Aqueous Extract of the seeds of *Pithecellobium dulce* (Fabaceae) Demonstrates Anti-inflammatory Effect Through Anti-oxidant Activity Enhancement in a Rodent Model of on Hemorrhoidal disease induced by Acetic Acid

Atsang A Kiki Gisèle, Takvou Francis, Egre Finsia, Zramah Mathieu, Dzeufiet DPD

**ABSTRACT**

After the last treatment, all animals received 5 % acetic acid anal route to the hemorrhoidal submucosa. Administration of acetic acid resulted in a significant reduction (p < 0.01; p < 0.001) in body weight and recto-anal coefficient at doses of 250 and 500 mg/kg compared to the negative control. In addition, the concentration of nitric oxide (NO), MDA were significantly reduced (p < 0.05 p < 0.01) in the groups pretreated with different doses of the extract (125, 250 and 500 mg/kg) compared to negative and normal control with a significant increase (p < 0.05 and p < 0.001) in the level of SOD, CAT and GHS compared to the negative control. Histological sections showed cellular regeneration in the anal mucosa.

**Keywords:** Inflammation, *Pithecellobium dulce*, Acetic acid.

**INTRODUCTION**

Hemorrhoids are normal vascular formations in the human body and are present in the fetus from the 28th week whose function is to contribute to anal continence [18]. Hemorrhoidal disease refers to all disease states resulting from the progressive dilation or rupture of the hemorrhoidal venous plexuses [7]. The pathogenesis of hemorrhoidal disease is multifactorial: Mechanical factors such as relaxation of the musculo-ligamentous suspension apparatus, alteration of the means of attachment of the hemorrhoidal plexuses and vascular factors such as increased pressure in the hemorrhoidal vessels vascular anatomical changes [10; 16]. The etiology of hemorrhoidal disease (HDD) remains uncertain; however unbalanced diet, particularly high residue and low fiber diets, constipation, prolonged sitting and episodes of female genital life are the most critical factors more incriminated. There are two major types of hemorrhoids namely internal hemorrhoids and external hemorrhoids. They are characterized by a painful, often blue-purple swelling outside the anus, which can appear immediately after physical exertion and sometimes a tear in the epidermis under the pressure of the underlying clot. Hemorrhoidal disease represents 40.83% of lower digestive pathologies [2]. Worldwide, the overall prevalence of hemorrhoidal diseases is estimated at 4.4 % [21], more specifically in Cameroon, hemorrhoidal disease represents 10 to 70 % of consultations in different health centers [3]. Hemorrhoidal pathology takes on a wide variety of clinical pictures, including rectal bleeding, pruritus, hemorrhoidal prolapse, and hemorrhoidal thrombosis leading to permanent and exacerbated pain, incontinence and cancer [4]. The treatment of this disease involves the adoption of hygienic and dietetic rules (the avoidance of spices, alcohol, tobacco and coffee), the use of topical (cream, suppositories), venotonic (diosmin, troxerutin, rutoside) as well as analgesics and non-steroidal anti-inflammatory drugs [23]. Endoscopic methods such as sclerosing injections, elastic ligatures of the base of a hemorrhoidal bundle, cryotherapy does not completely remove hemorrhoids and their effectiveness decreases over time [23]. However, surgical treatments present satisfactory results but sometimes cause inconvenient side effects and remain very expensive [14]. [25] compared to the low economic level of the population of underdeveloped countries. This therefore motivates the population to turn to medicinal plants as alternative solutions for the treatment of HDD. Many plants have also shown pronounced effects against hemorrhoidal conditions and whose use is recognized by WHO. It is *Hamamelis* whose leaves and bark contain 8 to 12 % tannins and flavonoids; *Ginkgo biloba*, *Hypericum perforatum* and *Aesculus hippocastanum* which are rich in ruscogenin and tannins. The venotonic, analgesic, anti-inflammatory and hemostatic effects which are recognized in these plants are attributed to these phenolic compounds. However, their availability in certain areas is a hindrance to their expansion on a global scale. *Pithecellobium dulce* is therefore noticed by its composition in phenol metabolite [31] in inflammatory diseases such as arthritis [3]. It is in view of the above that we set out in this study to evaluate the anti-hemorrhoidal properties of the aqueous extract of *P. dulce*.

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MATERIALS AND METHODS

Chemicals

To induce hemorrhoidal disease, 100 μL of acetic acid (Sigma-Aldrich, St. Louis, MO, USA) was introduced into the rectoanal part of the animal. Control animals received orally distilled water and daflon (from Sigma-Aldrich), drugs used to reduce hemorrhoidal disease.

