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RESEARCH ARTICLE

PHYTOCHEMICAL STUDY AND EVALUATION OF THE ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIAL OF HYDROETHANOLIC EXTRACTS OF PLUMBAGO ZEYLANICA

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ABSTRACT

Plumbago zeylanica is an herbaceous plant used in traditional medicine to treat microbial and parasitic infections, swelling, arthritis, and certain types of tumors. However, scientific data validating the pharmacological properties of the species found in Togo remain limited. The objective of this study was to evaluate the antioxidant and anti-inflammatory activities of leaf and root extracts of *P. zeylanica*. To this end, a phytochemical assay of flavonoids and total polyphenols, as well as a phytochemical analysis using DPPH and FRAP techniques, were performed on *P. zeylanica* extracts. The anti-inflammatory potential was evaluated in rat paw edema induced by 1% formaldehyde. The phytochemical analysis revealed a significantly higher total polyphenol content in the root extract (41.68 mgEqAG/g) compared to the leaf extract (23.18 mgEqAG/g) ($p<0.0001$). In contrast, the flavonoid concentration was significantly lower in the leaf extract (31.18±0.37 mgEqQ/g) than in the root extract of *P. zeylanica* (62.36±1.02 mgEqQ/g) ($p<0.0001$). The results obtained demonstrated the strong antioxidant power of the extracts examined. The root extract of *P. zeylanica* caused a significant inhibition of edema in rats treated with 400 mg/kg body weight (bw) ($p<0.001$). These pharmacological effects are thought to be attributable to the chemical molecules present in the extracts, particularly the flavonoids and polyphenols present in the extracts examined. *P. zeylanica* is undoubtedly a medicinal plant with antioxidant and anti-inflammatory properties, but caution should be exercised when using it in traditional medicine to prevent harmful side effects in users.

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INTRODUCTION

The use of plant-derived medicines for the treatment of many conditions is gaining interest within communities (1). This trend is the result of an increase in side effects associated with synthetic products, a decline in the effectiveness of certain existing medicines, the rapid emergence of new diseases, and the persistence of diseases that are difficult to treat (cancer) (2). Oxidative stress and chronic inflammation are the cause of many diseases (cancer, diabetes, cardiovascular disease), requiring antioxidant and anti-inflammatory agents. Medicinal plants and their active ingredients are increasingly being incorporated into alternative therapies, particularly for patients with chronic diseases such as inflammatory diseases and metabolic disorders (3). *P. zeylanica* is a perennial plant, a perennial shrub that grows mainly in the wild in Africa. This plant is renowned for its various therapeutic properties (4). Used for centuries in traditional Ayurvedic and African medicine, this species is renowned for treating various conditions such as rheumatism, swelling, liver disorders, and the prevention of atherogenic risks (5). *P. zeylanica* is known for its antioxidant and anti-inflammatory effects, particularly in the management of diabetes and neurodegenerative diseases (4). Its growing use, particularly in topical applications and in alcoholic tincture preparations, raises questions about its pharmacological effects on human and animal health (6). As such, promote the plant heritage of Togolese flora in the face of chronic diseases linked to oxidative stress and inflammation become necessary. It is with this in mind that the present study aims to evaluate the antioxidant and anti-inflammatory potential of hydroethanolic extracts of the leaves and roots of *P. zeylanica*.

MATERIALS AND METHODS

Plant material and extraction: The leaves and roots of *P. zeylanica*, collected in Avetonou (Agou), constituted the bulk of the plant material. These harvested organs were dried in the laboratory under air conditioning for two weeks and then finely ground in an electric mill. Extraction was carried out by macerating 100 g of dry powder from each organ in one liter of hydroethanolic solution (30/70) under continuous stirring for 72 hours at room temperature. The macerate obtained was filtered twice on cotton wool and then once on Whatman°1 paper.

The filtrate was finally evaporated under vacuum at 40°C and at reduced pressure between 999-1000 in a rotary evaporator (7).

