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Original Article

Pharmacology Section

Evaluation of Antidiabetic and Antioxidant Activities of L-carnosine using Enzyme Inhibition and Free Radical Scavenging Assays: An In-vitro Study

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ABSTRACT

Introduction: Diabetes is a chronic disease that causes dysfunction of various organs and tissues resulting in end organ damage and premature mortality. Oxidative stress is a main factor of diabetic complications. There is a need for the development of safer therapeutic agents for diabetes, due to the adverse effects of conventional drugs. L-carnosine is a dipeptide synthesised by the body from β-alanine and L-histidine. It is reported to have heavy metal chelating, pH buffering, anti-inflammatory property and neuroprotective effect making it a prospective drug target for chronic diseases like diabetes. The antidiabetic property of L-carnosine and its free radical scavenging potential have not yet been fully explored.

Aim: To evaluate the in-vitro antidiabetic and antioxidant activities of L-carnosine.

Materials and Methods: The present in-vitro study was conducted in the Department of Pharmacology at Sri Ramachandra Medical College and Research Institute, Porur, Chennai, India. The duration of the study was one month, done in December 2020. The in-vitro antidiabetic property of L-carnosine was evaluated using α-glucosidase and α-amylase inhibition. About 20, 40, 60, 80, 100 μg/mL concentrations of L-carnosine and acarbose were used for the study wherein, acarbose was used as the standard. The absorbance values were taken in spectrophotometer at 405 nm and 540 nm for α-amylase and α-glucosidase enzyme, respectively. Further, the antioxidant activity of L-carnosine was determined at the same concentrations using 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay with Butylated Hydroxytoluene (BHT) as standard. The spectrophotometric absorbance was read at 734 nm. The data analysed was presented as percentage inhibition. The percentages of enzyme inhibition for various concentrations were compared between the standard and L-carnosine.

Results: The inhibitory percentages for α-glucosidase enzyme at concentrations of 20,40,60,80 and 100 μg/mL of L-carnosine were 28.61%, 36.01%, 45.33%, 53.05%, 62.70% respectively. The percentages for α-amylase inhibition at concentrations of 20, 40, 60, 80 and 100 μg/mL were 18.18%, 31.81%, 45.45%, 59.09% and 72.12%, respectively. The free radical scavenging activity by ABTS assay for 20, 40, 60, 80 and 100 μg/mL concentrations of L-carnosine were 34.40%, 36.65%, 38.04%, 40.51% and 43.30%, respectively. L-carnosine exhibited significant inhibition of α-glucosidase and α-amylase enzyme in dose-dependent manner. The result of the ABTS assay showed that L-carnosine possessed significant free radical scavenging property in a concentration-dependent manner.

Conclusion: Results showed L-carnosine had considerable α-glucosidase inhibitory activity, α-amylase inhibitory activity, as well as, ABTS radical scavenging activity. The present findings indicate that L-carnosine has in-vitro antidiabetic and antioxidant activity. Hence, the present study supports further evaluation and use of L-carnosine for the management of diabetes and as an antioxidant in nutraceuticals.

Keywords: β-alanyl-L-histidine, Diabetes, Oxidative stress

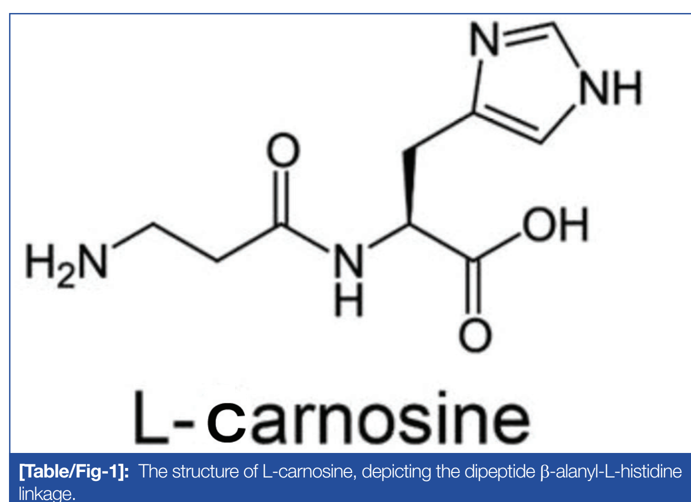
INTRODUCTION

Diabetes Mellitus (DM) has become a leading cause of morbidity and mortality factor amongst individuals in recent times. The most concerning factor is the increasing number of young adults becoming diabetic further leading to various chronic complications [1,2]. Epidemiological meta-analysis suggests an uncontrollable increase in global health expenditure due to diabetes, so the management of this disease must be given top priority [3]. There is a need to follow an integrated approach to achieve better control over diabetes. Research needs to be done on various cost-effective, as well as, efficacious newer drugs and dietary supplements in order to establish their antidiabetic property [4].

