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In vitro biological activity and total phenolic content of *Morus nigra* seeds

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ABSTRACT

The present study was designed to investigate phenolic content, antidiabetic and antioxidant activity of Morus nigra seeds. Extracts were prepared by soxhlet extraction using petroleum ether, ethyl acetate, ethanol and double distilled water as solvent in increasing order of polarity. The highest extractive yield was obtained in aqueous extract. Each extract was subjected to undergo phytochemical screening by standard protocol. Phytochemical screening revealed the presence of numerous bioactive secondary metabolites in seeds. Total phenolic content was estimated by Folin-Ciocalteau method. Ethanol extract contain highest phenolic content which was found to be 7.44 mg GAE/100 g dw. Antidiabetic activity was evaluated by α- amylase and α- glucosidase inhibitory assay while antioxidant activity was estimated by DPPH radical scavenging assay and ferric reducing antioxidant potential (FRAP) method. Ethanol extract demonstrated prominent α- amylase (IC_{50} = 679.50 µg ml^{-1}), α- glucosidase (IC_{50} = 327.90 µg ml^{-1}), DPPH radical scavenging (IC_{50} = 211.00 µg ml^{-1}) and FRAP activity (278.64 µM/ml, FRAP value 0.779). The study provides a scientific proof for Morus nigra seeds regarding their traditional therapeutic application. Seeds can be used to treat diabetes and its associated secondary complications that arise due to oxidative stress.

Keywords: *Morus nigra,* Total phenolic content, α-Amylase, α-Glucosidase, DPPH, FRAP

INTRODUCTION

The term "diabetes" is generally used for diseases marked by excessive urination and usually refers to diabetes mellitus [1]. Basically diabetes is of two type diabetes insipidus and diabetes mellitus (DM). Diabetes insipidus is less prevalent and occurs due to the deficiency of anti-diuretic hormone (ADH or Vasopressin) released from posterior pituitary (Neurohypophysis). On other hand diabetes mellitus (DM) is third most prevalent disease which arises from total or partial insulin deficiency, and is characterized by high blood sugar (hyperglycemia) and glycosuria and this leads to provide a pathway for other life threatening complications like atherosclerosis, hyperlipidemia, nephropathy, neuropathy and retinopathy [2]. It is the seventh leading cause of death in the United States. In India every fourth man is a diabetic or has the chance of becoming a diabetic patient. Indian with big bellies central obesity makes them more prone to diabetes. The global prevalence of diabetes is estimated to increase, from 4% in 1995 to 5.4% by the year 2025. It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025 [3]. As a very common chronic disease, it is becoming the third "killer" of the health of mankind all over the world along with cancer, cardiovascular and cerebrovascular diseases because of its high prevalence, morbidity and mortality [4].

Despite of substantial progress in the treatment of diabetes mellitus by oral hypoglycaemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The side effects associated with the prolonged use of hypoglycaemic agents have imposed the demand for safe and effective drugs especially of herbal origin [5, 6]. In different traditional medicine systems crude plant extracts or their active constituents are being used

for management of diabetes. Herbal drugs are considered free from side effects than synthetic one. They are less toxic, relatively cheap and well accepted [7]. Furthermore, after the recommendation made by WHO on diabetes mellitus, investigations on hypoglycaemic agents from medicinal plants have become more important [8]. The herbal drugs with antidiabetic activity are needed to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine [9]. It is estimated that more than 800 plant species having hypoglycaemic activity and more than 450 plants have been experimentally tested [10, 11, 12].

Morus nigra L. is commonly known as black mulberry and belongs to family Moraceae. It is traditionally used in ayurveda, unani and Chinese system of medicine with a number of pharmacological properties. The fruits, leaves, barks and roots of this plant are used as laxative, sedative, expectorant, refresher, emollient, calmative, diuretic, hypoglycaemic agent, antiseptic, anti-inflammatory, antioxidant, and in the treatment of eczema, and oral inflammation [13, 14, 15, 16]. In China, the leaves of *Morus sp*. are used as an antioxidant, antimicrobial agent and anti-inflammatory [17]. The leaves of *M. nigra* are commonly used by women in menopause as a substitute for the conventional hormonal replacement therapy, with a similar effect to that obtained after estrogen use [18, 19].

Till date, no work was previously reported on seeds of this plant. The present study aims at estimating total phenolic content, antidiabetic and antioxidant activity of seeds of *Morus nigra*, to prove its therapeutic efficacy in diabetes treatment, so that its traditional use in various medicine systems can be claimed.

EXPERIMENTAL SCETION

Reagents and Chemicals

Petroleum ether, ethyl acetate, ethanol, Folin's ciocalteau phenol reagent, Ascorbic acid, Tris buffer (Merck); P-Nitrophenyl-α-D-glucopyranoside [p-NPG], 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), α-amylase (Hi-media); Gallic Acid (Loba chemie), Dimethyl sulphoxide (Rankem); sodium carbonate (CDH); 1,1-diphenyl-2-picrylhydrazyl [DPPH] (Sigma Aldrich); 3,5-Dinitrosalicylic acid [DNSA] (S.d.fine-chem Ltd); α-glucosidase (SRL, Mumbai); acarbose (Bayer India Limited) were purchased. All other chemicals and reagent used were of analytical or HPLC grade.

Collection of Plant Material

Fruits of *Morus nigra* were collected from Dehradun, Uttarakhand (India) in May, 2012 and authenticated by Botanical Survey of India, Northern Regional Centre, Dehradun (BSD) under accession No. 114150. A voucher specimen has been retained in Department Of Chemistry, Gurukula Kangri Vishwavidyalaya, Haridwar under the registry No. 24/15. Collected fruits were harvested for seeds. They were washed with water to remove dirt, dried in shade and finally grinded in to powder form and stored in polythene bags for further use.

Preparation of Extracts

Extraction of *Morus nigra* seeds was done using soxhlet apparatus. Briefly 170 gm of powdered seeds were loaded in thimble of soxhlet extractor and extracted with petroleum ether, ethyl acetate, ethanol and double distilled water in increasing order of polarity. A minimum of 75 cycles of siphoning was done for each solvent, and the process was continued for 72 hours or until the solvent in the extractor siphon tube became colourless. Extracts were concentrated at reduced pressure in a rotary vacuum evaporator and refrigerated until further use.

