TO IDENTIFY THE ACTIVE PRINCIPLE OF FORMULATED ANTI DIABETIC DRUG SYNDREX® WITH STRONG ANTIOXIDANT POTENTIAL

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ABSTRACT

Diabetes mellitus is a complex chronic disease characterized by hyperglycemia, which via several mechanisms leads to an increase in production of ROS (Reactive Oxygen Species) and oxidative stress. Additionally, impaired synthesis of naturally occurring antioxidants under diabetic condition also results in an increased oxidative injury to biological molecules. Oxidative stress has been shown to be responsible for development of diabetic complications. Hence antioxidant supplementation along with a drug to control hyperglycemia would be a helpful therapy to combat diabetes. Therefore many products of herbal origin are being tested for both their antidiabetic and antioxidant properties. Syndrex® manufactured by Plethico Laboratories, Indore, is a formulated antidiabetic drug, which contains powder of germinated fenugreek seeds. The antioxidant potential of this drug was already demonstrated in our laboratory and among the different soxhlet fractions checked, the methanolic fraction was found to possess the highest antioxidant activity (Dixit et al, 2008). The aim of the present study is to sub fractionate the soxhlet methanolic fraction of Syndrex® and to purify the compounds with high antioxidant activity. Sub-fractionation of methanolic extract by silica gel column chromatography followed by flash column chromatography lead to elution of eight fractions, which were then assessed for their antioxidant potential using various in vitro standard biochemical assays and by pulse radiolysis. Among the eight fractions obtained, fraction one, two and three showed high antioxidant activity in all the biochemical assays corresponding to different levels of protection. Fraction 1 gave 91% protection against DPPH (2,2’-Diphenyl-1-picrylhydrazyl) radicals and 82% protection against deoxyribose degradation. Fraction 1, 2 and 3 also conferred protection to lipids and proteins against oxidative damage, using rat liver mitochondria as model systems. Pulse radiolysis study revealed the ability of fraction 1 to scavenge ABTS•⁺ (2,2’- azinobis (3-ethylbenzothiazolin-6-sulphonic acid)) and CO₂•⁻ radicals.
with a value of 27 μg/ml AEAC (ascorbic acid equivalent antioxidant capacity) and 4.7 μg/ml AEAC. HPLC analysis of fractions 1, 2 and 3 revealed peaks corresponding to the purity of each fraction. Thus we have succeeded in standardizing methods, for resolving the soxhlet methanolic fraction of Syndrex® into subfractions exhibiting high antioxidant activity.

Introduction

Diabetes is a metabolic disorder characterized by hyperglycemia. Persistent hyperglycemia is accompanied by increase in free radicals generated due to auto-oxidation of glucose, AGE (Advanced Glycation Endproducts) formation and their binding to respective receptors, increased substrate flux through the polyol pathway and stimulation of eicosanoid metabolism (De Vriese et al, 2001). Additionally hyperglycemia leads to generation of excess ROS (Reactive Oxygen Species) via oxidative phosphorylation, thereby inducing oxidative stress that plays a key role in the development of diabetic complications such as nephropathy, retinopathy and neuropathy (Valko et al, 2007). In addition to the increased generation of free radicals in diabetes, impaired generation of naturally occurring antioxidants also results in increased oxidative injury, by failure of protective mechanisms. Antioxidant defense system appears to be compromised in diabetic patients (Glugliano et al, 1996). Recent findings suggest that the same pathways used in the activation of GDIS (Glucose Dependent Insulin Secretion) can dramatically enhance ROS production and manifestations of oxidative stress and possibly apoptosis (Leonid et al, 2004). Thus free radicals play a significant role in the pathogenesis of chronic diabetic complications (Segal, 2004).

Many dietary components and their individual constituents have antioxidant properties and are capable of interacting with reactive molecules. One of the most potent groups possessing antioxidant properties includes polyphenols and flavonoids. These components are known to be present in seeds of fenugreek (Trigonella foenum-graecum) which is widely used as a spice and a rich source of fibre. Its sub-fractions are known to have hypoglycemic effects in different model systems (Srinivasan, 2006).