Animal material: We used Wistar rats (figure 3), aged 8 to 12 weeks and weighing an average of 120 g. These animals were obtained from the animal facility of the Faculty of Sciences of the University of Lomé, then brought to the animal facility of the Pharmaceutical Sciences Laboratory of the Faculty of Health Sciences of the same university, where they were placed in cages. Food and water were provided ad libitum. These animals were subjected to a natural photoperiodic cycle (12 hours, plus or minus 30 minutes, of light or darkness). The room temperature was 26°±2°C, with humidity ranging between 60 and 70%.

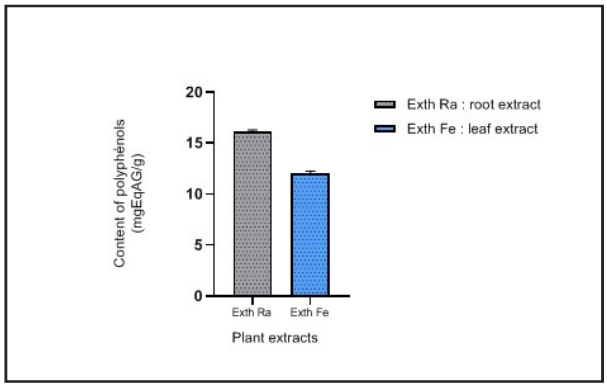

Figure 1. Total polyphenol content in hydroethanolic extracts from roots (Exth Ra) and leaves (Exth Fe) of *P. zeylanica*. The concentration of polyphenols in each extract is expressed as a mean ± standard deviation at 95%

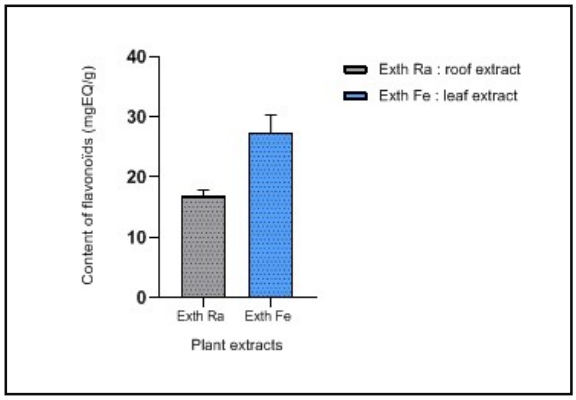

Figure 2. Flavonoid content in hydroethanolic extracts from the roots (Exth Ra) and leaves (Exth Fe) of *P. zeylanica*. The content of polyphenols in each extract is expressed as an average ± 95% confidence range

Figure 3. Edema of the rat paw induced by 1% formaldehyde. Left (A): normal paw; right (B): inflammatory edema

Total polyphenol assay: The Folin-Ciocalteu method was used to quantify the total polyphenols present in hydroethanolic extracts from the roots and leaves of *P. zeylanica*. This technique is based on the reducing power of the yellow Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid), which is converted into tungsten and molybdenum oxides (blue in color) during the oxidation of phenols. The intensity of this blue coloration is proportional to the amount of polyphenols present in the extracts examined (8). For this assay, 1 ml of Folin-Ciocalteu solution (diluted to 1/10) was added to 200 µl of each extract solution to be analyzed (1 mg/ml). The mixture was incubated at room temperature for 2 minutes. Next, 800 µl of 75 g/l sodium carbonate was added to the mixture, which was then homogenized and incubated for 30 minutes at room temperature and protected from light. The optical densities of each solution thus obtained were measured at 765 nm using a spectrophotometer. Gallic acid was used to establish the calibration curve with concentrations ranging from 0 to 100 µg/ml (9).

Total flavonoid assay: In the presence of flavonoids, aluminium trichloride ($AlCl_3$) forms a yellow complex whose color intensity is proportional to the amount of flavonoids present in the extract being examined (8). For the assay, one (1) ml of each 1 mg/ml *P. zeylanica* extract solution in methanol is mixed with 1 ml of a 2% AlCl3 methanolic solution. The absorbance of the reaction medium is measured at 415 nm using a spectrophotometer after incubation for 10 minutes at room temperature. The total flavonoid content was determined based on the calibration curve established with quercetin under the same conditions (9).

