Journal of Complementary Medicine
And
Alternative Healthcare
ISSN: 2572-1232

Juniper Publishers
key to the Researchers

Research Article
Volume 5 Issue 2 - January 2018
DOI: 10.19080/JCMAH.2018.05.555656

J Complement Med Alt Healthcare

Antioxidant, Anti-Diabetic and Hypolipidemic Effects of Aniseeds (*Pimpinella anisum L.*): *In vitro* and *in vivo* Studies

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Submission: December 19, 2017; **Published:** January 25, 2018

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Abstract

The involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of antioxidants in the maintenance of human health and prevention and/or treatment of diseases. Aniseed (*Pimpinella anisum L.*), commonly known as 'saunf,' a plant with potential health benefits is traditionally used as a mouth freshener to relieve toothache and also to relieve digestive problems. An *in vitro* study conducted on the methanolic extract and ethyl acetate fraction of methanolic extract of aniseeds revealed that methanolic extract and ethyl acetate fraction exhibited significant radical (ABTS and DPPH) scavenging activity, anti-diabetic and hypolipidemic effects (*in vitro*) by inhibiting the activities of α-amylase and α-glucosidase and HMG-CoA reductase and pancreatic lipase respectively. In vivo studies in diabetes patients by the supplementation of aniseeds (5g/day) for 60 days proved aniseeds to be anti-hyperglycemic, hypolipidemic and anti oxidative as evidenced by decreased blood glucose, lipid profile, lipid peroxidation and protein oxidation in aniseed-treated diabetes patients. Thus, aniseeds are the promising source of nutraceuticals which are efficient protective agents against stress-induced diseases such as diabetes.

Keywords: Oxidative stress; Antioxidants; Aniseeds; Antidiabetic; Hypolipidemic; Lipid peroxidation; Protein oxidation

Introduction

Oxygen molecule plays an adverse role by facilitating oxidative stress in biological systems though it is essential for the existence of aerobic organisms [1]. Even at the steady state, oxygen always undergoes metabolism in living systems to engender oxygen-derived free radicals (superoxide O_2^-, hydroxyl OH•, alkoxyl RO• and peroxyl RO•$_2$) and non-radicals (hydrogen peroxide H_2O_2, peroxynitrite $ONOO^-$, hypochlorous acid HOCl etc.). These two as a group are termed as reactive oxygen species (ROS) and are deemed as important factors for oxidative stress mediated cellular damages [2]. Antioxidants can delay, inhibit or prevent the oxidation of oxidizable substrates by scavenging free radicals and diminish oxidative stress. However, in disease conditions, the defence against ROS is weakened or damaged and the oxidant load increases. In such conditions, the external supply of antioxidants is essential to countervail the deleterious consequences of oxidative stress [3,4]. It has been proposed that polyphones can act as antioxidants by a number of potential mechanisms. Polyphenols break the free radical chain reaction, as well as suppress free radical formation by regulating enzyme activity or chelating metal ions involved in free radical production that are reported to be the most important mechanisms of the antioxidant activity [5].

Aniseed is one such spice that contains considerable amount of phenolic compounds [6] which possess varying degrees of antioxidative activity. Aniseed (*Pimpinella anisum L.*), a native of the Eastern Mediterranean region and is widely cultivated in southern and central Europe. In India, it is grown to a small extent as a culinary herb. Aniseed is one of the oldest spices and is also used as traditional medicine [7]. However, very few reports are available on the antioxidant and anti-hyperglycemic activities of aniseeds. Thus, the objective of this work was to investigate aniseeds for both in vitro and in vivo antioxidant as well as the anti-hyperglycemic effects.

Materials and Methods

Preparation of aniseed extract

Aniseeds (*Pimpinella anisum L.*) purchased in one lot from the local market were shade dried, powdered and extracted with 80% methanol(Me), thrice (1:1, w/v) at room temperature [8]. The combined extract was concentrated in a vacuum evaporator and the residue was dissolved in water and fractionated successively using the solvents with increasing polarity viz. hexane (He), benzene (Be), ethyl acetate (Ea), n-butanol (Nb) and water (Aq) and each fraction was evaporated to dryness.

Before use, a small amount of each fraction was re-dissolved in a suitable solvent as required at a concentration of 1mg/ml [9].

All the fractions were examined for total polyphenolics [10], total flavonoids [11] and total flavonols [12]. The multi-component extract i.e. methanolic extract and the fraction with highest polyphenolic content (polyphones, flavonoids, flavonols, etc.) i.e. ethyl acetate fraction were utilized for investigations.

Sub-fractionation of methanolic extract and the best fraction

Methanolic extract and ethyl acetate fraction of aniseeds were partitioned using column chromatography to obtain individual sub-fractions 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 gradiently. Sub-fractions were collected in 20 ml portions and monitored on thin layer chromatography using methanol: chloroform (5%) as the mobile phase and the sub-fractions showing similar spots were combined.

In vitro antioxidant and antidiabetic effects

Methanolic extract, ethyl acetate fraction and the sub-fractions were further analyzed for antioxidant activity in terms of scavenging of synthetic radicals ABTS [13], DPPH [14]. *In vitro* anti-diabetic and hypolipidemic effects were assessed in terms of inhibition of α-amylase [15], α-glucosidase [16] and HMG CoA reductase [17,18] and pancreatic lipase activities [19] respectively.

