Inhibition of Formation of Fructose Induced Advanced Glycation End Products by *Momordica chochinchinensis*

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ABSTRACT
The present study evaluates the antioxidant potential of hydro alcoholic pericarp extract of *Momordica chochinchinensis* (HAPMC) using different model systems. To establish the link between glycation and oxidation processes, the HAPMC extract has been evaluated for its in vitro radical scavenging activity against superoxide, hydroxyl, hydrogen peroxide, nitric oxide, ferric and ABTS\(^+\) radical and antiglycation activities like the inhibitory activities on bovine serum albumin (BSA) and as well as protein oxidation markers including protein carbonyl formation (PCO). The results indicated that the HAPMC possesses the highest antioxidant potential against superoxide, hydroxyl, hydrogen peroxide, nitric oxide, ferric radical and ABTS\(^+\) radical. The HAPMC extract at different concentrations (10–250 µg/ml) has significantly quenched the fluorescence intensity of glycated BSA and the glycoxidation measured in terms of advanced glycation end products (AGEs). Furthermore, the study demonstrated that the inhibitory effect of HAPMC extract in preventing oxidative protein damages including effect on PCO formation, which are believed to form under the glycoxidation processes. These results clearly demonstrate that the HAPMC, owning to its antioxidant content, is capable of suppressing the oxidative stress, carbonyl stress and formation of AGEs and protein oxidation in vitro.

**Keywords:** Advanced glycated end products, Oxidation, *Momordica chochinchinensis*

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INTRODUCTION

Diabetes mellitus is an endocrine disorder characterized by chronic hyperglycemia, which results from an absolute or relative deficiency of or resistance to insulin. According to the World Health Organization (WHO), 346 million people suffer from diabetes worldwide, whereas deaths due to this disease will double between 2005 and 2030. The increased morbidity and mortality in diabetics is mainly due to poor glycemic control over a long period of time\cite{1}.

Glycation is the non-enzymatic reaction between a protein and a reducing sugar, such as glucose and fructose \cite{2} resulting in the formation of a reversible structure called a Schiff ’s base and undergoes rearrangements to the Amadori products that induce further oxidation, generating dicarbonyl compounds and finally advanced glycation end products. Glycation stress is one of the risk factors for aging from inside and outside of the body \cite{3}. Evidence has shown that AGEs participate directly in the pathogenesis of diabetic complications by accumulation and cross linking of long lived proteins. \cite{4,5}

Aminoguanidine is an established antiglycation agent shown to have serious side effects such as flu-like symptoms, gastrointestinal problems, and anemia \cite{6}. In recent times researchers have been directed towards plant based medicine for the treatment of glycation and hyperglycemic conditions. Some herbal drugs like *Allium cepa* \cite{7}; *Garcinia indica* \cite{8}; *Salvia reuterana* \cite{9}; *Camellia sinensis* \cite{10}; *Astragalus radix* \cite{11,12}; *Teucrium polium* \cite{13}; *Feronia limonia* \cite{14} were reported to have antiglycation activity due to the presence of antioxidant principles. The quercitin, apigenin, kaempiferol; carotenoids like α, β- carotene,zeaxanin, lycopene, lutein and phenolic compounds like gallic acid, vanillic acid, ferulic acid, caffeic acid, proto catechuic acid were present in Gac (*Momordica chochinchinensis* \cite{15}) are reported to have biological activities like Immunomodulatory \cite{15}, antiinflammatory, antimicrobial \cite{16}, antioxidant \cite{17}, DNA protective \cite{18}, tumor growth and angiogenesis \cite{19} activities. Hence the present study was focused on the effect of hydroalcoholic pericarp extract of *Momordica chochinchinensis* against oxidative stress, carbonyl stress and fructose induced advanced glycation end products.