Free radical reduction test (DPPH): The antiradical activity of *P. zeylanica* extracts was measured using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) test. In the presence of a hydrogen-donating antioxidant, the unstable dark purple DPPH radical is reduced to a non-radical purple compound (DPPH-H) (9). This test was performed by adding 100 microliters (100 µl) of each extract to be examined at various concentrations (25, 50, and 100 µg/ml) to 2 ml of a DPPH solution (0.04 mg/ml) solubilized in methanol. The absorbances were measured at 517 nm using a spectrophotometer after 30 minutes of incubation in the dark (10). Three tests were performed for each assay. Quercetin was used as a reference molecule under the same conditions. The calibration curve generated was used to determine the percentage inhibition of the DPPH free radical by *P. zeylanica* extracts. The different percentages of DPPH radical inhibition were calculated using the following formula (11):

$$R\ (\%) = (1 - \left(\frac{AE}{AO}\right))X100$$

AO: absorbance of the reagent blank;
AE: absorbance of the extracts tested.
R: inhibition

Ferric reduction assay (FRAP): The revised protocol of Benzie and Strain (1996) (12) was used to evaluate the reducing potential of *P. zeylanica* extracts (13). This method is based on the ability of ferric ion (Fe^{3+}-TPTZ) to convert to ferrous ions at low pH, causing the formation of a blue-colored ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex. The increase in the intensity of the reaction medium's color at 593 nm is proportional to the Fe^{2+} content (12).

Preparation of reagents: To prepare the FRAP test reagent, three solutions were made: an acid buffer pH = 3.5 (50 ml), a solution of 2,4,6-tripyridyl-s-triazine (TPTZ) (5 ml), and a solution of ferrous chloride (5 ml). The acid buffer (pH = 3.5) was prepared by dissolving 0.310 g of CH_3COONa, $3H_2O$ in a 100 ml bottle, followed by the addition of 1.6 ml of pure acetic acid and filling to the mark with distilled water. The TPTZ solution was prepared by dissolving 156 mg of this compound in 50 ml of 40 mMol/l HCl. To prepare a ferric chloride solution (20 mMol/l), we dissolved 27 mg of $FeCl_3$, $6H_2O$ in a 50 ml bottle, then made up the volume with distilled water. A ferrous sulfate solution was prepared by dissolving 27 mg of $FeSO_4$, $7H_2O$ in 50 ml of methanol. The resulting solution (2000 µMol/l) is diluted to obtain a range of ferrous sulfate concentrations (13).

Experimental protocol: One hundred (100) µl of each extract to be analyzed was mixed with 2 ml of a freshly prepared FRAP solution.

The mixture was vigorously shaken using a vortex mixer, then the optical density was measured after 30 minutes using a spectrophotometer at 593 nm (Agbodan et al., 2014). The reducing power of each *P. zeylanica* extract was determined by the color change associated with the formation of the complex (TPTZ-Fe^{2+}). The calibration curve was constructed using the absorbance of the TPTZ-Fe^{2+} complex from the concentration range of the iron sulfate ($FeSO_4$, $7H_2O$) solution dissolved in methanol. The results are expressed in µMolEqFe^{2+}/mg of extract. Ascorbic acid was used as a reference molecule under the same conditions. Each test was repeated three times (13).

