Antihyperglycemic effect of Conocarpus erectus leaf extract in alloxan-induced diabetic mice

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Abstract: Synthetic drugs have widely been helpful in management of diabetes mellitus type 2. However, side effects associated with synthetic drugs serve as an impetus to explore plants as alternate mode of treatment. The hydroethanolic leaf extracts of Conocarpus erectus were evaluated for phenolic contents, flavonoid distribution, antioxidant activity and antidiabetic potential. The maximum extract yield, total phenolic and flavonoid contents were exposed by 60% ethanolic extract. The Antioxidant and anti α-glucosidase tendency of 60% ethanolic extract was the most promising and complemented by in-vivo antihyperglycemic impact on mice. The findings were substantial regarding suppression of blood glucose levels in alloxan induced diabetic mice establishing the Conocarpus erectus as proficient pool of nutraceuticals for diabetes mellitus type 2 management.

Keywords: Freeze drying, phytochemical, antioxidant, antidiabetic, Conocarpus erectus.

INTRODUCTION

Oxygen is an integral part of metabolic functions in living systems. The oxygen is often converted into reactive oxygen species (ROS) like singlet oxygen, super oxides, hydroxyl radicals and peroxides as result of various metabolic activities under aerobic environment (Lee & Min, 2004). The ROS are involved in cell signaling but the hazardous impacts also lie with them. The internal antioxidant defense system comprising of multifunctional enzymatic and non-enzymatic entities along with external sources of antioxidants, collectively encounters the ROS to maintain the proper physiology of biological systems (Poljsak et al., 2013; Pizzino et al., 2017). State of oxidative stress develops when the balance between ROS and antioxidant defense becomes out due to multidimensional reasons (Kurutas, 2016). Oxidative stress is associated with wide spectrum of chronic diseases and metabolic disorders including diabetes mellitus by altering the antioxidant enzymes status, lowering of vitamin C level and disturbed glutathione metabolism (Asmat et al., 2016). The diabetes mellitus is a metabolic disorder imprinted by persistent hyperglycemic physiological condition mainly due to low insulin secretion or insulin insensitivity (Patel and Group, 2007; Association, 2014). The diabetes mellitus is under intensive consideration due to its rapid rate of growth and associated side complications. According to estimation, the number of diabetic patients will jump up to 592 million till the year 2035 (Guariguata et al., 2014). Pakistan is also suffering from this medical menace and may be the 7th largest country in world with diabetic population. The disease has become a socioeconomic burden due to its wide and quick expansion in urban as well as rural population of Pakistan (Hussain and Ali, 2016). Diabetes mellitus type 2 (DMT2) is the major type of diabetes and 90-95% diabetic patients come under its coverage (Raz et al., 2013). The influence of modern urbanized life style and unhealthy dietary approaches are the key factors in development of DMT2 and oxidative stress further facilitates the diabetes pathogenesis (Charokopou et al., 2016; Sami et al., 2017). The synthetic drugs and treatments for DMT2 available in market are linked with side complications due to their mode of action at physiological level (Chaudhury et al., 2017). The critical drawbacks of synthetic agents for DMT2 cure serve as an impetus to develop safe and effective therapeutics from alternate sources like plants (Kooti et al., 2016). Plants are natural reserves of antioxidants and nutraceuticals which are effective to combat oxidative stress and to minimize the disease initiation and prolongation (Zaid et al., 2015; Xu et al., 2017). Plants are enriched with α-glucosidase inhibitors exposing their antidiabetic potential with improved physiological status under diseased conditions (Rouzbeh et al., 2017). Main focus to cure diabetes and to minimize its complications moves around management of postprandial hyperglycemia. The α-glucosidase is the key enzyme responsible for the breakdown of complex carbohydrates into simpler sugars. The inhibition of α-glucosidase restricts the hydrolysis of carbohydrates and delays their absorption resulting in controlled blood glucose level (Brunetti and Kalabalik, 2012). The efficient use of α-glucosidase inhibitors can be a...

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successful therapeutic approach to treat DMT2. Plants gain sound gravity for the DMT2 cure due to presence of natural \(\alpha\)-glucosidase inhibitors. The unconditional efficacy, cost-effectiveness, availability, natural and safe nature of nutra-pharmaceuticals emphasized on the need to explore plants for their hidden antidiabetic potential.