In vivo anti-diabetic effects

Selection of subjects: Both male and female mild non-insulin dependent (type 2) diabetes (fasting blood glucose ranging from 110-130mg/dl) patients with in the age group of 40-60 yrs were recruited into the study after a preliminary screening through a questionnaire consisting of a brief medical history, smoking and alcohol habits and physical examination by physician. Patients with any history of liver diseases, respiratory disorders and cardiovascular diseases and any other chronic diseases were excluded from the study. Written consent was obtained in a proforma for all the procedures from each participant included in the study. The proposal of this investigation was approved by Institutional Ethical Committee (IEC)(SSSIHL/IEC/ATP/HS/2010/03) of Sri Sathya Sai Institute of Higher Learning, Prasanthi Nilayam, A.P, India.

Study design: Out of the patients chosen for the study, the experimental group received aniseeds 5g per day in 2 equal doses for a period of 60 days based on the dose response trials conducted on diabetes patients prior to these clinical trials. The patients in the control group who did not receive aniseed supplementation received Metformin (500mg), antidiabetic drug twice a day as prescribed by the physician. Both the experimental and control groups were instructed to follow an isocaloric diet (moderate-carbohydrate, moderate-fat diet) and were followed up every week by the local physician. The experimental design is as shown in Figure 1.

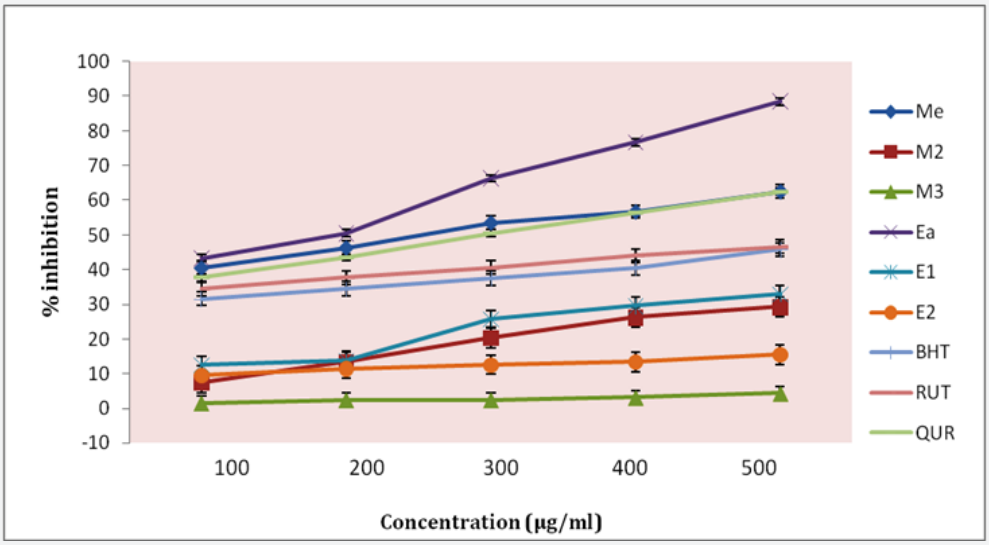

Figure 1: α-amylase inhibitory activity (%) of Me extract, Ea fraction, sub-fractions (M2 & M3; E1 & E2) and positive controls.
No activity was detected for sub-fractions M1, M4 & M5
Values are mean ± SEM of three replicates, p<0.001

nitially and at the end of the experimental period of 60 days, intravenous blood was drawn for the assay of various parameters viz. fasting blood glucose [20], serum cholesterol and triglycerides [21], lipid peroxidation in plasma and erythrocytes [22] and protein oxidation [23] in serum.

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Results and Discussion

Phenolic compounds in methanolic extract and other fractions of aniseeds

Phenolic compounds 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 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 also more in ethyl acetate fraction followed by methanolic extract, benzene, n-butanol, hexane and aqueous fractions.

Table 1: Phenolic compounds in Me extract, Ea fraction and the sub-fractions (M1-M5; E1 & E2).

Sample	Total Phenolics (Mg/100 GAE)	Total Flavonoids (Mg/100 RE)	Total Flavonols (Mg/100 RE)	Tannins (Mg/100 CE)
Me	502.7±1.1	221.7±0.7	74.7±0.4	53.2±0.6
M1	0.46±0.1	-ND-	-ND-	-ND-
M2	12.8±0.1	2.4±0.2	-ND-	-ND-
M3	0.16±0.01	-ND-	-ND-	ND
M4	-ND-	-ND-	-ND-	-ND-
M5	-ND-	-ND-	-ND-	-ND-
Ea	603.9±0.8	452.2±0.7	274.3±0.6	85.1±0.9
E1	64.2±0.5	26.6±0.8	15.4±0.3	-ND-
E2	12.8±0.2	12.4±0.3	8.5±0.6	-ND-

Me: methanolic extract, M1-M5: sub-fractions of methanolic extract, Ea: ethyl acetate fraction, E1 & E2: sub-fractions of ethyl acetate fraction

Column chromatography

Five sub-fractions obtained from methanolic extract and two from ethyl acetate fraction were designated as M1-M5 and E1 and E2 respectively. Methanolic extract, ethyl acetate fraction and all the sub-fractions were tested for their *in vitro* antioxidant, anti-diabetic and hypolipidemic activities.

In vitro antioxidant activity

Table 2: ABTS radical scavenging activity (%) of Me extract, Ea fraction, sub-fractions (M1-M5; E1 & E2) and positive controls.