Phytochemical analyses

Phytochemical analyses of the extract were tested using the following chemicals and reagents (Trease et al. 1983). Saponin (fothing test), tannins (FeCl₃), flavonoid (NaCl and HCl), phenol (FeCl₃ and K₂Fe(CN)₆) and lipids (filter paper).

Plant material

The plant material consisted of the seeds of *Pithecellobium dulce* harvested in May at the fruiting stage, in the Ouro-Iché district of the Maroua first district, Diamaré Division in the Far-North region of Cameroon. The plant was identified and authenticated at the Cameroon national herbarium in comparison with the material of GANESH Iyer, herbarium NO 33821 / HNC. After harvest, these seeds were dried in the shade for 7 days. They were then reduced to a fine powder using a traditional mortar. The resulting powder was stored in tightly closed glass vials and used for the preparation of the extract.

Extraction of plant material

Three hundred grams (300 g) of *P. dulce* was boiled in 2 L of distilled water for 15 minutes. After cooling, the mixture was filtered on Whatman number 4 paper and the resulting filtrate was placed in an oven at 50 °C for 24 hours to evaporate the water. A mass of 37.1 g was obtained after drying and the yield was 12.36 %.

Animal and experimental design

Animals (*Mus musculus* Swiss strain mice) of both sexes weighing 25 ± 5 g and aged 10 ± 2 weeks at the start of the experiment were used. The animals were kept in a room at room temperature in cages lined with litter before and during the period of the experiment. The mice were given free access to tap water and fed a standard diet.

After randomization according to their weight, 36 mice were distributed into 6 groups (6 rats each, 3 males and 3 females) and treated as follow. All treatments was administered orally thirty minutes before acetic acid induction (day 0), then the animals were treated daily for up to the 10th days.

Induction of hemorrhoidal disease by acetic acid 5 %

On the tenth day of treatment, after administration of the extract, the hemorrhoids were induced according to the protocol described by Wang et al., 2008. Briefly, 100 μL of 5 % acetic acid were applied to the rectal route in all groups except the normal control which received distilled water, followed by overnight fasting. Changes in body weight of mice were noted daily for 10 days. On the eleventh day, all animals were sacrificed by cervical dislocation. Blood was drawn by rupturing the jugular vein. The laparotomy was performed using a chisel and forceps. The liver was removed for the determinations of the parameters of oxidative stress and NO. Recto-anal tissues (20 mm long) was isolated and weighed to assess the severity score. Recto-anal tissues were fixed in 10% formaline for histological sections. The recto-anal coefficient (CRA) was calculated using the formula:

\[
\text{Recto-anal coefficient} = \frac{\text{rectoanal tissue weigh}}{\text{Animal body weigh}}
\]

Evaluation of the in vivo antioxidant activity of the aqueous extracts of the seeds of *Pithecellobium dulce*

The antioxidant potential of the extract was assessed by estimating the liver content of Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) according to the methods of Wilbur et al., In 1949; Misra and Fridovish in 1972; Sinha in 1972 and Ellman in 1959 respectively. The NO was assayed according to the method of Zainul et al., in 2016.

Histological study

The histological study was carried out according to the technique described by Hould in 1984. In short, it involves fixing the organs in a 10 % formalin solution. The samples were placed in special cassettes with inverted walls in order to allow the passage of liquids. Sections were stained with Hematoxylin-Eosin X 40.

Statistical analyzes

Results were expressed as the mean ± standard error of the mean (ESM) for each group. Number per group = 5. The one-way analysis of variance test (Anova) was used followed by the Student Newman Kells post-test to compare the values with each other. The results were significantly different for p <0.05.

RESULTS

Qualitative Phytochemistry

The phytochemical screening of the aqueous extract of the seeds of *Pithecellobium dulce* revealed the presence of certain classes of bioactive compounds such as alkaloids, flavonoids, coumarins, terpenoids, tannins and sugar

Anti-hemorrhoidal effects of aqueous extract of *P. dulce*

Macroscopic appearance of the anus after induction of hemorrhoidal disease by acetic acid

Figures 1 showed the effect of 5 % acetic acid on the anal canal. Acetic acid caused severe damage to the anal canal of mice in the negative control group, characterized by diarrhea, weight loss, and anal bleeding. Whereas in the groups pretreated with Daflon (10 mg/kg) and with the aqueous extract of *P. dulce* at different doses, we observed a significant protection of the structure of the anal canal compared to the negative control group.

