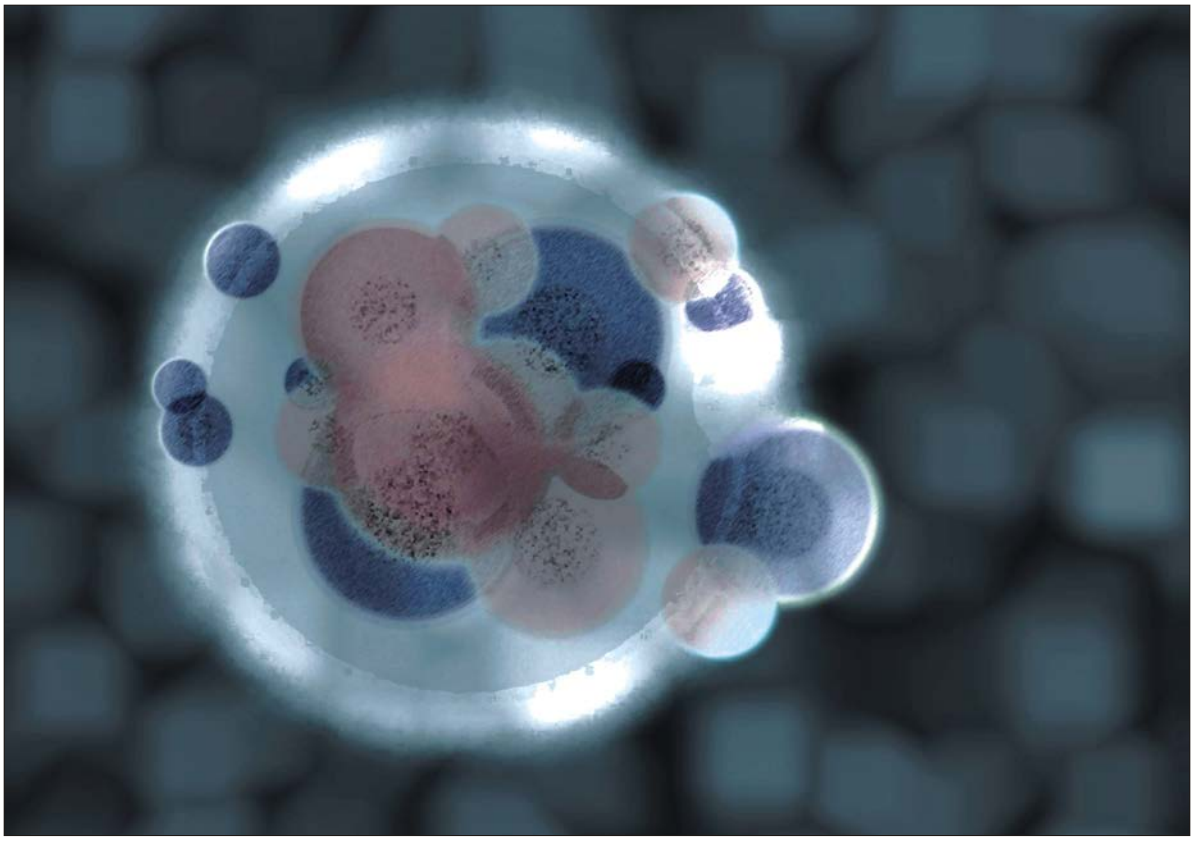

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Antidermatophytic Activity and Dermal Toxicity of Essential Oil from the Leaves of *Ageratum houstonianum* (Asteraceae)