Additionally, fenugreek seed extract was known to stimulate hypoglycemic effect by activation of an insulin signaling pathway (Vijaykumar et al, 2005). Supplementation of this seed in diet, enhances the antioxidant potential in control and diabetic rats (Anuradha and Ravikumar, 2001). The polyphenol rich extract of fenugreek seeds is shown to protect erythrocytes from oxidative damage demonstrating the potent antioxidant activities of this seed (Kaviarasan et al, 2004).

The present study was done to evaluate the antioxidant potential of one such anti-diabetic drug Syndrex®. Syndrex® is manufactured by Plethico laboratories, Indore, India. It contains powder of germinated fenugreek seeds. We have shown that germinated fenugreek seed extracts, exhibit high in vitro antioxidant potential (Dixit et al, 2005). Previous studies of this drug in our laboratory have shown, that the soxhlet methanolic extract of this drug has high in vitro antioxidant properties. Besides the whole drug extract has shown to improve the functionality and viability of pancreatic islet in vitro (Dixit et al, 2008). Additionally, in vivo studies of this drug also showed that pre-treatment of this drug, prevented STZ induced diabetes in mice (Dixit et al., Communicated). As the soxhlet methanolic extract of Syndrex® exhibited high antioxidant potential, we have tried to sub-fractionate the methanolic extract and identify pure compounds responsible for conferring high antioxidant activity.

Materials and Methods

Chemicals

Chemicals were either from one of the following companies: Sisco Research Laboratories (Mumbai-India), British Drug House (Mumbai-India), Hi-media (Mumbai-India), Sigma-Aldrich (U.S.A) and Merck (Mumbai-India).
Preparation of extract

Syndex® tablets were purchased from local market, finely powdered and subjected to sequential extraction using solvents in increasing order of polarity such as petroleum ether, chloroform and methanol. The powder was extracted with each solvent for 8-10 hrs to remove soluble matter. The methanolic extract obtained was used for further fractionation.

Chromatographic Procedures

Thin Layer Chromatography

TLC plates precoated with silica plates (Silica gel G) were used, to separate constituents of methanolic extracts. Methanolic extract was spotted on the TLC plates and air-dried. Several solvents were used for developing the plates. The solvent that gave best separation was chloroform : ethyl acetate : methanol : water (1:8:1.5:0.8, v/v/v). Bands were noted by UV (254 nm) and the TLC plate was captured using gel documentation system.

Column Chromatography

The methanolic extract was fractionated using silica gel (200-400 mesh size) as stationary phase and eluted following a stepwise gradient of chloroform, ethyl acetate, methanol and water in order of increasing polarity and in varying concentrations. Fractions were collected and TLC plate was developed using chloroform: ethyl acetate: methanol: water (1:8:1.5:0.8), observed under UV (254 nm) and captured using gel documentation system.

Flash Chromatography

Methanolic extract was separated using silica gel (200-400 mesh size) as stationary phase and chloroform: ethyl acetate: methanol: water (1:8:1.5:0.8) as mobile phase and under pressure. Fractions were collected, resolved on TLC plates and observed under UV (254 nm).

High Performance Liquid Chromatography

Reverse phase chromatographic separation of fractions of methanolic extract was carried out, by using C18 as the station phase and water (solvent A) and acetonitrile (solvent B) as the mobile phase with the following gradient programme:

<table>
<thead>
<tr>
<th>Time</th>
<th>A (%)</th>
<th>B (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
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<tr>
<td>6</td>
<td>90</td>
<td>10</td>
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<td>12</td>
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<td>24</td>
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<td>80</td>
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<tr>
<td>30</td>
<td>100</td>
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and a flow-rate of 1 ml/min. Chromatographic peaks were identified at 280 nm.