Effect of *P. dulce* aqueous extract on body weight

Figure 2 showed the effects of EAPD on the change in body weight in different groups of animals. After the 11 days of experimentation, a significant decrease (p < 0.001) in weight was noted in the animals of the negative control group compared to the animals of the normal control group throughout the experiment. In addition, a loss of body weight was observed in all the different groups pretreated with EAPD and the reference medicine. Animals treated at the doses of 250 and
500 mg/kg of the extract showed a significant difference (p < 0.01) compared to animals in the normal control group. A non-significant increase in the weight of the animals was also observed with daflon (10 mg/kg) compared to the negative control.

**Effect of aqueous extract of *P. dulce* on the recto-anal coefficient**

The effect of the aqueous extract of *P. dulce* on the recto-anal coefficient was shown in Table 1. Hemorrhoid disease caused a significant difference (p < 0.05 and p < 0.01) in the recto-anal coefficient of the animals in the positive control group and those in the test group at different doses (125, 250 and 500 mg/kg) in comparison with the negative control group. However, significance (p < 0.01) is also observed only on animals in the negative control group in comparison with the normal group receiving only distilled water.

**Effects of the aqueous extract of *P. dulce* seeds on some parameters of oxidative stress**

**Effects of aqueous extract of *P. dulce* seeds on the concentration of nitric oxide in liver tissue**

Figure 3 below showed the effect of aqueous extract of *P. dulce* on nitric oxide (NO) concentration in liver tissue in mice. It appears that no difference was noted in the negative control group compared to the normal control. However, a significant decrease (p < 0.05; p < 0.01) of NO in liver tissue was observed in the positive control and the groups pretreated at different doses of the extract (125, 250 and 500 mg/kg) compared to the negative and normal control. Likewise, a significant decrease (p < 0.01 and p < 0.001) in the hepatic NO concentration is noted in the animals of the positive control group and the test group at different dose.

**Effects of aqueous extract of *P. dulce* seeds on hepatic malondialdehyde (MDA) levels**

Figure 4 showed the effects of the aqueous extract of the seeds of *Pithecellobium dulce* on the concentration of malondialdehyde (MDA). There was a significant increase (p < 0.001) in the concentration of MDA in negative control group compared to normal animals. In addition, the aqueous extract of *Pithecellobium dulce* caused a significant decrease (p < 0.001) in the concentration of malondialdehyde (1.96 ± 0.52, 4.68 ± 0.53 and 4.24 ± 0.61 mmol / mg of organs) in mice treated with different doses (125, 250 and 500 mg/kg) of *P. dulce*. The same was true in animals treated with Daflon 10 mg/kg (1.60 ± 0.10 mmol / mg of organs).

**Effect of aqueous extract of *P. dulce* seeds on superoxide dismutase activity**

Figure 5 showed the effects of *Pithecellobium dulce* aqueous extract on superoxide dismutase (SOD) activity. After 10 days of treatment, SOD activity was significantly higher (p < 0.05; p < 0.001) (0.716 ± 0.0302; 0.765 ± 0.0210; 0.524 ± 0.0551 and 0.545 ± 0, 0581 U / mg of organs) in the animals of the positive group and of the three test groups (125, 250 and 500 mg/kg) compared to the animals of the negative control group. Similarly, this activity was also significantly high (p < 0.01 and p < 0.001) in the animals which received Daflon (10 mg/kg) on the one hand and the different doses of the aqueous extract of *P. dulce*, compared animals in the normal control group.

Each bar represents the mean ± SEM; n = 6. 00p < 0.01 000p < 0.001 significant difference compared to the animals of the normal control group; * p < 0.05; *** p < 0.001 significantly different from the negative control group.

Nor C= Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).

**Effect of aqueous extract of *P dulce* seeds on catalase activity (CAT)**

Catalase activity (CAT) was shown in Figure 6 below. A significant increase (p < 0.05; p < 0.01) in catalase activity was observed in animals treated at the dose of 500 mg/kg of the aqueous extract of *P. dulce* compared to the negative control and normal.

**Effects of aqueous extract of *P. dulce* seeds on recto-anal tissues in mice**

Histological analysis of the recto-anal tissue revealed in the normal control normal wall architecture showing lumen at the periphery, mucosa, submucosa, muscle and serosa (Figure 7). In the negative control group, pathological changes in the rectal wall marked by: denaturation of the mucosa as well as inflammation of the submucosa and muscle tissue were observed. The groups treated with the extract at different doses and with the reference substance showed a preservation of the recto-anal wall close to that of the normal control group.

