Antiinflammatory and antioxidant evaluation of *Maytenus senegalensis* hydroalcoholic roots extract fractions in allergic asthma

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**ABSTRACT**

The present work aims to evaluate the antiinflammatory and antioxidant activities of the *Maytenus senegalensis* (Lam.) roots hydroethanolic extract and to determine its active fraction. The total extract is fractioned in cold alcohol 75%. Phytochemical studies and *in vitro* antioxidant test are performed on the extract and obtained fractions. During 28 days, mice were sensitized and challenged with OVA to induce allergic airway inflammation. the anti-inflammatory effect of the plant extract and its fractions is assessed by differential white blood cell counts and histopathological examinations. Their antioxidant effect is evaluated *in vivo* by MDA and glutathione dosage. Qualitative and quantitative phytochemical tests showed that the extract and its fractions contain phenolics compounds. *In vitro*, both extract and its fractions expressed antioxidant activities. Treatment with *M. senegalensis* extract and its fractions significantly decreased inflammatory cells number in BALF, reduced MDA concentration and enhanced glutathione level (p<0.05; p<0.01; p<0.001) in lung homogenate. Histological observations confirmed the anti-inflammatory effect of the extract and its fractions. Comparatively, the supernatant fraction showed the best effect. This study may land up in establishing the scientific evidences for the traditional claims anti-asthmatic and anti-inflammatory activity of *Maytenus senegalensis* (Lam.). It also suggests that the phytochemical groups involved in this activity would be more concentrated in the supernatant fraction.

**Keywords:** Allergic asthma, *Maytenus senegalensis*, Antinflammatory, Antioxidant.

**INTRODUCTION**

Asthma is a chronic respiratory disease which include airways inflammation, bronchial smooth muscle hypersensibility leading to bronchoconstriction and airway remodeling [1]. Touching people of all ages round the world, asthma affects approximatively 339millions people since 2018 with at least 250,000 deaths each year [2].

The main feature implied in the pathogenesis of the disease is airways inflammation which is characterized by the infiltration of inflammatory cells such as eosinophils, neutrophils, T cells, basophiles, monocytes… from the circulation to the airways. These cells interact with their secreted products to control and perpetuate inflammation [3, 4]. Activated recruited cells including eosinophils and neutrophils produce and release reactive oxygen species in the airways and this result in oxidative stress that occurs in asthma [5]. Increased oxidative stress amplify inflammatory response in asthma and can be linked to less responsiveness to corticosteroids treatment and the severity of the disease [6].

Current synthetic drugs used in pharmacotherapy of asthma are unable to act at all the stages and targets of asthma and associated with side effects. In this junction several studies carried out on herbs have proven their promising activity in treatment of asthma [7]. One such plant is *Maytenus senegalensis* (Lam.) Exell (Celastraceae) which is used traditionally for the treatment of various ailments including malaria, rheumatism, dysmenorrhea, infections, chronic wounds, chest pains… [8, 9, 10]. Its roots are used in Togo against infections, simple or asthma cough. Pharmacologically, the plant is reported to be anti-inflammatory [10, 11], antimalarial and analgesic [12, 8, 13], antitussive [14], antibacterial and anthelminthic [15, 16, 17, 18]. In a previous study, the plant extract showed inhibitory effect on leukocyte influx in the bronchi and antioxidant potential [19].

The current study is the continuation of our earlier work to further explore the antiinflammatory and antioxidant effect of the extract and its ethanolic fractions in mouse exposed to OVA during longer period.
**MATERIAL AND METHODS**

**Chemicals**

Ovalbumin (grade V), aluminium hydroxide, bovine serum albumin (fraction V), standard MDA, Glutathione and DPPH were purchased from Sigma Aldrich. potassium ferricyanide and ammonium molybdate are also used for the study.

**Animals**

Female ICR mice (27 - 35g) from the Animal Physiology Department (University of Lomé) are used for this study. These mice are kept in a lighted room (12h:12h light:dark cycle) with free access to food and water. Experimental tests were conducted according to the WHO Guidelines for the care and use of human blood and laboratory animals and approved by the national bioethics committee of University of Lomé-Togo.