Sample	Concentration (µg/ml)					IC_{50} (µg/ml)
	100	200	300	400	500	
Me	45.1±0.8	50.7±0.4	57.3±0.5	63.6±0.5	65.5±0.1	198
M1	5.4±0.2	12.9±0.8	14.4±0.3	15.3±0.0	16.2±0.2	1572
M2	15.2±0.1	18.5±1.5	20.4±0.2	22.5±0.3	25.5±1.9	973
M3	9.5±0.1	10.3±0.2	10.4±1.2	11.4±0.1	13.5±0.2	1894
M4	2.5±0.3	3.4±1.3	4.1±0.4	4.7±0.1	5.6±0.1	4386
M5	0.3±0.2	1.2±0.1	1.7±0.1	2.4±0.5	3.6±0.1	6410
Ea	72.3±0.7	75.5±0.2	82.1±0.3	85.4±0.4	90.9±0.3	69
E1	35.3±0.6	39.2±0.1	41.1±0.5	49.2±0.3	58.3±0.2	402
E2	22.2±0.3	25.1±0.4	32.4±0.2	35.2±0.6	38.1±0.1	646
BHT	70.5±0.6	74.6±0.3	78.5±0.4	86.5±0.2	95.1±2.1	71
RUT	28.5±0.8	35.8±0.1	45.6±0.1	47.5±0.3	52.4±0.5	472
QUR	27.7±0.3	32.4±0.2	42.3±0.4	49.1±0.6	51.2±0.7	481

ABTS radical scavenging activity: Table 2 shows ABTS radical scavenging activity exhibited by Me extract, Ea fraction, the sub-fractions (M1-M5; E1 and E2) and positive controls. All the samples significantly ($p<0.001$) scavenged ABTS radicals in a concentration dependent manner. It was interesting to note that the scavenging activity exhibited by Ea fraction was better than that of positive controls BHT, rutin and quercetin. Positive correlation was noticed ($p<0.01$) between ABTS scavenging activity and total phenolics ($r=0.874$), flavonoids ($r=0.865$), flavonols ($r=0.919$) and tannins ($r=0.765$) in the sample extracts.

2,2'-azinobis(3-ethylbenzothiozoline-6-sulfoni acid) (ABTS) is a prorogated radical, which has a characteristic absorbance maxima at 734nm. Absorbance decreases with the scavenging of proton radical [24] resulting in decolourization of ABTS radical

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cation. In the present investigation, Ea fraction revealed the highest ABTS radical scavenging ability, followed by Me extract, E1 and E2 and M1-M5sub-fractions speculating the presence of hydrogen donating compounds that are extracted by the polar solvents especially phenolic compounds and tannins as observed by the quantitative analyses.

The highest scavenging ability exhibited by Ea fraction and the higher activities by Me extract, E1 and E2 indicate the presence of high molecular weight phenolics especially tannins which possess ability to quench free radicals (ABTS) which depends on the molecular weight, the no of aromatic rings and nature of hydroxyl group substitution than the specific functional groups as reported by Hagerman et al. [25].

Besides, higher activity exhibited by Ea fraction than the positive controls can also be due to the presence of many compounds that are extracted by ethyl acetate whereas positive controls like rutin and quercetin are single isolated compounds and this can be supported by lesser activity exhibited by E1 and E2, the sub-fractions of Ea fraction. The least activity exhibited by the sub-fractions of Me extract compared with that of Me extract also support that multi-component extract always performs better than one or two compounds present in the sub-fractions probably because of the synergism among the compounds.

DPPH radical scavenging activity: Table 3 shows concentration dependent DPPH radical scavenging activity ($p<0.001$) of Me extract, ethyl acetate fraction, the sub fractions (M1-M5; E1 and E2) and the positive controls BHT, rutin and quercetin. The scavenging potential of Ea fraction was almost similar to that of synthetic and natural antioxidants viz. BHT, rutin and quercetin used as positive controls. A positive correlation ($p<0.01$) was found between DPPH radical scavenging activity with total phenolics ($r=0.678$), total flavonoids ($r=0.802$), total flavonols ($r=0.889$) and tannins ($r=0.675$).

Table 3: DPPH radical scavenging activity (%) of Me extract, Ea fraction, sub-fractions (M1-M5; E1 & E2) and positive controls.

Sample	Concentration (µg/ml)					IC_{50} (µg/ml)
	100	200	300	400	500	
Me	72.4±0.3	80.1±0.1	85.2±0.1	86.3±0.2	87.2±0.1	69
M1	1.6±0.1	8.4±0.3	14.2±0.1	14.9±0.2	17.7±0.3	1429
M2	19.3±0.2	21.1±0.1	32.4±0.1	33.5±0.4	38.1±0.1	654
M3	3.1±0.9	3.6±1.3	6.5±0.1	6.5±0.1	7.5±0.2	3181
M4	2.4±0.1	9.2±0.3	10.6±0.1	10.7±0.7	15.6±0.5	1644
M5	3.6±1.2	8.6±0.8	9.2±0.1	9.5±0.4	12.5±0.2	1965
Ea	84.2±0.6	90.5±0.1	91.5±0.1	91.9±0.1	93.7±0.5	59
E1	35.5±0.7	49.6±0.8	52.6±0.2	52.7±0.1	57.7.±0.9	142
E2	18.7±0.2	26.5±0.4	28.4±0.1	28.9±0.2	30.4±0.7	833
BHT	87.5±0.6	90.4±0.1	90.5±0.0	90.7±0.5	90.8±0.6	57
RUT	90.5±0.1	91.3±0.2	91.6±0.1	91.8±0.7	92.1±0.3	55
QUR	90.7±0.5	91.6±0.4	92.1±0.6	92.2±0.4	92.6±0.4	56