Phytochemical Screening

Phytochemical analysis for various phytoconstituent present in extracts was undertaken using standard qualitative methods [20, 21]. Each extract was screened for the presence of biologically active compounds like alkaloids, carbohydrates, glycosides, amino acid, proteins, steroids and triterpenoids, flavonoids, phenolics and tannin, gums and mucilages, naphthoquinones etc.

Total Phenolic Content

Total phenolic content of each extract was determined by Folin-Ciocalteau method [22] with a little modification. Briefly 1ml of extract dilution in DMSO (1000 µg ml^{-1}) or gallic acid (100 - 700 µg ml^{-1}) was added to a 50 ml volumetric flask, containing 35 ml of distilled water. Distilled water serves as blank. 2.5 ml of Folin-Ciocalteau's phenol reagent was added to the mixture and shaken. After 8 min of incubation at room temperature, 7.5 ml of 20 % Na_2CO_3 was added to the mixture. The mixture was diluted to 50 ml with distilled water and mixed. After incubation for 2 hrs at room temperature, the absorbance was measured at 765 nm with UV-Vis spectrophotometer (Agilent Technologies cary-60). Calculations were performed using the calibration curve of gallic acid. The total phenolic content of extracts was expressed as milligrams of gallic acid equivalents (GAE) per 100 grams dry weight (mgGAE.$100g^{-1}$ dw).

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Antidiabetic Activity
Anti-diabetic activity of each extract was assessed by alpha amylase and alpha glucosidase inhibitory method

Alpha-Amylase Inhibition Activity
The α-amylase inhibitory activity of extracts was performed using DNSA method with a little modification [23]. Briefly, 1 ml of each solution of different concentration (1-5000 µg ml^{-1}) of extract or standard acarbose in DMSO was incubated with 1 ml of α-amylase (concentration 3 $mg.ml^{-1}$ in 20 mM phosphate buffer containing 6.7 mM NaCl, pH 6.9) for 30 min at 37ºC. After pre incubation, 1 ml of 1% starch solution in 20 mM phosphate buffer, pH 6.9, was added. The reaction mixtures were then incubated for 15 minutes at 37ºC. The reaction was stopped by adding 1 ml of DNSA color reagent (96 mM 3,5-dinitrosalicylic acid and 5.315 M sodium potassium tartrate in 2 M NaOH). The tubes containing resultant mixture were then incubated in a boiling water bath for 5 min and then cooled to room temperature. The absorbance was taken at 540 nm with a UV-Vis spectrophotometer (Agilent Technologies cary-60 spectrophotometer) after diluting each tube with 9 ml of deionised water. For correcting background absorbance (absorbance due to extracts or standard) the enzyme was replaced by 1 ml buffer solution with similar test procedure. The α-amylase inhibitory activity was calculated by equation [24].

$$\textbf{α-amylase inhibitory activity (\%Inhibition)} = \frac{[(A_{C}+ - A_{C}-) - (A_S - A_B)]}{(A_{C}+ - A_{C}-)} \times 100$$

Where A_C+ represents absorbance of pure control having 100% enzyme activity (DMSO and Enzyme), A_C- symbolize absorbance of blank for pure control having 0% enzyme activity (DMSO and Buffer), A_S represent absorbance of sample or standard (sample/standard and Enzyme) and A_B symbolize for background absorbance due to sample and standard (sample/standard and Buffer). IC_{50} of each extracts and standard acarbose was calculated by graphical method by plotting % inhibition *vs.* concentration.

Alpha-Glucosidase Inhibition Activity
The α-glucosidase inhibitory activity of extracts was determined according to cetto *et al.* with a little modification [25]. Briefly, 1 ml of each solution of different concentration (1-5000 µg ml^{-1}) of extracts or standard acarbose in DMSO was incubated with 1 ml of α-glucosidase (1$U.ml^{-1}$ in 100 mM phosphate buffer pH 6.8) for 30 min at 37ºC. After pre incubation, 1 ml of, p-NPG in 100 mM phosphate buffer, pH 6.8, was added. The reaction mixtures were then incubated for 15 minutes at 37ºC. The reaction was stopped by adding 4 ml 0.5 M tris buffer. The absorbance was taken by UV-VIS spectrophotometer (Agilent Technologies cary-60) at 410 nm. For correcting background absorbance the enzyme was replaced by 1 ml buffer solution with similar test procedure. The % inhibition and IC_{50} was calculated in similar way as mentioned in α-amylase activity. Earlier 0.1 M NaOH was used to stop the reaction but it brought auto degradation of p-NPG which causes a steady increase in absorbance at 410 nm.

Anti-Oxidant Activity
Anti-oxidant activity of extracts was evaluated by DPPH free radical scavenging assay and by ferric reducing antioxidant potential assay

DPPH Free Radical Scavenging Assay
The free radical scavenging assay extracts was performed by stable DPPH free radical according to the method of Brand-Williams with some modification [26]. A working solution of 0.004% was freshly prepared by dissolving 10 mg of DPPH in 250 ml of methanol. 1 ml of each solution of different concentration (1, 5, 10, 50, 100, 500 µg/ml in DMSO) of extracts was added to 3 ml working solution of DPPH. After 30 min the absorbance of the preparations were taken at 517 nm by UV-VIS spectrophotometer (Agilent Technologies cary-60) which was compared with the corresponding absorbance of standard ascorbic acid of similar concentrations (1-500 µg/ml). 1 ml of DMSO with 3 ml of working DPPH solution serves as blank. The % radical scavenging activity or % inhibition or % reduction in color or % antiradical activity was calculated by equation

$$\textbf{\% Inhibition} = \frac{\textbf{(Abs of blank – Abs of sample/ standard) after 30 min}}{\textbf{(Abs of blank) after 30 min}} \times 100$$

IC_{50} of each extract and standard ascorbic acid was calculated by graphical method by plotting % inhibition *vs* concentration.