MATERIALS AND METHOD

**Materials:**

Nitroblue tetrazolium (NBT), bovine serum albumin (BSA) were obtained from Merck. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4 dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO, USA). 2.4.6-Tri- (2ʹ-pyridyl)-1, 3, 5-triazine (TPTZ), 2, 2ʹ- Azinobis- (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) and Trolox were
obtained from Sigma Aldrich Chemical Co., Ltd. (England). All other reagents were of analytical reagent (AR) grade.

**Plant materials and preparation:**
The hydroalcoholic pericarp extract of *Momordica cochinchinensis* was obtained from Laila Implex, Vijayawada.

**Superoxide radical scavenging activity:**
The assay was based on the capacity of the aqueous extract to inhibit formazan formation by scavenging the superoxide radicals generated in a riboflavin-light-NBT system\(^{[20]}\). The reaction mixture contained 58 mM phosphate buffer, pH 7.6, 20μM riboflavin, 6mM EDTA, and 50μM NBT, final volume made up to 3 ml, added in that sequence. Reaction was started by illuminating 40 volts under fluorescent lamp, the reaction mixture with different concentrations of HAPMC for 15 minutes. Immediately after illumination, the absorbance was measured at 560 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes, with reaction mixture, were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following formula:

\[
\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100
\]

Where \(A_0\) was the absorbance of the control, and \(A_1\) was the absorbance of the aqueous extract/standard.

**Hydroxyl radical scavenging activity:**
Scavenging activity of hydroxyl radical was measured by the method of Halliwell et al., 1985\(^{[21]}\). Hydroxyl radicals were generated by a Fenton reaction (Fe\(^{3+}\)-ascorbate-EDTA-H\(_2\)O\(_2\) system), and the scavenging capacity of the extract and standard towards the hydroxyl radicals was measured by using deoxyribose degradation method. The reaction mixture contained 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), ferric chloride (20 μM), EDTA (100 μM), hydrogen peroxide (500 μM), ascorbic acid (100 μM) and various concentrations of the test sample in a final volume of 1 ml. The mixture was incubated for 1 h at 37 °C. After the incubation an aliquot of the reaction mixture (0.8 ml) was added to 2.8% TCA solution (1.5 ml), followed by TBA solution (1% in 50 mM sodium hydroxide, 1 ml) and sodium dodecyl sulphate (0.2ml). The mixture was then heated (20 min at 90 °C) to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicates. The percentage of inhibition was expressed, according to the following equation:
% Inhibition = \frac{A_O - A_1}{A_O} \times 100

Where A_0 was the absorbance of the control without a sample, A_1 is the absorbance in the presence of the sample.

**Hydrogen peroxide radical scavenging activity:**

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch et al., 1989 [22]. The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2. A solution of 43mM H_2O_2 was prepared in 0.1M phosphate buffer (pH 7.4). HAPMC at concentration in 3.4mL phosphate buffer was added to 0.6mL of H_2O_2 solution (43mM) and absorbance of the reaction mixture was recorded at 230 nm. A blank solution contained the sodium phosphate buffer without H_2O_2.

**DPPH radical scavenging activity:**

The potential AA of extracts, fractions and pure compounds was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Aliquots of 1ml of a methanolic solution containing each pure compound were added to 3ml of 0.004% MeOH solution of DPPH. Absorbance at 517 nm, against a blank of methanol without DPPH, was determined after 30 min (UV, Perkin-Elmer-Lambda 11 spectrophotometer) and the percent inhibition activity was calculated [23]. IC50 values denote the concentration of sample required to scavenge 50% DPPH free radicals. All tests were run in triplicate and averaged.

**Nitric oxide radical scavenging activity:**

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [24]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and IBPG and the reference compound in different concentrations (20, 40, 60, 80 and 100 μg) were incubated at 25°C for 150 min. After incubation 1.5ml of the Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance of the chromophore formed was measured at 546nm. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples.

