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Enzyme inhibition studies of purified bioactive fractions of *Cassia glauca* Lam leaves

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Abstract

Over centuries the use of medicinal plants for the treatment of human diseases has been gaining attention of researchers due to increasing side effects of synthetic drugs. Medicinal plants possess such therapeutic components which play significant role in treatment of human diseases. In the present research work, crude methanolic extract of *C. glauca* leaves extracted through soxhlet apparatus was purified into different fractions by using different organic solvents (*n*-hexane, ethyl acetate, methanol) in polarity order through silica based column chromatography. These fractions were assessed for enzyme inhibition activity of alpha amylase and lipase to check their potential against obesity and diabetes. Alpha amylase inhibition was checked by UV-visible spectrophotometer by using DNS method and lipase inhibition by phenol red using plate method. All fractions (F1-F13) showed alpha amylase and lipase inhibition activities. However among these fractions, F6 (*n*-hexane: Ethyl acetate, 1:10) and F9 (Ethyl acetate: Methanol, 5:1) showed maximum alpha amylase inhibition i.e. 27.52% and 27.17% respectively. Maximum lipase inhibition activity was shown by fractions F1 (*n*-hexane 100%) and F3 (*n*-Hexane: Ethyl-acetate, 5:1) i.e. 38.02% and 36.78% respectively. Results showed that alpha amylase and lipase inhibitors from plant extracts could be promising drug candidates for the treatment of diabetes and obesity. However, more research is needed to evaluate the enzyme inhibition potential of purified bioactive fractions of *C. glauca* leaves. Statistical analysis of data was done by one-way ANOVA.

Keywords: Amylase inhibition; *Cassia glauca* Lam; Diabetes; Lipase inhibition; Obesity

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1. Introduction

Globally, the occurrence of chronic and non-communicable diseases is increasing rapidly and now has reached at alarming rate. According to world health organization (WHO), approximately 18 million people are dying every year due to cardiovascular diseases, among which diabetes, obesity and hypertension are major ones [1]. In this century, the increasing rate of obesity has become one of the greatest threats to global health. About 1.1 billion adults worldwide are overweight and 312 million people of them are subjected to be obese. According to International Obesity Task Force (IOTF) approximately 155 million children worldwide are overweight or obese due to their changing life style or inadequate dietary habits. Due to increasing obesity rate, WHO has promoted to consider it as the widespread disease of 21st century [2].

The major cause of obesity is characterized by an imbalance between energy intake and its utilization. Visceral adipose tissues in body are metabolic and endocrine tissues that are not only composed of adipocytes but also have immune cells like fibroblasts, regulatory T cells and macrophages. In obesity, these adipose tissues start an overexpression of pro-inflammatory proteins like α-interlukin-6 or inducible nitric oxide synthase, tumor necrosis factor (TNF) etc. which may be involved in development of obesity related complications [3]. Lipids are essential compounds for all living organisms. They are the building blocks of cellular membranes and act as thermal insulators [4]. Due to their high calorie value, lipids represent an important ingredient of human diet. However, their prolonged intake contributes to the development of obesity and is associated with related morbidities [5].

Moreover, lipase enzyme enhances the metabolism of lipids breakdown and thus lead to storage of fats in body causing obesity. Diabetes mellitus is another chronic disease in worldwide which is caused by metabolic disturbance of glucose level in blood. Currently, it is estimated that more than 220 million people worldwide have diabetes [6]. Two types of diabetes are commonly known, diabetes type 1 and type 2. In type 1 diabetes, insufficient production of insulin occurs which leads to hypoglycemia while, type 2 diabetes occurs as a result of ineffectiveness of insulin, which leads to an abnormal postprandial increase in blood glucose