Ferric Reducing Antioxidant Potential Assay

The FRAP assay was done according to a Shukla *et al*. [27]. The stock solutions included 300 mM acetate buffer (3.1g NaOAc .$3H_2O$ and 16 ml Glacial HOAc), pH 3.6, 10 mM TPTZ (2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The working FRAP reagent was freshly prepared by mixing acetate buffer, TPTZ solution and $FeCl_3.6H_2O$ solution in proportion of 10:1:1 (v/v) and then warmed at 37^0C before using it. Antioxidant potential was determined by reacting a mixture 1 ml of each extracts of concentration 500 µg ml^{-1} in DMSO and 10 ml of working FRAP reagent. Absorbance of colored product (ferrous tripyridyltriazine complex) was then taken at 593 nm after 4 min of incubation at 37^0C. Ascorbic acid standard solution was tested in parallel process. The standard curve was linear between 100 - 800 µM ascorbic acid. Working FRAP reagent serves as blank and 1 ml of DMSO with 10 ml of working FRAP reagent act as control. Calculations were made by calibration curve. Results were expressed as µM ml^{-1}. FRAP value of each extracts was calculated by equation.

$$\text{FRAP value} = \frac{\text{Change in abs of Sample from 0 to 4 min}}{\text{Change in abs of Standard from 0 to 4 min}} \times \text{FRAP value of standard}$$

Statistical Analysis

Results were presented as mean ± standard deviation of triplicate measurements and analyzed as one-way analysis of variance (sigmastat ver. 2.0). The significant differences between means were calculated by a one way analysis of variance (ANOVA) using dunnett multiple-range test at $P<0.05$.

RESULTS AND DISCUSSION

Extractive Yield

Soxhlet extraction of seeds with different solvents in increasing order of polarity yielded different extracts. The extractive yield (in % w/w) with their consistency after complete removal of respective solvent is illustrated in Table 1. The maximum yield was obtained in aqueous (21.13%) followed by petroleum ether (17.97%) extract respectively.

Table 1: Extractive yield of different extracts of *Morus nigra* seeds

Extracts	Consistency	% Yield (w/w)
Petroleum Ether	Oily, Viscous	17.97
Ethyl Acetate	Semisolid	2.06
Ethanol	Semisolid, Sticky	2.62
Aqueous	Semisolid, Fluidy	21.13

Phytochemical Screening

Phytochemical screening (Table 2) of each extract revealed that seeds contain a number of secondary metabolites like alkaloids, carbohydrates, glycosides, inulin, protein, amino acid, Steroids, triterpenoids, fixed oils, fats, phenolic group, flavonoids, gums and mucliages and naphthoquinones. The presence of these phytoconstituents suggests that the seeds of this plant might be of medicinal importance to pharmaceutical industries. Seeds are devoid of hydroxyanthraquinones. Secondary metabolites and other chemical constituents of medicinal plants account for their medicinal value [28] and the results of phytochemical screening are indicative for its therapeutic value and also for the use of this plant traditionally in a number of disorders.

Table 2: Phytoconstituents present in different extracts of *Morus nigra* seeds

Phytoconstituents and Test performed			Extracts			
			PE	**EA**	**ET**	**AQ**
Alkaloids	Mayer's Test		-	-	-	-
	Wagner's Test		-	-	+	-
	Hager's Test		+	+	+	-
	Tannic acid Test		-	+	+	+
Carbohydrate	Molisch's Test		-	-	+	+
	Fehling's Test		-	-	+	+
	Benedict's Test		-	-	-	+
	Selivanoff's Test		-	-	-	+
Glycosides	Anthraquinone glycosides	Borntrager's Test	+	-	-	-
		Hydroxyanthraquinone	-	-	-	-
	Cardiac glycosides	Keller-Killiani Test	+	+	-	-
		Legal's Test	-	-	-	-
		Baljet's Test	-	-	-	-
	Saponin glycosides	Froth formation Test	-	-	-	+
	Flavanol glycosides	Mg and HCl reduction	-	-	+	-
Inulin			-	-	+	-
Protein	Heat Test		-	-	+	-
	Biuret Test		-	-	+	+
	Xanthoproteic Test		-	-	+	+
Amino Acid	Ninhydrin Test		-	-	-	+
Steroid/ Triterpenoid	Salkowski Test		+ (T)	+ (S)	+ (S)	-
Fixed oils and Fats	Spot Test		+	+	+	-
	Saponification Test		+	+	+	-
Flavonoids	Shinoda Test		-	-	+	-
	Alkaline reagent Test		-	-	-	-
	Zinc hydrochloride Test		-	-	+	-
Phenolic compounds and Tannins	Lead Acetate Test		-	-	+	+
	Ferric chloride Test		-	-	+	+
	Test for Catechin		-	-	-	-
	Test for Chlorogenic acid		-	-	-	-
Gums and Mucilage			-	-	+	+
Naphthoquinone	Juglone Test		-	+	+	+
	Dam-Karrer Test		-	-	-	-

+: Present, -: Absent, PE: Petroleum ether, EA: Ethyl acetate, ET: Ethanol, AQ: Aqueous, T: Triterpenoids, S: Steroids

Total Phenolic Content

Phytochemicals have various protective and therapeutic effects which is crucial to prevent diseases and maintain a state of well being. Phenolic constitutes a diverse class of chemical compounds and are well known for their powerful biological activity. The increasing interest in the potent biological activity of phenolics outlined the necessity of determining their content in medicinal plants. The total phenolic content of *Morus nigra* seed extracts is shown in Table 3. The total phenolic content was expressed as mg GAE/100 g dw using the standard curve (Figure 1) equation: $y = 0.0014x + 0.0278$, $R^2 = 0.9983$ where y is the absorbance at 765 nm and x is the total phenolic content in 1000 µg/ml of extract.