**Reducing power:**

The reducing power of the extract was determined according to the method of Oyaizu 1986 [25]. Various concentrations of the extracts (mg/ml) in distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% of potassium ferricyanide water solution (2.5 ml, K3[Fe(CN)6]). The mixture was incubated at 50 8C for 20 min. Aliquots of trichloracetic acid
(2.5 ml, 10% aqueous solution) were added to the mixture which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared FeCl3 solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. In this method, the higher the absorbance, the higher the reducing power.

**Phosphomolybdinum method:**

The antioxidant activity of HAPMC extract was evaluated by the phosphomolybdenum method of Prieto et al., 1999 [26]. An aliquot of 0.1 ml of sample solution (equivalent to 100 lg) was combined with 1 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In the case of the blank, 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in water bath at 95 °C for 90 min. After the samples were cooled to RT, the absorbance of the aqueous solution of each was measured at 695 nm.

**Fe²⁺ Chelating activity:**

The chelating activity of the extracts for ferrous ions Fe²⁺ was measured according to the method of Dinis et al., 1994 [27]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of FeCl2 (2 mM) was added. After 30 sec, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe²⁺Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe²⁺ was calculated as Chelating rate = (A₀ - A₁)/A₀×100, where A₀ was the absorbance of the control (blank, without extract) and A₁ was the absorbance in the presence of the extract.

**Ferric reducing ability power:**

The FRAP method measures the absorption change that appears when the TPTZ (2,4,6 -tripyridyl-s-triazine)-Fe³⁺ complex is reduced to the TPTZ-Fe²⁺ form in the presence of antioxidants [28]. An intense blue colour, with absorption maximum at 595 nm, develops. Briefly, the FRAP reagent contained 2.5 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20 mM FeCl3 and 25 ml of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The extracts were dissolved in ethanol at a concentration of 1 mg/ml. An aliquot of 0.2 ml of solution was mixed with 1.8 ml of FRAP reagent and the absorption of the reaction mixture was measured at 595 nm. Ethanolic solutions of known Fe (II) concentration, in the range of 50-1000 μM (FeSO4), were used for obtaining the calibration curve. The FRAP value represents the ratio
between the slope of the linear plot for reducing Fe$^{3+}$-TPTZ reagent by plant extract compared to the slope of the plot for FeSO$_4$.

**ABTS$^{+}$ Assay:**

ABTS assay was based on the method of Re et al., 1996$^{[29]}$ with slight modifications. ABTS radical cation (ABTS$^{+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS$^{+}$ solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. After addition of 25µl of sample or Trolox standard to 2 ml of diluted ABTS$^{+}$ solution, absorbance at 734 nm was measured at exactly 6 min. The decrease in absorption at 734 nm was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of ABTS$^{+}$ solution at different concentrations of Trolox. Appropriate blank measurements were carried out and the values recorded. Results were expressed as Trolox equivalent antioxidant capacity (TEAC).

**Estimation of protein carbonyl content:**

The effects of HAPMC extract on oxidative modification of BSA during glycoxidation process were carried out according to method described previously Ardestani and Yazdanparast, 2007$^{[30]}$. For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the samples (1 mg). Samples were incubated for 30 min at RT. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH. The data were expressed as nmol/mg protein.

**In Vitro protein glycoxidation method:**

This assay was adopted from the literature Wu & Yen, 2005 and used as an in vitro model for comparing the anti-glycation activities HAPMC$^{[31]}$. In brief, 5 g BSA and 14.4 g D-glucose were dissolved in phosphate buffer (1.5 M, pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. 2 mL of the control solution was incubated at 37$^\circ$C for 21 days in the presence or absence of 1 mL of plant extracts in phosphate buffer (1.5 M, pH 7.4) (the final concentration of plant extract in the 3 mL test solution was 500 ppm). The test solution also contained 0.2 g/L NaN$_3$ to assure an aseptic condition. After 7 days of incubation, fluorescent intensity (excitation, 330 nm; emission, 410 nm) was measured for the test solutions. Percent inhibition of AGE formation by each extract or compound was calculated using the following
equation, % inhibition = \[\frac{1 \times (fluorescence \ of \ the \ solution \ with \ inhibitors)}{(fluorescence \ of \ the \ solution \ without \ inhibitors)} \times 100\%\].

