Anti-inflammatory and radical scavenging properties of the ethyl acetate fraction from the leaves of *Agelanthus dodoneifolius* (DC) Polh. & Wiens (Loranthaceae)

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Abstract

The current research aimed to assess the anti-inflammatory and radical scavenging activities of the ethyl acetate (EtOAc) fraction from the leaves of *Agelanthus dodoneifolius*, a mistletoe belonging to the family of Loranthaceae. The inhibition of carrageenan-induced paw edema on NMRI mice was used to demonstrate the EtOAc fraction's *in vivo* acute anti-inflammatory effect. The anti-inflammatory effect was compared to dexamethasone at 10 mg/kg and rutin at 50 mg/kg. The radical scavenging activity was determined using free radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Moreover, the flavonoid content was obtained spectrophotometrically using the Neu reagent. Five hours after the carrageenan administration, the EtOAc fraction reduced the paw edema significantly ($p < 0.01$, $p < 0.001$, versus control). No significant ($p > 0.05$) statistical difference was observed between the fraction at 100 mg/kg and the reference dexamethasone.

Similarly, no statistical differences were seen between rutin and the EtOAc fraction at 25 and 50 mg/kg doses. The EtOAc fraction was potent at scavenging the DPPH radicals with an IC_{50} value of 22.03 ± 0.94 µg/mL. The flavonoid content was equal to 2.87 g Rutin Equivalent/100 g of the fraction.

The EtOAc fraction showed important anti-inflammatory and radical scavenging effects. These activities could be explained by flavonoids, compounds known to have several pharmacological effects. Further investigations are needed to highlight the mechanism of anti-inflammatory activity of the ethyl acetate fraction.

Keywords: *Agelanthus dodoneifolius*; ethyl acetate fraction; anti-inflammatory; radical scavenging; flavonoids

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Introduction

Medicinal plants and herbal-derived products have been used to treat various diseases for several millennia (1,2). Inflammation is involved in the body's healing process and may be triggered by many stimuli, including microorganisms, damaged cells, irradiation, or toxic compounds (3). The failure to eliminate the causative agents leads to the development of various inflammation-related diseases such as cancer, diabetes, and cardiovascular diseases (3).

Mistletoes are evergreen shrub hemiparasitic plants that occur ubiquitously in temperate zones, arid regions, and wet tropics (4). Mistletoes are represented by five families, including the Misodendronaceae, Eremolepidaceae, Santalaceae, Loranthaceae, and Viscaceae (4,5). Among this polyphyletic group, the most widely known are the Loranthaceae and Viscaceae (4,5).

Agelanthus dodoneifolius (DC) Polh. & Wiens (synonym *Tapinanthus dodoneifolius*) is a hemiparasitic plant belonging to the Loranthaceae family. Also called "African mistletoe," *Agelanthus dodoneifolius* (*A. dodoneifolius*) is distributed mainly in Africa (6,7). This plant is used in traditional medicine to treat various ailments, including gastrointestinal, gynecological, cardiovascular, and respiratory disorders (7,8). *A. dodoneifolius* is also used to treat malaria, cancer, and wounds (7). Some of the traditional uses of this plant, including antihypertensive, spasmolytic, antiplasmodial, anti-inflammatory, antioxidant, antimicrobial, larvicidal, and molluscicidal properties, have been demonstrated (6,8–12). The oral and intraperitoneal lethal dose (LD_{50}) of the 70% methanolic extract was higher than 5000 mg/kg of body weight (11). Moreover, our group found an LD_{50} equal to 368.96 mg/kg after an intraperitoneal administration of the aqueous decoction from *A. dodoneifolius*. The ethyl acetate fraction from the leaves of *A. dodoneifolius* has shown *in vitro* significant antiradical and anti-inflammatory activity on the equine myeloperoxidase enzyme involved in acute and chronic inflammation conditions (13).

The present research aimed to assess the *in vivo* anti-inflammatory and DPPH radical scavenging properties of the ethyl acetate fraction from the leaves of *A. dodoneifolius*.

Materials and Methods

Plant material collection and extract preparation

The leaves of *A. dodoneifolius* infecting the shea tree, *Vitellaria paradoxa* (Sapotaceae), were collected in peri-urban areas of Ouagadougou (1369592 N, 0664286 W). A botanist authenticated the sample by comparing it with the existing voucher specimen recorded under reference numbers 01 & 02 at the Herbarium of University Joseph KI-ZERBO. The leaves were shade-dried at room temperature before being reduced to powder with a

mechanical grinder. The ethyl acetate fraction was obtained using a method previously reported (13). In brief, 100 g of powdered leaves of *Agelanthus dodoneifolius* were boiled in methanol for 15 min in a hume hood. The extract was concentrated under reduced pressure. The residue was then resuspended in boiling water (100 mL) and successively exhausted with diethyl ether (3x100 mL), ethyl acetate (3x100 mL), and n-butanol (3x100 mL). Each fraction was dried under reduced pressure and stored for further use. Approximately 1.2 g of ethyl acetate fraction was obtained.

