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Analysis of phytochemical potential and investigation of hypoglycemic effect of Cassia fistula Linn fruit wall extracts by alpha-amylase assay and streptozotocin-induced diabetic rat model

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ABSTRACT

Objective: This study will estimate the proximate composition of phytochemicals and study the hypoglycemic effect of Cassia fistula Linn fruit wall extracts. Materials and Methods: The in vitro inhibition of alpha-amylase and in vivo streptozotocin-induced diabetes model was applied to investigate the anti-diabetic activity. For standardization, atomic absorption spectroscopy and Fourier transform infrared spectroscopy (FTIR) were used. Results: Proximate analysis values were found to be within the specific limits as recommended by the British Pharmacopeia. The FTIR spectrum and elemental analysis confirmed the occurrence of various functional groups and minerals. The phytochemical analysis indicates the higher fraction of the carbohydrates (69.89%) and relatively lesser proportion of the total proteins (10.55%) and the lipids (3.65%). Secondary metabolites such as polysaccharide, flavonoids, polyphenols, and glycosaponins were detected in higher proportions in chloroform extract. The percentage inhibition using alpha-amylase assay was found to be higher in n-hexane extract which is 93% followed by methanol and chloroform, 81% and 64.7%, respectively. In vivo activity involves the induction of diabetes in rats by streptozotocin and the results showed that a dose of 200 mg/kg of n-hexane extract has a maximal hypoglycemic effect. Conclusion: The present study supports the anti-diabetic activity of C. fistula Linn and further study on its safety is required.

Keywords: Alpha-amylase, Cassia fistula Linn, diabetes, hypoglycemia, phytochemicals, streptozotocin

INTRODUCTION

The importance of medicinal plants in the treatment of various diseases is undeniable. Since the olden days, plants remain a major source for cure of many diseases.[1] Curing diseases from drugs that are obtained from plant sources, also termed as phytotherapy, which includes all plants with therapeutic properties such as digitalis, belladonna, and strophanthin.[2] Drugs that are prescribed globally, constitutes 25% of the medicines having plant source.[3] Cassia fistula L. fits in to family Fabaceae and its renowned as Amalts. The herb has compound leaves and length of its leaflets is around 5–12 cm. Its fruit is long tubular in shape, fragmented by horizontal walls. Fruits are filled with black seed. The Amalts is enriched with many valuable metabolites, comprising; glycosides, flavonoids, alkaloids, anthraquinones, amino acids, and tannins.[4] Earlier studies have proved that the bark of the plant possesses anti-inflammatory and anti-oxidant activities. Their results have manifested that methanol and water extracts are being the major contributors to reduce inflammation.[5] A study was conducted in which the leaves were screened for anti-inflammatory activity by employing dextran and carrageenan-induced edema rat model. Results have displayed that the plant leaves possessed an exceptional anti-inflammatory potential.[6] It was revealed that C. fistula L. depresses central nervous system and displayed synergistic actions with sedatives, especially benzodiazepines. Whereas methanolic extract of the seed presented an analgesic effect.[7]
The antipyretic potential of *C. fistula* L. was further confirmed when a study results verified that the herb shoot methanol extract (200 mg/kg) lessened the fever up to 3 h and with the dose of 400 mg/kg this antipyretic effect would persist up to 6 h. Those results have demonstrated a dose-dependent anti-pyretic response of the medicinal herb. The methanolic extract of plant seed has revealed a substantial decline in tumor cells. The anti-cancer effects were reported against ascites tumor in mice, but the exact mechanism of action is still unknown. The anti-infectious effects of methanolic extract (200 mg/kg) lessened the fever up to 3 h.

The current study aims to highlight *C. fistula* L. fruit wall phytochemical potential. For this purpose, the *C. fistula* L. fruit wall was first standardized, screened for plant chemical constituents, and then proximate composition was estimated. Further, its anti-hyperglycemic potential was explored using both *in vitro* and *in vivo* antidiabetic assays to validate the understanding behind the medicinal herb use for the treatment of diabetes.