1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical, a stable organic free radical which at room temperature produces violet color in methanol with a strong absorption band at 570nm in visible spectrum. The scavenging activity of the extract was due to the amount of extractable phenolics, molecular weight of phenolics, the no of aromatic rings, nature of hydroxyl group substitution and formation of complexes with proteins. In the reaction, abstraction of a hydrogen atom from the hydroxyl group of phytoceuticals present in the extracts occurs leading to discolouration of purple colour of DPPH resulting in a decrease in the absorbance [26]. Very efficient scavenging of DPPH radicals by Ea fraction comparable to the positive controls and more than that of Me extract followed by E1 and E2, M1-M5 indicate the presence of phenolics, flavonoids and tannins present in the extracts and the activity can be positively correlated to the phenolics present in respective extracts as reported by Sasipriya et al. [27] that scavenging potential of DPPH radicals is reported to be positively correlated with the phenolics, flavonoids and tannins which can donate hydroxyl groups for reducing DPPH radicals.

However, one of the sub-fractions of Me extract (M2) and of ethyl acetate fraction (E1) were found to be better than the other sub-fractions indicating the presence of comparatively higher amounts of phenolics. Higher activity exhibited by Me extract and Ea fraction may contribute to the fact that hydrogen-donating compounds are more likely to be present in polar solvents which might possibly donate hydrogen from phenolic hydroxyl groups and discontinue the free radical chain reaction and hence, such compounds can prevent cellular damage due to the free radicals as reported by Al-Zubari et al. [28].

In vitro antidiabetic activity

α-amylase and α-glucosidase inhibitory activities: The results obtained for inhibition of the activities of carbohydrate hydrolyzing enzymes viz. α-amylase and α-glucosidase by Me extract, Ea fraction and the sub-fractions of aniseeds is as

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shown in Figure 1 & 2. All the samples significantly ($p<0.001$) inhibited the activity of α-amylase in a concentration dependent manner. However, highest activity was exhibited by Ea fraction with values ranging from 43.3 to 88.5%, followed by Me extract, 40.6 to 62.6%. Sub-fractions of Me extract, M2 and M3 showed poor activity with values ranging from 7.5 to 29.3% and 1.6 to 4.4% respectively. Other sub-fractions M1, M4 and M5 did not show any inhibitory activity. Sub-fractions of Ea fraction E1 and E2 also showed moderate inhibitory activity, E1 (12.5 to 32.9%) being more potent than E2 (9.6 to 15.5%). Synthetic antioxidant BHT and natural antioxidants rutin and quercetin used as positive controls also exhibited moderate inhibitory activity with values viz. 31.6 to 45.8%, 34.5 to 46.6% and 37.7 to 62.6% respectively but the activity was lesser than that of Me extract and Ea fraction. Inhibition of α-amylase activity was positively correlated ($p<0.01$) with the phytoceuticals in aniseeds viz. total phenolics ($r=0.968$), total flavonoids ($r=0.972$), total flavonols ($r=0.914$) and tannins ($r=0.265$).

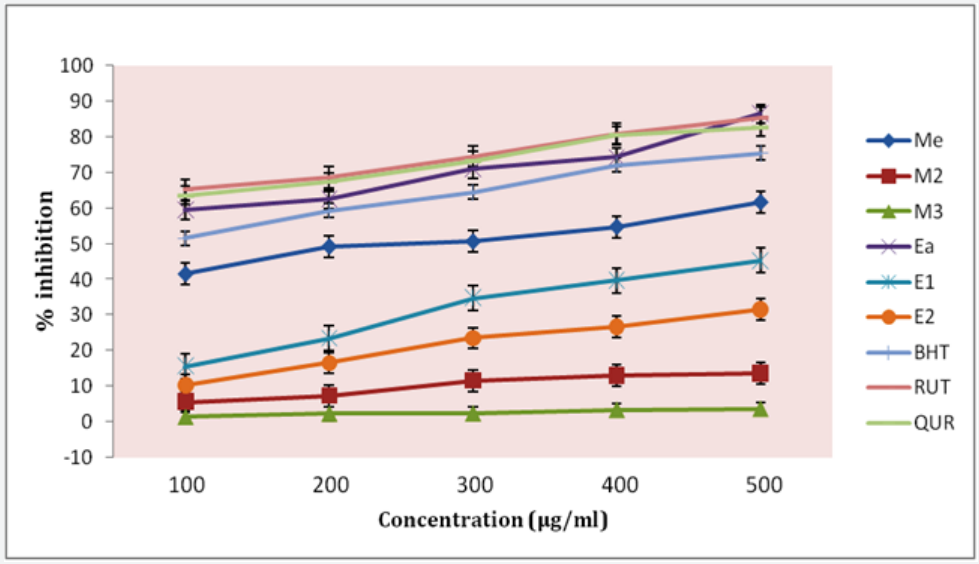

Figure 2: α-glucosidase inhibitory activity (%) of Me extract, Ea fraction, sub-fractions (M2 & M3; E1 & E2) and positive controls. No activity was detected for sub-fractions M1, M4 & M5, $p<0.001$ Values are mean ± SEM of three replicates

Similar response was observed for α-glucosidase inhibition ($p<0.001$). The sub-fractions M1, M4 and M5 did not show any inhibition. It was noticed that the activity of Ea fraction was comparable to natural antioxidants rutin and quercetin and was better than synthetic antioxidant BHT. Inhibition of α-glucosidase activity was positively correlated ($p<0.01$) with total phenolics ($r=0.936$), total flavonoids ($r=0.977$), total flavonols ($r=0.945$) and tannins ($r=0.627$) in the test samples.