known as hyperglycemia. Approximately, 90% cases of diabetes in worldwide are related to type 2. Type 2 diabetes is a major cause of complications of the body which are linked with cardiovascular diseases, blindness, neurological complications, renal failure etc. Recently, type 2 diabetes is also being found in children. Thus, the increasing trend in type 2 diabetes mellitus has become a serious medical concern worldwide [7]. The alpha-glucosidase enzymes such as α-amylase are responsible for the breakdown of polysaccharides, oligo and disaccharide to monosaccharides. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time causing a marked decrease in the rate of glucose absorption thereby reducing the chance of the post prandial plasma glucose rise [8]. The inhibition of carbohydrate hydrolyzing enzymes such as a-amylase can be an important strategy for treating the postprandial blood glucose level in patients with type II diabetes. Plants contains different chemical constituents with potential for inhibition of alpha-amylase and hence maybe used as therapeutic source. By controlling through such constituents the two key enzymes (α-amylase and α-glucosidase) in digestive system, the postprandial hyperglycemia condition can be controlled [19]. Many anti-obesity and anti-diabetic drugs are available in market for the treatment of obesity and diabetes. Such inhibitors which find application in the clinical practice for management of diabetes are acarbose, miglitol and voglibose. However, these drugs are known to be associated with various gastrointestinal side effects such as abdominal pain, flatulence and diarrhea in the patients [8]. However, due to limited effectiveness and significant side effects of synthetic drugs, there is a need to search drugs from plant source. Medicinal plants have been used as traditional medicines for thousands of years due to the bioactive constituents present in them [9, 24]. Approximately, 80% of the world population use herbal medicines primarily in the developing countries for primary health care. Natural products from medicinal plants provide unlimited opportunities for development of new drugs because of the unmatched availability of chemical diversity [10].

The experimental plant *Cassia glauca* belongs to caesalpiniaecae family and is ever green shrub with approximately 10 feet height. The phytochemicals reported from this plants are tannins, alkaloids, γ-sitosteroline, fatty acid and anthraquinones. The aerial part of *C. glauca* is being used as diuretic depressant. The leaves of this plant grinded in sugar and milk are used to cure gonorrhea, wound healing and blennorrhagia [11]. Many biological activities like antimicrobial and laxative are also reported from seeds of this medicinal plant [12]. The leaves and bark of *C. glauca* are reported to use it as folk medicines for the treatment of diabetes. The aqueous extract of *C. glauca* bark is also reported for its antidiabetic potential in diabetic rats [13]. Obesity is prevalent worldwide and it is associated with increased mortality, increased cardiovascular diseases, diabetes, colon cancers and gall bladder disease [14]. So, the purpose of this current research was to extract and purify bioactive fractions from *C. glauca* leaves and then to check their inhibitory potential against α-amylase and lipase enzymes.

2. Materials and methods

The present research work was conducted in Bioactive Molecules Research Lab (BMRL), University of Agriculture Faisalabad, Pakistan.

2.1. Collection and identification of Cassia glauca Lam *leaves*

From the old botanical garden of the University of Agriculture Faisalabad, Pakistan, the leaves of *Cassia glauca* Lam leaves were collected and confirmed by the Department of Botany, UAF.

2.2. Extraction of Cassia glauca lam leaves by soxhlet extractor

25 grams powdered sample of *C. glauca* leaves was filled in the thimble and extracted by using the suitable solvent, methanol, by Soxhlet extractor for 24 hours. The solvent (190 mL) was taken into the (250 mL) round bottom flask and brought to boiling using a heating mantle. The extract was evaporated in water bath at 45^0C and then dissolved in DMSO and stored in air tight bottles at 4^0C in a refrigerator for further use [18].

2.3. Purification of Cassia glauca by gel column chromatography

The extract obtained from Soxhlet extraction was further purified by silica gel column chromatography with the help of different solvents (*n*-hexane, ethyl acetate and methanol) by using gradient solvent method (from non-polar to polar solvent). Column of 50 mL capacity was used. Firstly a piece of cotton wool was taken and inserted into column then fixed the column into clamp. Then packing of column was done by making slurry of adsorbent silica gel 60 mesh (70-230). Slurry was made by mixing 20 gram of powdered silica with 10 mL of n-hexane which was used as a stationary phase. It was then subjected into the glass column with the help of funnel and allowed to settle.