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Abstract: In order to assess the antidermatophytic activity and the acute dermal toxicity of *Ageratum houstonianum* Mill., the essential oil was obtained from the leaves by hydrodistillation and tested against two clinical isolates of dermatophytes: *Microsporum gypseum* and *Trichophyton mentagrophytes*. The potentialisation of activity between griseofulvin and the essential oil was evaluated. The proportions of essential oil/griseofulvin tested were 8:2 and 10:1 (w/w). Also, the acute dermal toxicity of this oil was evaluated on guinea-pigs (*Cavia porcellus*) using a standard method. Agar dilution method with serial dilution of the oil and the mixtures was used for antidermatophytic tests. The percentage inhibition of the fungal growth at each concentration was determined. The MIC of the essential oil was 80 μg mL^{-1} for the tested dermatophytes. That of the 8:2 mixture was 20 μg mL^{-1}, whereas those of the 10:1 mixture were 8 and 10 μg mL^{-} for *Microsporum gypseum* and *Trichophyton mentagrophytes*, respectively. Compared to the control group, we noted no diarrhoea, no change in treated skin and on the fur appearance. In the contrary, the degree of sensitivity to noise, reaction to pinch, activity (locomotion) and reactivity decreased with increase in the dose. The LD_{50} was determined to be 5 g kg^{-1} b.wt. These data suggest that the essential oil of the leaves of *Ageratum houstonianum* contains antidermatophytic compound(s) and may not be toxic when used topically. A potentialisation effect was observed between the essential oil and griseofulvin.

Key words: Antidermatophytic, essential oil, *Ageratum houstonianum*, dermal toxicity, potentialisation, griseofulvin

INTRODUCTION

One of the most important preoccupations of mankind has been to fight against diseases. Unfortunately, during the last decades, the number of people suffering from infectious diseases has seriously increased (Clark and Hajjeh, 2002), principally due to the development of resistant strains of microorganisms (Bouree, 2008) and poverty that limit access to drugs (Sanglard and odds, 2002). Among these infectious diseases are dermatophytoses, the infections of the skin, hair or nails caused by a group of related filamentous fungi called dermatophytes (Feuilhade *et al*., 2002; Chen *et al*., 2004). These fungi attack the keratinized tissue of living host and belong to three genera: *Microsporum*, *Trichophyton* and *Epidermophyton* (Zacchino *et al*., 1998). A wide range of antifungal drugs (griseofulvin, mycostatin, terbinafin, kétoconazol) is available. However, their spectrum of action is narrow and also many of them have adverse side effects (Lorougnon *et al*., 1991). The treatment of these infections is long and expensive. This may explain why in developing countries, peoples use plants to fight against these types of diseases. Among the plants used in Cameroon is *A. houstonianum*, an annual or biannual herb belonging to the Asteraceae family. This plant is widely used in traditional medicine in many countries of the world. In Cameroon and Senegal, the juice of the leaves is instilled into the eyes to fight against conjunctivitis and ophthalmias and is used against skin diseases. Previous studies on this plant species showed the presence of chromenes and benzofurans in the leaves (Siebertz *et al*., 1990) as well as pyrrolizidine alkaloids (Wiedenfeld and Andrade-Cetto, 2001). The main constituents of an essential oil sample from the leaves of *A. houstonianum* collected in the town of Dschang in 1992 were found to be

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β-caryophyllene (27.5%), precocene I (32%) and precocene II (24%) (Menut *et al*., 1992). Precocene II was found to be fungistatic (Iqbal *et al*., 2004). To the best of our knowledge, no antidermatophytic activity of the essential oil of *A. houstonianum* has been studied against *Microsporum gypseum* and *Trichophyton mentagrophytes*.

This study was undertaken to assess the antidermatophytic activity and the acute dermal toxicity of the essential oil of the leaves of *A. houstonianum* in view of ascertaining its use in the treatment of dermatophytoses.

MATERIALS AND METHODS

Plant material and oil extraction procedure: Fresh leaves of non-flowered *A. houstonianum* were harvested in Dschang University campus (West-Cameroon) in May 2006. This plant was identified by Mr. Tadjouteu at the Cameroon National Herbarium (Yaoundé) where a voucher sample (No. 3114/39564/HNC) was deposited. Batches of 3 kg of fresh leaves were submitted to hydrodistillation for 4 h using a Clevenger-type apparatus. The essential oil obtained was dried over a column of anhydrous sodium sulphate.