Animals

NMRI strains mice weighing 24-40 g were obtained from our institute's animal house. The animals were raised under standard lab conditions of temperature (22 ± 3°C), humidity (50-70%), and a 12 h light-dark cycle. The animals have unlimited access to food and water. However, before experiments, animals were deprived of food and given free access to water. The experimental protocols followed the principles and regulations about laboratory animal care and ethical use set in the eighth edition of the Guide for the Care and Use of Laboratory Animals (Guidelines set by the European Union on animal protection) (CEC Council 86/609).

Drugs and reagents

Methanol, Diethyl ether, ethyl acetate, n-butanol, rutin, 2-aminoethyl diphenylborinate (Neu reagent), 2,2-Diphenyl-1-picrylhydrazyly (DPPH), dexamethasone, and carrageenan were purchased from Sigma (St. Louis, USA). Quercetin was purchased from ChromaDex (LGC Standard, France). Thin-layer chromatographic (TLC) silica gel 60 F_{254} plates were supplied by Merck (Belgium). All other substances were of technical grade.

Carrageenan-induced paw edema in mice

The carrageenan-induced paw edema model assessed the ethyl acetate fraction's *in vivo* anti-inflammatory effect (14). Mice were divided into seven (7) groups of five (5) mice each. Before the test, they were fasted for 16 hours. The control group (group I) was intraperitoneally injected with distilled water (10 mL/kg of body weight (b.w.)). In groups II, III, IV, and V, mice received the ethyl acetate fraction intraperitoneally at 10, 25, 50, and 100 mg/kg b.w. Groups VI and VII were given intraperitoneally 10 mg/kg b.w. and 50 mg/kg b.w. of dexamethasone and rutin, respectively. One hour after administration, 0.1 mL of 1% carrageenan prepared daily in normal saline was subcutaneously injected into each mouse's right dorsal hind paw. The Plethysmometer (Ugo Basile, Italy) was used to measure the extent of inflammation before treatment (T = 0 H) and 1, 3, and 5 H after carrageenan injection. The percentage of inhibition of inflammation (I%) was calculated as follows:

$I(\%) = \frac{(Dc-Dt)}{Dc} \times 100$, where Dc is the difference in paw volume of the control group, and Dt is the difference in paw volume in the treated group.

2,2-DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity was assessed in a 96-well microplate following a previous method (15). Quercetin was used as a reference compound. 100 µL of diluted methanolic samples (0.1 - 50 µg/mL) were mixed with 200 µL of 0.004% methanolic DPPH work solution. The mixture was incubated in the dark at room temperature for 30 min. For the control sample, extract or quercetin was replaced with 100 µL of methanol. Absorbances were recorded at 540 nm (Bio-Rad, Belgium), and measurements were conducted in triplicate. The DPPH radical scavenging activity (%) was calculated as follows:

DPPH scavenging activity (%) = $[(A_C - A_S) / A_B] \times 100$

Where A_C and A_S represent, respectively, the absorbance of the control and sample (extract/quercetin)

The IC_{50} (µg/mL), the concentration of sample or standard compound that produces half-maximal inhibition, was determined.

Thin-layer chromatographic (TLC) profile and Estimation of the flavonoid content

TLC analysis was performed on the silica gel pre-coated chromatographic plates TLC to determine the compounds in the ethyl acetate fraction. Based on previous results suggesting that several solvents, including ethyl acetate, are suitable for extracting phenolic compounds (16), the eluent used to develop the TLC plate was ethyl acetate/acid formic/water (90/1/1, v/v). After development, the plate was dried, and the compounds were visualized using the Neu reagent.

