Journal of Applied Pharmaceutical Science Vol. 4 (09), pp. 046-051, September, 2014
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2014.40908
ISSN 2231-3354 

In Vitro α-Amylase Inhibition and Antioxidant potential of Chloroform Fraction of Hydroalcoholic Extract Obtained from Hyptis Suaveolens

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ARTICLE INFO

Article history:
Received on: 27/07/2014
Revised on: 08/08/2014
Accepted on: 24/08/2014
Available online: 27/09/2014

Key words:
alpha-amylase inhibitory activity, *H. suaveolens*, DPPH, Super oxide radical Scavenging activity.

ABSTRACT

The plant Hyptis is an effective medicinal herb and a well known medicinal plant in herbal world. The present study was aimed to investigate the α-amylase inhibition and antioxidant activities of chloroform fraction of *H. suaveolens*. Chloroform fraction of *H. suaveolens* was screened for α-amylase inhibition activity by 3, 5-dinitrosalicylic acid (DNSA) method respectively. and antioxidant activity was evaluated by 1,1-diphenyl-2-picryl-hydrazile (DPPH) free radical scavenging, Super oxide radical Scavenging, nitric oxide (NO) radical scavenging, and 2.2'-azinobis-3-ethylbenzothiazole-6-sulfonic acid (ABTS) radical scavenging assays. The chloroform fraction of *H. suaveolens* showed effective α-amylase inhibition activity (IC_{50} 57.34µg/ml). Chloroform fraction demonstrated significant antioxidant activity in all the *in vitro* antioxidant models. Moreover, the chloroform fraction was found to be extremely effective in scavenging DPPH (IC_{50} 57.51 µg/ml) while compared to super oxide (IC_{50} 61.36 µg/ml), NO (IC_{50}76.3 µg/ml) and ABTS radical (IC_{50} 93.16 µg/ml) scavenging activity. In conclusion, from the results of present study it is established that antioxidant and alpha-amylase inhibitory activity of chloroform fraction of *H. suaveolens* may contribute in its earlier observed antidiabetic potential.

INTRODUCTION

Medicinal plants continue to be an important resource material for therapeutic agents both in developed and developing countries. Plants contain phytochemicals with various bioactivities including antioxidant, antidiabetic, and anticancer activities. At present, about 25% of the active component was identified from plants that are used as prescribed medicines (Gill *et al.,* 2011). Reactive oxygen species (ROS) use oxidative damaging effects by reacting with almost every molecules found in living cells including protein, lipid, amino acids and DNA, if excess ROS are not eliminated by antioxidant system. They play imperative roles in aging and in the pathogenesis of age related disorders such as diabetes, cancer, cardiovascular disease and renal failure, atherogenesis, Alzheimers disease and Parkinsons disease. The most practical way to fight degenerative ailments is to enhance antioxidant activity in our body and that might be achieved by consumption of vegetables, fruits or edible plants (Adefegha and Oboh, 2011). There is an increasing attention in natural antioxidants *e.g.* polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage. Natural antioxidants enhance the antioxidant capacity of the plasma and reduce the risk of diseases (Shekhar *et al.,* 2011). The little epidemiological evidence is available on the role of dietary antioxidant intake in prevention of type 2 diabetes. Various parts such as seeds, leaves and bark of stem and root well-known to contain significant amounts of phytoconstituents such as phenolics, flavonoids, tannins, sterols having the ability to inhibit the free radicals that are markedly produced, therefore can act as anti-oxidants (Samatha *et al.,* 2012). The continued search among plant secondary meta bolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic oxidants (Aliyu *et al.,* 2010).

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Obesity and physical inactivity are known to be main risk factors for type 2 diabetes, recent indication suggests that oxidative stress may contribute to the pathogenesis of diabetes by increasing insulin resistance or impairing insulin secretion (Oberley, 1988). Dietary antioxidants have been hypothesized to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions (Feskens *et al.*, 1995).

It seems possible that an adequate intake of antioxidants plays a significant role in protection against type 2 diabetes. Hyptis is a genus of Lamiaceae with about 400 species. One of the species from genus, Hyptis suaveolens Poit (referred as Hyptis hereafter) is naturalized in India (Raizada *et al.*, 2006). Its distribution is tropical and subtropical and it is not commonly found over 500 m. An abundance of traditional herbal medicinal practices have been used in the diagnosis, prevention and treatment of diabetes.

The plant, *Hyptis suaveolens* (L) Poit commonly known as *Wilayati tulsi.* Almost all parts of this plant are being used in traditional medicine to treat various diseases. The leaves of *H. suaveolens* have been utilized as a stimulant, carminative, sudorific, galactogogue and as a cure for parasitic cutaneous diseases (The Wealth of India, 1964).

Crude leaf extract is also used as a relief to colic and stomachache. Leaves and twigs are considered to be antispasmodic and used in antirheumatic and antisudorific baths, anti-inflammatory, antifertility agents (Kirtikar and Basu, 1991), and also applied as an antiseptic in burns, wounds, and various skin complaints. The decoction of the roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor (Chatterjee and Pakrashi, 1997). Fumes of the dried leaves are also used to repel mosquitoes and control insect pests of stored grains.