After packing of column 2.1 gram methanol extract of *Cassia glauca* Lam leaves were poured into the column and elution was performed from non-polar to polar solvent such as 100% *n*-hexane, (10:1) *n*-hexane: ethyl acetate, (5:1) *n*-hexane: ethyl acetate, (1:1) *n*-hexane: ethyl acetate, (1:5) *n*-hexane: ethyl acetate, (1:10) *n*-hexane: ethyl acetate, 100% ethyl-acetate, (10:1) ethyl acetate: methanol, (5:1) ethyl acetate: methanol, (1:1) ethyl acetate: methanol, (1:5) ethyl acetate: methanol, (1:10) ethyl acetate: methanol and 100% methanol. After uniform intervals the fractions (each of 30 mL) were collected and allowed to evaporate at water bath. After evaporation, residues obtained were further

dissolved in 1 mL methanol and again allowed to evaporate when evaporation was completed then added 1 mL of DMSO in each fraction and stored at 4^0C in refrigerator for further use in bioactivities. Each fraction was collected separately in a falcon tube for the checking of bioactive prospective by using different activities such as amylase and lipase enzyme inhibition activities [18].

2.4. α-Amylase inhibition activity

The α-amylase assay was performed by using a modified procedure [15, 19]. About 250 μL of extract was added in a test tube and then 250 μL of 0.02 M sodium phosphate buffer (pH 6.9) comprising α-amylase solution (0.5 mg/mL) was added into test tube. This solution was pre-incubated at about 25°C for almost 10 min, after incubation 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added after specific time intervals and then it was further incubated at 25°C for about 10 min. The reaction was then terminated by adding 500 μL of Dinitrosalicylic acid (DNS) reagent. The test tubes were then incubated in boiling water for about 5 min and after incubation it was cooled to room temperature. The reaction mixture was diluted with about 5 mL distilled water. The absorbance was then measured at 540 nm using visible spectrophotometer [21]. A control was also prepared using the same process replacing the extract with distilled water. The α-amylase inhibitory activity of sample was calculated as percentage inhibition.

Inhibition percentage=(A control-A extract)/Abs control x100

2.5. Lipase inhibition assay by plate method

Lipase inhibition assay by plate method was performed as described in literature [16, 20]. It was mainly accomplished by adding 2% (w/v) nutrient agar, 2.5% (w/v) olive oil and phenol red 1% (w/v) as indicator. 100 mM tris-HCL buffer, 30 mg/mL was used to prepare lipase solution. After this, pH was maintained to 7 and autoclaved it. When it attained room temperature, 500 μL phenol red was added and poured it in plate wells. Then poured 30 μL sample and 30 μL enzyme in each well. Enzyme itself was used as positive control. The plate with samples was then incubated overnight at 37°C. Lipase inhibitory activity of the sample was mainly calculated as percentage inhibition.

Percent inhibition=(Zone of extract -control zone)/zone of control x 100

3. Results and Discussion

3.1. Purified fractions and their yield

13 purified fractions were obtained after silica gel column chromatography by using different solvents such as hexane, ethyl-acetate, methanol and water and their yields are shown in Table 1. These extracts were further diluted in DMSO to make a concentration 20 mg/mL. Then these extracts were used to study lipase and amylase inhibition properties [18, 25].

3.2. Enzyme α-Amylase inhibition activity of Cassia glauca leaves extract

Enzyme inhibition activity of *Cassia glauca* Lam leave extracts for Sample F1-F13 was evaluated. Hexane soluble extract showed maximum %age inhibition as shown in Table 2.

All the thirteen bioactive fractions (figure 1) indicated inhibition activity against alpha amylase. Each bar shows data from at least three independent experiments with standard error. Maximum inhibition was shown by bioactive fraction F6 while F11 fraction shows minimum inhibition by using *Cassia glauca* plant. The overall Alpha-amylase inhibition percentage of *Cassia glauca* is quite high which indicates its importance to be used as amylase inhibitor. It was also reported that α-amylase inhibition is related to the presence of certain compounds such as tannins, phenols and flavonoids [22].