Evaluation of the anti-inflammatory activity of *P. zeylanica* extracts: The anti-inflammatory activity of *P. zeylanica* extracts was evaluated using the method of inhibiting rat paw edema induced by 1% formaldehyde (14). The rats were fasted for 12 hours before the experiment, weighed, and marked. For this test, six groups of five rats each were randomly selected. The initial thickness of the left hind paw of each rat was measured using callipers before oral administration of the treatments according to the schedule below:

Batch 1: physiological saline solution at a rate of 1 ml/100 g body weight (bw); batch 2: diclofenac solution at a concentration of 100 mg/kg bw; batch 3: root extract solution at 200 mg/kg bw; batch 4: root extract solution at 400 mg/kg bw; batch 5: leaf extract solution at 200 mg/kg bw; batch 6: leaf extract solution at 400 mg/kg bw. Half an hour after administration of the extracts, 5 µl of a 1% formaldehyde solution was injected subcutaneously into the aponeurosis of the rats' left hind paw (Lakache, 2019). The thickness of the paw was then measured at the first, third, and fifth hours. The percentage reduction in edema was calculated using the formula:

$$\text{Edema (\%)} = \frac{(\Delta T - \Delta E)}{\Delta T} X100$$

ΔT: difference between the average of the hind legs (left - right) for the control group (0.9% NaCl saline solution);

ΔE: difference between the average of the hind legs (left - right) for the test groups (Lakache, 2019).

Data analysis: Data analysis was performed using analysis of variance (ANOVA). Graphpad Prism 8.4.3 software was used to perform this analysis, applying a 95% confidence interval.

RESULTS

Total phénols contents: The total polyphenol content in the hydroethanolic leaf and root extracts of *P. zeylanica* was determined using the linear regression line for gallic acid, Y = 0.008397X + 0.1816; R^2 = 0.9791. These findings revealed a significantly higher total polyphenol content in the root extract (41.68 mgEqAG/g) compared to the leaf extract (23.18 mgEqAG/g) ($p<0.0001$).

Total flavonoids contents: The content of flavonoids in the extracts of *P. zeylanica* was determined from the linear regression line of quercetin Y = 0.03688X + 0.07347; R^2 = 0.9946. The flavonoid content was significantly lower in the leaf extract (31.18±0.37 mgEqQ/g) compared to that in the root extract (62.36±1.02 mgEqQ/g) of *P. zeylanica* ($p<0.0001$).

Antioxidant activity of extracts: According to our findings, *P. zeylanica* root extract at a concentration of 25 µg/ml demonstrated a reducing power of 9.27±0.39% for the 2,2'-diphenyl-1 -picrylhydrazyl radical, while the leaf extract showed a reducing power of 8.15±2.30% at the same concentration. The antioxidant potential of *P. zeylanica* was substantially higher in the root extract (32.24±1.95%) at a concentration of 100 µg/ml ($p<0.001$) than those of the leafextract (23.15±0.23%). The antioxidant activity of each of the extracts examined was significantly lower than that of quercetin (83.23±4.82%) ($p<0.001$). The inhibitory concentrations (IC_{50}) were determined from the linear regression lines: Y = 0.8179X + 7.7179; R^2 = 0.9549 for quercetin, Y = 0.3232X + 1.3364; R^2 = 0.9792 for the root extract, and Y = 0.2253X+2.2646; R^2 = 0.9444 for the leaf extract. The IC_{50} values were 141.63±1.56 µg/ml for root extract and 237.6±2.04 µg/ml for leaf extract of *P. zeylanica.* The iron reduction capacity (FRAP) was determined from the linear regression line for ferrous sulfate Y = 0.1434X + 0.0020, R^2 = 0.9842. Our findings revealed that the root extract of *P. zeylanica* showed significant overall antioxidant activity (348.66±2.37 µMolEqFe^{2+}/mg extract) compared to that of the leaf extract, which was 273.18±1.04 µMolEqFe^{2+}/mg extract ($p<0.001$).

Effect of *P. zeylanica* extracts on inflammatory edema in animals: The administration of 1% formaldehyde into the aponeurosis of animals caused progressive inflammation, manifested by an increase in paw volume (edema) in rats. Root extract at a dose of 400 mg/kg bw showed significant inhibition of inflammatory edema in animals at 5 hours after treatment($p<0.0001$). This inhibition percentage was 66.67%. However, this action remained moderate, significant, and significant compared to that observed in rats treated with diclofenac.