Hydrolysis of dietary carbohydrates such as starch is a major source of glucose in the blood. Pancreatic α-amylase and intestinal α-glucosidase are key enzymes in the digestive system and catalyze the first step in the digestion of starch, hydrolyzing the α-1-4-glucosidic linkages. The inhibition of these enzymes significantly decreases the digestion and uptake of carbohydrate, thereby decreasing the postprandial blood glucose level in the non-insulin dependent diabetes mellitus patients [29]. Therefore, the search for more effective and safer hypoglycemic compounds from plants has continued to be an important area of active research. Several studies have reported the ability of various medicinal plants in the inhibition of activities of α-glucosidase and α-amylase [30,31].

Present investigation revealed the presence of many secondary metabolites which get extracted differently in various solvents. It was observed by quantitative analyses that Ea fraction had maximum amount of phenolics, tannins, etc. which were reported to function by different routes to control hyperglycemia in diabetes patients. For instance, tannins in addition to their α-glucosidase inhibitory activity also inhibit insulin degradation and improve glucose utilization [32]. As oxidative stress is one of the important factors in tissue injury in diabetes mellitus, potent antioxidants like tannins may protect β-cells and increase insulin secretion [33]. Saponins present in some plants have been described to demonstrate glucagon decreasing effect which may enhance glucose utilization and lower blood glucose. It was equally reported that saponins stimulate insulin release from pancreas [34]. Phenolic compounds have electron donating capability and are readily oxidized to form phenolate ion or quinone which is an electron acceptor [35]. Thus, they have the ability to block or enhance specific enzymes responsible for digestion of carbohydrates [36]. Being good source of phytoceuticals viz. phenolics, tannins saponins etc., various extracts of aniseeds could inhibit carbohydrate hydrolysing enzymes viz. α-amylase and α-glucosidase indicating that

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aniseeds can control blood glucose levels which can be supported by antidiabetic effect exhibited by aniseeds in NIDDM patients as reported by Rajeshwari et al. [37].

In vitro hypolipidemic activity

HMG-CoA reductase inhibitory activity: The inhibitory activity of Me extract, Ea fraction, the sub-fractions (E1 and E2) of aniseeds and the positive controls against HMG-CoA reductase enzyme is displayed in Figure 3. The samples exhibited moderate inhibitory activity with Ea fraction showing highest inhibitory activity (IC50 243µg/ml). Sub-fractions M1-M5 did not exhibit any inhibitory activity. A positive correlation ($p<0.01$) was observed between inhibition of HMG-COA reductase activity and total phenolics (r=0.966), total flavonoids (r=0.832), total flavonols (r=0.685) and tannins (r=0.489).

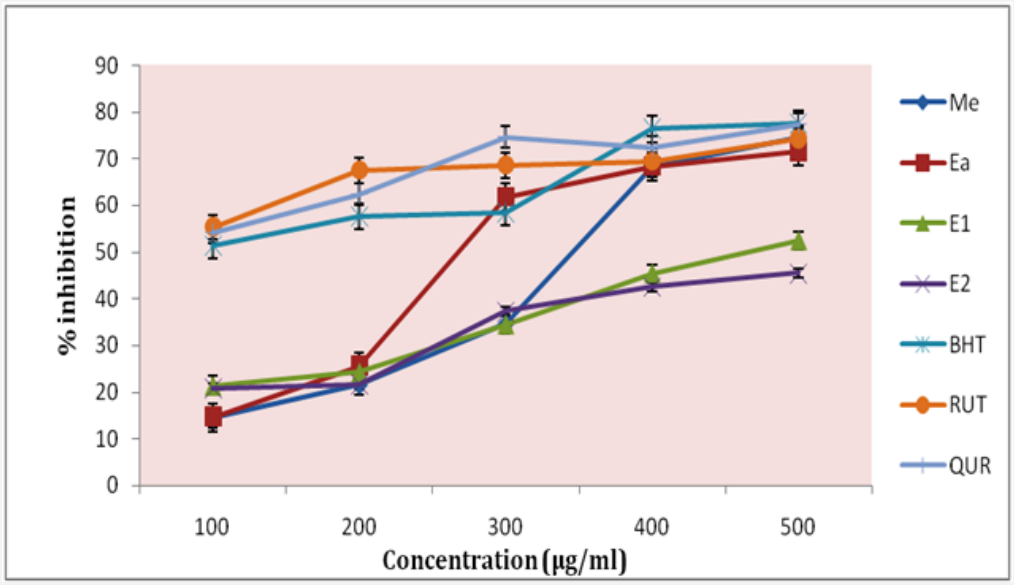

Figure 3: HMG-CoA reductase inhibitory activity (%) of Me extract, Ea fraction, sub-fractions (E1& E2) and positive controls

No activity was detected for sub-fractions M1 - M5

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is rate-limiting enzyme in cholesterol biosynthesis that catalyzes the conversion of HMG-CoA to mevalonate. The inhibition of HMG-CoA reductase effectively lowers the level of cholesterol in humans and most animals by the activation of sterol regulatory element-binding protein-2, which up regulates HMG-CoA reductase and LDL receptor that lead to decrease in cholesterol levels [38]. Although statins are well-known HMG-CoA reductase inhibitors, long term consumption of statins causes severe adverse effects such as muscle and liver damage, rhabdomyolysis and renal failure [39].