In vitro antioxidant assays

DPPH radical scavenging assay

Determination of scavenging effects on 2,2’-Diphenyl-1-picrylhydrazyl (DPPH) radical was carried out with different fractions of soxhlet methanolic extract. In this assay, a commercially available and stable free radical (DPPH) which is soluble in methanol was used. DPPH has a strong absorption band at 517 nm, which disappears on reduction by an antioxidant (Aquino et al, 2001). The calibration curve was plotted with %DPPH SCAVENGED versus concentration of the standard antioxidant ascorbic. The results were expressed as ascorbic acid equivalent antioxidant capacity (TEAC).

ABTS radical scavenging assay

In this assay, 2,2’-azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) (7 mM) was allowed to react with potassium persulphate (2.5 mM) for a duration of more than 16 hrs in dark, at room temperature (Lee et al, 2006). This leads to incomplete oxidation of ABTS by persulphate to form ABTS radical. The fractions were then allowed to react with this solution and were checked for their ability to reduce the free radical, by a decrease in absorbance at 734 nm.
**Ferric Reducing Antioxidant Power (FRAP)**

The ferric reducing ability of the fractions was assayed using the FRAP assay. In this assay, the ability of the fractions to reduce the ferric-tripryidyltrazine (Fe³⁺-TPTZ) to ferrous form was checked. The ferrous form gives intense blue colour, which is monitored spectrophotometrically at 595 nm (Pulido et al, 2000). The results are expressed as ascorbic acid equivalent antioxidant capacity.

**2-Deoxy-D-ribose degradation assay**

The ability of the fractions to scavenge hydroxyl radical (·OH) was conducted in Fe³⁺–EDTA–H₂O₂–deoxyribose system. In this assay OH are formed by the Fenton reaction which leads to oxidation of 2-deoxyribose (Halliwell et al, 1987). The oxidation products when heated with thiobarbituric acid (TBA) under acidic conditions, yield a pink chromogen with the maximum absorbance at 532 nm. Results are expressed as percent inhibition of hydroxyl radical formation.

**Isolation of rat liver mitochondria**

Three months old female Wistar rats (weighing about 250 ± 20 g) were used, for the preparation of mitochondria. In brief, rat livers were homogenized and the homogenate was centrifuged. The resultant supernatant was centrifuged at 10,000 x g for 10 min to sediment mitochondria. This pellet was washed thrice with 5 mM potassium phosphate buffer, pH 7.4, to remove sucrose. Protein was estimated and pellets were suspended in the above buffer at the concentration of 10 mg protein/ml. Mitochondria at a final concentration of 0.25 mg/ml were treated with ascorbate-Fe²⁺ to generate hydroxyl radical and to induce oxidative stress (Devasagayam et al, 1983).

**Lipid Peroxidation**

After exposure of mitochondria to oxidative stress, the products of lipid peroxidation were measured as lipid hydroperoxides (LOOH) and thiobarbituric reactive substances (TBARS). Lipid hydroperoxides were measured using FOX II (Ferric oxidation in xylene orange) method (Nourooz-Zadeh et al, 1996). FOX II contains 90% methanol which facilitates solubilization of lipids. Oxidation of ferric in xylene orange due to lipid hydroperoxides, results in change in colour from orange to blue which is read at 560 nm. Concentration of LOOH is then calculated with the help of standard graph using H₂O₂. One of the end products of lipid peroxidation is Malonaldehyde (MDA) and other aldehydes. These aldehydic products were measured spectrophotometrically using TBA reagent which reacts with aldehydes to give a pink coloured product, which is read at 532 nm (Devasagayam et al, 1983; Pushpendran et al, 1998). The standard was prepared by acid hydrolysis of tetramethoxypropane.

**Protein sulphydryl depletion assay**

Depletion in protein sulphydryls were quantitated using Ellman’s reagent (5, 5-dithiobis-(2-nitrobenzoic acid and expressed as nmoles protein sulphydryls/mg protein (Santos et al, 1998).

