expressed are mean of sample analyzed in triplicate ± standard error of means (SEM).

RESULTS AND DISCUSSION

Phenolic compounds in methanolic extract and the solvent fractions of aniseeds

Phenolic compounds viz. total phenolics, total flavonoids, total flavonols, tannins estimated in methanolic extract, hexane, benzene, ethyl acetate, n-butanol and aqueous fractions of methanolic extract of aniseeds are presented in **Table 1**. Ethyl acetate fraction had the highest phenolic content followed by benzene and aqueous fractions, methanolic extract, hexane and n-butanol fractions. Flavonoids were concentrated in ethyl acetate fraction followed by hexane fraction, methanolic extract, benzene, aqueous and n-butanol fractions. Similarly, flavonol content was maximum in ethyl acetate fraction followed by methanolic extract, benzene, n-butanol, hexane and aqueous fractions. n-butanol fraction had maximum tannin content followed by ethyl acetate, aqueous, hexane, benzene fractions and methanolic extract.

Ethyl acetate fraction was found to possess maximum amount of total phenolics, flavonoids and flavonols than methanolic extract and all the other fractions which was in second position in tannin content. The variation in the phytochemicals in methanolic extract and other fractions is due to the variation in the solubility of the compounds in the solvents of varying polarity.

Table 1:Phenolic compounds in methanolic extract and various fractions of methanolic extract of aniseeds

Sample extract	Total phenolics (mg/100g) GAE	Total flavonoids (mg/100g) RE	Total flavonols (mg/100g) RE	Tannins (mg/100g) CE
Methanol	0.48±2.3	0.22±0.9	0.23±1.9	0.005±0.8
Hexane	0.36±2.9	0.30±0.5	0.04±0.5	0.06±1.3
Benzene	0.60±0.6	0.12±1.4	0.19±0.3	0.05±0.8
Ethyl acetate	**0.77±0.3**	**0.45±1.6**	**0.55±0.1**	0.07±0.2
n-butanol	0.23±1.7	0.05±1.1	0.07±0.9	**0.08±0.6**
Aqueous	0.55±2.1	0.07±1.7	0.03±1.4	0.06±1.6

Values are mean ± SEM of three replicates

Anti-inflammatory effects

Lipoxidase (LOX) inhibitory effect

In the present study, the anti-inflammatory activity measured in terms of inhibition of lipoxidase (LOX), exhibited by methanolic extract and various fractions of methanolic extract of aniseeds is depicted in **Table 2**. All the fractions exhibited good inhibitory activity ($p<0.001$) at all the concentrations examined. Among the fractions, ethyl acetate fraction exhibited the highest inhibitory activity (52 to72%) at all the concentrations (IC_{50} 95µg/ml), benzene and n-butanol fractions showed moderate inhibitory activity (IC_{50} 191µg/ml and 275µg/ml respectively) and hexane fraction displayed the lowest activity (IC_{50} 375 µg/ml). Except ethyl acetate fraction all the test samples had lesser inhibitory activity than the positive control Quercetin which showed an inhibition of 52 to 78% at various concentrations (IC_{50} value of 97 µg/ml), slightly lesser than that of ethyl acetate fraction.

Table 2:Lipoxidase inhibitory activity (%) of methanolic extract and various fractions of methanolic extract of aniseeds

Conc (µg/ml)	Me	He	Be	Ea	nBu	Aq	Quercetin
100	36.4±0.1	32.3±0.8	48.1±1.4	52.4±0.9	38.3±2.8	36.2±0.6	51.5±0.1
200	46.9±1.5	34.6±0.5	52.2±0.6	59.2±0.2	42.4±1.0	42.3±0.2	58.2±0.5
300	42.0±2.4	41.5±0.9	57.0±0.1	60.1±0.1	54.5±1.2	48.4±0.4	59.1±2.4
400	58.2±1.4	53.1±0.5	59.6±0.7	68.4±1.9	58.1±1.1	54.9±0.5	64.3±2.2
500	60.5±0.3	56.4±0.2	64.2±0.3	72.1±0.2	62.5±0.1	59.3±0.6	78.4±1.3
IC_{50} (µg/ml)	**288**	**375**	**191**	**95**	**275**	**364**	**97**