## MATERIALS AND METHODS

### Procurement of Medicinal Herb, Verification and Drying

Fruits of the plant were obtained from the Botanical Garden of University of the Punjab, Lahore, in July 2018. *C. fistula* L. authentication was done by the taxonomist Professor Zaheer Khan, Government College University (GCU) Lahore, Pakistan. After verification, a voucher no. 3601-A was deposited in GUC herbarium in the botany department. First of all, the seeds were separated by fruit walls. The fruit walls of fruits have gained very less attention and consideration. The current study aims to highlight *C. fistula* L. fruit wall phytochemical potential. For this purpose, the *C. fistula* L. fruit wall was first standardized, screened for plant chemical constituents, and then proximate composition was estimated.

### Extraction, Drying, and Storage of the Fruit Wall Extracts

Extracts were obtained by performing the sequential extraction in a Soxhlet apparatus. Solvents such as n-hexane, chloroform, and methanol in order from lower to higher polarity were used for extraction. For drying of extracts, the rotary evaporator (Heidolph, Germany) was used. The temperature of rotary evaporator was kept lower than the boiling point of relevant solvents. The extracts were kept in properly labeled, clean, and tarred glass vials. The extracts were dried at a temperature 40°C in an oven and kept in a semi-solid state at 4°C in a refrigerator.

### Estimation of the Secondary Metabolites

Estimation of secondary metabolites in *C. fistula* L. fruit wall powder was estimated by evaluating the carbohydrates,[12] lipid contents,[13] and total protein content.[14]

### In vitro Anti-diabetic Assay

The hypoglycemic activity of *C. fistula* L. fruit wall was assessed by implementing *in vitro* alpha amylase assay. The buffer phosphate (pH = 6.9) was used to prepare the solutions of the alpha-amylase (1%) and starch (1%). The acarbose was used as a standard in this *in vitro* model. Next, for each extract, a stock solution of strength 1 mg/mL was made. After that three cleaned test tubes were taken, each containing 1 mL of each extract separately. Then 1 mL of alpha amylase solution was added in each test tube. The test tubes were kept under incubation for 10 min at 37°C followed by the addition of starch solution (1 mL) in each test tube. Test tubes were again incubated for about 15 min. Then, a solution of 1 mL 3, 5-dinitrosalicylic acid was prepared and added to each test tube. The test tubes were heated in a water bath for 10 min at 85°C. When test tubes were cooled down, the spectrophotometer was used to take absorbance at 540 nm wavelength. The percentage inhibition (% inhibition) was calculated using the following formula:

\[
\text{Percentage inhibition (% inhibition)} = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]
Absorbance (control) = \( A_{\text{control}} \)
Absorbance (test) = \( A_{\text{test}} \)

**In Vivo Anti-diabetic Assay**

*Streptozotocin-induced diabetes rat model*

In order to assess the anti-hyperglycemic properties of *C. fistula* L. fruit wall and efficacy of its extracts, a diabetic rat model was employed in which diabetes was induced in rats using streptozotocin.[19]

Experimental animals

In this streptozotocin-induced diabetes rat model, 24 female albino rats 10 weeks old, weighing 150–200 g were used. These female albino rats were procured from the University of Veterinary and Animal Sciences Lahore, Pakistan. Throughout 15 days of study, the rats were kept in well-controlled conditions in the animal house of the University College of Pharmacy, University of the Punjab Lahore, Pakistan. The rats were retained in iron cages using soft, warm bedding at temperatures between 65 and 85°F, provided with whole-wheat as food.

Induction of hyperglycemia

The streptozotocin (55 mg/kg), intraperitoneal injection was administered in albino rats for the instigation of diabetes. Streptozotocin is a poisonous, light-sensitive chemical, so its dose was synthesized with great caution using chilled normal saline as solvent. High blood glucose levels in rats were observed 5–6 days after administration of streptozotocin. For this *in vivo* anti-diabetic assay, rats with blood glucose levels higher than 200 mg/dL were chosen.

Experimental study design

Out of 24 female albino rat model six groups containing the same number of rats were created.