Antifungal assay: The tested fungi were clinical isolates of dermatophytes obtained in the Laboratory of Microbiology and Antimicrobial Substances (LAMAS) of the University of Dschang and identified in collaboration with Centre Pasteur in Yaoundé. The species concerned were *Microsporum gypseum* and *Trichophyton mentagrophytes*. The antidermatophytic activity of the oil was evaluated using the agar dilution method as reported by Zacchino *et al*. (1998). A stock solution of essential oil (1 mg mL^{-1}) was prepared using 10% Tween 20 solution in a final volume of 10 mL. Dilutions were made in such a way that, by mixing 1 mL of diluted solution with 9 mL of medium, the concentrations to be tested (obtained) were 5, 10, 20, 40, 80 and 160 μg mL^{-1}. Four Petri dishes (55 mm of diameter) were prepared for each oil concentration and the control (culture without oil). Griseofulvin was used as the reference drug and tested at 0.25, 0.5, 1, 2, 4, 5, 8, 10 and 20 μg mL^{-1}. Each Petri dish was inoculated with a colonial disc (6 mm in diameter), cut from the periphery of a 10-days old culture of dermatophytes. The plates were incubated at 30°C for 10 days. Diameters of the fungal growth (colonies) were measured every day. The percentage inhibition of the fungal growth was calculated at each dilution considering the control as 100% growth. The MICs were defined as the minimum concentration of oil that prevented visible growth of the tested fungal isolates (Zacchino *et al*., 1998). The fungicidal/fungistatic nature of the essential oil was determined by sub-culturing the fungal disc whose growth was entirely inhibited by the oil into fresh medium. Any revival of fungal growth within 2 weeks indicated fungistatic effect, while no growth implied fungicidal effect of the oil. For the potentialisation effect evaluation between oil and griseofulvin, a mixture of the essential oil and griseofulvin in the ratios 8:2 and 10:1 (w/w) was prepared and tested in the same concentrations as that of griseofulvin. The percentages of inhibition were then compared to those of pure essential oil and pure griseofulvin.

Acute dermal toxicity: Twenty male guinea pigs each of 3 months old and weighing 350-400 g were used. They were randomly divided into four groups of five animals each. The animals were housed individually in Perspex cages at the Research and Applied Farm of the Faculty of Agronomy and Agricultural Sciences of the University of Dschang. The OECD method (ECL, 1981) was used to evaluate the acute dermal toxicity. A single administration of the chosen doses of essential oil, 2.5, 5 and 10 g kg^{-1} of body weight, in a final volume of 1 mL was done on the glabrous skin (9 cm^2 representing about 10% of the total body surface) of the test animals in group 2, 3 and 4, respectively. The essential oil was prepared in palm kernel oil for various doses. Palm kernel oil only was applied to the control animals (group 1). The animals were observed daily for fourteen days to detect possible behavioural changes including salivation, appearance of the treated skin, respiration, state of the excrement, sleep, appearance of the fur, activity (locomotion), sensitivity to noise, reaction to pinch and reactivity. The liver, kidneys and heart of dead animals and those sacrificed at the end of the observation period were removed and observed to detect possible morphological and colour changes. The blood of the survival animals were collected for haematological and biochemical studies. The LD_{50} was determined using the method of Behrens and Karber (1983). Blood cell count and haematocrit were evaluated according to the method of WHO (1993). The serum total protein levels were determined by the Biuret method (Gornal *et al*., 1949) whereas the serum total creatinin level was evaluated by the method described by Newman and Price (1999). The organs were cleaned and crushed separately in 5 mL of NaCl 0.9% and the homogenate obtained was centrifuged at 5000 rpm for 15 min. The volume of the supernatant recovered was measured and the protein levels determined.

Statistical analysis: The statistical analysis was done using the one way Analysis of Variance (ANOVA). The Waller-Duncan test was used to compare the means in different groups at 5% significant level.

Ethics: The experiments were carried out respecting the welfare of animals as recommended by WHO (1993). Moreover, all procedures involving animals were carried out in strict compliance with the local ethical committee rules and regulations.

