Effect Of Oleanolic Acid Isolated From Garlic Leaves On Carbohydrate Metabolizing Enzymes, *In Vitro*

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Abstract:

Post-prandial hyperglycemia can be controlled by retarding the absorption of glucose through inhibition of the two main enzymes, α-amylase and glucoamylase. This action delays carbohydrate digestion causing reduction in the rate of glucose absorption and degradation of glycogen during starvation. *Allium sativum* (Garlic) is a medicinal plant used worldwide for flavoring as well as medicinal purpose. However, chemical examination and biological activity of compounds present in the leaves are less studied. The pharmacological effects of garlic leaves are still unexplored. Isolation and purification of the chloroform fraction from leaves of garlic resulted characterization of a triterpene, oleanolic acid. Oleanolic acid showed remarkable inhibition of pancreatic α-amylase and glucoamylase enzyme, *in vitro.*

Keywords: garlic leaves, oleanolic acid, α- amylase, glucoamylase.

Introduction:

Plant derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [1].

Diabetes mellitus is a metabolic disorder which results due to chronic hyperglycemia associated with the imbalance in carbohydrate, fat and protein metabolism resulting from the defect in insulin secretion, insulin action or both. One of the therapeutic approaches to treat diabetes is to decrease post prandial hyperglycemia. This is done by retarding and reducing the digestion and absorption of glucose through inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and glucoamylase in the digestive tract. Inhibition of these enzymes delays the digestion of the carbohydrates causing a reduction in the rate of glucose absorption [2]. Because of their purported ability to prevent starch breakdown and absorption, α-amylase inhibitors have been used for weight loss in humans.

Acarbose and voglibose are currently used as inhibitors of α-amylase and glucoamylase but they also induce side effects such as abdotension, bloating, flatulence and diarrhea [3]. It has been suggested that such adverse effects might be caused by excessive inhibition of pancreatic α-amylase resulting in abnormal bacterial fermentation [4]. Therefore, natural α-amylase inhibitors from the medicinal plants can prove an effective therapy for post prandial hyperglycemia with minimal side effects [5].

Garlic, bulbs of *A. sativum L*, (Liliaceae) has been used worldwide as a tonic, a bacteriocide and a popular remedy for various ailment [6]. In the literature survey, we have observed a paucity of research done on the leaves of garlic. In the earlier work, we have discussed about the effects of the garlic leaves extracts on the carbohydrate metabolizing enzymes where we have concluded that the chloroform extract is a potent inhibitor of α-amylase [7]. The present paper deals with the study on the leaves of garlic reporting the isolation and structural determination of triterpenoid and its effect on carbohydrate metabolizing enzyme, viz. α-amylase and glucoamylase. To the best of our knowledge, there has been no documented evidence for the isolation of a triterpenoid from garlic leaves.

Materials and Methods:

Chemicals

Porcine pancreatic α-amylase (EC 3.2.1.1) was procured from SRL Ltd. and *A. Niger* Glucoamylase (EC 3.2.1.3) were procured from SRL Ltd., Mumbai. All other chemicals were used of analytical grade. Glass double distilled water was used to carry out enzyme assays.

General

Melting point was determined in an open capillary tube using Buchi M-560 melting point instrument. IR spectra was obtained on Perkin-Elmer Frontier 91579 FT-IR spectrophotometer using ATR attachment. All ^{1}H and ^{13}C NMR (300 and 75 MHz respectively) spectra were recorded on BrukerAvance Spectrometer with 1% TMS as internal standard. Chemical shifts are expressed as δ. Abbreviations used in the splitting pattern were as follows:

s for singlet, d for doublet, t for triplet and dd for doublet of doublet. Mass spectra was obtained on Thermo-Finnigan Discovery- Max GC-MS. Elemental analysis was performed on Elemental Analyzer model 'EURO EA 3000'. UV-Double Beam Spectrophotometer on which absorbance was recorded by using 'Shimadzu UV 2400'model

Extraction

The fresh leaves of *Allium Sativum* were collected from a local market in Mumbai. The leaves were cleaned properly with glass distilled water.

300g garlic leaves were chopped and extracted with 500mL ethanol at room temperature for one week replacing the solvent after every 24 hours. The extract thus collected was further distilled in vacuo, to obtained a sticky green residue. This residue then was extracted with petroleum ether, chloroform and ethyl acetate. The extracts obtained were distilled to remove the solvents and the residues were weighed and stored.

The chloroform extract (3.8g) was subjected to silica gel column chromatography (60-120 mesh size) for the isolation of phytoconstituents. The column was eluted gradiently with chloroform, chloroform-methanol mixtures 90:10, 80:20, 70:30, 50:50 and methanol. At uniform intervals, the eluents were collected and the progress of separation was monitored by TLC (Silica gel H) using solvent system $CHCl_3$: MeOH (90:10) and iodine vapours as detecting agent.

Fractions eluted with $CHCl_3$: MeOH (70:30) which showed a single spot on TLC afforded the residue (35mg). The residue thus obtained was recrystallized in methanol and referred as Compound (**1**) and was subjected to spectroscopic analysis, GC-MS, IR, ^{13}C-NMR, ^{1}H-NMR, DEPT-135, Elemental analysis to elucidate the structure.