**Pulse radiolysis**

The ability of the fractions to scavenge ABTS·⁻ and CO₃⁻ radicals were determined by pulse radiolysis. ABTS·⁻ radical was produced by the reaction of radiolytically generated azide radicals with ABTS·⁻. CO₃⁻ radicals were generated using reaction mixture containing 0.05 M NaHCO₃ and 0.05 M Na₂CO₃ saturated with N₂O. In the presence of the extract, the decay of ABTS·⁻ and CO₃⁻ are correlated with the concentration of ascorbic acid equivalents (Dixit et al, 2005). Pulse radiolysis experiments were carried out at the National Centre for Free Radical Research, University of Pune.

**Results**

**Thin Layer Chromatography**

Thin layer chromatography of methanolic extract developed using chloroform: ethyl acetate: methanol: water (10:80:15:8) yielded 3 bands with R₅ value of 0.6, 0.5 and 0.4 (Fig.1). Excellent resolution of the methanolic extract was obtained in this system.
levels. The DPPH* and ABTS radical scavenging assay corresponds to scavenging ability, while the FRAP assay corresponds to the reducing ability of the fractions. As can be seen in Figs. 2a and 2b, 0.1% of fractions 10, 11, 12, 14, 18, 21 and 24 exhibited high DPPH as well as ABTS radical scavenging activity. The ferric complex reducing ability of the fractions is shown in Fig. 2c. 0.1% of fractions 10, 11, 12, 14, 18, 21 and 24 exhibited highest reducing ability. The TLC profile of fractions 10, 11, 12, 14, 18, 21 and 24 is shown in Fig. 2d. These fractions contained a mixture of components. Therefore to facilitate better separation of the components, soxhlet methanolic extract was subjected to flash chromatography.

**Flash Chromatography**

Fractionation of soxhlet methanolic extract of Syndrex using flash chromatography yielded eight fractions. The TLC profile of these fractions is shown in Fig. 3. These eight fractions contained different components in varying concentrations, thus exhibiting better separation of the methanolic extract. These 8 fractions were then subjected to various in vitro antioxidant assays.

**In vitro antioxidant assays of flash chromatography fractions**

Among the 8 fractions tested 0.01% of fractions 1, 2 and 3 exhibited high DPPH and ABTS radical scavenging activity as well as were effective in reducing the ferric complex in the FRAP assay (Figs. 4a, 4b and 4c). Among the three fractions, 0.01% of fraction 1 scavenged 91% of DPPH radicals and 68% of ABTS radicals, whereas in the FRAP assay 0.01% of fractions 3 showed highest AEAC of 0.402. The inhibition of deoxyribose degradation capacity is shown in Fig. 4d. Nearly all the fractions tested exhibited this ability with 0.01% fraction 1 showing 82% inhibition.

The ability of these fractions to prevent oxidative damage to mitochondrial lipids induced by ascorbate-Fe$^{2+}$ system was checked in terms of TBARS

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**Column Chromatography**

Column chromatography of methanolic extract of Syndrex methanolic extract using glass column packed with silica gel and eluted with chloroform following stepwise gradient of ethyl acetate, methanol and water, yielded 200 fractions of 1 ml each. These fractions were analyzed using TLC, fractions showing similar $R_f$ values on TLC plates were pooled to yield a total of 34 fractions. These fractions were evaluated for their antioxidant potential using in vitro antioxidant assays.