Physiological levels of reactive oxygen species are important for optimal cell signaling but at higher levels, it has detrimental effects [5]. Oxidative stress can be defined as an imbalance in the homeostatic levels of pro-oxidants and antioxidants resulting in excessive generation of free radicals, which can act as a major contributor to various chronic diseases [6,7]. In order to protect against this oxidative stress, antioxidants come into play. Various nutritional supplements and small molecules have been evaluated as antioxidants and found to have therapeutic potential in preclinical trials [8,9]. There is a necessity for better understanding of their mechanism of action to provide a rational approach to their pharmacological success.

L-carnosine is a dipeptide produced by the human body from β-alanine and L-histidine. It is present in large amounts in the skeletal muscles [10]. High concentrations of L-carnosine are also found in the heart, brain and gastrointestinal tissues. It is present only in vertebrates and not in plants. Dietary sources of L-carnosine are mainly meat, poultry and seafood [11]. The structure of L-carnosine, the dipeptide β-alanyl-L-histidine has been depicted in [Table/Fig-1] [12]. Carnosine occurs naturally as many variants of carnosine derivatives such as anserine and the ophidine/balenine-methylated imidazole ring of L-histidine, as homocarnosine where β-alanine is replaced by Gamma-Aminobutyric Acid (GABA), as carcinine where histamine replaces L-histidine but the most common ones are its methylated analogues, which are mainly from carnosine N-methyltransferase mediated methylation of its imidazole ring

[13]. Carnosine is degraded in the tissues through hydrolysis by the enzyme carnosinase (two isoforms are present in humans CN1 and CN2) [14,15]. It has many beneficial effects that have been well-established such as heavy metal chelation, buffering of pH, anti-inflammatory property and neuroprotection [16-18]. Carnosine is found to recover the glucolipotoxic inhibition of insulin-stimulated glucose uptake from the skeletal muscle cells, that can be further studied to evaluate its antidiabetic potential [19,20]. It also helps in non enzymatic detoxification of reactive aldehydes, which have been implicated in the causation of diabetes [21]. It has also been found to reduce lipid peroxidation, hence, can be evaluated for its antioxidant capacity [22,11]. Thus, these observations present substantial possibility of future therapeutic applications of carnosine. The present study was aimed to evaluate L-carnosine for its antidiabetic property by α-glucosidase inhibition, α-amylase inhibition and antioxidant property by ABTS assay.



[Table/Fig-1]: The structure of L-carnosine, depicting the dipeptide β-alanyl-L-histidine linkage.

MATERIALS AND METHODS

The present in-vitro study was conducted in the Department of Pharmacology at Sri Ramachandra Medical College and Research Institute, Porur, Chennai, India. The duration of the study was one month, in December 2020.

Study Procedure

Determination of α-glucosidase inhibitory activity: Phosphate buffer-50 mM, sodium carbonate-0.1 M, 4-Nitrophenyl-β-D-glucopyranoside (PNPG)-1 mM, α-glucosidase-1 u/mL and sample extract with range of concentrations 20-100 μg/mL α-glucosidase inhibitory activity was conducted in accordance with the method of Thengyai S et al., with slight modification [23]. Reaction mixture of 50 μL phosphate buffer, 10 μL alpha-glucosidase and 20 μL of various concentrations of extracts (20-100 μg/mL) were preincubated at 37°C for 15 minutes. A 20 μL p-nitrophenyl-α-D-Glucopyranoside (PNPG) was then added as a substrate and further incubated at 37°C for 30 minutes. The reaction was terminated, when the yellow colour was produced by addition of 50 μL sodium carbonate. The experiments for each concentration were performed with appropriate blanks in Ultraviolet (UV) spectrophotometer. The Optical Density (OD) was read at 405 nm as a measure of absorbance. Various concentrations of acarbose (20-100 μg/mL) were used as a standard and control tubes without extracts was setup as negatives in parallel. The results were expressed as percentage inhibition calculated by using the following formula:

