Research Article

ALPHA AMYLASE INHIBITOR FORMULATION DEVELOPMENT USING COWPEA A NOVEL ENTITIES

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ABSTRACT
Digestive enzyme lipase and amylase are responsible for catalyzing the digestion and absorption of lipid and carbohydrates. Alpha amylase inhibitors are substances which alter the catalytic action of alpha amylase on starch and consequently slow down or stop the breakdown of starch to maltose. The present research work describes the inhibition of mammalian alpha amylase (MAA) by extract obtained from cowpea (Vigna ungiculata) seed. Alpha amylase inhibitor was extracted in 0.1 N HCl by sonication method. Obtained extract was evaluated qualitatively for phytochemical constituents and total protein content by biuret method. Crude extract was also study for acute toxicity. In-vitro alpha amylase inhibition activity was performed with crude extract and prepared formulation (Chewable Tablet) which showed that it slower down the digestion of starch.

KEY WORDS Alpha amylase, Cowpea, Digestion, Chewable tablet

INTRODUCTION
Obesity has ailment consequences that are costly to society and to the individual. We need to understand more about obesity and how to manage obese individual. Obesity means deposition of excess fat in body. It is generally caused by ingestion of greater amount of food that can be utilized by the body for energy. Obesity is the greatest public health problem today and the rate of increase among all age group is alarming. Clinically, obesity is defined as a body mass index greater than thirty kg/m² and overweight as a body mass index more than 25kg/m². Obesity causes great embarrassment to a person, which may cause depression, agitation, irritation, aggressive, which also leads to various physical and mental problems. Thus, the treatment of obesity becomes very important for the person. Several substances can be used to block the digestion or absorption of food. Tetrahydrolipostatin is a lipase inhibitor that blocks the fat digestion and is potent, specific and long acting inhibitor of gastrointestinal lipases. It forms a covalent bond with the active serine of the gastric and pancreatic lipase. The inactivated enzyme are thus unable to hydrolyze dietary fat in the form of triglycerides, in the absorbable free fatty acids and mono glycerides as undigested glycerides are not absorbed, the resulting calorie deficiency has a positive effect on weight control. Systemic absorption is not needed for activity. Alpha amylase is an amylolytic enzyme present in salivary and pancreatic secretion with the specific activity for converting starch in dextrin and maltose. Salivary alpha amylase is capable of bringing about the hydrolysis of starch and glycogen to maltose and other oligosaccharides by attacking alpha 1-4 glycoside bonds. Pancreatic alpha amylase is similar to the action of salivary alpha amylase hydrolyzing starch and glycogen to maltose.

MATERIALS AND METHODS

Seeds, chemicals and reagents
Cowpea seed and required chemicals and reagents were provided by the central store house B.R. Nahata college of pharmacy, Mandsaur,(M.P)

Extraction Procedure
Cowpea seeds were extracted with 0.1M HCl, (1:5w/v, meal to buffer ratio) with continuous stirring for 5 hours at 4°C. The material was centrifuged at 10000 x g for 30 minutes. The clear supernatant was neutralized with 0.1M NaOH and submitted to a fractionation with ammonium sulphate (60%).

Qualitative Chemical Evaluation Of Crude Extract
Various chemical tests were performed for testing different chemical groups present in extracts.

Total Protein Estimation By Biuret Method
Preparation of Standard Curve of Bovine Serum Albumin (BSA)
Firstly 200 mg of BSA was dissolved in 10 ml of distilled water to make a stock solution of 20 mg/ml. From this stock solution different aliquots of BSA was prepared by mixing the water and biuret reagent and their absorbance was measured at 540 nm against blank
Table 1: Qualitative Chemical Evaluation of Crude Extract

<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Present</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Present</td>
</tr>
<tr>
<td>Fats and oils</td>
<td>Absent</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Absent</td>
</tr>
<tr>
<td>Steroids</td>
<td>Absent</td>
</tr>
<tr>
<td>Tannins</td>
<td>Absent</td>
</tr>
<tr>
<td>Glycosides</td>
<td></td>
</tr>
<tr>
<td>- Cardiac</td>
<td>Absent</td>
</tr>
<tr>
<td>- Saponins</td>
<td>Present</td>
</tr>
<tr>
<td>- Flavanoid</td>
<td>Present</td>
</tr>
<tr>
<td>- Anthraquinone</td>
<td>Absent</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Present</td>
</tr>
</tbody>
</table>

Estimation of Total Protein in Crude Extract

One ml Solution of extract of concentration 1mg/ml was taken and 0.5ml water and 6 ml of biuret reagent was added and exactly after 30 min absorbance was measured at 540 nm against the blank which contain 1.5 ml water and 6.0 ml biuret reagent only. Amount of protein in test solution was estimated with references to the plotted standard curve.

Principle

\[
\text{Starch} + \text{Water} \xrightarrow{\alpha-\text{Amylase}} \text{Reducing Groups (Maltose)}
\]

Condition: T=40°C, pH=5, \( \lambda_{\text{max}} = 620 \text{nm} \), Light path=1 cm.