*Conocarpus erectus* (C. erectus) belongs to family of Combretaceae and commonly exists in tropical and subtropical regions of world (Rosa Galdino Bandeira, 2003). *C. erectus* is well known for its folkloristic medicinal potential including treatment of diabetes (Nascimento et al., 2016). Some scientific confirmations are also presented in support of its curative activities (Abdel-Hameed et al., 2012; Abdel-Hameed et al., 2013). However, the role of *C. erectus* in DMT2 management needed to be explored to proceed for evidence based therapy from natural resource.

**MATERIAL AND METHODS**

**Green extract preparation**

Fresh leaves of *C. erectus* were immediately quenched in liquid nitrogen with continuous grinding to bring leaves in powdered form. The powder was freeze dried -68°C for 48 hours on Christ Alpha 1-4 LD (Germany). The powder was subjected to hydroethanol compositions ranging from 100% aqueous to 100% ethanol with multiple intervals of 20°C for 48 hours. The resultant mixtures were sonicated on Soniprep 150 ultrasound disintegrator (UK) below 10°C. Each fraction was shaken for 2 hours, filtered on suction assembly and again freeze dried at -68°C for 48 hours. The extract yields were calculated to discriminate extraction solvent systems.

**Determination of total phenolic contents**

Mixture of 1mL Folin Ciocalteu reagent and 0.1 mg of plant extract was prepared. Then 2mL of 10% Na\(_2\)CO\(_3\) were added to the mixture and stayed for two hours. The absorbance of mixture was noted at 765 nm on UV-1700 (Schimadzu Japan) spectrophotometer. Findings were represented as Gallic acid equivalent (GAE) mg/g plant extract (Zengin et al., 2010).

**Determination of total flavonoid contents**

Initially 100µL of crude plant extracts were dissolved in 2mL of methanol and diluted with 4mL of distilled water. Secondly 0.6mL of 5% NaNO\(_2\) solution and 0.6mL of 10% AlCl\(_3\) solution were added to previously obtained mixture and stayed for 10 minutes at ambient conditions. At the end 4mL of 1 molar NaOH was added and net volume of 20mL was obtained by adding distilled water. After 25 minutes stay, absorbance was measured at 510 nm and results were represented as Rutin equivalent mg/g plant extract (Zhishen et al., 1999).

**Free radical scavenging activity**

Free radical scavenging of extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Shahwar and Raza, 2012). Initially 25% DPPH solution in 10mL methanol was made. Each plant extract (50-200 µg) was re-suspended in methanol (1mL) to form extract concentrations of 50-200ppm. After that two hundred µL of each extract solution was then mixed to 1.0mL of 25% DPPH solution. Resultant reaction mixture was allowed to stand for 35 minutes in dark at ambient temperature condition to complete the reaction. The absorbance was measured at 517 nm on UV-1700 (Schimadzu Japan) spectrophotometer. Free radical scavenging potential was finally calculated by using the following formula.

\[
\% \text{DPPH inhibition} = \frac{A - B \times 100}{A}
\]

A = Absorbance of blank
B = Absorbance of Sample

BHA was used as standard antioxidant and results were expressed as µg/mL.

**In-vitro antidiabetic activity**

Inhibitory action of extracts against \(\alpha\)-glucosidase was checked to evaluate their antidiabetic potentials. Extracts (10-100µg) were dissolved in 70µL of (50 Mm) phosphate buffer having pH 6.8 and 1unit/mL of \(\alpha\)-glucosidase. Incubation of mixture at 37°C for 10 minutes was done and reaction was started by adding 10µL of \(\rho\)-nitrophenolglucopyranoside (5mM). After further incubation of 30 minutes, absorbance was measured at 405 nm for released \(\rho\)-nitrophenol. All the measurements were made in triplicate. Following formula was used to calculate percentage inhibition (Mediani et al., 2012)

\[
\% \text{inhibition} = \frac{Ab - As \times 100}{Ab}
\]

Ab = Absorbance of blank
As = Absorbance of Sample
Acarbose was used as standard reference and results were represented as IC\(_{50}\) (µg/mL) values for each extract.

