Wound healing activity of standardized extract of *Curculigo orchioides* in streptozotocin–induced diabetic mice

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**1. Introduction**

Plants constitute the richest source of natural antioxidants to counteract reactive oxygen species (ROS). ROS are continuously generated inside the human body as a result of contacting with excess of exogenous chemicals in our ambient environment and/or due to a number of endogenous metabolic processes involving redox enzymes[1]. ROS are thought to be harmful to human health and to trigger many diseases arteriosclerosis, inflammatory disorders, cancer, coronary disease and diabetes. Hence natural antioxidants due to their radical scavenging ability are considered as possible protection against many chronic diseases as well as lipid peroxidation (LPO)[2-3]. Many medicinal plants are found useful in treating wounds such as *Alternanthera sessilis*, *Morinda citrifolia*, *Lycopodium serratum*, *Sesamum indicum*, *Catha ranthrus roseus*, *Cecropia peltata*, *Euphorbia hirta*, *Ginkgo biloba*, *Clerodendrum serratum*, *Pterocarpus santalinus*, *Lawsonia alba*, *Napoleona imperialis*, *Kaempferia galangal*, *Radix paeoniae*, *Prosopis cineraria* and *Trigonella foenum-graecum*.

This study explores the wound healing activity of *Curculigo orchioides* Gaertn. (**Hypoxidaceae**). *Curculigo orchioides* with their recent advancement in treating...
chronic wounds in diabetic condition. Diabetes mellitus is one of the major contributors to chronic wound healing problems. When diabetic patients develop an ulcer, they are exposed to high risk for major complications including infection and amputation. It has been suggested that diabetes impairs wound healing through disruption of local cytokine production, notably platelet derived growth factor, tumor necrosis factor α, interleukin 1β, and vascular endothelial growth factor, reduced biosynthesis or accelerated degradation of newly synthesized collagen[4–6]. Wound healing process involves several steps, including contraction, granulation, epithelization and formation of collagen[7]. This is mainly achieved by synthesis of new connective tissue matrix. Collagen is a major protein of the extracellular matrix and is the major component that ultimately contributes to wound strength. Tannins promotes the wound healing through several cellular mechanisms, chelating of the free radicals and reactive species of oxygen, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts.

*C. orchioides* also known as black musali, is a tiny herbal plants widely distributed in China, Malaya, Japan, Australia and also in subtropical Himalayas region of India. *C. orchioides* included in the IUCN category of “lower risk near threatened” medicinal plants, so it requires ex-situ and in-situ conservation. This plant has been used to treat a wide assortment of diseases such as asthma, piles, jaundice, diabetes, leucorrhoea and urinary disorder. Root tuber is the main essential part of black musali, which is an essential component of several Ayurvedic formulations such as vidaryadhigta, vidarayi lehya and musalyadi churan[8]. Phenolic compounds such as phenolic acids, flavonoids and tannins are important plant metabolites that play a significant role in the diabetic wound healing. Tannins act as free radical scavengers, triterpenoids and flavonoids that promote wound healing due to their astringent and antimicrobial property, and saponins due to their antioxidant and antimicrobial activity which appear to be responsible for wound contraction and elevated rate of re-epithelialization. Flavonoids also possess potent antioxidant and free radical-scavenging effect, enhancing the level of antioxidant enzymes in granuloma tissue[9].

Wang and Li have isolated phenolic glycosides, crassifoside G and isocrassifoside G from the root stock of *C. orchioides*[10]. The root tubers are also reported to contain antioxidative phenols and certain phenolic glycosides such as orcinol glucoside (1), orcinol-1-O-beta-D-glucopyranosyl-(1-->6)-beta-D-glucopyranoside (2), orcinol-1-O-beta-D-apiofuranosyl-(1-->6)-beta-D-glucopyranoside (3), curculigoside (4), curculigoside B (5), curculigoside C (6), 2,6-dimethoxy benzoic acid (7), and syringic acid (8)[11]. These antioxidative phenolic compounds possess several pharmacological activities such as antidiabetic, antimicrobial, and anticancer properties. The methanolic extract of root tubers of *C. orchioides* was determined to investigate their wound healing activity in experimentally induced diabetic mice.