Method: Visible Spectroscopy

Alpha amylase which found in saliva, break down starch in to the simple sugar glucose and is one of the first step in digestion. Starch can be detected with a simple chemical stain. In the presence of starch, a solution of potassium iodide/iodide (KI/iodine) will turn violet colour. This is a quantitative reaction (darker the violet colour, the more starch is present). As amylase digest the starch, the intensity of the violet colour of solution decreases.

This study was carried out in three following parts

Part 1. Development of Standard Curve of Starch

One gram starch was dissolved in 100 ml of boiling water to have 1%w/v solution of starch. Appropriate quantity was taken from stock solution and solutions ranging from 100 to 700 µg/ml concentration were prepared. One drop of iodine solution was added to each of solution to develop violet colour and absorbance was taken in a UV-Visible spectrophotometer (Thermospectronic-uv model) at 620 nm absorption maxima. A standard curve was plotted between concentration and absorbance.

Part 2. Determination of amount of starch remains in reaction at different time intervals in absence of crude products.

- From a volunteer, collect at least 1 ml of saliva in a large test tube.
- Pipette out 1 ml of saliva into a test tube, added 9 ml of deionized water to a 1 ml saliva sample and mix well.
- This is the 1:10 saliva dilution.
- Remove 1 ml of the 1:10 saliva dilution and pipette into a different test tube.
- Filled each of the 7 test tubes with 2 ml deionozed water.
- Prepared 1%starch solution and added 1 ml of this solution in to test tube which contain 1 ml of the 1:10 saliva dilution, from 1 to 7 and remain it for 0, 2, 4, 6, 8, 10, 12 min respectively (reaction time).
- Added 1 drop of KI/Iodine reagents in each test tube after precise time.
- Mixed well and record the absorbance at 620 nm by using spectrophotometer.

Part 3. Determination of amount of starch remains in reaction at different time intervals in presence of crude products. (Establishment of amylase inhibition activity of crude extract)

- From a volunteer, collect at least 1 ml of saliva in a large test tube.
- Pipette out 1 ml of saliva into a test tube, added 9 ml of deionized water to a 1 ml saliva sample and mix well.
- This is the 1:10 saliva dilution.
- Remove 1 ml of the 1:10 saliva dilution and pipette into different test tubes.
- Filled each of the 7 test tubes with 2 ml deionized water.
- Now 1 ml solution of 1% w/v of crude extract was added in each test tube.
- Prepared 1%starch solution and 1 ml of this solution was added in to test tube which contain 1 ml of the 1:10 saliva dilution and 1
ml of 1% w/v of solution of crude extract, from 1 to 7 and remain it for 0, 2, 4, 6, 8, 10, 12 min respectively (reaction time).

- Added 1 drop of KI/Iodine reagents in each test tube after precise time. Mixed well and record the absorbance at 620 nm by using spectrophotometer.

**Acute Toxicity Study**

Acute toxicity study was done according to OECD (organization for economic cooperation and development) guideline no 420. Animals were acclimatized to laboratory condition five day prior to the experiment body weight of animals was recorded and individual identification was done. Starting dose of 2000mg/kg body weight was adopted. Starting dose of 2000mg/kg of extract was given to 5 animals following p.o and i.p route, and animals were observed for behavioral changes and death. No animals were found dead after 14 days. The study was repeated with same dose and again no death was observed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Number of Animals</th>
<th>Mortality After 24 hrs</th>
<th>Mortality After 7 Days</th>
<th>Mortality After 14 Days</th>
<th>Toxicity Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>2000</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Safe</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>2000</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Safe</td>
</tr>
</tbody>
</table>

**Formulation Development and Evaluation**

**Formulation of Chewable Tablet**

The dried extract and other ingredients were mixed uniformly and granules were prepared by wet granulation technique. Different concentration of PVP in isopropyl alcohol was used as a binding agent. The mass was forced manually through sieve no 12. The resulting granules were mixed thoroughly with magnesium stearate and talc. The lubricated granules were compressed into tablet in an 8-station machine with 500 mg die cavity.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity per Tablet (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug Extract</td>
<td>200</td>
</tr>
<tr>
<td>PVP</td>
<td>q.s.</td>
</tr>
<tr>
<td>Mannitol</td>
<td>285</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>1%</td>
</tr>
<tr>
<td>Talc</td>
<td>2%</td>
</tr>
<tr>
<td>Flavouring Agent (Vanilla)</td>
<td>q.s.</td>
</tr>
</tbody>
</table>

**Evaluation of Chewable Tablet**

**Enzyme Inhibition Activity of Developed Formulation Procedure**

- From a volunteer, collect at least 1 ml of saliva in a large test tube.
- Pipette out 1 ml of saliva into a test tube, added 9 ml of deionized water to a 1 ml saliva sample and mix well.
- This is the 1:10 saliva dilution.
- Remove 1 ml of the 1:10 saliva dilution and pipette into a different test tube.
- Filled each of the 7 test tubes with 2 ml deionized water.
- Prepared 1% starch solution and added 1 ml of this solution in to test tube which contain 1 ml of the 1:10 saliva dilution, from 1 to 7 test tubes.
- Prepared tablets are triturated and dissolved in distilled water and then filtered.
- Added 1 ml of this solution in above test tubes and remain for about 0, 2, 4, 6, 8, 10, 12 min.
- Added 1 drop of KI/Iodine reagents in each test tube after precise time.
- Mixed well and record the absorbance at 620 nm by using spectrophotometer.