Compound (**1**) was studied for its effect on the enzymes, glucoamylase and α-amylase, in varying concentrations (10-100µg/mL).

In vitro porcine pancreatic α-amylase assay

The α-amylase activity was assayed according to the method described by Miller[8]with slight modifications.Briefly, the total assay mixture containing 300µL of 20mM phosphate buffer (pH 7.0), 100µL of α-amylase and 100µL of modulator in the concentration range10-100µg/mL were incubated for 30 min at 37^0C followed by addition of 500µL of starch solution (10mg/mL prepared in 20mM phosphate buffer pH 7.0). The reaction was terminated by keeping the test-tubes in boiling water bath for 1-2minutes and cooled under running tap water. 2mL of 3,5-dinitrosalicylic acid (DNS) reagent was added, placed in boiling water bath for 15 min., cooled to room temperature and diluted with 7mL distilled water to make a total volume of 10mL. The absorbance was measured at 540nm using UV Spectrophotometer. Acarbose was used as positive control. A unit activity (U) is defined as the mg of glucose liberated per mg of protein per minute.

The maximum inhibition was determined from plots of percent inhibition versus modulator and calculated as below,

% Activity = (enzyme activity of test / enzyme activity of control) X 100,

% Inhibition = (100 - % activity)

Glucoamylase inhibition assay

The glucoamylase inhibition was determined as per the method described by Miller[8] with slight modifications. The assay mixture containing 300µL of 100mM acetate buffer (pH 4.5), 100µL of glucoamylase and 100µL of modulator in the concentration range 20-100µg/mL were incubated for 30 min at 37°C followed by addition of 500µL of starch solution (5mg/mL prepared in 100mM phosphate buffer pH 4.5). After 30 min, the reaction was terminated by keeping the test-tubes in boiling water bath for 1-2minutes and cooled under running tap water. 2mL of 3,5-dinitrosalicylic acid (DNS) color reagent was added, placed in boiling water bath for 15 min., cooled to room temperature and diluted to 7mL with distilled water. The absorbance was measured at 540nm using UV Spectrophotometer. Acarbose was used as positive control. A unit activity (U) is defined as the mg of glucose liberated per mg of protein per minute.

The maximum inhibition was determined from plots of percent inhibition versus modulator and calculated as below,

% Activity = (enzyme activity of test / enzyme activity of control) X 100,

% Inhibition = (100 - % activity)

The IC_{50} values (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) were determined by performing the assay as above with varying concentrations of the modulator ranging from 20-100µg. The IC_{50} values were determined from plots of percent inhibition vs inhibitor concentration and calculated by linear regression analysis from the mean inhibitory values.

Statistical Analysis

All the experiments were performed in triplicates and the results were expressed as mean ±standard error of mean.

Results and Discussion:

Compound 1

Elemental Analysis

Found values: C – 78.58 %, H -10.52%,

Required values for $C_{30}H_{48}O_3$: C – 78.94%, H – 10.52%,

IR (V_{max}) (cm^{-1}): 3400, 3123, 2920, 2880, 1690, 1440, 1373, 1360, 1450

^{1}H-NMR (300MHz, $CDCl_3$): δ 0.63 – 1.07 (7 ‘ –$CH_{3'}$), 2.83 (1H, dd, J=3.6,13 H**z,),** 3.20 (dd, 1H,J=10.2, 5.7Hz, H-3), 5.22 (1H, t, H-12)

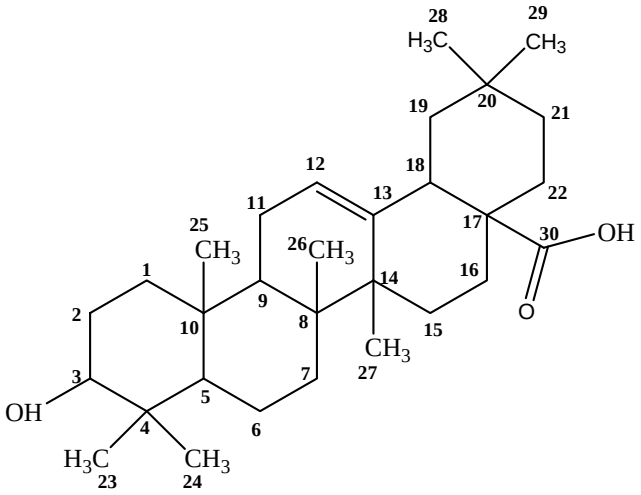
^{13}C-NMR (300 MHz, $CDCl_3$, ppm) : 39.0(C-1), 28.1 (C-2), 78.2 (C-3), 39.2 (C-4), 55.9 (C-5), 18.8 (C-6), 33.4 (C-7), 39.8 (C-8), 48.2 (C-9), 37.4 (C-10), 23.8 (C-11), 122.6 (C-12), 144.8 (C-13), 42.2 (C-14), 28.4 (C-15), 23.8 (C-16), 42.7 (C-17), 46.7 (C-18), 31 (C-19), 34.3 (C-20), 33.2 (C-21), 28.8 (C-22), 16.5 (C-23), 15.6 (C-24), 17.5 (C-25), 15.6 (C-26), 26.2 (C-27), 180.0 (C-28), 33.4 (C-29), 23.8 (C-30))

EI-MS: m/z 456.23, [M^+] 410,289, 248, 207, 203.