Table 3: Total phenolic content in of *Morus nigra* seed extracts

Extracts	Total Phenolic Content (mg GAE/100 g dw)*
Petroleum Ether	5.44 ± 0.90
Ethyl Acetate	5.08 ± 1.92
Ethanol	7.44 ± 0.69
Aqueous	4.80 ± 1.22

** Results are expressed as mean of 3 values ± standard deviation*

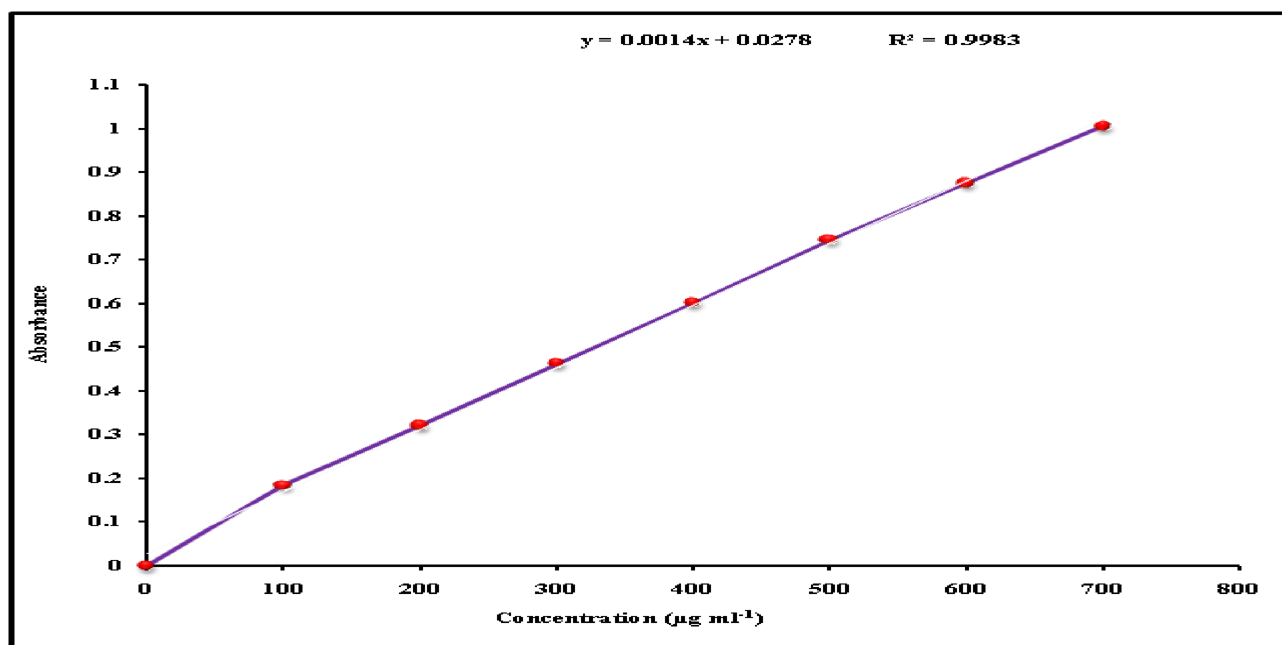

Figure 1: Standard Curve of Gallic acid for estimating total phenolic content

The phenolic content was found to be highest in ethanol extract followed by petroleum ether extract. It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health beneficial effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores [27].

Antidiabetic Activity

The rapid absorption of glucose is recognized as a risk factor for type II diabetes mellitus, and modulation of glucose absorption is therefore a potential strategy for the control of hyperglycaemia. Postprandial glucose control is one of the possible six mechanisms that can be potentially used for controlling diabetes. Thus, the therapeutic approach pertaining to lower postprandial blood sugar can be achieved by delaying the absorption of glucose by inhibiting carbohydrate hydrolysis enzymes such as α- amylase and α-glucosidase in the gastrointestinal tract. Till now there is no study available on anti-diabetic activity of seeds of this plant with special reference to carbohydrates hydrolysing enzyme inhibition, this is the first ever investigation performed to evaluate antidiabetic activity of *Morus nigra* seeds using α-amylase and α-glucosidase inhibitory method.

Alpha-Amylase Inhibition Activity

The *in vitro* α- amylase inhibitory activity of *Morus nigra* seed extracts compared with acarbose is illustrated in Table 4. Figure 2 graphically represents the alpha amylase inhibition on changing the concentration of each extract and helps in estimation of IC_{50} value of each extract as well as standard acarbose. IC_{50} value is the concentration of extract or standard drug which is required to inhibit 50 percent of the enzyme in reaction mixture**.** All the extracts signifies a dose dependent α- amylase inhibitory activity. Acarbose showed percentage alpha amylase inhibition of 3.756% - 89.239% on varying concentration from 1-5000 µg ml^{-1} with an IC_{50} value 617.23 µg $ml^{-1.}$ Lower IC_{50} value corresponds to greater potency and better therapeutic efficacy. Ethanol extract reflects the highest alpha amylase inhibitory activity (IC_{50} = 679.50 µg ml^{-1}) followed by ethyl acetate extract (IC_{50} = 780.90 µg ml^{-1}). The IC_{50} value of ethanol extract is nearly comparable with acarbose and thus can be regarded as an excellent alpha amylase inhibitor.

Table 4: Alpha amylase inhibitory activity of *Morus nigra* seed extracts compared with acarbose

Extract/Standard	IC_{50} value (µg ml^{-1})
Acarbose	617.23 ± 3.25
Petroleum Ether	2013.60 ± 4.78
Ethyl Acetate	780.90 ± 6.28
Ethanol	679.50 ± 2.44
Aqueous	1452.50 ± 3.02