**Plant**

The roots of *Maytenus senegalensis* harvested in August 2016 in Tsévié-Boloumondji consist the plant material. A specimen is authenticated at the Department of Ecology and Botany and then deposited at the Herbarium of the University of Lomé (number TG 15182). *Maytenus senegalensis* extract is prepared by macerating the roots in distilled water/ethanol 1:1 and evaporation of the macerate like described previously [19]. The crude extract obtained is fractioned in cold alcohol. Briefly, thirty grams of extract were dissolved in 400 ml of 75% alcohol. The solution was refrigerated at 4°C for 24 hours. After centrifugation at 2500 rpm, the supernatant was separated from the pellet and then evaporated under vacuum at 45°C. The pellet was dried in ambient air [20].

**Phytochemical studies**

**Phytochemical screening**

Chemical group such as Alkaloids, flavonoids, tannins, saponins, anthracenes, carbohydrate and reducing compounds were researched in the extract’s fractions by standard staining tests [21, 22].

**Quantitative assay**

**Total phenols and tannins content**

Total phenols and tannins were determined in the extract and its fractions by folin ciocalteu and tannin fixation method [23].

- **Total phenols**
  
  200µl of the Folin-Ciocalteu reagent (10%) are added to either 200µl of the gallic acid solution (25-100μg/ml) or extract or its fractions (1mg/ml). After 30 min incubation at room temperature, 800µl of a sodium carbonate solution (700 Mm) is added. The absorbance of each sample is read at 735 nm after 2 h incubation. The absorbance of the extract and its fractions is referred to the standard gallic acid curve to determine the quantity of total phenols they contain. This quantity is expressed in mg gallic acid equivalent per gram of extract.

- **Tannins**
  
  500µl extract or fractions are first added to 10mg PVP and the mixture incubated on ice for 30 minutes. The supernatants obtained were treated in the same manner. After centrifugation, the phenol assay is performed on the subsequent supernatants. For each sample, the difference between the absorbance obtained from the total phenol determination and this second determination was calibrated with gallic acid curve to express the tannins content.

**Total flavonoids**

The determination of total flavonoids is carried out by adding 2 ml of aluminum chloride (20 mg/ml) and then 6 ml of sodium acetate (50 mg/ml) to 2 ml of the rutin range (5 to 100 µg/ml) or extract and its fractions. Absorbance is read at 440 nm against blank after 150 minutes’ incubation. Calibration with rutin curve serves to determine total flavonoid content in samples [24].

**In vitro antioxidant tests**

**DPPH test**

DPPH reduction ability of the extract and its fractions is evaluated by adding these samples to DPPH solution as reported previously [19].

**Determination of the reducing power**

The reducing potential of the extract and its fractions is assessed according to the Fc3+/ferricyanide complex reduction method [25].

To 0.5 ml of extract, fractions or ascorbic acid (25 to 600 µg/ml), 2.5 ml of phosphate buffer and 2.5 ml of potassium ferricyanide are added respectively. After 20 min incubation, 2.5ml trichloroacetic acid is added and the mixture centrifuged at 3000 rpm for 10min. to 2.5ml of supernatant from each tube, 2.5ml distilled water and 0.25ml ferric chloride were added respectively. The mixtures are homogenized and then read at 700nm. The blank is prepared in the same way by replacing the tested samples with methanol.

**Total antioxidant capacity**

To 0.3 ml of various concentrations (25 - 250 µg/ml) of ascorbic acid or the extract solution and its fractions (1 mg/ml) prepared with methanol, 3 ml of the prepared reagent (0.6 M sulphuric acid; 28 mM sodium phosphate and 4 mM ammonium molybdate) was added. The whole was incubated at 95°C for 90 min and then brought back to laboratory temperature. Absorbance was measured at 695 nm against blank. For the blank, the extract was replaced by methanol. Antioxidant activity is expressed as ascorbic acid equivalence [26].

**Evaluation of antiinflammatory activity in asthma model**

**OVA sensitization/challenge and drug treatment**

Animal are exposed to OVA during 28days according to the procedure described by [27].

Eight groups of 6 mice are formed. On days 0, 3, 7 and 21, all animals were sensitized by intraperitoneal injection of 100 µl of a mixture of ovalbumin and aluminum hydroxide (50 µg+ 2mg) in 0.9% NaCl). On days 24, 25, 26 and 27, the mice were challenged by intranasal route with 12.5 µl of ovalbumin (0.4mg/ml) except the control group which received saline. Two hours before the challenge, animals were treated orally (5ml/kg) with distilled water (Normal and OVA groups), Crude extract (250 and 500mg/kg), Supernatant fraction (125 and 250mg/kg), pellet (250mg/kg) and betamethasone (2mg/kg).