**Tablet Physical Appearance**

As one of the quality control procedure, tablet should be inspected for smoothness, absence of cracks, chips, and other undesirable characteristics.

**Hardness**

The hardness test is performed to provide a measure of tablet strength. Tablet should be hard enough to withstand packaging and shipping but not so hard as to create undue difficulty upon chewing. Hardness of chewable tablets was measured with the help of Monsanto hardness tester. The tablet to be tested was held between a fixed and a moving jaw and reading of the indicator was adjusted to zero. Moving the screw knob forward until the tablet broke gradually increased the forced applied to the edge of tablet. The reading was noted from scale, which indicated the pressure required in kg to break the tablet.

**Tablet Weight Variation**

The USP weight variation test is run by weighing 20 tablets individually, calculating the average weight and comparing the individual tablet weights to the average. The tablets meet the USP test if no more
than 2 tablets are outside the percentage limit and if no tablet differs by more than 2 times the percentage limit. The weight variation tolerances for uncoated tablets differ depending on average tablet weight. For weight variation twenty tablets were weighed individually and calculated for average weight of tablet, the average was compared with individual tablet weight and % weight variation was determine

**Friability**

The friability test give an indication of the tablets ability to resists chipping and abrasion on handling during packaging and shipping. Usually for conventional tablets a friability value of 1% or less is desirable, while for chewable tablet (due to the lower hardness of the tablets) friability values of up to 4% are acceptable. Roche friabilator was used for the determination of friability. Pre-weighed 6 tablets were placed in the friabilator, which was then operated for 100 revolutions. Tablets were dusted and reweighed. The percent friability was measured using the formula;

\[
\% F = \left(\frac{W_0 - W}{W_0}\right) \times 100
\]

Where,
- \(\% F\) = Friability in percent,
- \(W_0\) = Initial weight of tablet
- \(W\) = Weight of tablet after test

**Disintegration**

This test initially may not appear appropriate for chewable tablet as these tablets are to be chewed before being swallowed. However, patients, especially pediatric and geriatric, have been known to swallow these chewable dosage forms.

**Stability studies of formulation**

To ensure the quality, safety and efficiency of drug product up to their expiration date (ICH-QIC). Two primary groups were prepared in each group 30 tablets were taken and kept for 3 month in different environmental conditions.

**Figure 1: Relationship between Concentration of Starch and Reaction Time Absence of Alpha Amylase Inhibitor**

![Graph showing the relationship between starch concentration and reaction time](image-url)
Figure 2: Relationship between Concentration of Starch and Reaction Time in Presence of Alpha Amylase Inhibitor

Figure 3: Comparison of Concentration of Starch in Absence and Presence of Alpha Amylase Inhibitor
RESULTS AND DISCUSSION
Cowpea seeds were extracted with 0.1M HCl, (1:5w/v, meal to buffer ratio) with continuous stirring for 5 hours at 4°C. The material was centrifuged at 10000 x g 4°C for 30 minutes. The clear supernatant was neutralized with 0.1M NaOH and submitted to a fractionation with ammonium sulphate (60%). The qualitative chemical examination showed the presence of protein, amino acid, glycosides, carbohydrates, flavonoids and saponins. Total protein content in extract was determined by spectrophotometrically using bovine serum albumin as a standard protein at 540 nm by the help of biuret reagent and % yield was calculated and it was found to be 51.99%. The enzyme inhibition activity of crude extract and developed formulation was studied.
and the results were shown that digestion of starch was delayed with time in the presence of amylase inhibitor that was extracted from cowpea seeds. The result suggests that this formulation can be taken as a safe adjuvant for weight loss management. This allows an individual to enjoy carbohydrates-containing food while still avoiding the absorption of carbohydrates. The acute toxicity study showed that the extract is safe up to 2000mg/kg body weight in both routes. Therefore 2000mg/kg dose was considered as a safe dose, so $1/10^{th}$ (200mg) of that was selected for formulation development. The chewable tablets were evaluated for different pharmaceutical parameters such as physical appearance, hardness, weight variation and friability. All these parameters were found to be under limits. The stability study of developed formulation was performed and the results were shown that there was no significant difference in evaluated parameters.

**REFERENCES**

10. ICHQ1C: “Stability Testing of New Dosage Forms” Recommended for adoption of the ICH process.