* *Results are expressed as mean of 3 values ± standard deviation*

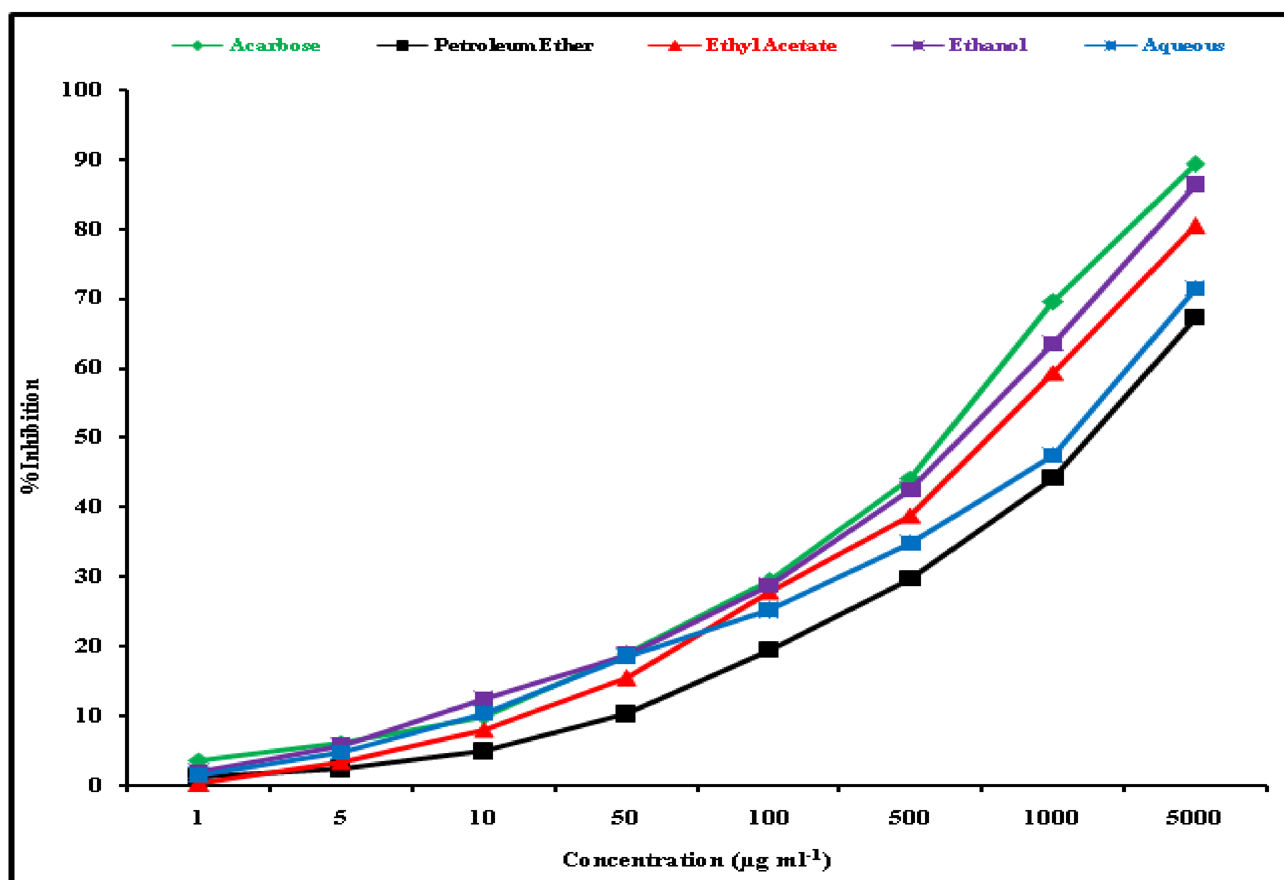

Figure 2: Alpha amylase inhibitory activity and estimation of IC_{50} value of *Morus nigra* seed extracts and acarbose

The alpha amylase inhibitory activity shown by all extracts might be due to a various phytoconstituents present in each extract. Furthermore the potent activity of ethanol extract must be attributed to its high phenolic content and majority of phytoconstituents present in it. The available literature depicts that alkaloids, terpenoids, glycosides, flavonoids and phenolic components were known to show alpha amylase inhibitory activity [29, 30].

Alpha-Glucosidase Inhibition Activity

The *in vitro* α- glucosidase inhibitory activity of *Morus nigra* seed extracts compared with acarbose is illustrated in Table 5. Figure 3 graphically represents the alpha glucosidase inhibition on changing the concentration of each extract and helps in estimation of IC_{50} value of each extract as well as standard acarbose. Again the highest alpha glucosidase inhibitory activity was demonstrated by ethanol extract (IC_{50} = 327.90 µg ml^{-1}) followed by aqueous extract (IC_{50} = 652.80 µg ml^{-1}). The IC_{50} value of ethanol extract is even better than acarbose indicating its extremely potent nature.

Table 5: Alpha glucosidase inhibitory activity of *Morus nigra* seed extracts compared with acarbose

Extract/Standard	IC_{50} value (µg ml^{-1})
Acarbose	358.42 ± 2.48
Petroleum Ether	904.56 ± 8.17
Ethyl Acetate	716.00 ± 5.66
Ethanol	327.90 ± 2.75
Aqueous	652.80 ± 3.15

** Results are expressed as mean of 3 values ± standard deviation*

Previous literature evidenced alkaloids, phenolics, terpenoids and flavonoids as potent α-glucosidase inhibitors [31] and these phytoconstituents can be well cited in ethanol extract. Further naturally occurring extracts could be well tolerated as antidiabetic agent in comparison to synthetic inhibitors with a number of side effects like bloating, abdominal discomfort, diarrhoea and flatulence [32].