RESULTS

Antifungal effects: In contrast to griseofulvin and mixtures, the inhibitory effect of the essential oil of *A. houstonianum* on the fungal isolates decreased with time irrespective of the fungal isolate. In addition, this inhibitory effect increased as the concentration of essential oil increased (Table 1, 2). It was noted that at 5 μg mL^{-1} of essential oil, percentage inhibition was negative (-17.83%) meaning that, at that concentration, the radial growth was more than that in the control (i.e., culture without oil).

Table 1: Inhibition percentages on *Trichophyton mentagrophytes* by the essential oil of *Ageratum*

Substances	Concentration (μg mL^{-1})	%I±SEM
Essential	5	-17.83±2.28^{a}
	10	6.06±1.58^{b}
	20	34.56±3.19^{cd}
	40	61.41±3.61f^{ghij}
	80	100.00±0.00^{m}
	160	100.00±0.00^{m}
Griseofulvin	0.25	43.48±2.71^{defgh}
	0.5	58.25±2.70^{efghi}
	1	58.52±2.13^{efghij}
	2	71.26±1.83^{hijkl}
	4	60.66±2.20^{ghijk}
	5	61.64±2.26^{ghijk}
	8	88.91±1.11^{ijkl}
	10	91.05±1.32^{jkl}
	20	100.00±0.00^{m}
Mixture 8:2	0.25	30.76±4.58^{c}
	0.5	31.77±3.28^{cdef}
	1	48.34±2.71^{efgh}
	2	66.06±1.73^{ghijk}
	4	82.32±1.72^{ijkl}
	5	87.74±1.59^{ijkl}
	8	88.36±1.51^{ijkl}
	10	91.62±0.36^{kl}
	20	100.00±0.00^{m}
Mixture 10:1	0.25	36.76±3.84^{cdefg}
	0.5	38.79±3.31^{cdefg}
	1	46.89±3.49^{efgh}
	2	63.52±2.58^{fghijk}
	4	84.92±0.35^{ijk}
	5	88.62±1.60^{ijk}
	8	99.27±0.21^{m}
	10	100.00±0.00^{m}
	20	100.00±0.00^{m}

Tabulated values are Mean±SEM of 3 determinations. In the columns, values affected by the same letter(s) are not significantly different at 5% significance level (Test of Waller-Duncan)

Similar effect of essential oil on the 2 dermatophytes was observed, with MIC values of 80 μg mL^{-1} on both isolates.

As far as the antifungal activity of the mixture in 8:2 proportion is concerned, the susceptibility of the two isolates was the same with regards to its MIC (20 μg mL^{-1}) on them. *T. merntogrophytes* was less sensitive (MIC = 10 μg mL^{-1}) to the mixture 10:1 than *M. gypseum* (MIC = 8 μg mL^{-1}). In general, the mixture 10:1 was more active than 8:2 (Table 1, 2). As shown by Table 1 and 2, the essential oil at 5 μg mL^{-1} exhibited an inhibitory effect of -17.83 and 18.71% on *T. mentagrophytes* and *M. gypseum*, respectively. On the other hand, griseofulvin at 0.5 μg mL^{-1} exhibited an inhibitory effect of 58.25 and 59.93% on *T. mentagrophytes* and *M. gypseum,* respectively. But when the two substances were mixed in 8:2 or 10:1 proportions, the mixture exhibited an inhibitory effect of 87.74 or 88.62% and 82.47 or 93.83% at 5 μg mL^{-1} on *T. mentagrophytes* and *M. gypseum*, respectively.

Table 2: Inhibition percentages on *Microsporum gypseum* by the essential oil of *Ageratum houstonianum* and other substances tested as a function of concentration