**Bronchoalveolar lavage and inflammatory cell count**

On day 28, 18 to 20 hours after the last challenge, bronchoalveolar lavage was performed via trachea cannulation. For each mouse, part of the BALF served for total leukocyte count using malassez cell. The rest is centrifuged and the cells pellet stained with May-Grunwald-Giemsa for differential cells count of eosinophils and neutrophils based on morphological criteria.

After bronchoalveolar lavage, the lungs of the animals were removed and used for antioxidant assays and histological studies.
Assessment of in vivo antioxidant activity

Lipid peroxidation measurement

To 1.3ml of activated phenylindole solution, 500 µL of standard MDA or lung homogenate and 300 µL of concentrated HCl (37%) are added respectively. After incubation for 1 hour, the contents of each tube are centrifuged at 4000 rpm for 10 minutes and the absorbance is read at 586 nm. The absorbance of each homogenate is referred to the standard MDA curve to determine the MDA concentration (nM) in each lung [28]. Total proteins amount was assessed in each homogenate using Bradford method. Finally, the MDA concentration in each organ is divided by the total protein amount in the organ to obtain the concentration of MDA/mg protein. 2 assays are performed for each homogenate.

Glutathione assay

To 50 µl of lung homogenate or GSH range (100 – 400µg/ml), 150 µl of Tris buffer (0.2 M; pH = 8.2) and 10 µl 5,5'-dithiobis-2-nitrobenzoic acid (0.01 M) were added. The mixture was completed to 1 ml with 790 µl of absolute methanol. All tubes were homogenized and incubated for half hour. They were then centrifuged at 3000 rpm for 15 min. The absorbance was read at 412 nm after five (5) minutes of incubation. The GSH level in each organ is divided by the total protein amount. 2 assays are performed for each homogenate [20].

Statistical analysis

All the data are expressed as mean±SEM (n = 6). Statistical analysis of the values was performed using one-way ANOVA followed by Tukey’s multiple comparison test. GraphPad prism software (USA) version 6.02 was used for this analysis. P value < 0.05 was considered to be statistically significant.

RESULTS

Fractionation

The fractions obtained after separation in 75% ethanol of the Maytenus senegalensis extract (MSE) are the supernatant fraction (SF) and the pellet (Pe), with yields of 68.53% and 14% respectively.

Phytochemical studies

Phytochemical screening

The results of the staining tests carried out on the hydro-ethanolic extract of Maytenus senegalensis’s fractions are presented in the table1.

Table 1: Chemical groups in the fractions of hydroethanolic extract of M. senegalensis.

<table>
<thead>
<tr>
<th>Phytochemical groups</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins/ Triterpens</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthracenes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Absent; + Present

Quantitative determination of total phenols, tannins and flavonoids

Total phenols and tannins quantities present in the extract and its fractions were determined using the gallic acid range. Rutin was used to determine total flavonoids (Table 2).

Table 2: Amounts of total phenols, tannins and flavonoids in the hydroethanolic extract of M. senegalensis and its fractions.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Phenols (mgGAE/g)</th>
<th>Tannins (mgRE/g)</th>
<th>Total Flavonoids (mgRE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE</td>
<td>68.75 ± 0.25</td>
<td>52.75 ± 1.25</td>
<td>29.50 ± 1.5</td>
</tr>
<tr>
<td>SF</td>
<td>67.50 ± 0.50</td>
<td>62.05 ± 0.45</td>
<td>57.5 ± 0.5</td>
</tr>
<tr>
<td>Pe</td>
<td>14.50 ± 2</td>
<td>4.25 ± 0.75</td>
<td>59.75 ± 1.25</td>
</tr>
</tbody>
</table>

The results represent Mean ± SEM (n =3). GAE = gallic acid equivalent RE = rutin equivalent. MSE= M. senegalensis extract; SF= Supernatant Fraction; Pe= Pellet

In vitro antioxidant tests

DPPH test

The DPPH test showed that the extract and its fractions are all able to reduce this radical. When compared, this antiradical effect is more pronounced in the supernatant and weaker in the pellet. Ascorbic acid used as reference molecule showed a better reducing activity of the DPPH radical (Table 3).

Table 3: Respective IC50s of ascorbic acid, hydro-ethanolic extract of M. senegalensis and its fractions showing their DPPH-reducing effect.