The inhibitory activity of ethyl acetate and Me extract gradually increased as the concentration increased resulting in higher IC50 values while synthetic antioxidant and isolated rutin and quercetin exhibited higher activity at both lower and higher concentrations resulting in lower IC50 values. The inhibitory effect of aniseed extracts is attributed to flavonoids, tannins which are identified to be present in the extracts by qualitative and quantitative analyses that are reported to be strong antiplatelet [40] and anti-hypercholesterolemia agents in animal studies. Saponins identified to be present in aniseeds were also reported to be hypocholesterolemic but the mode of action was reported to be inhibition of absorption of cholesterol in the intestines of experimental animals [41]. Luteolin and apigenin of Ea fraction and Me extract of aniseeds were reported to possess vasoprotective effect whereby the compounds protect arteries from injuries by superoxide anions and hence, were reported to be potentially useful as therapeutic agents for cardiovascular disease patients [42]. Luteolin has been reported to possess antihypercholesterolemic effect since it reduced the concentration of total cholesterol, triglycerides and free fatty acids as well as decreased the levels of cardiac marker enzymes troponin I and troponin T in rat-enzymes that exist during myocardial injury [43]. Possessing flavonoids such as luteolin and apigenin, aniseeds may be useful in controlling blood lipids and is supported by the reports of Rajeshwari et al. [37] in diabetes patients.

Pancreatic lipase inhibitory activity: Methanolic extract, Ea fraction, the sub-fractions of Ea fraction (E1 and E2) of aniseeds and the positive controls moderately inhibited the activity of pancreatic lipase while no activity was shown by the sub-fractions M1-M5 (Figure 4). Pancreatic lipase inhibitory activity was positively correlated ($p<0.01$) with total phenolics (r=0.987), total flavonoids (r=0.935), total flavonols (r=0.839) and tannins (r=0.657) present in the respective test samples.

The way inhibition of CHO hydrolyzing enzymes like α-amylase and α-glucosidase is used as a therapeutic approach

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for controlling hyperglycemia, one of the strategies used in the discovery of anti-obesity drug is through inhibition of activity of pancreatic lipase. The synthetic drugs caused several side effects. Obesity is caused by excess calorie intake entry of which to the blood stream can be overcome by inhibiting the activity of pancreatic lipase or by delaying lipid absorption [44]. The inhibitory capacity of various extracts though only up to 40% at 500µg/ml concentration, might be coincident with total phenolics as polyphenols have been reported to have anti-obesity effects by way of inhibiting the activity of lipase [45].

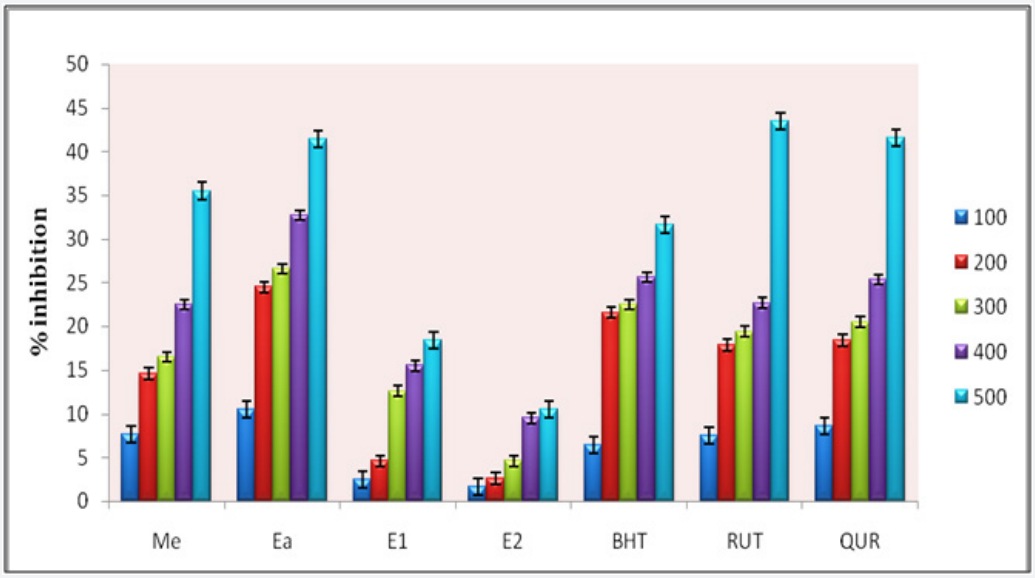

Figure 4: Pancreatic lipase inhibitory activity (%) of Me extract, Ea fraction, sub-fractions (E1& E2) and positive controls.

No activity was detected for sub-fractions M1 - M5, Values are mean ± SEM of three replicates, p<0.001

In vivo anti-hyperglycemic, hypolipidemic and antioxidant activities

Table 4: Fasting blood glucose and lipid profile of control and experimental diabetes patients.

Groups	Fasting Blood Glucose (Mg/Dl)	Cholesterol (Mg/Dl)	Triglyceride (Mg/Dl)
Control			
Initial	185.23+0.7	238.03+0.7	214.01+0.7
Final	206.04+1.0*** [11]	239.04+0.4	217.12+0.8
Experimental			
Initial	190.53+0.8	238.11+0.7	222.36+0.8
Final	122.16+0.9*** [36]	220.12+0.6*** [8]	189.21+0.6*** [15]

Values are mean +SEM of 20 subjects in each group

The figures in parentheses indicate per cent increase /decrease over respective initial values.