Substances	Concentration (μg mL^{-1})	%I±SEM
Essential oil	5	18.71±1.44^{a}
	10	38.58±1.17^{c}
	20	64.63±2.43^{j}
	40	84.73±2.34^{o}
	80	100.00±0.00^{t}
	160	100.00±0.00^{t}
Griseofulvin	0.25	50.63±2.57^{g}
	0.5	59.93±0.91^{i}
	1	61.30±0.94^{i}
	2	69.55±2.04^{k}
	4	80.12±1.28^{m}
	5	86.46±1.27^{op}
	8	93.06±0.73^{r}
	10	98.34±0.32^{t}
	20	100.00±0.00^{t}
Mixture 8:2	0.25	35.85±3.02^{b}
	0.5	46.01±1.42^{d}
	1	49.15±1.17^{fg}
	2	66.59±2.34^{j}
	4	79.86±1.32^{m}
	5	82.47±1.31^{n}
	8	87.28±1.67^{p}
	10	95.65±0.65^{s}
	20	100.00±0.00^{t}
Mixture 10:1	0.25	47.85±2.03^{de}
	0.5	48.50±2.74 ^{ef}
	1	54.95±1.69^{h}
	2	72.83±1.83^{l}
	4	90.76±0.91^{q}
	5	93.83±0.85^{rs}
	8	100.00±0.00^{t}
	10	100.00±0.00^{t}
	20	100.00±0.00^{t}

Tabulated values are Mean±SEM of 3 determinations. In the columns, values affected by the same letter(s) are not significantly different at 5% significance level (Test of Waller-Duncan)

Acute dermal toxicity: Effect of essential oil on the guinea-pigs (*Cavia porcellus*): Compared to the control group we noted no diarrhoea, no change in treated skin and on the fur. On the contrary, the reaction to noise, reaction to pinch, activity and the reactivity decreased with increase in the dose.

In addition, as far as group 4 animals were concerned, a little foam was also noticed from the mouth of some of the animals a few hours before death. Moreover, in group 3 and 4, some of these animals showed somnolence and presented signs of tiredness. In general, the food and water intakes decreased with time but after 8 days, for survival animals, the foods and water intakes increased with time although the total amount of food and water taken by these guinea pigs was still lower than that taken by the control group.

This situation could be confirmed by the weight variations observed in these animals (Fig. 1).

The dermal LD_{100} and LD_{50}: The mortality was observed over 48 h after a single oral administration of the essential oil to guinea pigs. The LD_{50} of the essential oil was 5 g kg^{-1} of body weight. The LD_{100} was considered to be 10 g kg^{-1}, corresponding to the dose which killed all the animals of group 4.

Anatomical examination of the organs: A macroscopic examination of the test organs compared to the control showed a slight darkening of the liver, kidneys and heart with an intensity which increased with the doses (Fig. 2a-c).

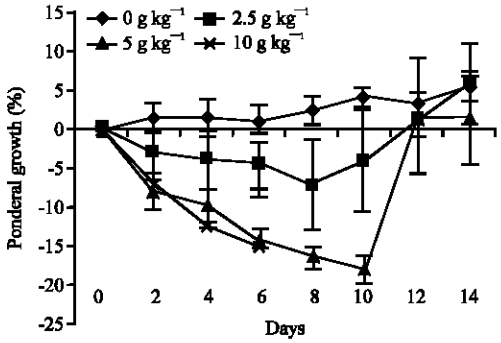

Fig. 1: Ponderal growth (%) of animals as a function of the dose of essential oil of *A. houstonianum*