### 2. Materials and methods

#### 2.1. Collection and identification of plant material

The roots of *C. orchioides* were collected from the forest areas of Naugarh block in Chandauli district Uttar Pradesh, India. The plant was identified and authenticated by the Department of Botany, Banaras Hindu University, Varanasi. The material was shade dried, pulverized and preserved in air tight containers.

#### 2.2. Extraction of plant material

##### 2.2.1. Methanolic extraction

The methanolic extract of dried powder (1 kg) of the roots was prepared by using Soxhlet apparatus at 65 °C. The extracted materials was then kept in water bath to evaporate solvent totally and then kept on a rotary shaker at 190–220 r/min for 6 h to make the final volume one fourth of the original volume and stored at 4 °C in airtight bottles. The yield of the extract was 5.6 %. The methanolic extract was then subjected to phytochemical analysis and wound healing activity of diabetic mice.

##### 2.2.2. Phytochemical analysis

Quantitative analysis of various phytochemicals such as alkaloids (Harbone, 1973), flavonoids (Boham and Kocipai, 1994), phenols (Harbone, 1973; Obadoni and Ochuko, 2001), saponin (Obadoni and Ochuko, 2001) and tannins (Van-Burden and Robinson, 1981) of roots of *C. orchioides* were performed.

#### 2.3. Animals

All experiments were performed on 7 to 8 week–old male Swiss albino mice with an average weight of (25±1) g. The animals were individually kept under laboratory conditions at the Department of Biochemistry, Banaras Hindu University, Varanasi. The mice were divided into two groups diabetic (D) and non–diabetic (ND) comprising five animals in each group. The first five groups were considered as diabetic and assigned as DC, DM, DM+E1, D+E1 and D+E2 where M for metformin and E, respectively represented the different concentration of plant extracts. Diabetes was induced by giving intraperitoneal streptozotocin injection (50 mg/kg body weight) in cold 0.1 mol/L citrate buffer, pH 4.5 for 5 consecutive days. The animals were confirmed for diabetes before the start of experiment. The serum glucose level was measured by glucose oxidase-peroxidase method using glucose test kit (Span diagnostics Ltd., India). The other five groups of mice were considered as non–diabetic and assigned as NDC, NDM, NDM+E1, ND+E1, and ND+E2.

##### 2.3.1. Wound creation

To develop wounds, a single full thickness 1.0 cm diameter superficial excision was made on the mid-dorsum of each diabetic and non–diabetic mouse at Day 0. The measurement
of the wound diameter was taken on Day 1, Day 7 and Day 13 by using transparency paper and permanent marker.

2.3.2. Extract administration

The DC and NDC (control), DM and NDM (60 mg/kg body weight in 200 µL ddH2O), DM+E1 and NDM+E1 (30 mg/kg body weight metformin in 100 µL ddH2O+100 mg/kg body weight extract in 200 µL ddH2O), D+E and N+D+E (400 mg/kg body weight extract in 200 µL ddH2O) were injected for 14 consecutive days starting from Day 0 through intraperitoneal tube. The level of glucose, superoxide dismutase (SOD), LPO and nitric oxide (NO) were measured on 1st, 7th and 13th post wounding days of both diabetic and non-diabetic mice.

2.4. Determination of LPO and SOD activity

The levels of malondialdehyde (MDA) for LPO and SOD were estimated in the supernatant of wound tissue homogenates. To obtain supernatant, the homogenized wound tissues were centrifuged at 5 000 r/min for 10 min to remove the cell debris. The level of MDA was estimated according to the method of Ohkawa et al.[12] and was expressed as nanomole per milliliter of MDA conjugate formed in the reaction. The estimation of SOD was done by the method of Mishra and Fridovich[13].

2.5. Determination of NOx activity

The levels of NOx were estimated by the method of Ghasemi et al.[14] and Miranda et al.[15]. A total of 100 µL of tissue supernatant was mixed with Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl) ethylene diammine with 5% phosphoric acid] and OD was taken at 570 nm.