The leaves of the plant have been shown to contain alkaloids, terpenes and volatile oils (Gills, 1992). Its leaf methanolic extract has shown various activities like antimicrobial and (Satish *et al.*, 2010) antibacterial activity (Kavitha and Satish, 2010) it was found effective against methicillinresistant Staphylococcus aureus (Daniyan *et al.*, 2011) similarly its toxicity and hypoglycemic activity was studied (Danmalam *et al.*, 2009). It was also screened for antifungal activity (Varaprasad *et al.*, 2009) while leaf and seed methanolic extracts was screened for insecticidal activity (Musa *et al.*, 2009). Various researchers have studied its antioxidant activity (Pradeep et.al 2011). found that aqueous extract of Hyptis suaveolens L.showed a protective effect on the antioxidant status of the animals which was evident from the low lipid peroxidation levels, similarly the alcoholic extract (Bhagwat *et al.*, 2003) and petroleum ether leaf extract (Iwalokun *et al.*, 2009) showed antioxidant activity. Previously anti oxidant and antidiabetic activity of various extract of *hyptis suaveolens* was reported. But not any work has been done on its fractions for keeping all these into account, the present study was undertaken to evaluate chloroform fraction of *hyptis suaveolens* for in vitro antioxidant activities as well as in vitro antidiabetic activity by using α-amylase inhibition assay method.

MATERIAL AND METHODS

Collection and authentification of plant

The plant material used in this study was aerial parts of *H. suaveolens*, collected from Kasrawad dist Khargone M.P., India, during spring (mid-March to mid-April) and was authenticated by the Former Taxonomist Dr. S. K. Mahajan, 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 $(37 \pm 1)^{o}C$ 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. therefore 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

The radical scavenging effect of chloroform fraction was 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 test fraction 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 (Liyana-Pathiranan *et al.*, 2005).

Lower absorbance of spectrophotometer indicated higher experimental free radical scavenging activity. The percent of DPPH decoloration of the samples was calculated according to the formula:

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

The antioxidant activity of sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

Superoxide radical scavenging

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 (Kumaran and Karunakaran, 2007).

The percentage inhibition of superoxide anion generation was calculated from

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

ABTS radical scavenging activity

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 (Arona *et al.,* 2001).

The percentage inhibition of ABTS radical generation was calculated from

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

Where $A_{control}$ is the absorbance of ABTS radical+methanol; A_{test} is the absorbance of ABTS radical+sample extract/standard.

Nitric oxide radical scavenging assay

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 (Sreejayan *et al.,* 1997).

In Vitro α- Amylase Inhibitory Assay

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 (Hansawasdi *et al.,* 2000).

Method for Calculation of α-amylase Inhibitory Activity Absorbance was calculated by using following formula

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

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.

RESULTS AND DISCUSSION

The earlier literature reports indicated that *H. suaveolens* have prospective 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 *H. suaveolens* 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. Hyperglycemia has been a classical risk in the development of diabetes and the complications associated with diabetes. Therefore control of blood glucose levels is critical in the early treatment of diabetes mellitus and reduction of macro- and microvascular complications. One therapeutic approach is the prevention of carbohydrate absorption after food intake, which is facilitated by inhibition of enteric enzymes including α-amylase present in the brush borders of intestine.

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 (Sood *et al.,* 2008). Chloroform fraction and standard ascorbic acid show decrease in absorbance i.e. it has the experimental free radical scavenging activity; which is measured in terms of IC_{50} (57.51 (µg/ml)) (Table-1).

Table 1: Effect of chloroform fraction of *H. suaveolens* in DPPH radical scavenging activity.

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	18.34 ±0.32	57.51
	10	26.58 ±0.21	
	20	39.32 ±0.19	
	40	48.33 ±0.22	
	80	68.62 ±0.27	
	160	86.56 ±0.15	
Ascorbic Acid			10.05

The data are displayed with mean ± standard deviation of thrice replications

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} (61.36 µg/ml) (Table -2).

Table 2: Effect of chloroform fraction of *H. suaveolens* in Super oxide radical Scavenging activity.

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	12.69 ±0.19	61.36
	10	21.49 ±0.15	
	20	32.36 ±0.11	
	40	56.43 ±0.16	
	80	73.08 ±0.19	
	160	81.57 ±0.14	
Ascorbic Acid			15.54

The data are displayed with mean ± standard deviation of thrice replications

Table 3: Effect of chloroform fraction of *H. suaveolens* in ABTS radical cation scavenging activity.

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	9.01 ± 0.32	93.16
	10	17.81 ± 0.26	
	20	24.32 ± 0.14	
	40	38.10 ± 0.32	
	80	48.47 ±0.12	
	160	71.57 ± 0.16	
Ascorbic Acid			7.71

The data are displayed with mean ± standard deviation of thrice replications

In vitro antioxidant activity by ABTS

As presented in (Table- 3). Effect of ABTS free radical scavenging activity of chloroform fraction of *H. suaveolens* 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 of chloroform fraction was evident Its IC_{50} was 93.16µg/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 (Sessa and Harrison, 1993). Chloroform fraction significantly decreased with IC_{50} value 76.3mg/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.