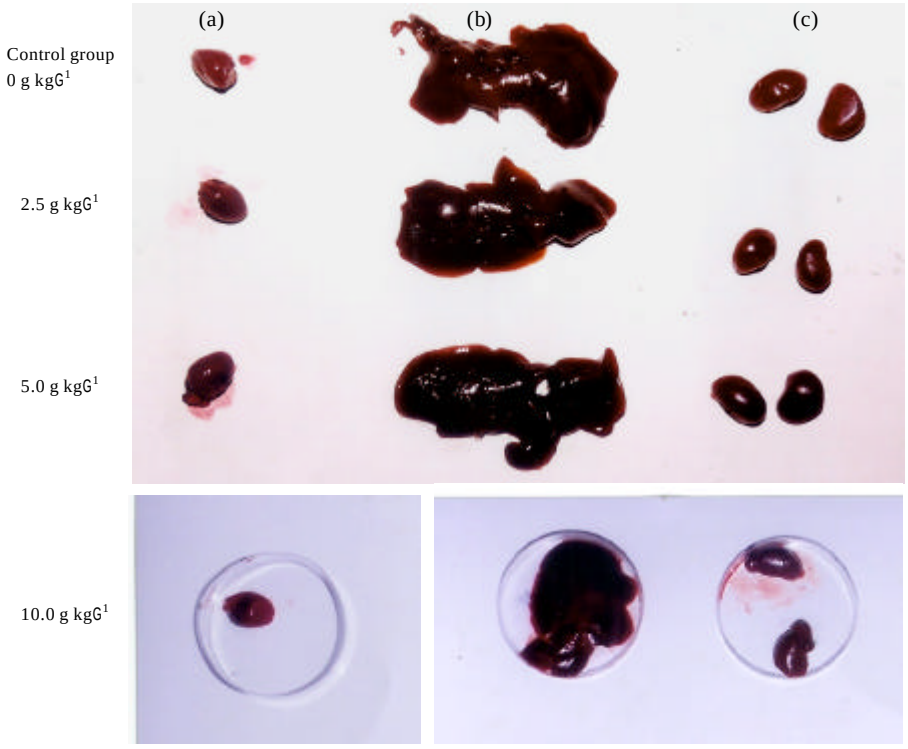

Fig. 2: Photographs presenting the effects of the essential oil of *A. houstonianum* (acute dermal administration) on the organs of the animals of the various groups, (a) Heart, (b) liver and (c) kidney

Table 3: Effects of essential oil of *A. houstonianum* on the rates of blood cells and of haematocrit of the guinea-pigs

Groups	N	Doses (mg kg^{-1})	Red blood cells±SEM	White blood cells±SEM	MH (%) ±SEM
1	4	0	4131225±189074^{a}	3550±288^{a}	45.48±1.78^{a}
2	3	2500	5878867±1218541^{a}	3034±197^{a}	45.25±1.50^{a}
3	2	5000	5326650±33550^{a}	3625±18^{a}	50.24±0.00^{a}

In the columns, values affected by the same letter(s) are not significantly different at 5% significance level (Test of Waller-Duncan); N: No. of animals; MH: Average of haematocrits

Table 4: Effects of essential oil of *A. houstonianum* on the serum and organ protein levels and on the serum creatinin level of the guinea-pigs

Doses (mg kg^{-1})	N	Serum P. (g L^{-1})	Hepatic P. (mg g^{-1})	Renal P. (mg g^{-}1)	Cardiac P. (mg g^{-1})	Creatinin (g L^{-1})
0	4	96.22±2.90^{a}	101.89±17.32^{a}	94.87±7.25^{a}	65.29±3.02^{a}	0.036±0.004^{a}
2500	3	91.62±2.76^{a}	109.37±19.95^{a}	91.46±6.36^{a}	65.49±4.89^{a}	0.026±0.013^{a}
5000	2	98.61±0.14^{a}	165.73±0.48^{a}	93.76±0.98^{a}	78.97±0.98^{a}	0.025±0.005^{a}

In the columns, values affected by the same superscript are not significantly different at 5% significance level (Test of Waller-Duncan); N: No. of animals; P: Protein

Haematological and biochemical parameters: The acute dermal administration of the essential oil of *A. houstonianum* did not cause any significant ($p>0.05$) modifications of the levels of red blood cells, white blood cells and haematocrite in the oil-treated animals compared to the control (Table 3). Also, the essential oil did not have any significant effect on the various protein levels in the serum and in the organs as well as the creatinin concentration (Table 4).

DISCUSSION

Antifungal effects: The essential oil of *A. houstonianum* showed inhibitory effect on the fungal isolates. This inhibitory effect could be mainly attributed to β-caryophyllene, precocene I and precocene II known to be its main constituents (Menut *et al*., 1993).