3.2. Lipase inhibition assay by using Cassia glauca lam leaves extractions after purification

The inhibition of pancreatic lipase was done by using the modified method (plate method) of phenol red. In this method 20 mg/mL of sample was used with enzyme itself as a positive control. Lipase inhibition activity of bioactive fractions of *Cassia glauca* Lam leaves extracts are shown in Table 3. The obtained results are in relevance with the study performed by different authors [17, 20] in which the fractions and extracts were dissolved in DMSO/water.

Enzyme inhibition activity of *Cassia glauca* Lam leaves extracts for Sample 1-13 were all significant. Lipase was used as positive control that showed 30.5 mm zone of inhibition. F1 showed the maximum zone of inhibition (19.3mm). These zones mm values were later changed to %age inhibitions.

Table 1: Concentration of purified fractions of *Cassia glauca* Lam leaves extracted [18, 25]

Sr.#	Name of solvents	Name of diluted fraction	Concentration mg/mL
1	Hexane 100%	F1	340mg/mL
2	Hexane: Ethyl acetate 10:1	F2	160mg/mL
3	Hexane: Ethyl acetate 5:1	F3	130mg/mL
4	Hexane: Ethyl acetate 1:1	F4	150mg/mL
5	Hexane: Ethyl acetate 1:5	F5	610mg/mL
6	Hexane: Ethyl acetate 1:10	F6	560mg/mL
7	Ethyl acetate 100%	F7	180mg/mL
8	Ethyl acetate:Methanol 10:1	F8	20mg/mL
9	Ethyl acetate:Methanol 5:1	F9	310mg/mL
10	Ethyl acetate:Methanol 1:1	F10	210mg/mL
11	Ethyl acetate:Methanol 1:5	F11	410mg/mL
12	Ethyl acetate:Methanol 1: 10	F12	50mg/mL
13	Methanol 100%	F13	460mg/mL

Table. 2: α-Amylase inhibition activity of bioactive fractions of *Cassia glauca* Lam leaves extract after purification

Sr.#	Sample Name	% age inhibition Mean±S.D
1	F1	25.63^{abc}±0.94
2	F2	23.62^{cde}±0.91
3	F3	23.88^{bcd}±1.40
4	F4	20.21^{efgh}±1.4
5	F5	22.88^{cdef}±0.92
6	F6	27.52^{a}±0.35
7	F7	22.69^{cdef}±0.31
8	F8	26.99^{ab}±0.56
9	F9	27.17^{a}±0.82
10	F10	20.94^{defg}±0.36
11	F11	17.65^{h}±1.28
12	F12	20.14^{fgh}±0.49
13	F13	19.12^{gh}±0.20

Means within column with different superscripts show significant difference according to Tukey test ($P \leq 0.05$).

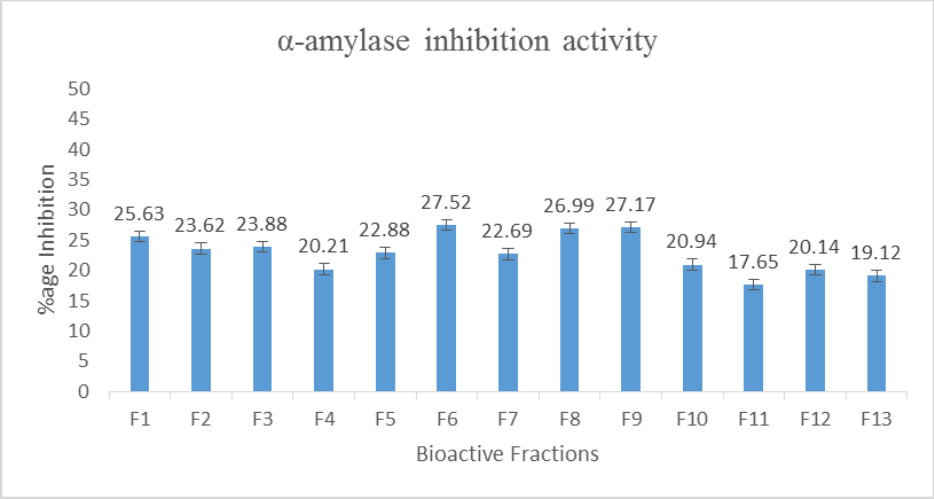

Figure 1: Graphical representation of alpha-amylase inhibition activity of *Cassia glauca* lam leaves extract