**In-vivo antidiabetic activity**

A mice model based experiment was designed comprising of 60 number of male mice of 5 to 6 week age having an average body weight of 27.35±1.90g. A prior permission from the ethical committee of GC University Lahore was obtained to run experiment. Mice were shifted to animal house and stayed for ten days acclimatization period at 28°C±2.0°C temperature and relative humidity of 68.75% ±4.5%. Mice were grouped into experimental sets after feeding on normal chow for ten days. The clinical trial was comprised of 10 weeks excluding adaptation period. After eight weeks of ad libitum supply to food and water, 24 hours fasted high fat diet (HFD) group was injected intraperitoneally with alloxan in 10% saline at concentration of 150mg/Kg body weight to induce diabetes. After three days of injection, blood glucose
level (BGL) of alloxan treated mice was measured by glucometer and mice having blood glucose level ≥200 mg/dL were considered as diabetic (Emordi et al., 2016). Diabetic mice were categorized into four groups namely untreated diabetic group (DUG), metformin treated (250 mg/kg body weight) group (MFG), low dose group (LDG, treated with 250 mg/kg body weight of plant extract) and high dose group (HDG) treated with plant extract at rate of 450 mg/kg of body weight (each group comprised of 12 mice). The clinical trial was completed in period of eight week after differentiation into experimental groups.

Statistical Analysis
Analysis of variance (ANOVA) was used to evaluate the level of statistical significance among values of experimental findings. Minitab 18 statistical software was utilized for statistical analysis.

Results

Extract yield (%), TPC and TFC
Maximum extract yield of 21.5±0.03% was obtained when extracted with 60% ethanol followed by 18.14±0.01% with 80% ethanol. The difference in extract yield was might be due to polarity of solvent system and maximum yield with 60% ethanol was supported by some previous studies (Nehete et al., 2010; AL-Zuaidy et al., 2017). Extract yield continuously increased and attained maximum value at 60% ethanol solvent composition, then gradually decreased with further increase in ethanol concentration (table 1).

Weight measurement
Body weight (g) measurement was carried out from the start of experiment and variations in weight were noticed weekly. The % weight gain was calculated by following relationship:

\[
\%\text{ body weight} = \frac{\text{Weight gain on specific week} \times \text{baseline weight}}{\text{Baseline body weight}}
\]

Blood glucose level
The blood glucose levels were calculated on weekly basis and were compared to evaluate the efficacy of treatments to manage DMT2. The blood from lateral vein in tail of mice was taken and blood glucose level was measured by glucometer and represented in mg/dL.
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Free radical scavenging activity
The DPPH assay was used as conventional tool to determine the antiradical potential of plant extracts. The IC-50 (µg/mL) values were statistically compared to evaluate the significance level of efficiency among hydroethanolic leaf extracts (fig. 1).

The findings were statistically significant and 60% ethanolic leaf extract exhibited maximum radical scavenging as compared to other fractions but less effective than the BHA.

In-vitro antidiabetic potential
Antidiabetic potential of C. erectus leaf extracts was evaluated by measuring the inhibition of α-glucosidase spectrophotometrically. The results for enzymatic inhibition were statistically significant (ρ<0.05) discriminating the fractions and acarbose as antidiabetic agents (fig. 2).

Obesity development
The weights of mice fed with high fat diet (HFD) were compared with normal diet groups (NDG) weekly (fig. 3). An increase of 98.21% was observed for HFD group being much higher than 48.38% (NRD) indicated the substantial obesity development due to diet rich in fat contents. Ad libitum water and food supply was provided to mice during the experiment. Obese mice group was injected with alloxan to induce diabetes successfully.

Blood glucose levels (BGL)
Impact of 60% ethanolic leaf extract on blood glucose concentrations at two doses (250 and 450 mg/kg body weight) was measured and compared with NG and MFG.

DISCUSSION
Findings revealed that TPC were also influenced by the polarity differences among the designed solvent composition. Maximum TPC value of 117±2.05 mg GAE/g was obtained from extract obtained by 60% ethanol. Similarly, the highest TFC were also extracted in 60% ethanol with the value of 70.10± 2.10. The values of TPC and TFC harmonized the extract yield generated by combinational solvent approach for extraction. This emphasized that the phenolic compounds extraction affected by solvent composition was highly important due to biological significance of phenolics especially as antioxidants (Shahzadi et al., 2011).