Comparison between initial and final: *** p<0.001

Fasting blood glucose: Data presented in Table 4 indicates 19% (p<0.001) decrease in fasting blood glucose levels in control and 36% (p<0.001) decrease in aniseed-treated type 2 diabetes patients. Hyperglycemia resulting from unregulated glucose level is widely recognized as a link between diabetes and diabetic complications [46]. Hyperglycemia causes tissue damage by mechanisms involving repeated changes in the cellular metabolism [47]. One of the key metabolic pathways, a major contributor to hyperglycemia-induced cell damage is the non-enzymatic reaction between excess glucose and proteins to form advanced glycation end products (AGEs) [48]. Production of AGEs interferes with cell integrity by modifying protein function or by inducing receptor mediated production of reactive oxygen species (ROS) [49].

Hyperglycemia-evoked oxidative stress plays an important role in the development of diabetic complications, including nephropathy, neuropathy, retinopathy and hepatopathy which is considered to result from augmented reactive oxygen species generation and decrease in antioxidant defense [50]. Implication of oxidative stress in the pathogenesis of diabetes mellitus is not only by oxygen free radical but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes and formation of lipid hydroperoxides [51,52].

In the present study, significant decrease (36%) in fasting blood glucose in aniseed-treated group is a result of control over hyperglycemia due to decreased oxidative stress caused by reactive oxygen species as oxidative stress is reported [53] to be responsible for the development and progression of diabetes. A number of phytoceuticals (phenolics, flavonoids, flavonols) present in aniseeds would have controlled hyperglycemia as

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some of the phenolic compounds interfere with the absorption of glucose or dietary carbohydrates in the small intestine [54], facilitate utilization of glucose by peripheral tissues mediated by an insulin dependent glucose transporter [55], restore insulin sensitivity [56] as reported by Hosseini et al. [57] for phenolic acids and flavonoids in the leaves of Juglan regia which also inhibit the activity of carbohydrate hydrolyzing enzymes viz. α-amylase and α-glucosidase [58] which is evidenced by in vitro analyses in the present study. Besides, the free radical scavengers present in aniseeds would have played an important role in controlling hyperglycemia by preventing oxidation of glucose as reported by Kumarappan et al. [59].

Phytoceuticals in aniseeds can prevent development of diabetic complications by controlling hyperglycemia thereby oxidative stress as hyperglycemia-evoked oxidative stress is reported [60] to play a crucial role in the development of diabetic complications. The phytoceuticals in aniseeds by controlling blood glucose levels, decrease the production of free radicals as increase in blood glucose levels i.e. hyperglycemia generates free radicals due to auto-oxidation of glucose [61]. This is very much evidenced by significantly decreased lipid peroxidation in the in vitro models, protein oxidation as well as lipid peroxidation in aniseed-treated diabetes patients (Table 5).

Table 5: Lipid peroxidation and protein oxidation in control and experimental diabetes patients.

Groups	Protein Oxidation (Nmol/Ml)	Lipid Peroxidation	
		Erythrocytes (Nmol MDA/ Ghb)	Plasma (Nmol MDA/Dl)
Control			
Initial	51.0+0.8	5.12+0.6	419.81+0.2
Final	54.0+1.0** [6]	10.09+0.9*** [97]	420.32+3.0
Experimental			
Initial	68.0+0.4	10.83+0.5	434.11+0.7
Final	42.0+3.0*** [38]	5.32+0.5*** [51]	341.22+1.0*** [21]

Values are mean + SEM of 20 subjects in each group

Controlled blood glucose levels can also be due to the effect of quercetin, the aglycone of rutin which is reported to decrease blood glucose concentration and increase insulin release in STZ diabetic rats [62]. Quercetin protected pancreatic β-cells by decreasing oxidative stress and by preventing pancreatic β-cell integrity [63,64] and also showed increase in the no. of pancreatic islets in quercetin treated animals. Hence, quercetin present in aniseeds [65] would have played an important role in the present study to control oxidative stress and there by blood glucose levels.

Serum cholesterol, triglycerides and lipoproteins: In the present study, significant decrease (8% and 15%; p<0.001) was observed in serum cholesterol and triglycerides in aniseed-treated diabetes patients while no significant decrease was observed in the control group. A significant decrease of 10% (p<0.01) in VLDL-C and 6% (p<0.001) in LDL-C levels and a significant rise in HDL-C (34% , p<0.001) were observed in aniseed treated group whereas no much change was observed with respect to VLDL, LDL and HDL-C in control group (Table 6).

Table 6: Serum lipoproteins in control and experimental diabetes patients.

Groups	Very Low Density Lipoprotein (VLDL-C) (Mg/Dl)	Low Density Lipoprotein (LDL-C) (Mg/ Dl)	High Density Lipoprotein (HDL-C) (Mg/Dl)
Control			
Initial	43.16+ 0.1	165.0+0.5	27.6+0.8
Final	43.21+0.1 [1]	169.6+1.1* [2]	27.3+ 0.9 [2]
Experimental			
Initial	44.62+0.3	168.1+0.6	26.1+0.8
Final	40.11+0.9** [11]	157.3+0.6*** [7]	35.1+0.4*** [34]

Insulin deficiency (IDDM)/inadequate function (NIDDM) leads to a variety of derangements in the metabolic and regulatory processes which in turn lead to accumulation of triglycerides (TG), cholesterol, lipoproteins except HDL-C which is a significant and independent marker of possible coronary problems. Lowering triacyglycerol, total cholesterol and low density lipoprotein cholesterol (LDL-C) levels or increase in high density lipoprotein cholesterol (HDL-C) levels may prevent, control and even reverse lipid metabolic outcomes [66]. The actions exhibited by aniseeds in diabetes patients are in agreement with the aforesaid. Aniseeds decreased LDL-C, VLDL-C and increased HDL-C significantly (p<0.001) in the experimental group as shown in Table 3. The percent decrease in LDL-C and VLDL-C and increase in HDL-C were more pronounced in diabetes patients treated with aniseeds than the patients in control group treated with allopathic drug.