Table 3: Lipase inhibition activity of bioactive fractions of *Cassia glauca* Lam leaves extracts

Sr.#	Sample	% age inhibition Mean ± S.D
1	F1	38.02^{a} ± 3.23
2	F2	32.95^{ab} ± 4.28
3	F3	36.78^{a} ± 1.27
4	F4	29.46^{ab} ± 1.08
5	F5	32.61^{ab} ± 2.68
6	F6	34.3^{ab} ± 1.41
7	F7	35.77^{a} ± 1.38
8	F8	35.88^{a} ± 0.69
9	F9	31.04^{ab} ± 1.47
10	F10	25.63^{b} ± 3.44
11	F11	30.36^{ab} ± 2.19
12	F12	33.63^{ab} ±2.77
13	F13	32.73^{ab} ± 2.80

Means within column with different superscripts show significant difference according to Tukey test ($P \leq 0.05$)

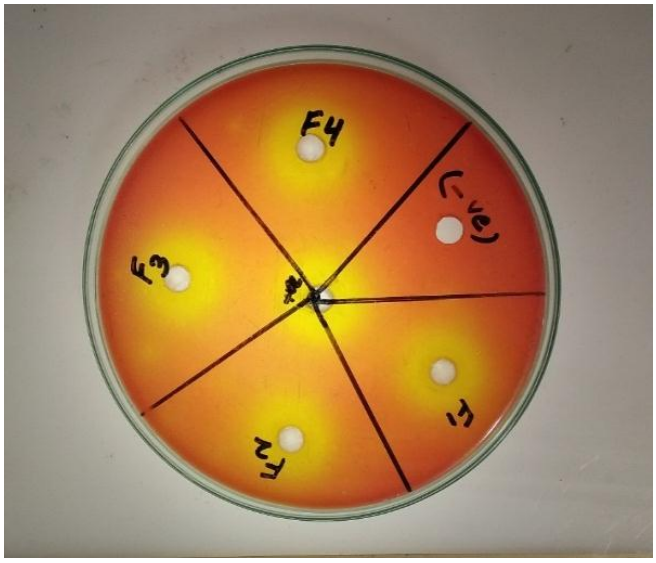

Figure. 2.1: (a) Lipase enzyme inhibition activity of *Cassia gluaca* lame leave extract

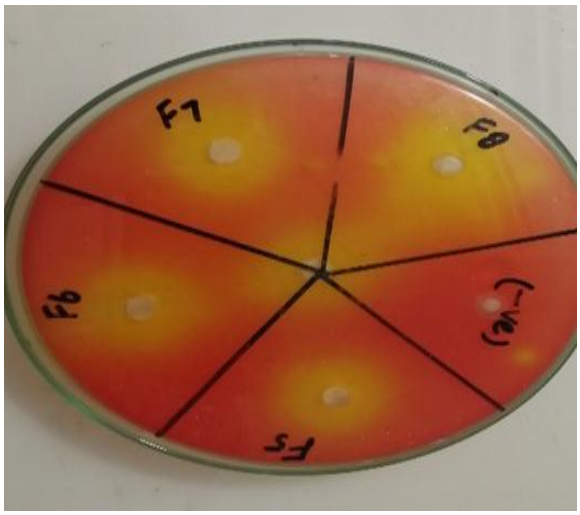

Figure. 2.2: (b) Enzyme inhibition activity of *Cassia gluaca* lam leaves extracts (Sample 5-8). Lipase was used as Positive control that showed 30.5mm zone of inhibition. F8 showed the maximum zone of inhibition (19.2 mm)