The location of the double bond at C12- C13 position was supported by the diagnostic mass spectral fragmentation pattern. The fragment ion peaks at m/z 248 $[C_{16}H_{24}O_2]^+$, 203 $[C_{15}H_{23}]^+$ derived from D, E rings via Retro Diels-Alder fission indicating that the compounds possesses one hydroxyl group in the A-B ring and a carboxyl group in D-E ring on the Olean-12-ene-skeleton[9].The isolated compound was a white powder, showing characteristic absorption band for –OH function at 3400 cm^{-1}, for –OH of carboxylic at 3123cm^{-1}, C-H stretching at 2920$cm^{-1}$and 2880cm^{-1}, 1690cm^{-1} and presence of double bond at 1450cm^{-1}. The ^{1}H-NMR spectrum of the compound exhibited presence of methyl groups at δ 0.99, 0.80, 0.68 and 1.35, characteristic olefinic proton of C12-C13, double bonded pentacyclic triterpenoid at δ 5.39 (1H, t). The spectrum also showed a downfield signal for oxygenated methine proton at δ 3.20 (1H, dd, J=10.2, 5.7Hz), which was assigned to H-3 proton. A doublet of doublet at δ 2.85 (J=3.6, 13.6Hz), indicated coupling between a single proton at C-18 and two protons at C-19. This is a characteristic of β – type triterpenes (where only two protons are attached to C-19).

The ^{13}C signal at δ-182.38 indicated the presence of carbonyl group assigned to C-28. The two peaks at δ-122.66 and δ-143.58 represent the presence of a pair of sp2 hybridized carbon atoms assigned to C-12 and C-13 while the seven peaks at δ-28.11, 15.55, 15.33, 17.11, 25.92, 33.07 and 23.58 are assigned to the seven methyl groups which are assigned to C-23, C-24, C-25, C-26, C-27, C-29 and C-30 respectively. DEPT 135 shows ten methylene (CH_2 negative) groups with signals at δ-38.41, 27.20, 18.31, 32.65, 23.00, 27.69, 23.40, 45.89, 33.81 and 32.44 which are attributed to C-1, C-2, C-6, C-7, C-11, C-15, C-16, C-19, C-21 and C-22 respectively.

On the basis of above spectral and chemical evidences, the isolated compound was identified as Oleanolic acid [10].



Oleanolic acid

The isolated compound was further studied for its effect on the carbohydrate metabolizing enzymes, α-amylase and glucoamylase in the concentration range 10-100µg/mL.Acarbose was used as a reference glucosidase inhibitor.

Table 1.Effect of the Oleanolic acid on glucoamylase as compared to Acarbose

Concentration (µg/mL)	Acarbose % Inhibition	Oleanolic Acid % Inhibition
10	14.07	17.36
20	22.89	20.92
40	32.76	26.44
60	46.97	40.65
80	57.23	48.81
100	58.94	57.50

Table 2. Effect of the Oleanolic Acid on α-amylase as compared to Acarbose

Concentration (µg/mL)	Acarbose % Inhibition	Oleanolic Acid % Inhibition
10	18.75	30.49
20	22.41	38.89
40	29.06	49.63
60	38.74	53.90
80	47.72	57.07
100	58.45	62.43

Table 1 and 2 summarizes the effect of oleanolic acid on α-amylase and glucoamylase, invitro. The studies demonstrated that the oleanolic acid has both glucoamylase and α-amylase inhibitory activity.

The percentage inhibition at 10, 20, 40,60,80 and 100µg/mL concentrations of oleanolic acid showed a concentration dependent increase in percentage inhibition. Thus, the highest concentration of 100µg/mL tested showed a maximum inhibition of 57.50% and the standard drug acarbose showed inhibition of 58.94% (Table 1.) with IC_{50} values of 83.56 and 73.15µg/mL respectively, in the case of glucoamylase whereas in the case ofα-amylase, the percentage inhibition of 62.43% was observed at a concentration of 100µg/mL and that of acarbose was 58.45% with IC_{50} values of 55.51 and 83.89µg/mL respectively.

Conclusion

The present study was undertaken to evaluate the effect of oleanolic acid on α-amylase and glucoamylase activity in vitro. To the best of our knowledge, first time report on the isolation of oleanolic acid from garlic leaves and it's biological evaluation from the leaves of *Allium Sativum*. Oleanolic acid from *Allium Sativum* leaves showed strong inhibitory activity against α-amylase and glucoamylase compared to Acarbose as the control. Thus, *Allium Sativum* leaves may be a good natural source of glucosidase inhibitor used to control post prandial blood glucose and its complications.

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