Significant increase in HDL-C in aniseed treated group is beneficial to the patients against atherosclerosis and cardiovascular diseases (CVD) [67] because HDL removes cholesterol from at heroma within arteries and transports the same back to the liver for excretion or reutilization while LDL transports cholesterol through the arteries where it can be retained by arterial proteoglycans thus initiating and sustaining plaque formation. Besides, LDL-C increases the risk of cardiovascular disease when it invades the endothelium and becomes oxidized. The radical scavengers/antioxidants identified to be present in aniseeds in the present study, by preventing oxidation of LDL, probably prevent atherosclerosis and peripheral vascular diseases as reported by Cromwell and Otvos [68] that LDL-C becomes atherogenic when it is modified by oxidative reaction and retention of oxidized form of LDL by

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the proteoglycans and increased levels of LDL-C are associated with atherosclerosis, heart attack, stroke and peripheral vascular disease. Treatment with aniseeds could produce significant decrease in TG, cholesterol, LDL-C, VLDL-C and significant increase in HDL-C levels that could not be noticed in controls-treated with the drug. The lipid lowering effects of aniseeds may also be credited to the possible action on the pancreas since, apart from the regulation of CHO metabolism, insulin is also known to play an important role in lipid metabolism as insulin activates lipoprotein lipase in the metabolism of lipids [69].

Lipid peroxidation and protein oxidation: Table 5 shows lipid peroxidation in erythrocytes and plasma and protein oxidation in control and experimental diabetes subjects. Increase in the erythrocyte and plasma lipid peroxidation was observed in the control group. Treatment with aniseeds decreased lipid peroxidation in erythrocytes by 51% ($p<0.001$) and plasma by 21%, ($p<0.001$) in diabetics. Protein oxidation increased in both the control groups indicating oxidative stress which was significantly ($p<0.001$) decreased in aniseed-treated diabetics indicating control over oxidative stress.

Free radicals attack membranes and cause peroxidation of unsaturated fatty acids. Lipid peroxidation (LPO) leads to extensive membrane damage and dysfunction [70]. Increased oxidation of lipids induces oxidative damage by increasing peroxyl and hydroxyl radicals [71]. Thus, lipid peroxidation is one of the characteristic features of chronic diseases. The most commonly used indicator of LPO is thiobarbituric acid reactive substances (TBARS) [72]. Increase in lipid peroxidation is observed as a remarkable increase in the concentration of TBARS and MDA as the main product of LPO in the plasma [73].

In the present study, significant elevation was observed in TBARS in both the control groups. Administration of aniseeds significantly decreased TBARS in both the experimental groups. The phytoceuticals present in aniseeds by scavenging free radicals would have decreased lipid peroxidation in plasma and erythrocytes of diabetes and arthritis patients as evidenced by in vitro studies wherein aniseeds scavenged radicals (ABTS, DPPH, hydroxyl, peroxyl, etc.). Flavonoids such as rutin identified to be present in aniseeds by HPLC analysis [65]would have decreased TBARS and hydroperoxides in the treated patients as rutin has been reported to decrease peroxidation of lipids in the in vitro [74] and in vivo [75] analyses.

Under conditions of severe oxidative stress, free radical generation leads to protein modification. Proteins may be damaged directly by specific interactions of oxidants or free radicals with susceptible amino acids. They are also modified indirectly, with reactive carbonyl compounds formed by the auto-oxidation of carbohydrates and lipids with eventual formation of advanced glycation end products/lip oxidation end products [76]. Reactive oxygen species can cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross linking of proteins and oxidation of specific amino acids and therefore leading to increased susceptibility to proteolysis by specific proteases [77]. Protein carbonyl content is the most widely used marker of oxidative modification of proteins and suggested to be reliable marker of oxidative stress [78]. A significant decrease in the protein oxidation along with significant decrease in LPO in the present study, confirms control over oxidative stress in aniseed-treated diabetes and arthritis patients owing to the antioxidant potential of aniseeds which can be supported by in vitro radical scavenging, reducing potential and anti-peroxidative efficacy of aniseeds [65]. Controlled oxidation of proteins in the treated groups is due to radical scavenging effect of rutin which protected proteins such as hemoglobin from oxidation as reported by Grinberg et al. [79]. Nagasawa et al. [80] had shown that rutin suppressed the accumulation of glycation products in serum and tissue attributable to the antioxidant activity of rutin. Thus, rutin in aniseeds would have also acted in a similar manner to control protein oxidation in the current study.

Conclusion

Ethyl acetate fraction exhibited efficient antioxidant, anti-diabetic and hypolipidemic activities in vitro by virtue of different photochemical (phenolics, flavonoids, and flavanols) present in ethyl acetate fraction of methanolic extract of aniseeds. Besides, aniseeds decreased blood glucose in type 2 diabetes patients but not brought down below normal, indicating the seeds to be anti-hyperglycemic. the seeds decreased serum lipids, lipoproteins (LDL and VLDL) and improved HDL and controlled lipid peroxidation and protein oxidation, markers of oxidative stress proving the seeds to be hypolipidemic and antioxidative. The versatile activities exhibited by the seeds are a result of the synergistic action of bioactive compounds present in the seeds.

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