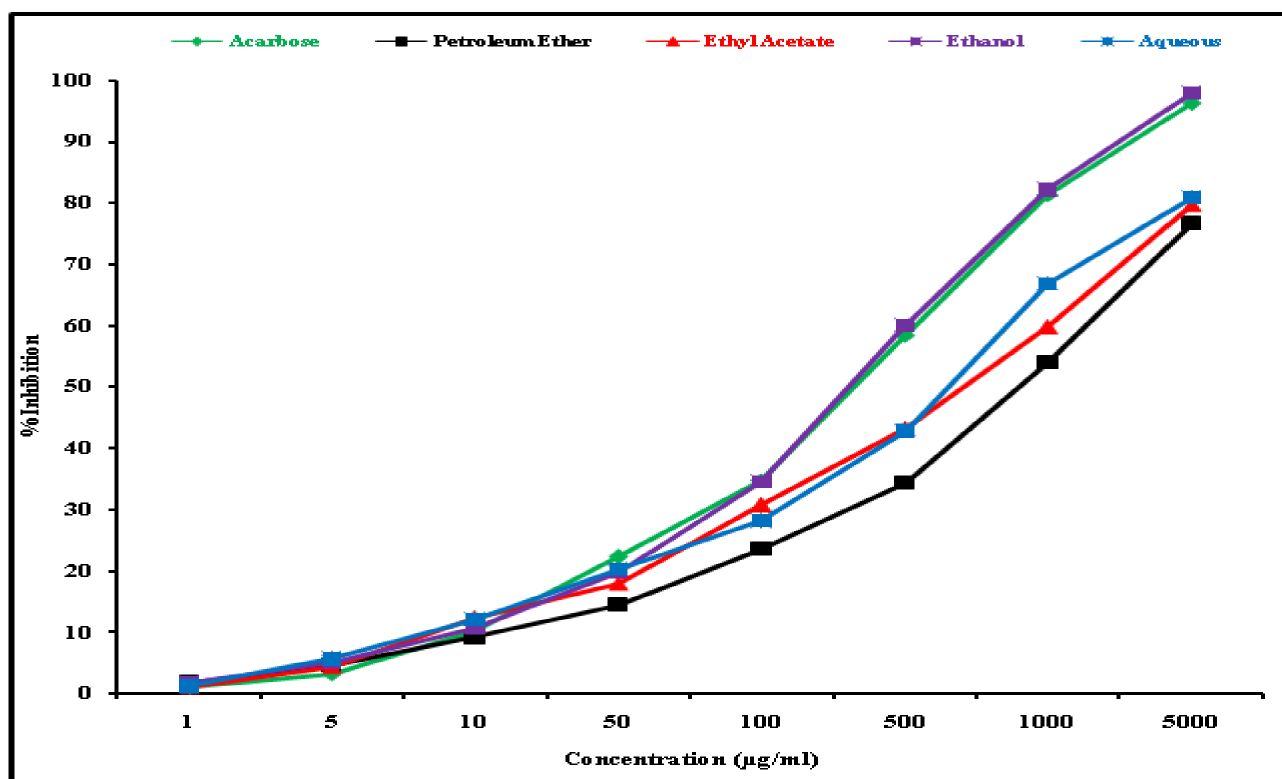

Figure 3: Alpha glucosidase inhibitory activity and estimation of IC_{50} value of *Morus nigra* seed extracts and acarbose

Anti-Oxidant Activity

Hyperglycaemia causes oxidative stress, which further aggravates the progression of diabetes mellitus and its complications. The role of reactive oxygen species (ROS) in diabetes is well known and recently studies on the connection between oxidative stress, diabetes and diabetic complications are efficiently going on [33]. In diabetes as a consequence of oxidative stress, free radical production increases while antioxidant production decreases. So, increased free radical concentration can be considered as one of the major complication of diabetes [34]. So the role of antioxidants in treatment of diabetes is always crucial.

DPPH Free Radical Scavenging Assay

DPPH is a nitrogen centred free radical with a characteristic absorption at 517 nm, antioxidants reacts with DPPH and convert it in to1,1-diphenyl-2-picryl hydrazine, due to their hydrogen donating or electron releasing ability at a very rapid rate [35]. As antioxidants donate proton or provide electron to these radicals the absorption decreases. The decrease in absorption is taken as a measure of the radical scavenging activity. DPPH radical scavenging activity of *Morus nigra* seed extracts and their comparison with standard ascorbic acid is in illustrated in Table 6. Figure 4 depicts the concentration versus percentage inhibition graph and estimation of IC_{50} value of extracts and ascorbic acid. Ethanol extract (IC_{50} = 211.00 µg ml^{-1}) showed remarkable activity followed by ethyl acetate extract (IC_{50} = 257.00 µg ml^{-1}).

Table 6: DPPH radical scavenging activity of *Morus nigra* seed extracts compared with ascorbic acid

Extract/Standard	IC_{50} value (µg ml^{-1})
Ascorbic Acid	22.72 ± 0.09
Petroleum Ether	489.00 ± 0.02
Ethyl Acetate	257.00± 0.06
Ethanol	211.00± 0.11
Aqueous	353.00 ± 0.26

** Results are expressed as mean of 3 values ± standard deviation*

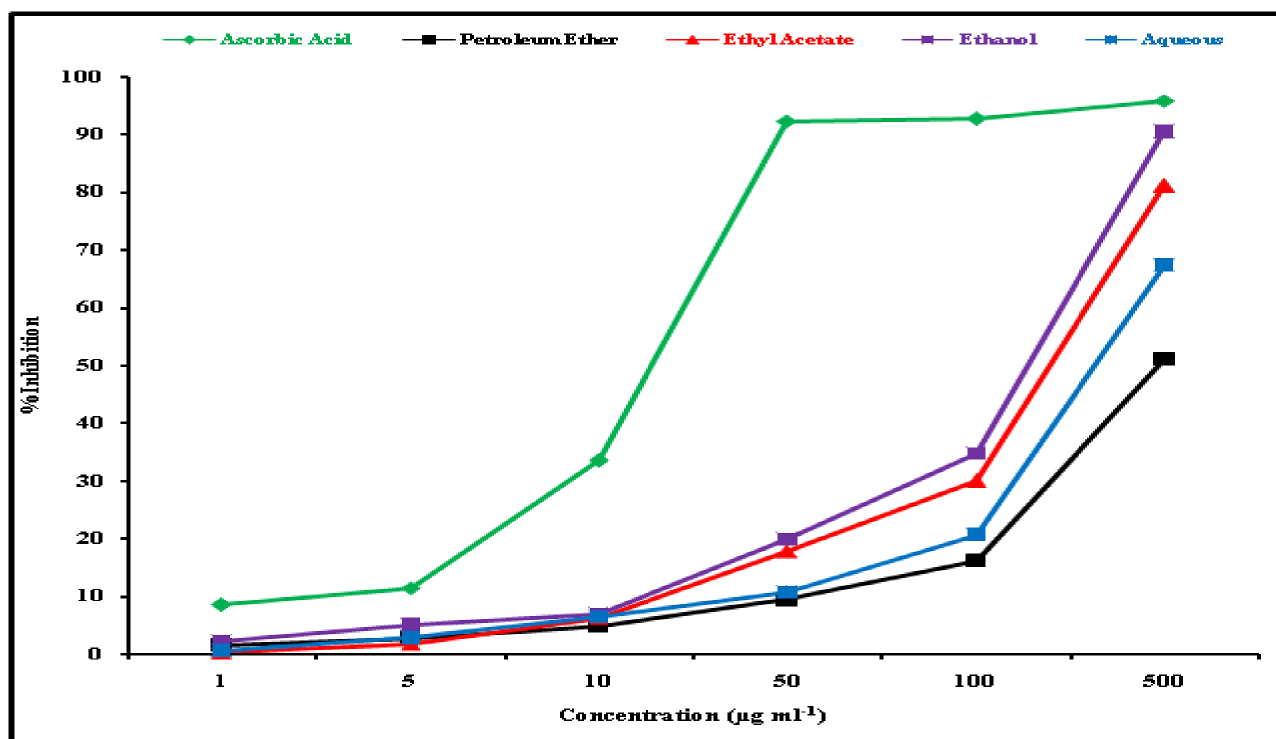

Figure 4: DPPH radical scavenging activity and estimation of IC_{50} value of *Morus nigra* seed extracts and ascorbic acid

Generally DPPH radical scavenging method assesses the antioxidant activity of those sample or component which scavenges free radical by proton donation or by electron transfer. Phenolic compounds and flavonoids have been demonstrated to exhibit a scavenging effect for free radicals [36]. Ascorbic acid is a potent free radical scavenger and in comparison to such a pure component, ethanol extract showed significant radical scavenging activity.