Table 1. Antioxidant capacity of *P. zeylanica* extracts

Plant extracts	FRAP (µMolEqFe^{2+}/mg)	DPPH (µg/ml)
Exth Ra	348.66±2.37	141.63±1.56
Exth Fe	273.18±1.04	237.6±2.04

Exth Ra: hydroethanolic extract from the roots;
Exth Fe: hydroethanolic extract from the leaves

DISCUSSION

Oxidation is a biochemical process essential to the optimal functioning of an organism. This biological process leads to a loss of electrons, which causes the formation of endogenous electrophilic substances, the excessive production of which is very harmful to the individual (15). However, defense mechanisms such as antioxidants help to limit the excessive formation of these reactive oxygen species. The use of medicinal plants with antioxidant properties could be an effective alternative for treating chronic diseases and improving quality of life. To this end, exploring naturally occurring antioxidant molecules would be an asset (16). In this study, we examined the antioxidant potential of hydroethanolic extracts from the leaves and roots of *P. zeylanica* using FRAP and DPPH techniques. The findings revealed significant overall antioxidant capacity in the hydroethanolic leaf and root extracts of *P. zeylanica*. These observations suggest that the compounds present in the extracts are likely to donate hydrogen atoms and electrons to unstable or highly reactive chemical entities. These data confirm the observations reported by other authors in the literature (17). Preliminary phytochemical tests had indicated the presence of phenolic compounds, more specifically flavonoids and terpenic compounds, in the extracts examined (18). These compounds are thought to play a significant role in the adsorption, capture, and neutralization of free radicals. Indeed, the antioxidant effect of flavonoids is attributable to their low redox potential, which enables them to reduce free radicals by transferring hydrogen atoms from the hydroxyl groups present in the phenolic nucleus (15). Polyphenols can regulate the oxidative state of cells by inhibiting oxidative enzymes (xanthine oxidase, cyclooxygenase, etc.) responsible for the production of superoxide radicals (19). In numerous studies, secondary plant metabolites such as myricetin and resveratrol have inhibited free radical generation, limited lipid peroxidation, and prevented glutathione depletion and loss of mitochondrial membrane potential in several cell models (20). From the above, the pharmacological properties of the extracts examined could help in the prevention and reduction of chronic pathologies related to oxidative stress (21).

The application of 1% formaldehyde to the aponeurosis of rats triggered an acute inflammatory reaction characterized by vasodilation, extravasation, and infiltration of

Table 2. Average diameter of paw edema in rats over time

Treatments	Doses	Measurement of edema (mm)		
		1 hour	3 hours	5 hours
Control (formaldehyde)	1%	5.00 ± 0.24	7.20 ± 0.00	7.80 ± 0.24
Diclofenac	100 mg/kg	5.80 ± 0.10	6.00 ± 0.24*	5.80 ± 0.20*
Rootextract	200 mg/kg	5.00 ± 0.32	7.80 ± 0.20	7.80 ± 0.10
Leafextract	200 mg/kg	6.00 ± 0.18	7.80 ± 0.10	8.00 ± 0.00
Rootextract	400 mg/kg	5.00 ± 0.00	7.20 ± 0.20	6.00 ± 0.20*
Leafextract	400 mg/kg	5.00 ± 0.00	7.20 ± 0.32	8.00 ± 0.20

Comparisons were made between the control group and the test groups, * Significant difference.

Table 3. Percentage inhibition of inflammatory edema in rat paws

Traitements	Doses	Edema inhibition (%)		
		1 hour	3 hours	5 hours
Diclofenac	100 mg/kg	0.00±0.00	33.33±0.00*	66.67±0.00*
Rootextract	200 mg/kg	0.00±0.00	0.00±0.00	0.00±0.00
Leafextract	200 mg/kg	0.00±0.00	0.00±0.00	0.00±0.00
Rootextract	400 mg/kg	0.00±0.00	0.00±0.00	40.00±0.10*
Leafextract	400 mg/kg	0.00±0.00	0.00±0.00	0.00±0.00

Comparisons were made between the control group and the test groups.
* Significant difference.