The method described by (15) was used to determine the flavonoid content of the ethyl acetate fraction. The procedure uses the 2-aminoethyl diphenylborinate (Neu's reagent) and rutin as reference compound. In brief, 2 mL of 0.25% (g/v) methanolic extract was mixed with 100 µL of Neu's reagent. Rutin was treated with the same reagent. The absorbance was measured at 405 nm using a microplate reader (Bio-Rad, Belgium). The total flavonoid content (TFC) was expressed as g Rutin Equivalent per 100 g of extract and calculated using the following equation:

$$TFC = [(0.05 \times Ae)/(Ar \times Ce)] \times 100$$

with Ae: absorbance of the extract; Ar: absorbance of rutin; Ce: concentration of the extract (2.5 mg/mL)

Data and statistical analyses

The data were expressed as means ± standard deviation (SD) of three replicate experiments. GraphPad Prism (version 8.0) was used to determine the IC_{50} values of the radical scavenging activity. A two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests was used to compare the ethyl acetate fraction to the control (Anti-inflammatory activity). An unpaired t-test was used to assess the difference regarding the DPPH radical scavenging's IC_{50} values between quercetin and the ethyl acetate fraction. The difference was considered statistically significant for a *p*-value < *0.05*.

Results

Anti-inflammatory activity

The subcutaneous injection of carrageenan in mice increased the paw volume in the control group. The maximum increase was observed after 5 H with a mean volume of 0.23 mL. The ethyl acetate fraction significantly reduced the carrageenan-induced mice paw edema (Figure 1 and Table 1) in a time and dose-dependent manner.

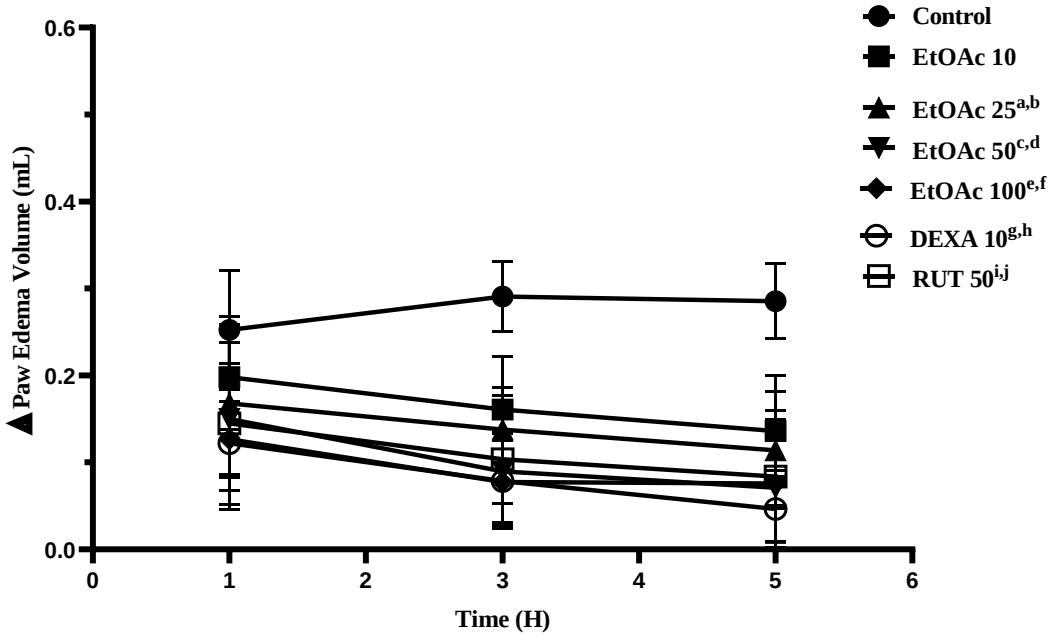

Figure 1. Effect of the samples on carrageenan-induced mice hind paw edema.

Values are expressed as mean ± SD of three experiments. A Two-way ANOVA followed by Dunnett's test was used to compare groups to the control. Meaning of the letters in superscript in

the legend: $^{a}p < 0.05$ and $^{b}p < 0.01$ respectively at T=3 and 5H; $^{c}p < 0.01$ and $^{d}p < 0.001$ respectively at T=3 and 5H; $^{e}p < 0.001$ and $^{f}p < 0.001$ respectively at T=3 and 5H; $^{g}p < 0.001$ and $^{h}p < 0.001$ respectively at T=3 and 5H; $^{i}p < 0.01$ and $^{j}p < 0.01$ respectively at T=3 and 5H. DEXA: dexamethasone at 10 mg/kg of body weight (b.w.); RUT: rutin at 50 mg/kg b.w.; EtOAc: ethyl acetate fraction at 10, 25, 50, and 100 mg/kg b.w.

There was no significant difference ($p > 0.05$) between dexamethasone and the ethyl acetate fraction at 100 mg/kg b.w. (Figure 1). Five hours after carrageenan administration, no significant differences were seen between the ethyl acetate fraction at doses of 25 and 50 mg/kg b.w. and the flavonoid rutin.

Table I : Percentage inhibition of carrageenan-induced edema by the ethyl acetate fraction, dexamethasone, and rutin. The fraction and rutin were compared to dexamethasone.