Ferric Reducing Antioxidant Potential Assay

The total antioxidant capacity of extracts was evaluated by ferric reducing antioxidant potential assay (FRAP). FRAP-method was initially developed to assay plasma antioxidant capacity, but can be used to measure the antioxidant capacity from a wide range of biological samples and pure compounds [37]. The results for FRAP assay of *Morus nigra* seed extracts are illustrated in Table 7. The results were expressed as µM ml^{-1} using the standard curve (Figure 5) equation: $y = 0.0014x + 0.0649$, $R^2 = 0.9885$ where y is the absorbance at 593 nm and x is the ferric reducing antioxidant ability in 500 µg ml^{-1} of extracts. The unit µM ml^{-1} means the quantity of Fe^{3+} in µM that can be reduced to Fe^{2+} by per ml of extract or ascorbic acid. The highest FRAP activity was shown by ethanol extract (278.64 µM/ml, FRAP value 0.779) followed by petroleum ether extract (227.92 µM/ml, FRAP value 0.657).

Table 7: Ferric reducing antioxidant potential (FRAP Assay) of *Morus nigra* seeds compared with ascorbic acid

Extracts	**Ferric reducing antioxidant power (µM/ml)***	**FRAP Value**
Ascorbic acid	787.92 ± 0.14	2.000
Petroleum ether	227.92 ± 1.16	0.657
Ethyl acetate	139.35 ± 0.51	0.445
Ethanol	278.64 ± 0.60	0.779
aqueous	87.21 ± 1.60	0.320

** Results are expressed as mean of 3 values ± standard deviation.*

Even though the DPPH radical scavenging activity of ethyl acetate extract is better than petroleum ether extract, the FRAP activity of latter is superior to the former. Thus it is indicative that petroleum ether contains such constituents which act other than by radical quenching mechanism. The tremendous total antioxidant activity will maintain the level of antioxidant in body and definitely support in the treatment of diabetes associated complication that may arise due to elevated oxidative stress.

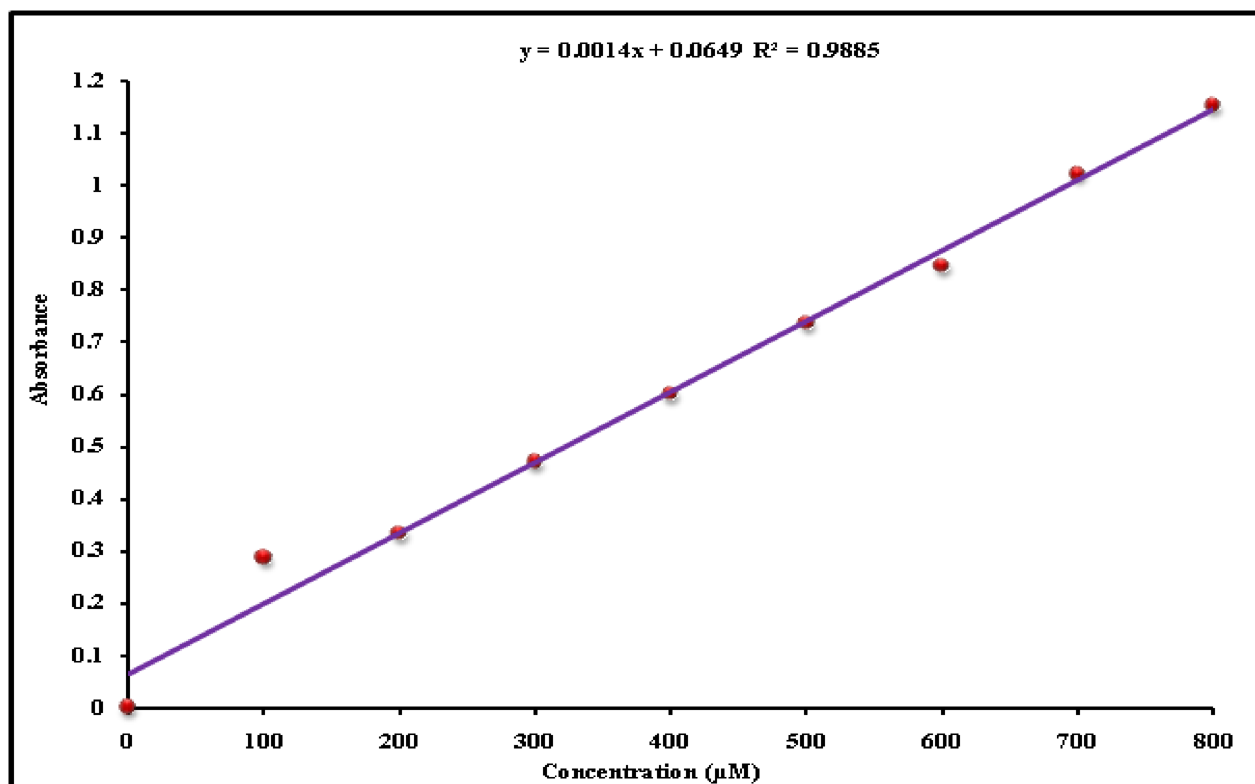

Figure 5: Standard Curve of Ascorbic acid for estimating Ferric reducing antioxidant potential

CONCLUSION

This study is the first comprehensive documentation demonstrating anti-diabetic and antioxidant activity along with estimation of total phenolic content of *Morus nigra* seeds. The seeds of this plant can be used to treat diabetes and its associated secondary complications that arise due to oxidative stress. Further phytochemical investigation will facilitate to isolate active molecule/molecules which could be potent α-amylase and α-glucosidase inhibitor of plant origin and an attractive strategy for treating diabetes could be developed. Phytochemical studies will also lead to isolate some active principles that might have anti-oxidant potential also.