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**Figure 1:** Photograph of the recto-anal regions of hemorrhoidal mice. A: negative control; B: positive control (Daflon 10 mg/kg); C: AEPD 125 mg/kg; D: AEPD 250 mg/kg and E: AEPD 500 mg/kg.; Inflammation of the recto-anal region

AEPD: Aqueous extract of *Pithecellobium dulce*
Figure 2: Change in body weight of mice. The values are expressed in terms of the mean ± SEM (n = 6). **: comparison with the normal control group (** p < 0.01, *** p < 0.001). Nor C = Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).

Figure 3: The protective effect of aqueous *P. dulce* extract on the expression of NO activity. The values are expressed in terms of the mean ± SEM (n = 6). **: comparison with the negative control group (* p <0.05, ** p <0.01). **: comparison with the normal control group (** p <0.01, *** p <0.001).

Nor C = Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).

Figure 4: Effects of the aqueous extract of the seeds of *Pithecellobium dulce* on the concentration of malondialdehyde. Each bar represents the mean ± SEM; n = 6 0 p < 0.05, 000 p < 0.001 significant difference compared to the animals of the normal control group; *** p <0.001, significant difference compared to the negative control group.
Figure 5: Effects of aqueous extract of *Pithecellobium dulce* seeds on superoxide dismutase (SOD) activity. Each bar represents the mean ± SEM; n = 6. **p < 0.01; ***p < 0.001** significant difference compared to the animals of the normal control group; * p < 0.05; ** **p < 0.001** significantly different from the negative control group.

Nor C= Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).

Figure 6: Effects of aqueous extract of *Pithecellobium dulce* on the concentration of liver tissue in catalase in experiments. Each bar represents the mean ± SEM, (n = 6) * p < 0.05: significant difference compared to the negative control; **p < 0.01: significant difference compared to the normal control.

Nor C= Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).
Figure 7: Effect of aqueous extract of *Pithecellobium dulce* seeds on the hepatic concentration of reduced glutathione (GHS). Each bar represents the mean ± SEM, (n = 6); *** p < 0.001: significant difference compared to the negative control; θθp < 0.01: significant difference compared to the normal control.

Nor C= Normal control; NC = Negative control; PC = Positive control (Daflon 10mg/kg); EAPD 125, 250, 500 = aqueous extract of *Pithecellobium dulce* (125, 250, 500 mg/kg).

Figure 8: Photomicrograph of recto-anal tissue (Hematoxylin-eosin X 40): Normal control; B: Negative control; C: Positive control; D, E, F: Test lots treated with *Pithecellobium dulce* extract at different doses; Lu = Light; Mu = Mucosa; SMu = Submucosa; Mus = Muscular; Se = Serious; Gi = Inflammatory granuloma.

Table 1: Distribution of Animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Distilled water</td>
<td>10 mL/Kg</td>
</tr>
<tr>
<td>Positif control</td>
<td>Daflon</td>
<td>10 mg/Kg + acetic acid</td>
</tr>
<tr>
<td>Negative control</td>
<td>Distilled water</td>
<td>10 mL/Kg + acetic acid</td>
</tr>
<tr>
<td>Test 1</td>
<td>Extract</td>
<td>125 mg/Kg + acetic acid</td>
</tr>
<tr>
<td>Test 2</td>
<td>Extract</td>
<td>250 mg/Kg + acetic acid</td>
</tr>
<tr>
<td>Test 3</td>
<td>Extract</td>
<td>500 mg/Kg + acetic acid</td>
</tr>
</tbody>
</table>

Table 2: Qualitative phytochemical of the aqueous extract of the seeds of *P. dulce*

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaloids</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
</tr>
<tr>
<td>Quinons</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Tanins</td>
<td>+</td>
</tr>
</tbody>
</table>