RESULTS AND DISCUSSION

The human body defense mechanisms protects the body against free radicals under normal physiological conditions \[^{32}\]. The free radicals were generated in the body during a number of the physiological and biochemical processes, which leads to the production of several radicals. The reactive oxygen species are superoxide radical (O$_2^-$); hydrogen peroxide (H$_2$O$_2$); hydroxyl radical (HO$^-$); peroxyl radical (ROO$^-$) and hypochlorous acid (HOCl) \[^{33}\]. The reactive nitrogen species (RNS): nitric oxide (\textit{NO}) and peroxynitrite (ONOO$^-$) which are formed by the reaction of \textit{NO} and superoxide during inflammatory processes \[^{33}\]. These accumulated reaction products of both oxygen and nitrogen free radical products undergoes protein glycation by nonenzymatic reaction of proteins with glucose and other reducing sugars in living tissues leads to generation of advanced glycation end products (AGEs) \[^{34}\]. These AGEs causes change in molecular pathways that interrupt the pathogenesis of several important diseases including carcinogenesis, cardiovascular diseases, neurodegenerative diseases and also in the process of physiological ageing \[^{35}\].

Therefore, the present investigation is an attempt to focus the effect of hydroalcoholic pericarp extract of \textit{Momordica chochinchinenesis} (HAPMC) role on oxidative stress, carbonyl stress and in fructose induced advanced glycation end products.

Antioxidants works as in various pathways by scavenging the radicals and decomposing peroxides by binding to the metal ions and some of them producing synergistic action. These free radicals act by damaging proteins, DNA, and other small molecules. These antioxidants work by three different mechanisms as antioxygen radicals, reducing substances and antiradicals. They work as metal chelators by chain breaking and reducing the chain initiation. Therefore antioxidant activity can be evaluated by different \textit{in vitro} methods for different mechanisms.

There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host’s total capacity to withstand free radical stress \[^{36}\]. Superoxide anions are thus precursors to active free radicals that have potential for reacting with biological macromolecules and thereby inducing tissue damage \[^{14}\]. In the presence of superoxide dismutase (SOD) as a cellular antioxidant enzyme, which removes this ubiquitous superoxide metabolic product by converting it into hydrogen peroxide and oxygen and this hydrogen peroxide radical readily decomposed into hydroxyl radical in the presence of Catalase.
in biological systems\textsuperscript{[37]}. The HAPMC shown to have scavenging property against superoxide, hydroxyl and hydrogen peroxide radicals in a concentration dependent manner as shown in table 1. The HAPMC shown to had reducing Mo (VI) to subsequent formation of a green phosphate/Mo (V) complex at acidic pH with increasing concentration (Table 1). Nitric oxide is a free radical in terms of its unpaired electron. It reacts with $O_2^-$ in termination reactions in the mitochondrial matrix, yielding peroxynitrite (ONOO). These oxyradical and peroxynitrite induce oxidative damage to mitochondrial DNA damage and protein inactivation and ATP synthesis\textsuperscript{[38]}. The HAPMC had shown better scavenging activity in scavenging nitric oxide free radical as ascorbic acid (Table 1).