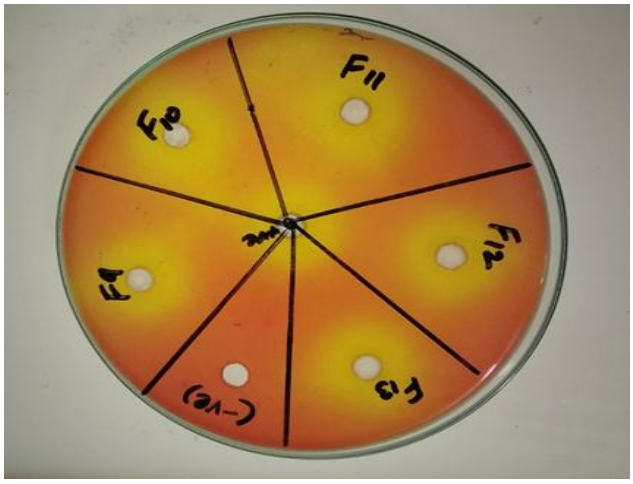

Figure. 2.3: (c) Enzyme inhibition activity of *Cassia glauca* lame leaves extracts (Sample 5-8). Lipase is used as Positive control that showed 30.5mm zone of inhibition. F12 showed the maximum zone of inhibition (20.4 mm)

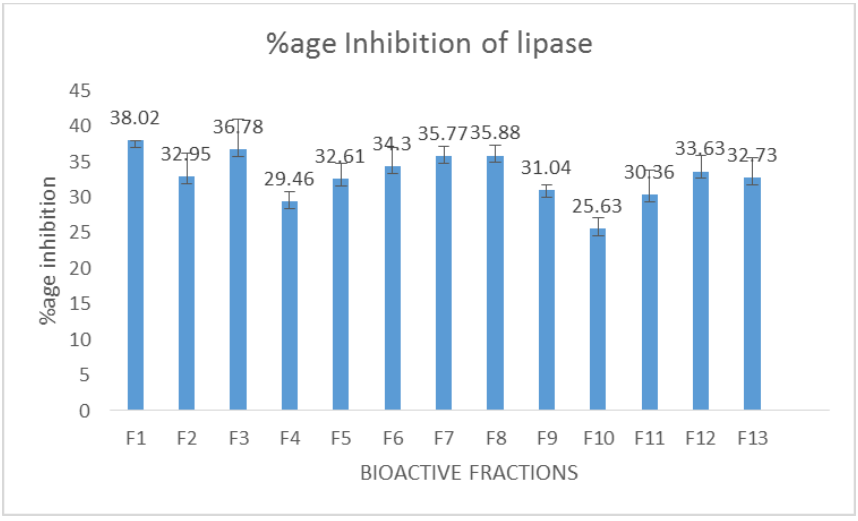

Figure. 3: Percentage of Lipase enzyme inhibition activity of C*assia glauca*

All the thirteen bioactive fractions indicated inhibition activities against lipase. F1 fraction showed maximum inhibition while F10 fraction of plant showed minimum activity of lipase inhibition. Enzyme inhibition activity of *Cassia glauca* Lam leaves extract was performed by using plate method in which Lipase enzyme was used as a positive control. Each of the bar represents data from at least three independent experiments with the standard error shown in graphs. Maximum zone of inhibition was shown by hexane soluble extract (20.6 mm).

Human beings are facing diverse types of serious problems related to health and environment, that is why the development of new therapeutic agents especially from plant source has become necessary to overcome these problems. All fractions (F1-F13) showed alpha amylase and lipase inhibition activity. However among these fractions, F6 (n-hexane: Ethyl acetate, 1:10) and F9 (Ethyl acetate: Methanol, 5:1) showed maximum alpha amylase inhibition i.e. 27.52% and 27.17% respectively. Maximum lipase inhibition activity was shown by fractions F1 (n-hexane 100%) and F3 (Hexane: Ethyl, 5:1) i.e. 38.02% and 36.78% respectively. Results showed that alpha amylase and lipase inhibitors from plant extracts could be promising drug candidates for the treatment of diabetes and obesity [21]. Several inhibitors such as amylase inhibitor are known as starch blockers because they inhibit the breakdown, and slowdown the digestion of dietary starch which is being absorbed by the body and lowers the blood glucose level, and have beneficial effects on insulin resistance and glycemic index control in people with diabetes. Progressive metabolic disorder in β-cells of the pancreas or insulin resistance result in Type I and Type II diabetes [23]. However, more research is needed to evaluate the enzyme inhibition potential of purified bioactive fractions of *C. glauca* leaves.

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