<table>
<thead>
<tr>
<th>M. senegalensis</th>
<th>Ascorbic acid</th>
<th>MSE</th>
<th>SF</th>
<th>Pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>26.20</td>
<td>152</td>
<td>87.10</td>
<td>372.78</td>
</tr>
<tr>
<td>MSE</td>
<td>152</td>
<td>87.10</td>
<td>372.78</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>87.10</td>
<td>372.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pe</td>
<td>372.78</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DPPH= 1,1-diphenyl-2-picrylhydrazyl; IC50= half concentration of DPPH reduction; MSE= M. senegalensis extract; SF= Supernatant Fraction; Pe= Pellet

Reducing power

This test reveals that the hydroethanolic extract of M. senegalensis and its fractions have a low reducing potential of potassium ferricyanide compared to that of ascorbic acid. When compared, the supernatant showed the highest reducing power (Fig 1).

Figure 1: Evolution of absorbance with concentrations showing the reducing potential of the hydroethanolic extract of M. senegalensis and its fractions.

Each value represents Mean ± SEM, n = 3.

Total antioxidant capacity

The range of ascorbic acid was used to determine the total antioxidant capacity of the extract and its fractions. From the results obtained, the TAC of the crude extract is slightly higher than the one of the supernatant fractions. The pellet also has an important antioxidant capacity but less than the SF and the MSE (table 4).
Table 4: Total antioxidant capacity of the M. senegalensis extract and its fractions

<table>
<thead>
<tr>
<th></th>
<th>MSE</th>
<th>SF</th>
<th>Pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAC (mgAAE/g)</td>
<td>157.5 ± 0.5</td>
<td>150 ± 1.0</td>
<td>105.5 ± 0.5</td>
</tr>
</tbody>
</table>

The values represent Mean ± SEM (n =3). TAC= Total antioxidant capacity; AAE= ascorbic acid equivalent; MSE= M. senegalensis extract; SF= Supernatant Fraction; Pe= Pellet.

Antiinflammatory activity of MSE and its fractions

**Effect on inflammatory cells in BALF**

Treatment with MSE and its fraction decreased inflammatory cells number which were significatively enhanced in BALF with OVA sensitization (p<0.01; p<0.001). Total leukocytes reduction is significant in MSE 500mg/kg (50.68%) and SF 250mg/kg (62.10%) groups (p<0.01; p<0.001) while eosinophils and neutrophils numbers are significantly decreased in all treated groups (p<0.05; p<0.01; p<0.001). Comparatively, SF 250mg/kg induced the best reduction of cells number in BALF (91.61% eosinophils and 71.47% neutrophils). Betamethasone was used as reference drug (fig 2).

![Figure 2](image)

**Histology**

The histological examination of the lung sections showed that exposition to OVA promoted cells infiltration and mucus production when compared to the normal group which did not shows any significant damage. Inflammatory damages are less expressed in all treated groups compared to OVA (fig 3).

![Figure 3](image)

**In vivo effect of MSE and its fractions on lung lipoperoxidation and glutathione level.**

The figure 4 showed that OVA exposure increased MDA concentration in lung (p<0.001). The extract and its supernatant fraction significantly reduced this concentration in treated animals (p<0.05; p<0.01; p<0.001). The GSH level which is significantly reduced (p<0.001) in OVA group have increased in MSE and its SF groups (p<0.05; p<0.01; p<0.001). These antioxidant effects are more expressed in the SF 250mg/kg group: 70.77% reduction of MDA and 332.24% augmentation of GSH amount when compared to OVA group.
Inflammatory cells
oxidant
lipid peroxidation (LPO) intermediates and views in Fe3+/ferricyanide act as antioxidants donors which can reduce l fraction. Compounds which have reducing potential are electrons complex. The results of the TAC test revealed that the total antioxidant showed their ability to reduce DPPH and th fractions both in vitro and in vivo. The study also investigates the antioxidant effect of the extract and its fractions control in asthma remain to be assessed.

5, IL the airway by down regulating of Th2 cells activation and/or IL4, IL-4, IL-4, IL-4, IL-4, IL-4. The authors declare that there are no conflicts of interest on this work.

DISCUSSION
Modelling allergic asthma in mice with OVA results in Th2 response with a significant increase of leukocytes, particularly eosinophils in the lung tissue [29]. The present study evaluates the effect of the extract and its ethanolic fractions on inflammatory cells infiltration in mice airways after 28days exposition to OVA. The MSE and its fractions significantly reduced inflammatory cells influx into the airways, particularly eosinophils and neutrophils. This antiinflammatory effect of the extract is corroborated by the histological evaluation.