Negative control group

In a negative control group, rats were given distilled water while the other groups were administered with oral dose of extracts.

Diabetic control group

In this group, rats received no treatment.

n-hexane extract treated diabetic group

In this group, rats received n-hexane (an oral dose, 200 mg/kg) for about 7 days after the instigation of diabetes. For n-hexane extract 20 % (v/v) of tween 20 was used as a solvent.

Methanol extract treated diabetic group

In this group, rats received methanol (an oral dose, 200 mg/kg) for about 7 days after the instigation of diabetes. For the methanol distilled water was used as solvent.

Chloroform extract treated diabetic group

In this group, rats received chloroform (an oral dose, 200 mg/kg) for about 7 days after the instigation of diabetes. For chloroform extract 20 % (v/v) of tween 20 was used as a solvent.

Standard control group (Glibenclamide control)

In this group, diabetic rats received glibenclamide (an oral dose, 600 μg/kg) for about 7 days after the instigation of diabetes. For glibenclamide oral dose, distilled water was used as a solvent.

Sampling and testing

The blood was withdrawn from the tail vein of rats to measure the blood glucose levels by using On Call Plus Glucometer. The blood sugar levels of rats were daily monitored for 7 days before administrating the next dose followed by the provision of food.

**RESULTS AND DISCUSSIONS**

The physical and chemical assessment of *C. fistula* L. was done by estimation of moisture content, ash test, and evaluation of extractive values. The results of all tests were compiled within specified limits. The results are shown in Table 1.

The purpose of the moisture content test is to determine water content in a given sample. This test serves as a valuable means to find out the purity, effectiveness, and safety of natural material.[20] Although in the ash test, unprocessed drugs are ignited until they are converted into ash. The resultant ash is a source of useful minerals like magnesium, sodium, silicates, and phosphates. The main purpose of ash test is not only limited to analyze quality attributes and purity of organic drugs but to also provide valuable information about their storage and drying conditions. There are various factors that contribute to the validity of ash test results such as fertilizers, water contents, and soil conditions.[21] The results have manifested, that the extractive value of water was less than that of methanol. These extractive values are used to find out polar and non-polar nature of the sample under consideration.[22]

The FTIR spectra of powder and methanolic extract of the fruit wall are revealed in Figures 1 and 2, respectively.

**Table 1: Proximate investigation of Cassia fistula L. powdered material**

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Percentage content±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content</td>
<td>8.45±0.13</td>
</tr>
<tr>
<td>Total ash</td>
<td>8.75±0.08</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>7.0±0.20</td>
</tr>
<tr>
<td>Water soluble ash</td>
<td>7.3±0.25</td>
</tr>
<tr>
<td>Sulfated ash</td>
<td>10.5±0.15</td>
</tr>
<tr>
<td>Alcohol soluble extractive</td>
<td>2.6±0.20</td>
</tr>
<tr>
<td>Water soluble extractive</td>
<td>1.28±0.30</td>
</tr>
</tbody>
</table>

**Figure 1:** Fourier-transform infrared spectroscopy spectrum of Cassia fistula Linn fruit wall powder
The FTIR spectra were obtained by adopting the potassium bromide (KBr) disc technique using FTIR spectrophotometer. This test is used to assess the qualitative nature of fruit wall of *C. fistula* L.

A peak at 3378.07 cm\(^{-1}\) represented a strong O-H stretch which is a distinctive feature for the incidence of phenolic and alcoholic compounds. However, moderate peaks between 2817.80 cm\(^{-1}\) and 2957.75 cm\(^{-1}\) were due to C-H stretch which confirmed the existence of alkanes.