Table 4: Effect of chloroform fraction of *H. suaveolens* in Nitric oxide radical scavenging activity.

Sample	Concentration (µg/ml)	% Reduction	IC_{50} Value (µg/ml)
Chloroform fraction	5	14.11 ± 0.67	76.3
	10	24.08 ± 0.48	
	20	32.18 ± 0.28	
	40	40.14 ± 0.25	
	80	59.48 ± 0.64	
	160	76.56 ± 1.64	
Ascorbic Acid			7.92

The data are displayed with mean ± standard deviation of thrice replications

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 (McEwan *et al.,* 2010). 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.

Table 5: alpha amylase inhibitory effect of chloroform fraction of *H. suaveolens*

Sample	Concentration (µg/ml)	% Reduction	IC_{50} value µg/ml
Chloroform fraction	**20**	16.79±0.18	57.34
	40	36.48±0.16	
	60	57.83±0.22	
	80	70.88±0.14	
	100	78.63±0.09	
Acrobose			83.33

The data are displayed with mean ± standard deviation of thrice replications

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 57.34µg/ml in comparison with acrobose IC_{50} value 83.33µg/ml.

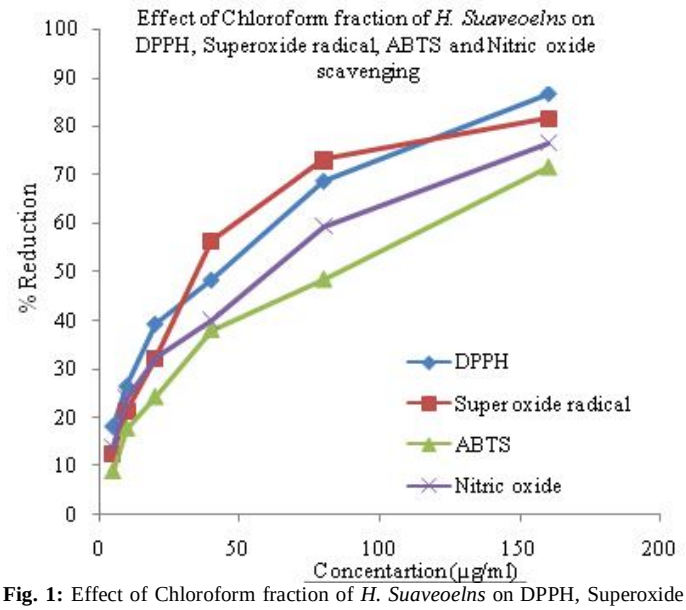

Fig. 1: Effect of Chloroform fraction of *H. Suaveoelns* on DPPH, Superoxide radical, ABTS and Nitric oxide scavenging.

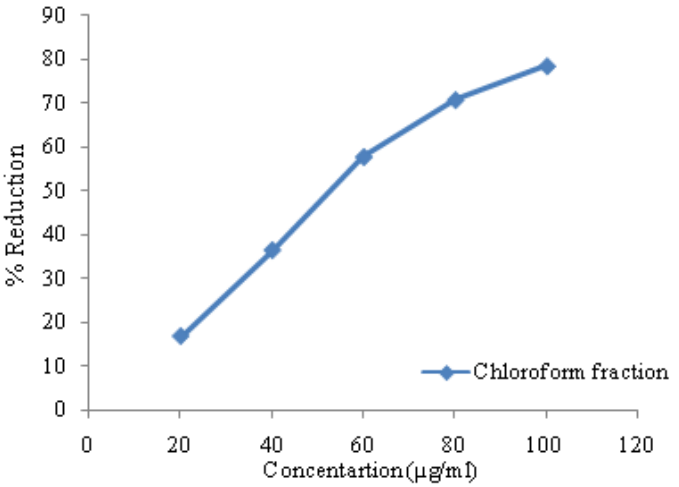

Fig. 2: alpha amylase inhibitory effect of chloroform fraction of *H. suaveolens.*

CONCLUSION

In conclusion, results of present study demonstrate that chloroform fraction of *H. suaveolens* possess antioxidant potential in different studied models. Though, 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. Thus this study confirms that the aerial parts of *H. suaveolens* can alleviate postprandial hyperglycemia and improve oxidative stress and therefore assist in combating diabetic complications. For further work on the profile and nature of chemical constituents of *H. suaveolens* will provide more information on the active principles responsible for their pharmacological properties. However, based on the above presented results, chloroform fraction of aerial parts of *H. suaveolens* could be investigated as a possible new source of antidiabetic drugs and natural antioxidants in the food, nutraceuticals and cosmetic industry.

ACKNOWLEDGEMENTS

The authors are grateful to SPS, SOA University, Bhubaneswar for providing necessary facilities to carry out the research work in the faculty of pharmacy, SOA University.

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How to cite this article:

Shweta P Nayak, Durga M Kar. In Vitro α-Amylase Inhibition and Antioxidant potential of Chloroform Fraction of Hydroalcoholic Extract Obtained from Hyptis Suaveolens. App Pharm Sci, 2014; 4 (09): 046-051.