Inhibition (%)=Absorbance of control-Absorbance of sample/Absorbance of control×100

Determination of α-amylase inhibitory activity: Sodium phosphate buffer-0.02 M, α-amylase-10 u/mL, soluble starch-0.05%, Dinitrosalicylic Acid (DNS)- as required and sample extract with range of concentrations 20-100 μg/mL α-amylase inhibitory activity was carried out according to a modified method of Akoro S et al., [24]. Preparation of assay mixture was done by adding 200 μL of sodium phosphate buffer, 20 μL of enzyme and 20 μL of extracts, which was then incubated at room temperature for 10 minutes. Then 200 μL of starch was added. A 400 μL of 3,5 DNS reagent was added and the reaction was terminated. After placing for five minutes in boiling water bath, it was cooled and 15 mL of distilled water was added. The negatives were prepared without any extracts. The absorbance produced, for each concentration of the sample, standard and control were measured in terms of OD read at 540 nm. The % inhibition was calculated using the following formula:

Inhibition (%)=Absorbance of control-Absorbance of sample/Absorbance of control×100

Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity: The spectrophotometric analysis of ABTS radical scavenging activity was determined according to the modified method of Palmieri S et al., [25]. The principle of this, is dependent on the antioxidant ability to scavenge the ABTS cation which produces a blue/green coloured compound. BHT was used as standard. ABTS decolourisation assay involves the generation of the ABTS chromophore by the oxidation of ABTS with ammonium persulphate. It can be applied for hydrophilic and lipophilic substances. Reagents used were 7 mM ABTS, 2.45 mM ammonium per sulphate, ethanol and ABTS solution, which was prepared using 7 mM of ABTS mixed with 2.45 mM ammonium per sulphate and then the mixture was kept in dark for 12-16 hours at room temperature before use. ABTS solution was diluted with ethanol and absorbance was measured at 734 nm. Samples were diluted to produce 20, 40, 60, 80, 100 μg/mL. The reaction was initiated by the addition of 1.0 mL of diluted ABTS to 10 μL of different concentration of the sample and 10 μL of methanol as control. BHT was used as standard. The percentage inhibition was calculated after the absorbance, as a measure of the OD, was read at 734 nm. The % inhibition was then calculated using the following equation:

$$\text{ABTS scavenging activity (\%)}=(A_0-A_1)/A_0\times100$$

where,

A_0 is the control absorbance and A_1 is the sample absorbance.

Optical Density (OD) measurement: OD is measured using a UV spectrophotometer. It is based on the amount of light scattered by the chemical substance rather than the amount of light absorbed at a particular wavelength. It is the logarithmic ratio of the intensity of incident light to the intensity of the transmitted light [26]. For all the above-mentioned assays, the OD was calculated using the formula:

Optical Density (OD)=Log (maximum power density output/maximum permissible exposure)

STATISTICAL ANALYSIS

The data analysed were presented as percentage inhibition. The percentages of enzyme inhibition for various concentrations were compared between the standard and L-carnosine.

RESULTS

The inhibitory effect of L-carnosine and acarbose for α-glucosidase activity shown in [Table/Fig-2,3].

Sample	Concentration (μg/mL)	OD (log ratio)	Inhibition (%)
L-carnosine	20	0.222	28.61
	40	0.199	36.01
	60	0.170	45.33
	80	0.146	53.05
	100	0.116	62.70

[Table/Fig-2]: Shows the percentage inhibitory activity of α-glucosidase enzyme by the test compound, L-carnosine at various concentrations.
Control Optical Density (OD): 0.311 (log ratio)

Sample	Concentration (μg/mL)	OD (log ratio)	Inhibition (%)
Acarbose	20	0.195	39.29
	40	0.167	46.30
	60	0.135	56.59
	80	0.107	65.59
	100	0.072	76.84

[Table/Fig-3]: Represents the percentage inhibitory activity of α-glucosidase enzyme by the standard, acarbose at various concentrations.
Control Optical Density (OD): 0.311 (log ratio)