2.6. Statistical analysis

The data were analyzed by one way analysis of variance (ANOVA) using SNK test (Students–Newmann–Keuls) with sigma stat 3.5. The P value less then 0.05 were considered to be significant (level of significance =0.05, **=0.01, ***=0.001). Data were represented as mean±SD. All the studies were performed in quadruplicate.

3. Results

3.1. Phytochemical analysis

The phytochemical analysis of C. orchioides revealed the presence of phenols, tannins, alkaloids, saponin and flavonoids. The presence of these phytochemicals were considered to be responsible for wound healing activity in diabetes (Table 1). The maximum saponin content (2.24%) was recorded in methanolic extract of root tubers of C. orchioides.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Alkaloids (%)</th>
<th>Phenols (%)</th>
<th>Tannin (%)</th>
<th>Flavonoids (%)</th>
<th>Saponin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. orchioides</td>
<td>1.29±0.12</td>
<td>1.46±0.03</td>
<td>0.09±0.09</td>
<td>1.35±0.22</td>
<td>2.24±0.02</td>
</tr>
</tbody>
</table>

3.2. Anti-diabetic plant extract decreases serum glucose level in diabetic mice

Administration of plant extract resulted in a significant decrease (P<0.001) in serum glucose level on Day 7 and 13 in case of diabetic mice as compared to the diabetic controls. A less significant decrease (P<0.05) was observed in case of mice treated with metformin on Day 7, but on Day 13, this decrease was highly significant (P<0.001) mice (Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Level of serum glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>NDC</td>
<td>122.00±3.10</td>
</tr>
<tr>
<td>NDM</td>
<td>125.00±3.13</td>
</tr>
<tr>
<td>NDM+E1</td>
<td>124.00±3.82</td>
</tr>
<tr>
<td>ND+E1</td>
<td>128.00±3.58</td>
</tr>
<tr>
<td>NDM+E2</td>
<td>121.00±3.12</td>
</tr>
<tr>
<td>DC</td>
<td>245.00±4.86</td>
</tr>
<tr>
<td>DM</td>
<td>220.00±3.24</td>
</tr>
<tr>
<td>D+E1</td>
<td>230.00±3.33</td>
</tr>
<tr>
<td>D+E2</td>
<td>240.00±3.36</td>
</tr>
<tr>
<td>D+E3</td>
<td>238.00±2.87</td>
</tr>
</tbody>
</table>

3.3. Calculation of wound contraction

The measurement of the wound areas were taken on the Day 1, 7 and 13 using transparency paper and a permanent marker. The wound areas were recorded and measured on graph paper. The plant extract increased the rate of wound healing in the diabetic mice. The wound closure was optimal in the diabetic group of D+E2. The results are summarized in Table 3 and Figures 1–3.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Wound contraction (mm2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>NDC</td>
<td>100.00±2.92</td>
</tr>
<tr>
<td>NDM</td>
<td>100.00±2.80</td>
</tr>
<tr>
<td>NDM+E1</td>
<td>100.00±2.45</td>
</tr>
<tr>
<td>ND+E1</td>
<td>100.00±2.67</td>
</tr>
<tr>
<td>NDM+E2</td>
<td>100.00±2.89</td>
</tr>
<tr>
<td>DC</td>
<td>100.00±2.81</td>
</tr>
<tr>
<td>DM</td>
<td>100.00±2.79</td>
</tr>
<tr>
<td>D+E1</td>
<td>100.00±2.65</td>
</tr>
<tr>
<td>D+E2</td>
<td>100.00±2.98</td>
</tr>
<tr>
<td>D+E3</td>
<td>100.00±2.96</td>
</tr>
</tbody>
</table>
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Figure 2. Photograph showing various stages of wound healing activity of C. orchioides extract at a dose of 200 mg/kg in streptozotocin induced diabetic mice.

Figure 3. Photograph showing various stages of wound healing activity of C. orchioides extract at a dose of 400 mg/kg in streptozotocin induced diabetic mice.