The presence of esters and saturated aliphatic groups was confirmed by carbonyl stretch at 1823.80 cm\(^{-1}\). A peak at 1659 cm\(^{-1}\) confirmed that aromatic groups are present in sample under consideration. The stretch at 1461 cm\(^{-1}\) and 1427.57 cm\(^{-1}\) is the characteristic bend of nitro groups. The bends at 666.87 cm\(^{-1}\) and 932.85 cm\(^{-1}\) confirmed the presence of alkenes.[23] Hence, the FTIR investigation of *C. fistula* L. established the manifestations of alkenes, nitro compounds, esters, hydrogen-bonded phenols, and alcohols. The mineral content investigation of *C. fistula* L. was done by atomic absorption spectrophotometer. Table 2 represents the results of atomic absorption spectra showing the presence of iron, zinc, and potassium.

The minerals detected through elemental analysis play a very significant role in various pharmacological and therapeutic activities such as anti-cancer, anti-hyperglycemic, and anti-oxidant activities. Iron is the essential part of hemoglobin so it is necessary for appropriate functions of red blood cells. It is not only needed for the oxygenation of primary hemoglobin so it is necessary for appropriate functions of red blood cells. It is not only needed for the oxygenation of primary hemoglobin but also plays a key role in smooth activities of the nervous system.[24] Furthermore, Zn is responsible for protein metabolic reactions and is a vital part of various immunological reactions.[25] Nevertheless, potassium is the vital element to carry out various cardiovascular functions and controlling blood pressure.[26]

The results of primary metabolites presented in Table 3 clearly indicate the higher fraction of carbohydrates (69.89%) and relatively lesser proportion of total proteins (10.55%). The reason is, as compared to other plant parts the fruits bear lesser concentrations of protein than carbohydrates.

Estimation of secondary metabolites was carried out using linear regression equations such as for total polyphenols, total flavonoids, glycosaponins, and total polysaccharides (y = −0.019x + 3.539, R\(^2\) = 0.991), (y = 0.006x + 0.074, R\(^2\) = 0.991), (y = 0.012x − 0.014, R\(^2\) = 0.903), and (y = 0.002x + 0.154, R\(^2\) = 0.909), respectively. The results are shown in Table 4.

The results have revealed that among all, the proportion of secondary metabolites was maximum in chloroform extract. Various findings have established the therapeutic implication and vital role of these secondary metabolites in various pharmacological activities.[11]

For determination of anti-hyperglycemic effects of *C. fistula* L. fruit wall in vitro alpha amylase inhibition assay was employed. Table 5 embodies the findings of α amylase inhibition assay.

The results have clearly indicated that the highest percentage of inhibition was shown by n-hexane extract (93%).

The percentage inhibition of methanolic and chloroform extracts were 81% and 64.7% respectively. The alpha-amylase inhibition assay is based upon hydrolysis of 1, 4-glycosidic linkage that results in suppression of starch absorption.[27]

To confirm in vitro results, in vivo anti-hyperglycemic assay was also carried out. Table 6 presents the findings of in vivo hypoglycemic potential of fruit wall of plant under investigation.

In this in vivo anti-diabetic assay, streptozotocin, a cytotoxic chemical was used to induce diabetes in rats. The assay results have disclosed that streptozotocin-induced diabetic rat group showed an intermittent rise in blood sugar levels. The intraperitoneal route was used to administer streptozotocin. It destroys the beta cells of pancreas and induces swelling and diabetes.[28] The rats developed symptoms of diabetes 6 days after administration of streptozotocin as their serum glucose levels were reported >200 mg/dL. According to the results, as mentioned in Table 6, all 3 extracts displayed noticeable anti-hyperglycemic activity. Graphical interpretation of results is represented in Figure 3. On 11\(^{th}\), 12\(^{th}\), and 13\(^{th}\) day of the study, extracts treated groups showed a striking drop in their serum glucose levels in contrast to the control group. Among all, the n-hexane-treated group demonstrated the most reassuring results followed by chloroform and methanol extract-treated groups. The findings have revealed that a marked deviation

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**Table 2:** A mineral content analysis of fruit wall powder of *Cassia fistula* Linn

<table>
<thead>
<tr>
<th>Element</th>
<th>Quantity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>1.73</td>
</tr>
<tr>
<td>Iron</td>
<td>0.0053</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0085</td>
</tr>
</tbody>
</table>