**In vitro antioxidant assays of column chromatography fractions**

The fractions were assayed for their antioxidant potential by various in vitro antioxidant assays, which corresponded to action of antioxidants at different
**Fig. 2: In vitro antioxidant assays**

**Fig. 2a:** Radical scavenging activity of fractions as measured by DPPH assay

**Fig. 2b:** Radical scavenging activity of fractions as measured by ABTS assay

**Fig. 2c:** Ferric complex reducing ability of fractions by FRAP assay
and lipid hydroperoxides formed. 0.1% of fraction 1 inhibited formation of TBARS as well as lipid LOOH (Figs. 5a and 5b). Similarly 0.01% of fraction 1 prevented depletion in protein sulphydryls in mitochondria, in response to oxidative damage (Fig. 5c). Therefore as fractions 1, 2 and 3 exhibited high antioxidant activity, the radical scavenging activity was quantified using pulse radiolysis. Decay of ABTS·−
ABTS· and CO₃⁻ radicals were determined for the known concentrations of extracts and from the calibration curve, the ascorbate equivalents present in different extracts were determined. Fig. 6a shows the ABTS· radical scavenging activity by fraction 1, 2 and 3 respectively. Maximum ABTS· radical scavenging activity was exhibited by 1% of fraction 1 with a value of 27 μg/ml of AEAC and maximum CO₃⁻ radical scavenging activity was exhibited by 1% methanolic soxhlet extract with a value of 4.4 μg/ml of AEAC.

**High Performance Liquid Chromatography**

HPLC analysis of fraction 1 using 0-80% acetonitrile revealed three peaks having different retention times (Fig. 7a). The chromatograms for fractions 2 and 3 (Figs. 7b & 7c) showed peaks corresponding to the TLC profile of the fractions. The area under curve for the obtained peaks co-related to the visible concentrations of these fractions as seen in their respective TLC profiles.

**Discussion and Conclusion**

Hyperglycemia causes auto-oxidation of glucose, glycation of proteins and activation of polyol metabolism. These changes accelerate generation of ROS and increases oxidative chemical modification of lipids, DNA and proteins in various tissues. Oxidative stress plays an important role in the development of complications in diabetes such as lens cataracts, nephropathy and neuropathy (Valko et al, 2007).
Therefore supplementation with antioxidants to diabetic patients would help in reducing the oxidative load. At present, the treatment of diabetes mainly involves a sustained reduction in hyperglycaemia by the use of biguanides, thiazolidinediones, sulphonylureas, D-phenylalanine derivatives, meglitinides and α-glucosidase inhibitors in addition to insulin. However, due to unwanted side effects, the efficacies of these compounds are debatable and thus there is a demand for new compounds for the treatment of diabetes. Hence, plants have been suggested as a rich and unexplored source of potentially useful antidiabetic as well as antioxidative drugs (Saxena et al, 2004).
Fenugreek is one of the oldest herbs known to have beneficial therapeutic effects. Seeds of fenugreek have been shown to have multiple benefits in patients with diabetes such as reduction in blood sugar and its complications (Madar et al, 1998; Preet et al, 2006). Many earlier studies whether using whole seeds (Shani et al, 1974) or extracts (Ribes et al, 1989) showed that fenugreek seeds decreased fasting blood sugar levels in animals. Additionally fenugreek seeds also possess antioxidant activity, which can be attributed to the presence of various polyphenols. (Dixit et al, 2005) have shown that among the various extracts tested the soxhlet methanolic extract of germinated fenugreek seeds exhibited strong antioxidant activity. Evaluation of antioxidant activity of antidiabetic drug Syndrex, which contains powder of germinated fenugreek seeds, revealed that the soxhlet methanolic extract possessed strong antioxidant activity. Additionally this extract contained highest amount of flavonoids and polyphenols. This prompted us to further sub-fractionate the extract and identify the active principle conferring high in vitro antioxidant activity.