3.4. Levels of SOD in wound tissue after extract administration

The wound tissue from diabetic mice showed decreased extracellular SOD activity as compared to non-diabetic mice. After plant extract administration, the SOD activity was found increased on both Day 7 and Day 13 in all diabetic groups being significant. However, no significant changes were observed in non-diabetic groups. (Table 4)

3.5. Levels of LPO in wound tissue after plant extract administration

In diabetic wounds, the level of LPO in terms of MDA (nmol/mL) was increased as compared to non-diabetic wounds. The administration of plant extract was effective in preventing LPO as observed by decrease in the level of MDA content in diabetic groups. The decrease in the level of LPO in non-diabetic was not significant (Figure 4). The estimated levels of lipid oxidation in wound tissue supernatant from various diabetic and non-diabetic plant extract treated groups of mice were as depicted in Table 4.

3.6. Levels of NO in wound tissue after extract administration

The wound tissue from diabetic mice showed decreased nitric oxide level as compared to non-diabetic mice (Table 4). After plant extract administration, the NO was found increased significantly ($P<0.001$) on Day 7 and Day 13 in all diabetic groups being significant (Table 4).

4. Discussion

ROS is produced during normal cellular function. ROS is produced by neutrophils and other leukocytes damaged cells. It prevents cell proliferation and wound closure by damaging DNA, lipids, proteins, the extracellular matrix and cytokines that speed healing[16,17]. ROS includes hydroxyl radicals (OH), superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and NO[18,19]. They are very transient species due to their high chemical reactivity that leads to LPO and oxidation.

Table 4

<table>
<thead>
<tr>
<th>Groups</th>
<th>Level of SOD$^a$</th>
<th>Level of LPO$^b$</th>
<th>Level of NO$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 13</td>
</tr>
<tr>
<td>NDC</td>
<td>1.40±0.12</td>
<td>1.24±0.11</td>
<td>1.45±0.10</td>
</tr>
<tr>
<td>NDM</td>
<td>1.20±0.10</td>
<td>1.33±0.14</td>
<td>1.34±0.16</td>
</tr>
<tr>
<td>NDM+E$_1$</td>
<td>1.35±0.15</td>
<td>1.37±0.12</td>
<td>1.33±0.12</td>
</tr>
<tr>
<td>NDM+E$_2$</td>
<td>1.47±0.12</td>
<td>1.41±0.16</td>
<td>1.45±0.13</td>
</tr>
<tr>
<td>NDM+E$_3$</td>
<td>1.22±0.17</td>
<td>1.32±0.17</td>
<td>1.33±0.18</td>
</tr>
<tr>
<td>DC</td>
<td>0.57±0.13</td>
<td>0.78±0.12</td>
<td>1.20±0.13</td>
</tr>
<tr>
<td>DM</td>
<td>0.64±0.11</td>
<td>1.13±0.15</td>
<td>1.53±0.14</td>
</tr>
<tr>
<td>DM+E$_1$</td>
<td>0.79±0.18</td>
<td>1.19±0.13</td>
<td>1.64±0.12</td>
</tr>
<tr>
<td>DM+E$_2$</td>
<td>0.89±0.13</td>
<td>1.60±0.19</td>
<td>1.84±0.19</td>
</tr>
<tr>
<td>DM+E$_3$</td>
<td>0.91±0.14</td>
<td>1.70±0.10</td>
<td>1.97±0.14</td>
</tr>
</tbody>
</table>

$^a$: Level of SOD (inhibition percentage of NBT reduction/mL of wound tissue supernatant) from various diabetic and non diabetic groups of mice.

$^b$: Level of LPO in progression of MDA (nmol/mL) of wound tissue supernatant from various diabetic and non-diabetic groups of mice.

$^c$: Level of total NO content (µmol/L) in wound tissue supernatant from various diabetic and non-diabetic groups of mice.
of DNA and proteins\textsuperscript{[20,21]}. The role of oxygen–derived species in causing cell injury or death is increasingly recognized and linked to low antioxidant concentration\textsuperscript{[22]}.