Airway eosinophilia is the key feature of allergic airway inflammation. Through the release of their products, eosinophils are associated to airway hyperresponsiveness, mucosal and epithelial damages. Neutrophils number also increase in some asthmatics airways and is associated to the severe form of the disease [1, 6]. Inflammatory cells activation and influx in airway is controlled by bioactive mediators such as IgE, cytokines and chemokines mainly coordinated by Th2 lymphocytes [4]. In allergic asthma, Th2 cytokines essentially IL-4, IL-5, and IL-13 and eosinax are the main molecules implied in Th2 cells activation and IgE production, mast cells activation, neutrophils and eosinophils recruitment and degranulation [30]. Several studies show that the attenuation of inflammatory cells infiltration into the airway is correlated to the reduction of these Th2 cytokines, eosinax and IgE level and enhancement of INFγ, a Th1 cytokine in the BALF [31, 32, 33, 34]. The MSE and its fractions would have reduced inflammatory cells influx in the airway by down regulating of Th2 cells activation and/or IL-4, IL-5, IL-13 and eosinax expression or rather by promoting Th1 cells activation. The precise inflammatory parameters which the extract and its fractions control in asthma remain to be assessed.

The study also investigates the antioxidant effect of the extract and its fractions both in vitro and in vivo. In vitro, the extract and its fractions showed their ability to reduce DPPH and the Fe3+/ferricyanide complex. The results of the TAC test revealed that the total antioxidant activity of the crude extract is almost entirely found in its supernatant fraction. Compounds which have reducing potential are electrons donors which can reduce lipid peroxidation (LPO) intermediates and act as antioxidants [25]. In vivo, the MSE and its SF significantly reduced LPO and enhanced glutathione content in lung. Evidence that oxidative stress occur in asthma is provided by an increased level of lipid peroxidation products in the expired air of asthmatics patients [6]. LPO is thought to be the major mechanisms of cells damage and is often used as an index of tissue oxidative stress [35] through the MDA dosage. According to the results, the extract and its SF would have inhibited the LPO process induced in lung by OVA exposition. Glutathione (GSH) is an antioxidant which plays an important role in several respiratory diseases by acting against oxidative inflammation along with other enzymatic/non-enzymatic antioxidants [36]. It is known to protect cells against LPO as its depletion in vivo lead to LPO increase [37]. Thus, by enhancing the GSH level in our model, the MSE and its SF contribute to the reduction of LPO and vice versa. Inflammation and oxidative stress are two separates, but highly interdependent processes in asthma. ROS produced by airway structural cells and recruited inflammatory cells contribute to the persistence of airway inflammation and are correlated with AHR, inflammatory cells infiltration, goblet cell metaplasia, mucus hypersecretion... [38, 39]. Therefore, the antioxidant property of the MSE and its fractions would have contributed to their observed anti-inflammatory effect.

This work corroborated the antioxidant and anti-inflammatory effect of the MSE demonstrated earlier in 8 days’ asthma model [19]. According to the results obtained on the ethanolic fractions, the SF shows more ability to inhibit inflammatory cells influx and oxidative stress at the dose of 250mg/kg while the pellet is almost inactive at the same dose. Quantitative dosages of total polyphenol, tannins and flavonoids show that the amount of the three is higher in the SF. As polyphenolic compounds have antioxidant and anti-inflammatory properties, they would have contributed in the observed effect of the SF. This suggests that the phytochemical compounds (including polyphenolics) involved in the effect of *Maytenus senegalensis* on bronchial inflammation and oxidative stress would be more concentrated in the supernatant fraction. Yet, there is more investigation to do before claiming that the SF is the best form on which the effect of the plant is potentiated.

CONCLUSION
In summary, this study shows that the hydroethanolic extract of *Maytenus senegalensis* and its supernatant fraction inhibit inflammatory cells influx in the airway and oxidative stress in allergic asthma induced in mice. This suggest that the plant would have anti-inflammatory effect in asthma. As airway inflammation is the main feature of allergic asthma and a particular focus in the development of therapeutic agents, *Maytenus senegalensis* would be useful in asthma treatment.

Conflict of Interest
The authors declare that there are no conflicts of interest on this work.

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