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule\textsuperscript{[26]}. It is an endogenous free radical. The HAPMC can able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine in a dose dependent manner by donating hydrogen atom donating capability thus neutralizing its free radical character (Table 1)\textsuperscript{[39]}. Iron, in nature, can be found as either ferrous (Fe$^{2+}$) or ferric ion (Fe$^{3+}$), with the latter form predominating in foods. This study, the iron reducing power of the HAPMC was estimated from their ability to reduce the Fe$^{3+}$-ferricyanide complex to the Fe$^{2+}$/ferrous form by reducing capability as donating an electron (Table 1)\textsuperscript{[40]}. Ferrous chelation may render important antioxidative effects by retarding metal-catalysed oxidation\textsuperscript{[41]}. Iron is known as the most important lipid oxidation pro-oxidant among the transition metals due to its high reactive nature. The effective ferrous ion chelators may also afford protection against oxidative damage by removing iron that may otherwise participate in HO• generating Fenton type reactions as discussed earlier (Table 1). The HAPMC shown to have Fe$^{2+}$ chelating property in a concentration dependent manner might be due to their hydroxyl radical scavenging property (Graph 1)\textsuperscript{[42]}. In this assay, HAPMC interfered with the formation of the ferrous–ferrozine complex, indicating that HAPMC has chelating activity and is able to capture ferrous ion with a higher binding affinity than ferrozine due to its ability to compete with ferrozine for ferrous ion in the solution mixture. In FRAP assay, the capability of test compound that react with ferric tripyridyltriazine (Fe$^{3+}$-TPTZ) complex and produce a coloured ferrous tripyridyltriazine (Fe$^{2+}$-TPTZ)\textsuperscript{[28]}. In this assay, HAPMC shown to increasing FRAP value in dose dependent effects shown in Graph 2.

In ABTS radical scavenging activity, ABTS•+ radicals involved in the electron-transfer process reactions. This is a direct generation of a stable form of radical to create a blue-green ABTS•+ chromophore prior to the reaction with antioxidants\textsuperscript{[43]}. The HAPMC shown to have ABTS•+
radical scavenging property might be due to electron donating capability to prevent the formation of stable radical formation (Graph 3).

During the early stage of glycation, Schiff bases are prone to oxidation leads to generating variety of free radicals, reactive carbonyl groups and formation of AGEs. Oxidative modifications of BSA incubated with fructose were demonstrated using a combination of protein carbonyl assay [44]. To evaluate HAPMC can reduce the protein glycation and extent of protein carbonyl formation after 21 days by DNPH reagent. As shown in Graph 4, 5 glycation elicited a significant increase of carbonylation of BSA in the presence of fructose compared to the control sample without reducing sugar. However a significant effect on the inhibition of protein oxidation due to glycation was exerted in a dose-dependent manner with increasing the concentrations of HAPMC.

Table1: Effect of Ascorbic acid and HAPMC on IC_{50} values of different in vitro models.

<table>
<thead>
<tr>
<th>Method</th>
<th>Ascorbic acid</th>
<th>HAPMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical</td>
<td>06.46±0.31</td>
<td>05.92±0.29</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>29.70±0.21</td>
<td>32.41±0.84</td>
</tr>
<tr>
<td>Hydrogen peroxide radical</td>
<td>09.08±0.29</td>
<td>08.15±0.19</td>
</tr>
<tr>
<td>DPPH radical</td>
<td>77.92±4.23</td>
<td>69.82±3.91</td>
</tr>
<tr>
<td>Nitric oxide radical</td>
<td>26.23±0.19</td>
<td>35.69±1.41</td>
</tr>
<tr>
<td>Reducing power</td>
<td>05.41±0.14</td>
<td>07.44±0.26</td>
</tr>
<tr>
<td>Phosphomolybdinum method</td>
<td>04.82±0.11</td>
<td>05.77±0.23</td>
</tr>
</tbody>
</table>

Figure 1: Effect of various concentrations of ascorbic acid and HAPMC on Fe^{+2} chelating activity.
Figure 2: Effect of various concentrations of ascorbic acid and HAPMC on ferric reducing ability power (FRAP).

Figure 3: Effect of various concentrations of ascorbic acid and HAPMC on ABTS assay
CONCLUSION

It is concluded that, using different in vitro models for estimation the antioxidant potential of *Momordica chochinchenensis* showed better effect in scavenging the different free radicals formed during oxidative stress and carbonyl stress. In addition to antioxidant activity it also showed better activity against the formation of protein carbonyl content and protect the structural changes in BSA during glycation processes.
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