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REFERENCES

[1] Kar A. Medicinal Chemistry, Fourth Edition, New age International (P) limited publishers, New Delhi, India, **2007**; 668–669.
[2] M Yadav; R Tomar. *Asian J. Biochem*., **2003**; 3(3), 182–187.
[3] HA Ahad; CS Kumar; PS Nanda; U Bhanu; BV Ravindra; V Mohan. *J Innov. Trends in Pharma. Sci*., **2010**; 1(2), 69–78.
[4] WL Li; HC Zheng; J Bukuru; DN Kimpe. *J. Ethnopharmacol*. **2004**; 92, 1–21.
[5] PJ Watkins. *Brit. Med. J*., **2003**; 326, 874–876.
[6] JK Grover; S Yadav; V Vats. *J. Ethnopharmacol*., **2002**; 8, 81–100.
[7] Momin. Role of indigenous medicine in primary health care. 1st International Seminar on Unani Medicine, New Delhi, **1987**; 54.
[8] Anonymous. WHO Expert Committee on Diabetes mellitus, Technical report series. World Health Organization, Geneva, **1980**.
[9] KA Wadkar; CS Magdum; SS Patil; NS Naikwade. *J. Herbal Med. Toxicol*., **2008**; 2, 45–50.
[10] O Bever. Oral hypoglycemic action of medicinal plants in tropical West Africa, Cambridge University Press London, **1986**.
[11] MK Rai. *Ancient Science of Life*, **1995**; 14, 42–54.

[12] FJA Aguilara; RR Ramos; SP Gutierrez; AA Contreras; CCC Weber; JLF Saenz. *J. Ethnopharmacol.*, **1998**; 61, 101–110.
[13] S Oryan; M Eydi; E Yazdi; A Eydi; J Soulati. *J. Med. Plants*, **2003;** 2, 27–32.
[14] AG Naderi; S Asgary; N Sarraf-Zadegan; H Oroojy; F Afshin-Nia. *Phytother. Res.*, **2004**; 18, 365–369.
[15] S Ercisli; E Orhan. *Food Chem.*, **2007**; 103, 1380–1384.
[16] MM Padilha; FC Vilela; CQ Rocha; MJ Dias; R Soncini; G Alves-da-Silva; A Giusti-Paiva. *Phytother. Res.*, **2010**; 24, 1496–1500.
[17] W Song; H Wang; P Bucheli; P Zhang; D Wei; Y Lu. *J. Agr. Food Chem.*, **2009**; 57, 9133–9140.
[18] IO Silva; IC Santos; VM Peters; REF Faria; ATL Andrade. *Bulletin of the Centre for Biology of Reproduction*, **2003**; 22, 49–57.
[19] MA Miranda; GD Vieira; MS Alves; CH Yamamoto; JJRG Pinho; OV Sousa. *Univ. Hosp. Mag.*, **2010**; 36, 61–68.
[20] JB Harborne. A Guide to Modern Techniques of Plant Analysis, Chapman and Hall, London, **1980**; 4–80.
[21] CK Kokate; AP Purohit; SB Gokhale. Pharmacognos, 35th Edition, Nirali prakashan, India, **2006**; 593–597.
[22] AL Waterhouse. Determination of total phenolics. In: Current protocols in food and analytical chemistry, ed: RE Wrolstad, John Wiley & Sons, New York, **2002**; 1.1.1- 1.1.8.
[23] RM Subramanian; Z Asmawi; A Sadikun. *Acta Biochim. Pol.*, **2008**; 55(2), 391–398.
[24] C Hansawasdi; J Kawabata; T Kasai. *Biosci. Biotech. Biochem.*, **2000**; 64(5), 1041–1043.
[25] AA Cetto; JB Jimenez; RC Vazquez. *J. Ethnopharmacol.*, **2008**; 116, 27–32.
[26] W Brand-Williams; ME C Cuvelier; Berset. *Food Sci. Technol.*, **1998**; 28, 25–30.
[27] RK Shukla; D Painuly; A Porval; A Shukla. Journal of Herbs, Spices and Medicinal Plants, **2014**; 20, 196–208.
[28] P Varadarajan; G Rathinaswamy; D Asirvatahm. *Ethnobot. Leaflet*, **2008**; 12: 841–845.
[29] GT Inyan; DB Kumar; M Nandhakumar; M Senthilkumar; A Mitra. *Ind. J. Pharmacol.*, **2010**; 42(5), 280–282.
[30] PMD Sales; PMD Souza; LA Simeoni; PDO Magalhaes; D Silveira. *J. Pharm. Pharm. Sci.*, **2012**; 15(1), 141–183.
[31] S Kumar; S Narwal; V Kumar; O Prakash. *Pharmacog. Rev.,* **2011**; 5(9), 19–29.
[32] AYY Cheng; IG Fantus. *Can. Med. Assoc. J.*, **2005**; 172(2), 213–226.
[33] R Srivatsan; S Das; R Gadde; M Kumar; S Taduri; N Rao; B Ramesh; A Baharani; K Shah; SC Kamireddy; G Priyatham; TA Balakumaran; SS Balakumaran; A Kamath; A Rao. *Arch. Iran. Med.,* **2009**; 12(2), 121–127.
[34] S Amaral; PJ Oliveira; J Ramalho-Santos. *Curr. Diabetes Rev.*, **2008**; 4(1), 46–54.
[35] T Yamaguchi; H Takamura; T Matoba; J Terao. *Biosci. Biotech. Biochem.*, **1998**; 62, 1201–1204.
[36] HY Lai; YY Yau; KH Kim. *BMC Comp. Alt. Med.*, **2010**; 10(15), 1–8.
[37] A Ghiselli; M Nardini; A Baldi; C Scaccini. *J. Agr. Food Chem.*, **1996**; 46, 361–367.