Treatment	Dose (mg/kg b.w.)	Inhibition percentage (%)		
		1 H	3 H	5 H
Control	-----	-----	-----	-----
DEXA	10	55.94	82.45	86.21
EtOAc	10	14.85[a]	27.72[a]	39.82[a]
EtOAc	25	37.70[b]	55.54[b]	63.76[b]
EtOAc	50	37.89[b]	58.23[b]	68.61[b]
EtOAc	100	51.90	63.76[b]	86.39
Rutin	50	44.22[b]	53.37[b]	62.99[b]

Values are presented as mean ± SD of three replications. A Two-way ANOVA followed by Dunnett's test evaluated differences within groups. Within each column, $^{a}p < 0.0001$ and $^{b}p < 0.001$ compared to dexamethasone. DEXA: dexamethasone; EtOAc: ethyl acetate fraction.

Five hours after the edema induction, dexamethasone elicited a percentage inhibition equal to 86.21%, which was significantly not different from the ethyl acetate fraction at a dose of 100 mg/kg b.w.

Radical scavenging activity, flavonoid content, and TLC separation

The ethyl acetate fraction inhibited the DPPH radicals in a concentration-dependent manner. The IC_{50} values were respectively 22.03 ± 0.94 µg/mL and 11.71 ± 2.69 µg/mL for the ethyl acetate fraction and quercetin (Table II).

Table II IC_{50} values of DPPH radical scavenging and Flavonoid content

Sample	DPPH	Flavonoid content[$]
	IC_{50} (µg/mL)	-----
Quercetin	11.71 ± 2.69[a]	-----
Ethyl acetate fraction	22.03 ± 0.94[b]	2.87± 0.01

Values bearing different superscript letters ([a, b]) are considered statistically significant ($p = 0.0157$) (Unpaired t-test). [$]: the flavonoid content was expressed in g Rutin Equivalent/100 g of the ethyl acetate fraction.

The antiradical activity of the ethyl acetate fraction and quercetin was determined using the DPPH radical. Figure 1 shows that the highest DPPH radical scavenging effect was obtained at 50 µg/mL with 92.60 ± 0.14 % and 91.92 ± 0.26 % for quercetin and the fraction, respectively.

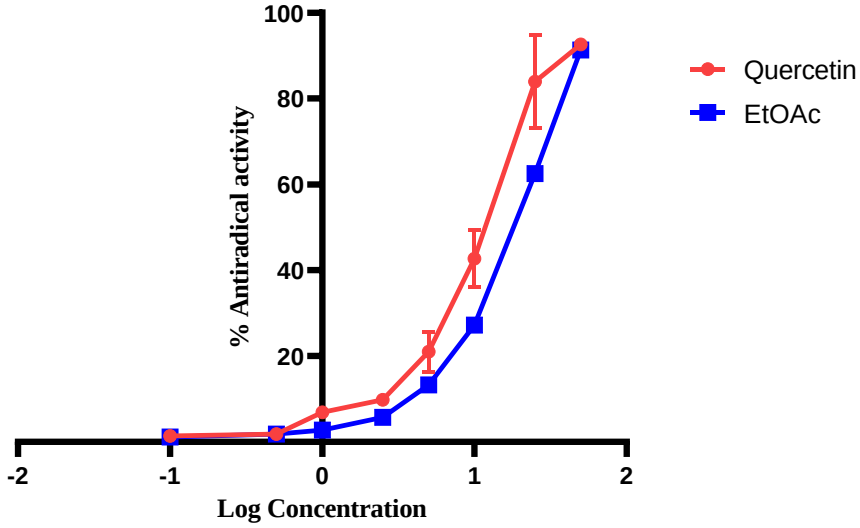

Figure 1 : Percentage of DPPH radical scavenging activity of the ethyl acetate fraction and quercetin

Figure 2 shows the separation of the ethyl acetate fraction. The TLC profile indicates several compounds in the ethyl acetate fraction. The chromatogram confirms the presence of quercetin, even if it's in a small amount. The compound (materialized with an arrow) and quercetin have the same retention factor (Rf) value, i.e., 0.90.

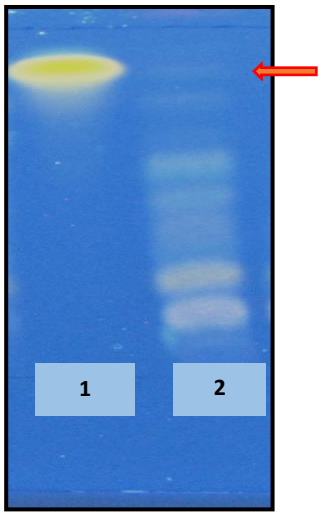

Figure 2 : Thin-layer chromatographic separation of quercetin (1) and the ethyl acetate fraction (2). The plate was developed in a mixture of ethyl acetate/formic acid/water (90/1/1, v/v). The arrow indicates the presence of quercetin in the fraction since the compound has the same Rf as quercetin (Rf = 0.90).

