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In Vitro Antioxidant and Enzymes Inhibitory activity of Chloroform Fraction of Hydroalcoholic extract obtained from *Argemone mexicana*

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Abstract

In the present investigation antioxidant and alpha-amylase inhibitory activity of chloroform fraction of *Argemone mexicana* were evaluated. The antioxidant activity of chloroform fraction of *A. mexicana* was evaluated by DPPH, Super oxide radical Scavenging activity, ABTS radical cation scavenging activity and Nitric oxide radical scavenging activity. Alpha-amylase inhibitory activity of chloroform fraction was evaluated by DNS method respectively. The observed resultant antioxidant activity of chloroform fraction in all studied models was moderate as compared with reference standard Ascorbic acid. The chloroform fraction exhibited appreciable α-amylase inhibitory activity with an IC_{50} value 48.92µg/ml respectively, when compared with acarbose (IC_{50} value 83.33µg/ml).In conclusion, from the results of present study it is confirmed that antioxidant and alpha-amylase inhibitory activity of chloroform fraction of *A. mexicana* may contribute in its earlier observed antidiabetic potential.

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INTRODUCTION

The little epidemiological evidence is available on the role of dietary antioxidant intake in prevention of type 2 diabetes. Previously comparison of various extract of *Argemone Mexicana* leaves was reported for anti oxidant activity. Herbs, spices and medicinal plants have been cherished by many ancient cultures for their use in curing common ailments and promoting good health[1]. Although obesity and physical inactivity are known to be major risk factors

for type 2 diabetes, recent evidence suggests that oxidative stress may contribute to the pathogenesis of type 2 diabetes by increasing insulin resistance or impairing insulin secretion[2]. Dietary antioxidants have been hypothesized to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions[3]. It seems plausible that a sufficient intake of antioxidants plays an important role in protection against type 2 diabetes. *Argemone mexicana* L. (Papaveraceae), commonly known as prickly poppy, is an indigenous herb used as a medicinal plant in several countries. In Mexico, the seeds are considered as an antidote to snake venom. In India, the smokes of the seeds are used to relieve toothache. The fresh yellow, milky seed extract contains protein-dissolving substances, effective in the treatment of warts, cold sores, cutaneous infections, skin diseases, itches, and also dropsy and jaundice[4]. The plant contains alkaloids as berberine, protopine, sarguinarine, optisine, chelerytherine etc. Medicinal plants being the effective source of both traditional and modern medicines are genuinely useful for primary health care. Over the years, World Health Organization (WHO) advocated traditional medicines as safe remedies for ailments of both microbial and non-microbial origins[5]. In USA, some plant based compounds as well as herbal remedies are used along with other medications. In some cases, patients used these treatments instead of conventional medications, and severe complications including increased hospitalizations, ketoacidosis, and acute hyperglycaemia occurred[6].

MATERIAL AND METHODS

Collection and authentification of plant:

The plant material used in this study was aerial parts of *A. mexicana,* collected from road side area from Kasrawad dist Khargone M.P., India, during spring (mid-March to mid-April 2012) and was authenticated by the Taxonomist Dr. S. K. Mahajan Former Taxonomist, department Botany, Government P G College Khargone M.P. The plant materials were initially rinsed with distilled water and dried on paper towel in laboratory at (37 ± 1) ^{o}C for 24 h.

Preparation and fractionation of crude extracts

The coarse powder was submerged in ethyl alcohol and water (50:50) and allowed to stand for continuous hot extraction. After extraction the solvents were allowed to evaporate using rotary evaporator at temperature 40-45°C. Thus the highly concentrated crude hydroalcoholic extract were obtained. They were then fractionated using Pet-ether, Chloroform and water soluble portion. The dried chloroform soluble fraction was then preserved in the refrigerator for the experimental use.

Antioxidant activity

DPPH radical scavenging assay[7]

The radical scavenging effect of chloroform and aqueous fractions were determined by using the DPPH method. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of both test fractions in methanol with different concentrations (5-160 µg/ml). The reaction mixture was mixed thoroughly and kept in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The % scavenging activity at different concentrations was determined and the IC_{50} value of the fraction was compared with that of ascorbic acid, which was used as the standard. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity. The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control - Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Superoxide radical scavenging[8]

Each 3ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 µg riboflavin, and 12 mM EDTA and 0.1 mg NBT and 1ml of sample solution. Reaction was started by illuminating the reaction mixture with different concentrations of test fractions and standard ascorbic acid solution (5-160 µg/ml) for 5min. immediately after illumination, the absorbance was measured at 590 nm. Identical tubes with reaction mixture and 1ml of methanol were kept in the dark along and served as control. The % scavenging activity at different concentrations was determined and the IC_{50} value of the chloroform fraction was compared with that of ascorbic acid, which was used as the standard.