The decreased in inhibitory effect of the essential oil of *A. houstonianum* on the fungi with time irrespective of the fungal isolate was observed. This result corroborates those of Zacchino *et al*. (1998) and Barrera-Necha *et al*. (2009), who attributed it to the evaporation of certain active components of the essential oil, a natural or fungi-induced degradation of the active principle responsible for the antifungal activity, or the development of an adaptation by the fungus to its new environment, characterized by the hostility of the active molecules of the tested substance. This adaptation can be done either through the modification of the active ingredient or its target, through the reduction of the access of this active ingredient to its target or through the combination of these mechanisms (Ghannoum and Rice, 1999). It is also characterized very often, by morphological changes of the cells of the fungus and the development of resistance structures (Ghannoum and Rice, 1999). This decrease in activity with time shows that if this essential oil is to be used for a treatment, it should be applied in a repeated manner (Valnet, 1980).

The radial growth which was more than that in the control at 5 μg mL^{-1} of essential oil was previously reported by Pfaller *et al*. (2004). According to them, this poses the problem of judicious choice of concentration of an antifungal compound sufficient enough to treat a dermatophytose, since an inadequate choice may contribute to the development of resistant strains.

The difference in sensitivity of *T. mentogrophytes* to the mixture 10:1 compared to *M. gypseum* could be due to the difference in their genetic constitutions (Kopecek *et al*., 1999).

The difference in activity of the mixture 10:1 and that of 8:2 could mean that the active principle present in the essential oil is highly potentiated in presence of a small amount of griseofulvin. This is in line with the results obtained by Giordani *et al*. (2006) with the essential oil from *Cinnamomum cassia*.

Acute dermal toxicity: Effect of essential oil on the guinea-pigs (*Cavia porcellus*): Reduced activity, reactivity and reaction to noise observed in treated animals compared to the control group were previously obtained by Gatsing *et al*. (2005) with the aqueous extract of *Allium sativum* bulbs. This result could suggest that the essential oil of *A. houstonianum* have a depressant or sedative effect on the central nervous system (Gatsing *et al*., 2009) at high doses. This oil may act as myorelaxant or tranquiliser on the nervous centres or on the motor fibres (Schmitt, 1973).

The reduction of reaction to pinch observed may be due to its action on the nociceptors or to the inhibition of the production of algogenic substances (e.g., prostaglandins, histamines), or to the inhibition of the painful message transmission, at the central level (Gatsing *et al*., 2010).

The foam noticed from the mouth of some of the animals of group 5 a few hours before death, the somnolence and signs of tiredness showed by some

animal of group 3 and 4 would result from a shock related to the administration of essential oil at high doses.

The dermal LD_{100} and LD_{50}: With a LD_{50} of 5 g kg^{-1}, in acute dermal administration, the essential oil of the fresh leaves of *A. houstonianum* could be considered almost not toxic based on the scale of Hodge and Sterner (Delongeas *et al*., 1983).

Anatomical examination of the organs: The change in colour of the organs of these animals could have resulted from the deleterious effects of the essential oil on these animals. These effects were comparable with those obtained by Goyal and Kadnur (2006). Through oral administration of high doses of essential oil of *Zingiber officinalis* Roscoe to Wistar albino rats. It is worth noting that essential oils have high concentrations of active compounds and consequently act therapeutically at very low dose (Valnet, 1980).

Haematological and biochemical parameters: The absence of significant effects observed for haematological and biochemical parameters could suggest that the essential oil of *A. houstonianum* has no effect on haematopoiesis and on the white blood cells synthesis. As far as serum protein level is concerned, it was indicated that any increase is an indicator of tissues damage and a significant decrease in hepatic protein level is an indicator of hepatotoxicity (Gatsing *et al*., 2005).

Creatinin result from metabolism of creatin in the skeletal muscles. In normal physiological conditions, it is filtered through the kidney and rejected in urine. Any increase in its serum concentration is an indicator of kidney damage (Abcar *et al*., 2004). According to James and Kathleen (1992), the measure of serum creatinin level can also permit to appreciate muscle mass. The results obtained in this study did not show any significant changes in the serum creatinin level of the treated animals, compared to the controls. Therefore, the essential oil of leaves of *A. houstonianum* may not affect the kidney function and muscle mass of guinea pigs after topical application.

In the light of the above, it is clear that these results confirmed the traditional use of the juice of the plant leaves to fight against skin diseases. It is also noted that if this essential oil is to be used for a treatment, it should be applied in a repeated manner.

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