To resolve the soxhlet methanolic fraction, various chromatographic procedures were used. The extract was first separated onto a thin layer of silica gel using a combination of polar and non-polar solvents. Among the various combinations tried, 3 bands of Rf value 0.6, 0.5 and 0.45 were obtained in a solvent system comprising of chloroform: ethyl acetate: methanol: water in
Flash chromatography makes use of pressure to facilitate better separation of components of a mixture. A methanol: water (10: 80: 15: 8) containing different amounts of compounds were obtained (Fig. 3). To further narrow down the search for active antioxidant principle, the 8 fractions were tested for their antioxidant ability. Low concentration (0.01%) of fractions 1, 2 and 3 showed differential radical scavenging and ferric reducing activities. Besides checking the direct radical scavenging activity, antioxidant activity can be evaluated by checking the ability of the compounds to inhibit the damage caused by free radicals. Various human diseases, such as heart ailments, diabetes, cancer and Alzheimer’s disease are known to be either consequence or cause of ROS production. The major consequence of ROS production is damage to membrane lipids by formation of lipid peroxides, which via chain reaction enhances membrane damage (Hirokazu, 2007). One of major sites of free radical production in the cell, is mitochondria. Therefore the ability of the fractions to prevent oxidative damage to membrane lipids was checked by measuring the aldehydic products (in terms of TBARS) and lipid hydroperoxides (LOOH) formed. Oxidative damage to rat liver mitochondria was induced by ascorbate-Fe²⁺ system that generates OH-radical like species on incubation at physiological temperature of 37°C. Fraction 1 was the best at preventing ascorbate-Fe²⁺ induced damaged to lipids as well as proteins in rat liver mitochondria. Pulse radiolysis reveals the ability of antioxidants to scavenge secondary radicals such as ABTS•⁺ and CO₃•⁻. Fraction 1 efficiently scavenged the generated secondary radicals exhibiting the possible mechanism of its action. Thus fraction 1 exhibited high antioxidant activity in all the assays performed. TLC profile of fraction 1 showed that it is a mixture of 3 compounds in different amounts. The observed antioxidant activity of fraction 1 could be due to the additive effect of the compounds present in the fraction. HPLC analysis of fraction 1, 2 and 3 revealed presence of peaks at retention time from 14-15 minutes, suggesting that the different fractions contain overlapping compounds. Chromatogram of fraction 1 showed three different peaks with different area under curve, which correlated with the differential amount of bands seen in the TLC profile of fraction 1 (Fig. 7a). Similar analysis was

Fig. 7c: HPLC analysis of fractions from flash chromatography
observed for fraction 2 and 3. All of these fractions eluted in 30% acetonitrile, suggesting the polar nature of the compounds in the fractions.

The above antioxidant activity and functional analysis of fractions indicate the likely presence of polyphenolic compounds in these fractions. Polyphenolic compounds are well known to play an important role in antioxidant activity. Naturally occurring polyphenols are known to have numerous biological activities and found to be potential candidates for use as drugs, in diseases like heart ailments, ulcer, diabetes and neuronal disorders. From a chemical point of view, polyphenols can react with one-electron oxidants which prevent free radical formation in biological systems. Such single electron oxidation products are considered to be key steps of structural analysis of polyphenolic compounds in these fractions. The above antioxidant activity and functional analysis of fractions indicate the likely presence of polyphenolic compounds in these fractions.

Our results thus suggest, that we were successful in standardizing the method for sub-fractionating the soxhlet methanolic extract. The obtained fractions revealed antioxidant activity comparable to that of known antioxidant such as ascorbic acid. Further studies such as purification of the fractions, characterization of the functional groups and identification of the pure compounds from the fractions would help us to give insights in the structure activity relationship of the compounds. Additionally in vivo studies would help to further understanding the mechanism of action of the purified compounds.

References


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**ABOUT THE AUTHOR**

Dr. T.P.A. Devasagayam joined BARC in 1975, from BARC Training School in Biology and Radiobiology, subsequent to his completion of Masters in Zoology from the American College, Madurai. At present he is Scientific Officer (H) and Head, Radiation Biochemistry Section in the Radiation Biology and Health Division of BARC. His major area of research relates to the role of free radicals in human health and radiation biology and potential uses of natural antioxidants. He has done his post-doctoral research in Germany and USA. He has over 130 publications in refereed journals with over 1500 citations. He has played a central role in the organization and development of the Society for Free Radical Research-India (SFRR-India). He was the first Secretary-General of SFRR-India and currently holds the position of President. He is also the current President of SFRR-Asia.