The percentage inhibition of superoxide anion generation was calculated from

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control - Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

ABTS radical scavenging activity[9]

The two stock solutions included 7.4 mM ABTS and 2.6 mM potassium persulphate was prepared as described by Arnao, Cano and Asota**.** The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hr at room temperature in dark. The solution was diluted by mixing with 1 ml ABTS solution prepared using 50 ml of methanol, in order to obtain absorbance 1.1 ± 0.02 units at 734 nm. Samples (1.5 ml) were mixed with 2.850 ml of ABTS solution and the mixture was left at room temperature for 2 hr in dark. The absorbance was then measured at 734 nm. The % scavenging activity at different concentrations was determined and the IC_{50} value of the chloroform fraction was compared with that of ascorbic acid, which was used as the standard.

Nitric oxide radical scavenging assay[10]

This assay was performed according to the method described by Sreejayan et al. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate buffered saline, and the fractions or the reference compound (ascorbic acid) at different concentrations (5-160 µg/ml) were incubated at 25°C for 150 min. About 0.5 ml aliquot of the incubated sample was removed at 30 min intervals and 0.5 ml Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. The % scavenging activity at different concentrations was determined and the IC_{50} value of the chloroform fraction was compared with that of ascorbic acid, which was used as the standard.

In Vitro α- Amylase Inhibitory Assay[11]

Starch azure (2 mg) was suspended in a tube containing 0.2ml of 0.5 M Tris-Hcl buffer (pH 6.9) containing 0.01 M calcium chloride (substrate). The tubes were boiled for 5 min and then pre incubated at 37°C for 5 min. 1ml of 0.1% of dimethyl sulfoxide was used to dissolve 1mg of chloroform and aqueous fractions in order to obtain concentrations of 20, 40, 60, 80 and 100µg/ml. Then 0.2 ml of test fractions of a particular concentration was added in the tube containing the substrate solution. 0.1 ml of porcine pancreatic amylase in Tris-Hcl buffer (2units/ml) was added to the tube containing the fractions and substrate solution, all the process was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4°C and the absorbance of resulting supernatant was measured at 595 nm spectrometrically.

Method for Calculation of α-amylase Inhibitory Activity

Absorbance was calculated by using following formula

$$\text{The } \alpha\text{-amylase inhibitory activity} = \frac{(Ac+) - (Ac-) - (As- Ab)}{(Ac+) - (Ac-)} \times 100$$

Where, Ac+, Ac-, As, Ab are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively.

RESULT AND DISCUSSION

The earlier literature reports indicated that *A mexicana* have potential application in the treatment of diabetes. In this study in order to understand its role in treating and management of complications of diabetes, antioxidant and alpha-amylase inhibitory activity of chloroform fraction of *A mexicana* was evaluated.

Now day's antioxidants are the most studied class of functional ingredients due to their protective role in various degenerative diseases such as diabetes, cancer, coronary diseases, inflammatory disorders etc., caused by increased oxidative stress by free radicals such reactive oxygen and nitrogen species (ROS/RNS). Antioxidants are known to neutralize these free radicals by donating an electron or hydrogen atom.

DPPH radical scavenging assay: DPPH is a stable free radical at normal temperature. It shows the specific absorbance at 517 nm due to colour of methanolic solution of DPPH. Body also contains the many free radicals, which assumed same as DPPH. Decrease in the absorbance of mixture indicates that scavenging of free radicals. Due to rapid hydrogen acceptable ability of DPPH, it reacts with antioxidants and gets converted into 1, 1-diphenyl-2-picrylhydrazine and hence shows decrease in absorbance[12]. Chloroform fraction and standard ascorbic acid show decrease in absorbance i.e. it has the radical scavenging activity; which is measured in terms of IC_{50} (61.89 (µg/ml)) (Table-1).