Values are mean ± SEM of three replicates p<0.001

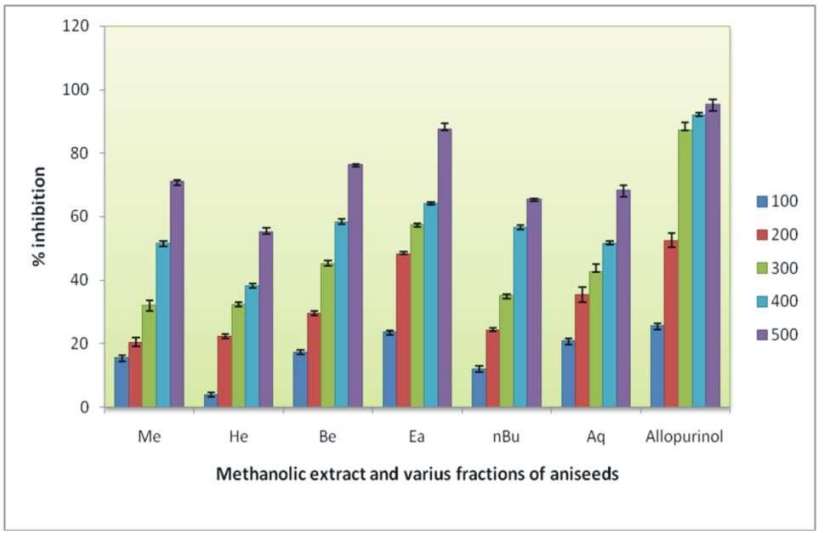
Me-Methanolic extract, He-Hexane fraction, Be- Benzene fraction, nBu- n-butanol fraction, Aq- Aqueous fraction

Elevated activities of lipoxidase / lipoxygenase at the progression of inflammation and cancer led the researchers towards the development of drugs targeting their activity. Inhibitors of LOX are reported to possess antiproliferative effects against various cancer cells and thereby have protective effect against various types of cancer[15]. It is worthy observation in the study that ethyl acetate fraction inhibited the activity of LOX better than that of standard, quercetin, reflecting the presence of more amount of potential phenolics (**Table 1**) than quercetin and/or synergistic action of phenolic compounds extracted in to ethyl acetate fraction reflecting efficient fractionation of the phytochemicals present in aniseeds as phenolic compounds are reported to inhibit both the cyclooxygenase and 5-lipoxygenase pathways, diminishing the formation of inflammatory metabolites[16]. Polyphenolic compounds present in the ethyl acetate fraction of methanolic extract of aniseeds acted as efficient lipoxygenase inhibitors as reported for phenolics present in various extracts of pumpkin seeds by Xanthopoulou et al.,[17].

Xanthine oxidase inhibitory effect

Assay of xanthine oxidase (XO) inhibitory activity of methanolic extract and various fractions of methanolic extract of aniseeds, showed perfect proportionality with the concentration (**Fig. 1).** All the fractions exhibited significantly (p<0.001) good inhibitory activity (4% to 88%) with the highest activity by ethyl acetate fraction (IC_{50} 260 µg/ml) followed by benzene fraction. All the other fractions showed moderate XO inhibitory activities with IC_{50} values ranging from 325µg/ml to 453 µg/ml). In the present study, allopurinol used as a positive control exhibited higher % inhibition (26 to 96) (IC_{50} 175µg/ml) of xanthine oxidase than that of ethyl acetate fraction as allopurinol is a drug used to treat gouty arthritis as it effectively inhibits xanthine oxidase and decreases the production of uric acid[18].

Fig. 1 Xanthine oxidase inhibitory activity of methanolic extract and various fractions of methanolic extract of aniseeds



Values are mean ± SEM of three replicates p<0.05

Xanthine oxidase is a flavoprotein, under oxidative conditions, it catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid. The accumulation of uric acid leads to hyperuricemia and gout and hence, inhibitors of uric acid formation could be useful as therapeutic agents for these diseases. In addition, a large amount of superoxide anions generated by xanthine oxidase leads to peroxidative damages of cells and inhibitors of the generation and scavengers of superoxide anion are useful for the prevention of oxidative damages[19]. Ethyl acetate fraction exhibited good XO inhibitory activity, but lesser than that of positive control allopurinol, and better than methanolic extract and all the other fractions by virtue of high amount of polyphenolic compounds present in ethyl acetate fraction (**Table 1**) than the other fractions as flavonoids are reported to be strong inhibitors of XO, a molybdenum containing enzyme, catalyses the reaction that proceeds via transfer of oxygen atom via xanthine from the molybdenum centre. The inhibitory action of flavonoids may be by way of competitively binding xanthine binding site in xanthine oxidase thereby inhibiting the activity of xanthine oxidase as reported by Lin et al., [20]. The flavonoids quercetin, myricetin, rutin, kaempferol, luteolin are reported to efficiently inhibit xanthine oxidase by binding with xanthine binding site of the enzyme[21]. As aniseeds are reported[5] to contain all the mentioned flavonoids, the XO inhibitory activity exhibited by aniseed extract can be assumed due to the binding of flavonoids to the xanthine binding site of the enzyme.

CONCLUSIONS

Methanolic extract and various solvent fractions of methanolic extract of aniseeds are rich in phenolic compounds. Ethyl acetate fraction of aniseeds possessed highest anti-inflammatory effects due to the phenolic compounds and/or the other bioactive compounds present therein. In light of these effects, aniseeds possess broad prospects for prospective applications in the food and drug industry. Further research is warranted on the mode of action of bioactive phenolics in aniseeds.

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