The prevention of LPO is an essential process in all the aerobic organisms, as lipid peroxidation products can cause DNA damage\textsuperscript{[23]}. Increased LPO and decreased antioxidant protection frequently occurs when ratio of ROS produced exceeds the level of antioxidants available. Such a reaction may lead to cytotoxicity, allergy, mutagenicity, carcinogenicity and delayed diabetic wound healing. Under normal conditions, antioxidant systems of the cell minimize the perturbations caused by ROS. When ROS generation is increased to an extent that it overcomes the cellular antioxidants, the result is oxidative stress. Antioxidants like SOD are substances that delay or prevent the oxidation of cellular oxidizable substrates from ROS species. The prevention of oxidation is an essential process in all the aerobic organisms. Nowadays in the field of biomedical sciences, plants are the poten source of antioxidant compounds that protect the cells from oxidative stress in vitro\textsuperscript{[24,25]}.

\textit{C. orchioides} is one of the highly useful plants in the indigenous system of medicine for wound healing due to presence of natural antioxidative phenolic compounds\textsuperscript{[26–28]}. \textit{C. orchioides} contains a major group of phenolic glycosides compounds such as corchioside A, isoscrassifoside G, curculigosaponin A–F, sitosterol, linolenic, palmitic acid & orenol glucoside. These phenolic glycosides can significantly inhibit the generation of reactive oxygen species such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) that promote the delayed wound healing in diabetic condition\textsuperscript{[29–31]}.

The constituents such as alkaloids, triterpenoids and tannins of roots of \textit{C. orchioides} may play a major role in the process of wound healing in diabetic rats. Tannins\textsuperscript{[32]} and triterpenoids\textsuperscript{[17]} are also known to promote the wound healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of re–epithelization. The vascular and lymphatic systems are of primary importance during the process of wound healing. Failure or delay of vascular regeneration decrease oxygen transport to the wound, which subsequently depresses the mobilization of excessive fluids from the wound site. The wound becomes edematous, leading to further damage, infection and eventually cell death. The roots of \textit{C. orchioides} may thus achieve the following effects to improve tissue healing: an increased blood supply which increases the oxygen supply to the wound by blocking vasoconstructive compounds; greater migration of epidermal cells and extensive reorientation of collagen fibers caused by a stronger cross linking\textsuperscript{[33]}. Several studies also reported this type of pro–healing action with the extract of many indigenous medicinal plants \textsuperscript{[34,35]}.

The results of this study can be justified by the facts that the methanolic extract of roots tubers of \textit{C. orchioides} enhances the faster lay down of collagen fibers and improves the antioxidant status in the wound of diabetic animals.

In conclusion, the results of study showed that the extract of root tubers of \textit{C. orchioides} effectively stimulate wound contraction as compared to control and other groups. This finding suggests the positive aspects of inclusion of this plant in management of diabetic wound healing.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgements**

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**Comments**

**Background**

This study was performed to calculate the effect of methanolic extract of \textit{C. orchioides} on rate of wound contraction and estimation of various biochemical parameters such as SOD, LPO and NO levels in the granulation tissue of diabetic and non–diabetic mice.

**Research frontiers**

The methanolic extract of \textit{C. orchioides} with the concentration of 200 mg/kg and 400 mg/kg body weight was induced through intraperitoneal injections in diabetic and non–diabetic mice. The results were compared with control and metformin induced diabetic and non–diabetic mice.

**Related reports**

The results showed that root tubers of \textit{C. orchioides} are a potent source of antioxidative phenolic compounds that counteract with ROS species responsible for delayed wound healing. The root tubers of \textit{C. orchioides} significantly increased the level of SOD, NO and decreased LPO in granuloma tissue of diabetic mice. Ozturk \textit{et al.} studied the wound healing activity of \textit{Hypericum perforatum} L. on chicken embryonic fibroblasts.

**Innovations & breakthroughs**

This study has showed that wound healing activity of \textit{C. orchioides} in streptozotocin induced diabetic mice positively.

**Applications**

It is important to estimate and to monitor the role of
herbal medicine in the case of delay wound healing in diabetic persons.

**Peer review**

The is an interesting study in which the results suggested that *C. orchioides* promoted the faster wound healing in the case of diabetic persons due to present of flavonoides, tannin, etc.

**References**