Polyphenols can regulate the oxidative state of cells by inhibiting oxidative enzymes (xanthine oxidase, cyclooxygenase, etc.) responsible for the production of superoxide radicals (19). In numerous studies, secondary plant metabolites such as myricetin and resveratrol have inhibited free radical generation, limited lipid peroxidation, and prevented glutathione depletion and loss of mitochondrial membrane potential in several cell models (20). From the above, the pharmacological properties of the extracts examined could help in the prevention and reduction of chronic pathologies related to oxidative stress (21). The application of 1% formaldehyde to the aponeurosis of rats triggered an acute inflammatory reaction characterized by vasodilation, extravasation, and infiltration of polynuclear cells, leading to the formation of paw edema in rats. The results indicated a significant suppression of paw edema in rats treated with root extract at a dose of 400 mg/kg bw ($p<0.005$). The leaf extract had no effect on the animals. These observations suggest that the hydroethanolic extracts of *P. zeylanica* roots examined have an anti-edematous effect (22). However, this effect remained significantly lower than that of diclofenac ($p<0.0001$). These results are consistent with those of other authors in the literature (23). These authors reported the ability of *P. zeylanica* extracts to induce anti-inflammatory effects in several cellular models (4). This remarkable pharmacological activity of the hydroethanolic root extract of *P. zeylanica* could be due to the secondary metabolites present in the extracts examined. Indeed, previous studies have indicated that molecules such as flavones, flavonols, quercetin, kaempferol, and myricetin induced significant inhibition of cyclooxygenases (COX-1, COX-2), considerably limiting plasma extravasation processes in animals (20). In addition, these molecules are likely to modulate the enzymatic and molecular systems involved in the inflammatory cascade. By inhibiting the metabolism of membrane phospholipids, these molecules prevent the phosphorylation of NF-kB (14), leading to the inactivation of transcription factors and resulting in negative feedback control of TNF-α and IL-1β gene expression. This results in the suppression of the initiation of the inflammatory process (25). Our data therefore suggest that herbal medicines derived from *P. zeylanica* preparations could be a potential source of antioxidant and anti-inflammatory molecules.

CONCLUSION

The purpose of this study is to evaluate the antioxidant and anti-inflammatory activities of the hydroethanolic extracts from leaves and roots of *P. zeylanica*. The findings highlighted the significant overall antioxidant potential of hydroethanolic extracts derived from the leaves and roots of *P. zeylanica*. A significant inhibition of paw edema by *P. zeylanica* root extract at a dose of 400 mg/kg bw in animals was revealed. The root extract of *P. zeylanica* therefore exhibited an anti-inflammatory effect at high doses.

In summary, hydroethanolic extract from *P. zeylanica* could be an alternative for the management of chronic metabolic and inflammatory disorders. However, further studies, particularly toxicological and clinical studies, are still needed to validate these results.

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Abbreviations

AG:	Gallic acid
AlCl:	Aluminum trichloride
ANOVA:	Analysis of variance
Bw:	Body weight
Cl:	Chlorine
COX-:	Cyclooxygenase-1
COX-2:	Cyclooxygenase-2
DPPH:	2,2-diphenyl-1-picrylhydrazyl
Eq:	Equivalent
Exth Fe:	Hydroethanolic extract from leaves
Exth Ra:	Hydroethanolic extract from roots
Fe^{2+}:	Ferrous ion
Fe^{3+}:	Ferric ion
$FeCl_3$:	Iron chloride
FRAP:	Ferric Reducing Antioxidant Power
NF-kB:	Nuclear factor kappa B
pH:	Hydrogen potential
Q:	Quercetin
TNF:	Tumor necrosis factor
IC_{50}:	Inhibitory concentration 50
IL-1β:	Inhibitory concentration

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