The extracts reduced stable nitrogen centered DPPH radical to different extents indicating the reduction potential. The spectroscopic measurements were based upon change in color intensity due to shift from violet to yellow, a bathochromic shift (Dehpour et al., 2009). Reduction potential was the measure of antioxidant activity.

The antiradical potential of 60% ethanolic fraction was probably due to the comparatively high TPC and TFC (Baba and Malik, 2015). The strong antioxidant activity by the leaf extracts of C. erectus made this plant a natural enriched pool of functional agents.

The functionalities associated with the 60% ethanolic extract were might be due to the high presentation of TPC as compared with other fractions. The aqueous fraction was the least effectiv e to restrict the enzymatic activity. The 40% and 80% ethanolic extracts were statistically non-significant opted as higher ones after 60% extract. However, no extract could meet the acarbose to inhibit the α-glucosidase as acarbose was associated with minimum IC-50 value. The recent study also established the exceptionally high antidiabetic potential of acarbose when used as standard antidiabetic reference agent (AL-Zuaidy et al., 2016; Safithri and Sari, 2016). The obesity and diabetes are closely linked due to physiological alternations caused by oxidative stress in system (Walton, 2017).

CONCLUSION
The 60% ethanol was proved as best choice for enhanced extraction of TPC and TFC from leaves of C. erectus. The extract was efficient α-glucosidase inhibitor and suppressed glycemic concentrations in blood of diabetic mice. The leaves of C. erectus may serve as natural source of bioactive entities for nutraceutical development to combat DMT2. The findings revealed the efficacy of plant extract to control blood glucose level. The HDG

Table 1: Extract yields, TPC and TFC from leaves of C. erectus under different solvent compositions.

<table>
<thead>
<tr>
<th>Solvent composition</th>
<th>Extract yield (%)</th>
<th>TPC in mg GAE/g PE</th>
<th>TFC in mg Rutin/g PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (100 % water)</td>
<td>11.05 ± 0.04 d</td>
<td>29.10 ± 1.53 d</td>
<td>11.25 ± 0.75 d</td>
</tr>
<tr>
<td>20% Ethanol</td>
<td>12.04 ± 0.1 cd</td>
<td>58.50 ± 1.30 c</td>
<td>27.30 ± 1.15 c</td>
</tr>
<tr>
<td>40% Ethanol</td>
<td>14.12 ± 0.02 c</td>
<td>87.50 ± 2.12 b</td>
<td>40.75 ± 1.25 b</td>
</tr>
<tr>
<td>60% Ethanol</td>
<td>21.5 ± 0.03 a</td>
<td>117.00 ± 2.05 a</td>
<td>70.10 ± 2.10 a</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>18.14 ± 0.01 b</td>
<td>88.29 ± 1.95 b</td>
<td>42.15 ± 1.05 b</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>14.05 ± 0.02 c</td>
<td>61.30 ± 1.45 bc</td>
<td>30.33 ± 1.11 bc</td>
</tr>
</tbody>
</table>

Results were represented with standard deviation values (±) and significant level was indicated by letter as superscript. PE = Plant extract.
exhibited sufficient reduction in BGL when compared
with NG but less effective as was the case with MFG (fig.
4). Both extract concentrations restricted the rise in BGL
above 200 mg/dL, the threshold level but HDG was more
promising. The proficient efficacy of leaf extracts to
suppress BGL proved as novel tool to treat DMT2 in
alternate mode to get rid of synthetic drugs. The
antihyperglycemic attributes of 60% Ethanolic extract
were probably due to comparatively higher amounts of
TPC and TFC, the valuable chemical entities to treat
chronic ailments (Asgar, 2013; Vinayagam and Xu,
2015). The role of bioactive ingredients of plants may
interfere with the redox reactions in living system to
minimize the oxidative stress and pathogenesis (Al-Waili
et al., 2017). The heterogeneous chronicity of DMT2 can
be overcome by considering the integrative and
alternative mode of treatment with minimum side
complications and C. erectus may be a workable choice.

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