At the initial concentration of 20 μg/mL, L-carnosine demonstrated a percentage inhibition of 28.61% as compared with acarbose, which showed 39.29%. The highest concentration of 100 μg/mL, L-carnosine demonstrated 62.7% inhibition as compared with acarbose, which showed 76.84%. L-carnosine inhibited α-glucosidase activity in a concentration dependent manner. Considering the percentage of inhibition at increased concentrations, it can be postulated that, L-carnosine can be a potential inhibitor of α-glucosidase activity. L-carnosine at the concentration of 100 μg/mL showed 72.12% inhibitory effect on the α-amylase activity, which was similar to acarbose, which showed 77.27% inhibitory effect at the same concentration. Acarbose is a well-known α-amylase inhibitor [27] and almost similar effect observed with L-carnosine validates its significant α-amylase inhibitory activity in increasing concentrations. [Table/Fig-4,5] shows the α-amylase inhibitory activity of L-carnosine and acarbose at various concentrations (20-100 μg/mL).

Sample	Concentration (μg/mL)	OD (log ratio)	Inhibition (%)
L-carnosine	20	0.018	18.18
	40	0.015	31.81
	60	0.012	45.45
	80	0.009	59.09
	100	0.006	72.12

[Table/Fig-4]: Depicts the Percentage inhibitory activity of α-amylase enzyme by the test compound, L-carnosine at various concentrations.
Control Optical Density (OD): 0.022 (log ratio)

Sample	Concentration (μg/mL)	OD (log ratio)	Inhibition (%)
Acarbose	20	0.014	36.66
	40	0.011	50.00
	60	0.009	59.09
	80	0.007	68.18
	100	0.005	77.27

[Table/Fig-5]: Shows the percentage inhibitory activity of α-amylase enzyme by the standard, acarbose, at various concentrations.
Control Optical Density (OD): 0.022 (log ratio)

L-carnosine showed effective ABTS radical scavenging activity in a concentration dependent manner (20-100 μg/mL). At the concentration of 20 μg/mL, L-carnosine produced a potential scavenging effect of 34.40% as compared with the standard BHT, that produced only 3.399%. As the concentration of L-carnosine increases, its scavenging activity was also found to increase. [Table/Fig-6,7] shows representation of the inhibition percentage of ABTS generation by 20-100 μg/mL concentrations of L-carnosine and BHT.

Sample	Concentration (μg/mL)	OD (log ratio)	ABTS activity (%)
L-carnosine	20	0.612	34.40
	40	0.591	36.65
	60	0.578	38.04
	80	0.555	40.51
	100	0.529	43.30

[Table/Fig-6]: Depicts the free radical scavenging activity of L-carnosine using ABTS assay, at various concentrations.
Control Optical Density (OD): 0.933 (log ratio)

Sample	Concentration (μg/mL)	OD (log ratio)	ABTS activity (%)
BHT	20	0.682	3.399
	40	0.657	6.940
	60	0.645	8.640
	80	0.618	12.464
	100	0.602	14.730

[Table/Fig-7]: Represents the free radical scavenging activity of the standard, BHT at various concentrations.
Control Optical Density (OD): 0.706 (log ratio)

The percentage inhibition of ABTS activity was significantly higher at various concentrations of L-carnosine starting with 34.4% inhibition at 20 μg/mL and the highest inhibition of 43.3% was recorded at 100 μg/mL, as compared to the standard which exhibited only 14.73% at the same concentration. These results showed, L-carnosine had a markedly higher ABTS inhibition as compared with BHT.

DISCUSSION

The L-carnosine is naturally synthesised mainly by skeletal muscles, which is used by various tissues and organs for their growth and development. It serves as the main building block of protein synthesis. L-carnosine is absorbed in the gut and when it reaches the bloodstream, it is metabolised by the enzyme, carnosinase. Carnosinase splits this back into its precursors, histidine and alanine [28]. Sugars when bound to proteins cause destruction of these proteins and also triggers inflammatory processes. It can help to prevent this damage. L-carnosine levels have been found to be reduced in chronic diabetes patients [29]. Hence, its supplementation can be a potential prospective strategy to maintain the blood glucose levels in these patients. Previous studies have shown L-carnosine has anti-inflammatory potential. Tang W et al., has reported that L-carnosine has metal chelation and anticell death properties [30]. Turner MD et al., have reported the reactive oxygen species scavenging potential of L-carnosine, which contributes to its anticancer property [31]. Hipkiss AR et al., reported that L-carnosine can reduce lipid peroxidation and senescence [32,33]. Studies have postulated that, it has haepatoprotective effect [34-36]. Therapeutic observation has showed dietary carnosine supplementation seems to prevent atherogenesis [37]. In the present study, the potential benefits of L-carnosine have been extended to evaluate the antihyperglycaemic and antioxidant property.