Superoxide radical scavenging

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant fraction and the reference compound indicates their abilities to quench superoxide radicals in the reaction mixture. Percentage inhibition of superoxide radical was determined. The chloroform fraction was found to be an effective superoxide anion scavenger to scavenge the superoxide anions as compared to ascorbic acid which is measured in terms of IC_{50} (87.16 µg/ml)) (Table -2)

In vitro antioxidant activity by ABTS

As presented in (Table- 3). Effect of ABTS free radical scavenging activity of chloroform fraction of A. mexicana was assayed at various concentrations. ABTS was used as a free radical to evaluate antioxidant activity of extracts. The method was based on the ability of antioxidant molecules to quench the long lived ABTS radical cation (ABTS). Significant ABTS scavenging activity was evident Its IC_{50} was 115.29µg/ml in comparison of ascorbic acid.

Nitric oxide radical scavenging assay

Nitric oxide exhibits numerous physiological properties and it is also implicated in several pathological states. It is an important second messenger, acts as a neurotransmitter and plays an important role in the defense against pathogens as well as in the control of blood pressure. NO is produced in various cells including neurons, endothelial cells and neutrophils by three isoforms of NO synthase enzyme (encoded by a unique gene),

from nitrogen of the guanidine group of l-arginine and from molecular oxygen[13]. Chloroform fraction significantly decreased with IC_{50} value 82.12mg/ml, in comparison with ascorbic acid, (Table- 4) the concentration of nitrite after spontaneous decomposition of sodium nitroprusside, indicating that chloroform fraction may contain compounds able to scavenging NO.

Alpha amylase inhibitory activity

It is well known that amylase inhibitors prevent dietary starches from being digested and absorbed by the body. This could be useful for treating diabetes mellitus[14]. The α-amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates and potentially useful in control of obesity and diabetes. Acarbose is complex Oligosaccharides that delay the digestion of carbohydrates. It inhibits the action of pancreatic amylase in breakdown of starch. Synthetic inhibitor causes side effect such as abdominal pain, diarrhoea and soft faces in the colon. The percent salivary alpha-amylase inhibitory activity of chloroform fraction at different concentration was shown (Table-5). The observed IC_{50} value i.e. the concentration of the fraction, containing the alpha-amylase inhibitor that inhibited 50% of the enzyme activity was 48.45 µg/ml in comparison with acrobose.

CONCLUSION:

In conclusion, results of present study show that chloroform fraction of A. mexicana possess antioxidant potential in different studied models. However, the Observed antioxidant potential of chloroform fraction is moderate as compared to reference standard ascorbic acid. Result of salivary alpha-amylase inhibitory assay also reveals that chloroform fraction possess Salivary alpha-amylase inhibitory potential. The observed alpha-amylase inhibitory potential of chloroform fraction is moderate as compared to reference standard acrobose.

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Table1: Effect of chloroform fraction of *A. mexicana* in DPPH radical scavenging activity

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	15.58 ± 0.33	61.89
	10	24.58 ± 0.12	
	20	37.98 ± 0.19	
	40	47.55 ± 0.25	
	80	67.63 ± 0.28	
	160	83.52 ± 0.16	
Ascorbic Acid			10.05

n=3, values are mean ± S.D

Table 2: Effect of chloroform fraction of *A. mexicana* in Super oxide radical Scavenging activity

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	11.75 ± 0.19	87.76
	10	19.52 ± 0.15	
	20	28.11 ± 0.02	
	40	34.56 ± 0.03	
	80	58.43 ± 0.16	
	160	71.03 ± 0.16	
Ascorbic Acid			15.54

n=3, values are mean ± S.D

Table 3: Effect of chloroform fraction of *A. mexicana* in ABTS radical cation scavenging activity

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	8.01 ± 0.30	115.29
	10	14.81 ± 0.28	
	20	21.22 ± 0.11	
	40	32.10 ± 0.22	
	80	47.47 ±0.12	
	160	58.57 ± 0.14	
Ascorbic Acid			7.71

n=3, values are mean ± S.D

Table 4: Effect of chloroform fraction of *A. mexicana* in Nitric oxide radical scavenging activity

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	12.11 ± 0.64	82.12
	10	22.08 ± 0.47	
	20	29.16 ± 0.29	
	40	38.14 ± 0.26	
	80	58.48 ± 0.64	
	160	73.56 ± 1.75	
Ascorbic Acid			7.92

n=3, values are mean ± S.D

Table 4: alpha amylase inhibitory effect of chloroform fraction of *A. Mexicana*

Sample	Concentration (µg/ml)	% Reduction	IC_{50} value µg/ml
Chloroform fraction	20	36.48	48.45
	40	48.23	
	60	65.82	
	80	71.55	
	100	75.22	
Acrobose			83.33

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