Discussion

Inflammation appears to be a defense mechanism by which organisms act to remove various stimuli (3). However, failing to eradicate the causative agents may result in various inflammation-related diseases (17). Inflammation activates various intracellular signaling pathways, leading to the overproduction of a large number of mediators, including cytokines, proteases, prostaglandins, leukotrienes, and reactive oxygen species (ROS) (1,3). Plant-derived products have been used successfully to treat many ailments, including inflammation-related disorders (2,17). The present manuscript presents the *in vivo* anti-inflammatory and radical scavenging properties of the ethyl acetate fraction of *Agelanthus dodoneifolius*.

The carrageenan-induced paw edema model is a well-known and characterized acute inflammatory model used in most laboratories to assess the anti-edematous effect of a product (18,19). The increase in the paw thickness observed in the control group after carrageenan administration was significantly ($p < 0.01$ and $p < 0.001$) reduced compared with all the treated groups. The ethyl acetate fraction exhibits a potent anti-edema effect

in a dose-dependent manner, and the highest inhibition percentage was observed at a dose of 100 mg/kg b.w. No statistical difference was seen between the EtOAc 100 and dexamethasone at 10 mg/kg regarding the inhibition percentage. However, the difference in active doses is due to dexamethasone being a pure compound and EtOAc, a composition of compounds.

Similarly, no statistical differences were found between the EtOAc at 25 and 50 mg/kg doses and rutin, a flavonoid retrieved in various plants with anti-edema properties (22).

According to various reports, the mechanism of carrageenan-induced edema involves a biphasic curve divided into early and late phases (19–21). The early phase, 30 min-2 H after edema induction, involves mainly vasoactive amines, including serotonin, histamine, and bradykinin. The late phase, from 3-4 H, was attributed to prostaglandins, leukotrienes, and cytokines. Therefore, the results indicate that the ethyl acetate fraction probably inhibits the early and late phases of the edema.

Moreover, scavenging ROS also participates in mitigating the inflammation process. DPPH assay is one of the most frequently used methods to evaluate plant-based products' antioxidant activity (23,24). The ethyl acetate fraction showed an important ability in scavenging the DPPH radical with IC_{50} and a maximal effect of 22.03 µg/mL and approximately 92%, respectively. The main free radicals scavengers have been reported to include phenolic compounds such as flavonoids and phenolic acids (23). Using the Neu reagent, the total flavonoid content was not statistically different from that was found in this fraction using the aluminum chloride ($AlCl_3$) reagent. Indeed, in a previous report, the total flavonoid content determined with the $AlCl_3$ reagent was 3.2 g Rutine equivalent per 100 g of ethyl acetate fraction; in the current report, it is approximately equal to 2.9 g Rutine equivalent per 100 g of extract (13). Therefore, It can be hypothesized that these two methods are complementary. However, this result differed from that obtained in the aqueous extracts from the leaves of *A. dodoneifolius* (15), although the reference compounds were different. For instance, the aqueous decoction of *A. dodoneifolius* contains 1.48 ± 0.01 g Quercetin Equivalent per 100 g of extract. Consequently, this result suggests that organic solvents such as ethyl acetate have more ability to extract polyphenol compounds. The ethyl acetate fraction contains numerous compounds, as revealed by the TLC profile. Moreover, it confirms the presence of quercetin in the fraction. As a result, flavonoids may be responsible for the radical scavenging effect of the fraction.

Furthermore, previous studies revealed that the ethyl acetate fraction contains several polyphenol compounds, including catechin, gallic acid, isoquercitrin, quercetin, and rutin (13). These compounds' pharmacological effects, including anti-inflammatory and antioxidant, have been previously demonstrated (25–28).

Conclusion

This current study demonstrated the potential of the ethyl acetate fraction from methanol decoction of the leaves of *Agelanthus dodoneifolius* in reducing the carrageenan-induced edema and scavenging the DPPH radicals. The high polyphenol compound content may be responsible for this fraction's anti-inflammatory and antiradical properties. Further *in vitro* and *in vivo* studies are required to understand the ethyl acetate fraction's anti-inflammatory and antioxidant mechanisms of action. Moreover, the isolation of flavonoid compounds could be performed using mainly chromatographic methods.

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