In the present study, L-carnosine was evaluated for its effect on α-glucosidase and α-amylase enzymes using in-vitro studies. L-carnosine was also evaluated for its antioxidant potential using ABTS assay. The main antidiabetic strategy is inhibiting carbohydrate digesting enzymes like α-glucosidase and alpha amylase. Inhibition of such enzymes results in postprandial antihyperglycaemic effect by decreasing the rate and the extent to which glucose absorption takes place in the small intestine [38]. Inhibition of these enzymes brings down the absorption of carbohydrates from the gastrointestinal system and slows down the rate of increase in postprandial glucose. The delay in digestion and breakdown of starch can also have a beneficial effect on insulin resistance. Glucosidase enzyme is considered one of the main therapeutic targets for the treatment of DM. It prevents the transformation of oligosaccharides and disaccharides to glucose and delays the absorption of monosaccharides from the small intestine inhibition of α-glucosidase enzyme can prevent undue fluctuations in blood glucose levels [39]. In order to develop a functional physiological food supplement or a chemical compound for diabetic therapy, the extent of glucosidase inhibition can be evaluated. In-vitro α-glucosidase enzyme inhibitory assay has merits such as minimal sample consumption and short but effective analysis time [40].

Complex polysaccharides are hydrolysed by amylases to dextrins, which are further hydrolysed to glucose. α-amylase inhibitors are also called as starch blockers, as they decrease the starch absorption by inhibiting the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides [41]. Hence, α-amylase inhibition plays a significant role in the treatment of postprandial hyperglycaemia. The present study also requires minimal screening time and small amount of sample. Synthetic compounds that inhibit α-glucosidase and α-amylase such as acarbose, are widely used in the treatment of DM. Due to increased incidence of various side-effects, safer α-glucosidase and α-amylase inhibitors are desired [42]. L-carnosine is a nutritional supplement present in meat products, which can prove to a be safe alternative to these synthetic compounds [44]. The current study utilises α-glucosidase and α-amylase inhibition to prove L-carnosine as a potential drug target for the treatment of DM [43]. Free radicals are not only generated exogenously, but also by endogenous physiological and biochemical processes. These are reactive oxygen species such as superoxide, hydroxyl and peroxyl radicals that damage the biomolecules. Increased production of free radicals and oxidative stress is a key player in the development of complications of DM [44].

Radical scavenging activity plays a vital role in maintaining the homeostasis of the biological system. Chemical assays have the ability to scavenge synthetic free radicals, by a variety of radical-generating systems and methods for detection of the oxidation end-point [45]. ABTS assay is a simple, direct antioxidant assay based on the reduction of ABTS radical cation. Bingol Z et al., states that generation of the ABTS radical cation is one of the widely used spectrophotometric method for the effective measurement of antioxidant activity of pure substances, solutions and aqueous mixtures [46]. The present assay is advantageous in terms of cost, reaction time and requirement of a simple equipment such as a spectrophotometer. The present study investigated the antioxidant potential of L-carnosine warranting its therapeutic potential in diabetes and other degenerative conditions.

With the positive findings from the present study, it can be definitely established that, L-carnosine has significant antidiabetic and antioxidant properties. But more assays are needed to further confirm these findings. The results of the present study, imply that L-carnosine, a nutraceutical, can be used as a therapeutic drug target for DM and other degenerative diseases, provided it shows similar positive results in, in-vivo and clinical trials in the future.

Limitation(s)

Further studies are required to establish the cellular mechanism, responsible for α-glucosidase and α-amylase enzyme inhibition. The antioxidant activity of L-carnosine and the mechanism could be further confirmed with other methods of antioxidant assays.

CONCLUSION(S)

Within the limits of the present study, it can be implied that, L-carnosine has demonstrated significant antidiabetic and antioxidant properties using α-glucosidase inhibitory assay, α-amylase inhibitory assay and ABTS antioxidant assay. However, more in-depth exploratory research is required both in-vitro and in-vivo to warrant the proof of these findings thereby, providing a necessity for further exploration of the potential use of L-carnosine for diabetic patients and patients with various inflammatory and degenerative disorders.

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