- (not present), + (present),
Hemorrhoidal diseases are characterized by severe vasodilation in the recto-anal region, which thus constitutes inflammation of the tissues with secondary complications such as extravasation of fluid in the interstitial space, mainly due to an increase in vascular permeability and migration of a large quantity of inflammatory cells (granulocytes and monocytes) thus justifying weight loss in experimental animals [19]. Acetic acid causes inflammation due to the release of soluble factors involving the inflammatory lipid metabolites, nitric oxide. These factors, alone and/or in combination, regulate the activation of resident cells (fibroblasts, endothelial cells, macrophages and mast cells) and newly recruited inflammatory cells (monocytes, lymphocytes, neutrophils and eosinophils) leading to a systemic response to the inflammation (fever) [11]. In the present study, an estimate of nitric oxide NO (quantification of inflammatory factor) and histopathology evaluations (presence of inflammatory cells) were performed to relate the series of inflammatory reactions involved in the development of hemorrhoids due to acetic acid. NO plays numerous regulatory roles at each stage of the development of inflammation: it has apoptotic effects and can induce DNA alterations while inhibiting repair systems [8]. The level of iNOS activation and NO expression may reflect the inflammatory state; Therefore, NO remains a potential target for the development of therapeutics for inflammatory diseases [13]. iNOS is an enzyme that increases in inflamed tissue in patients with hemorrhoidal disease. A significant decrease (p < 0.05; p < 0.01) of NO in liver tissue in the positive control and the groups pretreated at different doses of the extract (125, 250 and 500 mg/kg) was observed compared to the negative and normal control. Acetic acid induces hemorrhoid by generating reactive oxygen species [9]. The liver exhibits increased susceptibility to oxidative stress because it contains high concentrations of polyunsaturated fatty acids vulnerable to lipid peroxidation, and also has antioxidant capacity [30]. As the formation of ROS in liver cells is numerous, cells must therefore maintain an effective antioxidant system in order to protect themselves against ROS overload and the damage that results from it [9]. During oxidative stress, ROS production increases and the capacity of endogenous free radical scavengers such as GSH, CAT and SOD is activated [15]. Recto-anal administration of acetic acid caused a significant decrease in MDA content. This decrease was correlated with increased levels of GSH and activities of CAT and SOD in the liver. SODs are metalloproteins possessing enzymatic activity catalyzing the disproportionation of superoxide anions into dioxygen and hydrogen peroxide [24]. CAT is a heme oxidoreductase that catalyzes the disproportionation of hydrogen peroxide into water and oxygen [24]. GSH is vital for detoxifying heavy metals; the thiol group reacts with the salts of these heavy metals, creating with them a very strong sulfur-metal bond so that they are then excreted without causing damage to the body [20]. The observed effect may lie in the radical scavenging properties of the flavonoids in P. dulce. The present results therefore demonstrated the correlation between the anti-hemorrhoidal effects of the aqueous extract of P. dulce seeds against acetic acid-induced hemorrhoid and its antioxidant capacity. The histology of the recto-anal tissue shows in the normal control group a normal architecture of the wall showing lumen at the periphery, a mucosa, a submucosa, a muscular layer and a serosa. In the negative control group, pathological changes were observed in the rectal wall marked by: denaturation of the mucosa as well as inflammation of the submucosa and muscle tissue due to the pronounced effect of acetic acid. Unlike the animals treated at the dose of 125, 250 and 500 mg/kg of the extract and those treated with Daflon 10 mg/kg which showed an architecture similar to those of the normal control group. The aqueous extract of the seeds of P. dulce therefore protects hemorrhoids against the lesions that acetic acid can induce. The aqueous extract of the seeds of P. dulce would therefore act either as an antagonist of the norepinephrine receptors, or would act either by inhibiting lipid peroxidation while increasing the antioxidant status. All these properties could be attributed to the different compounds (alkaloids, flavonoids, tannins, terpenoids, sugars and coumarins) that the plant contains.

CONCLUSION

At the end of this work, it appears that our plant is rich in a bioactive compound responsible for restoring weight, reducing the recto-anal coefficient and as well as nitrogen monoxide in pre-treated animals. This plant would have a strong antioxidant power responsible for the cure of hemorrhoidal disease.

Credit authorship contribution statement

Atsang à kiki gisèle, Takvou Francis conceived and designed the experiments and carried out the major part of experiments. Egre finsia, Zramah Mathieu: Havested the plant and pre carried out the major part of experiments. Egre finsia, Zramah Mathieu, Dzeufiet Djomein Paul Désiré: Performed histopathological examination. Dzeufiet Djomein Paul Désiré: Made provision of reagent and proofread the paper. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that they have no conflict of interest to disclose.

Data availability

Data will be made available on request.

Ethical considerations

The study was approved by ethic Committee of the Faculty of Sciences of the University of Maroua (Ref N°14/0261/Uma/D/FS/VD-RC), according to the guidelines of Cameroonian bioethics committee (Reg N.° FWA-IRB00001954).
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