**Table 3:** Primary metabolites in powdered fruit wall of *Cassia fistula* Linn

<table>
<thead>
<tr>
<th>Primary metabolites</th>
<th>% Content±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proteins</td>
<td>10.55±0.66</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>69.89±0.01</td>
</tr>
<tr>
<td>Total lipids</td>
<td>3.65±0.53</td>
</tr>
</tbody>
</table>

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**Figure 2:** Fourier-transform infrared spectroscopy spectrum of *Cassia fistula* Linn fruit wall methanolic extract

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**Figure 3:** Graphical representation of plasma glucose level (mg/dL) of negative control, diabetic control, standard control, n-hexane, methanol, and chloroform-treated groups.

**Figure 4:** Graphical representation of the per day analysis of plasma glucose level (mg/dL) of diabetic control, n-hexane, methanol, and chloroform-treated groups for 7 days.
Table 4: Secondary metabolites in the fruit wall of Cassia fistula Linn

<table>
<thead>
<tr>
<th>Cassia fistula Linn extract, mg/g</th>
<th>Total polyphenols, mg/g</th>
<th>Total flavonoids, mg/g</th>
<th>Total polysaccharide, mg/g</th>
<th>Glycosaponins, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>30.82±0.030</td>
<td>20.27±0.0015</td>
<td>4.33±0.011</td>
<td>11.75±0.003</td>
</tr>
<tr>
<td>Chloroform</td>
<td>48.39±0.026</td>
<td>49.77±0.001</td>
<td>112.94±0.0015</td>
<td>24.47±0.004</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.807±0.0015</td>
<td>3.77±0.002</td>
<td>11.66±0.0015</td>
<td>4.67±0.003</td>
</tr>
</tbody>
</table>

Table 5: Percentage alpha-amylase inhibitory activity of n-hexane, chloroform, and methanol extract

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Experimental group with alpha amylase (absorbance)</th>
<th>Blank sample group (without alpha amylase) (absorbance)</th>
<th>Final absorbance</th>
<th>% Age activity Ac–As/Ac×100</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>0.143</td>
<td>0.098</td>
<td>0.045</td>
<td>93</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.264</td>
<td>0.012</td>
<td>0.252</td>
<td>64.7</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.220</td>
<td>0.09</td>
<td>0.130</td>
<td>81</td>
</tr>
</tbody>
</table>

Table 6: Blood glucose levels (mg/dL) of negative control, diabetic control, standard control, n-hexane, methanol, and chloroform treated groups for 7 days

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antidiabetic activity in streptozotocin-induced diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day-7, mg/dL</td>
</tr>
<tr>
<td>Negative control</td>
<td>70</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>267</td>
</tr>
<tr>
<td>Standard control (Glibenclamide)</td>
<td>261</td>
</tr>
<tr>
<td>n-hexane extract</td>
<td>342</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>321</td>
</tr>
<tr>
<td>Chloroform extract</td>
<td>400</td>
</tr>
</tbody>
</table>

was reported that is (P < 0.05) amongst serum levels of extract treated and control groups.[29] Hence, in vivo anti-diabetic assay also favors findings of in vitro alpha-amylase inhibition results. Figure 4 shows a graphical representation of per day analysis of plasma glucose level (mg/dL) of all groups for 7 days.

**CONCLUSION**

From the current study, it was concluded that *C. fistula* L. fruit wall is enriched with valuable phytochemicals including primary and secondary metabolites. This study has also disclosed that *C. fistula* L. fruit wall possesses marked anti-diabetic potential. According to findings of in vitro anti-diabetic assay, anti-diabetic activity of n-hexane extract was comparatively greater than that of methanol and chloroform extracts. These results were further confirmed by in vivo anti-diabetic assay findings as the latter demonstrated the same descending trend of anti-diabetic activity (n-hexane > Methanol > Chloroform) at a dose of 200 mg/ML. This trend was complementary to alpha amylase inhibition assay. The current study supports the anti-diabetic potential of *C. fistula* L. fruit wall and further spectroscopic investigations are required to validate its efficacy, safety, and standardization.

**REFERENCES**