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Research Article

Formulation and Evaluation of Safety and Antifungal Efficacy of *Syzigium Aromaticum*-Base Cream on Guinea Pigs Infected with *Trichophyton Mentagrophytes*

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ABSTRACT

Background: The increasing incidence of dermatophytoses in the world and the side effects of the current therapies encouraged the search of alternative drugs. Hence the objective of this work was to determine antidermatophytes activity of *Syzigium aromaticum* formulate antidermatophytic cream. **Materials and Methods:** The extracts were prepared by maceration of plant materials into methanol. Three formulations of creams were made, and the best was chosen according to its physicochemical stability and appearance. The acute dermal toxicity and antidermatophytic efficacy of the cream was performed on guinea-pig. **Results:** The methanolic extract of *S. aromaticum* was incorporated in the final cream formulation. The formulation containing shea-butter 58.5%, acetylic alcohol 2.5%, stearic acid 1.5%, bee-wax 10%, borax 1.5%, polysorbate 60 2.5%, 2 drops of lactic acid and water was chosen because of its good appearance and stability. The cream with methanolic extract of *S. aromaticum* did not reveal any dermal toxic effect. The cream efficacy was dose-dependent. The treatment with cream at 5% methanolic extracts of *S. aromaticum* revealed the best potency after 14 days of treatment. **Conclusion:** These results show that the cream at 5% methanolic extract of *S. aromaticum* seed is promising in the treatment of dermatophytoses and could be used as an alternative in the development of a new therapy.

Key Words: Dermatophytes, Antidermatophytes activity, Formulation, Cream, *S. aromaticum*, Toxicity.

INTRODUCTION

Dermatophytes are microscopic filamentous fungi which have an affinity for keratin. They affect the skin, nails or hair and cause superficial lesions in humans called dermatophytosis. Afflictions are among the most common forms of superficial mycosis in the world.[1] Despite many climatic factors offering enormous potential for development, their contagiousness varies according to the species responsible. The incidence increases overnight.[2] Dermatophytosis is the major cause of morbidity associated with superficial mycoses, with frequent relapses often refractory to therapy.[3]

Today, many conventional medicines are used for the treatment of these lesions. However, the impact of dermatophytosis is always increasing in the world.[4,5] Thus, the search for new therapeutic alternatives for better management is necessary. Therefore, to combat newly borne spectrum of fungal infections, step should be taken to make the benefits of successful pharmaceutical research available to all and especially to those who are in the greatest need. In fact, it is the need of hour to search for new antifungal agents of herbal origin which are relatively economically affordable, safer and easily available to common men. The World Health Organization (WHO) estimates that up to 80 percent of the world's population in general, and in particular the African population, use traditional herbal medicine to meet their health needs, because of their accessibility and reduced cost.[6] Their use for the treatment of skin infections is a historical practice in most countries in the world.[7]

Only 10 to 20% of the world's flora has been studied from a therapeutic point of view and the potential of this natural resource remains very important. Moreover, a perusal of literature indicates that many investigators have been reported fungi static and bacteriostatic properties of phytochemicals of higher plants such as *Syzigium aromaticum*. There are many reports on the *in vitro* anti-dermatophytes activity of its essential oil.[8] The essential oil showed is highly antifungal towards *M. gypseum* and *T. rubrum* with inhibition zone diameter ranging from 12-22 mm and MIC value of 9 µl/ml.[9] Besides, Park *et al.*[10] identified that eugenol is the most effective antifungal constituent against the dermatophytes *T. mentagrophytes* and *M. canis*. As well, the association of *S. aromaticum* oleoresin with concentrated sugar demonstrated strong fungicidal effect against *T. mentagrophytes.*[11] Its major constituents, eugenol and nerolidol showed efficacy in a guinea pig model infected by *M. gypseum.*[12]

As far as we know, no attempt has been made to test *S. aromaticum* organic solvent extracts-based cream in animal model. Thus, with the aim of enhancing

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and strengthening the empirical knowledge on medicinal plants of the Cameroonian pharmacopoeia, we evaluated the antidermatophyte activity of *Syzigium aromaticum*-based cream in guinea pigs.

MATERIALS AND METHODS

Plant collection and preparation of total extracts

Syzigium aromaticum (L.) Merr. cloves was bought at the Yaounde market, 8th Market, Cameroon and authenticated at the National Herbarium of Cameroon where specimens are stored under reference number of 1858/SRFK.

Fresh plant parts were dried in the laboratory and grind using commercial miller. Hundred grams (100 g) of each plant powder were macerated either in 1L of methanol for 72 hours with mechanical stirring at room temperature. The mixture was then filtered using Whatman No. 1 paper. The process was repeated several times until the plant material was exhausted (Figures 1 and 2). The resulting filtrates were concentrated on a rotary evaporator (BÜCHI 011) at 80 °C. The crude extracts obtained were weighed and their extraction yield calculated according to the formula below.

$$Extraction\ Yield = \frac{Crude\ extract\ (g)}{Raw\ material\ (g)} \times 100$$

The obtained extracts with a yield of 20.96% was stored at 4°C still the experiment.

Formulation and evaluation of the physico-chemical stability of cream vehicles

Formulation of vehicles

The formulation was made according to the protocol described by Heyam *et al.*[13] with some modifications. The base creams consisting in oil and water phases were prepared: the oily phase composed of beeswax, shea butter, cetyl alcohol, stearic acid, polysorbate 60 and an aqueous phase composed of borax and purified water were incorporated at different concentrations (Tables 1 and 2). Each phase was heated separately to 75 °C and then mixed and stirred for 15 minutes. Three different formulations (F1, F2, F3) were obtained and compared according to their consistency, softness and physicochemical stabilities. The composition and amounts of the formulated ingredients are shown in the following Table 1.

Evaluation of the physico-chemical stability of vehicles

Homogeneity test

The homogeneity of each formulation was tested by visual observation of their classified cream miscibility score using the criteria of Heyam *et al*. 2013 which are as follows: +++ excellence, ++ very good, + good, - no.

Physical stability

A 10 g sample of each formulation was placed in a beaker and stored at room temperature for 3 months. The stability of the emulsion or the absence of coalescence was observed after zero, one and three months of storage.[14]

Determination of pH

A 10 g sample of each formulation was placed in a beaker and stored at room temperature for 3 months. The pH was measured using pH paper after zero and three months of storage. The change in pH that may occur involves the chemical degradation of the cream.[15]

The F3 formulation being the most stable of the three formulations it has chosen to formulate a *S. aromaticum* extract-based cream that was further assess for skin toxicity.

Toxicity and efficacy of *S. aromaticum-based* cream in guinea pig model

Dermal toxicity: Repeated dose

Dermal toxicity was assessed according to OECD protocol No. 410 through repeated daily administration to the animal's skin, the dose of 1000 mg of the cream / kg of body weight over a 21-day period.[16]

Ninety (90) healthy young guinea pigs weighing between 350 g and 450 g (45 males and 45 females) were used to evaluate the skin toxicity of the cream formulation. The guinea-pigs were individually caged and acclimatized to laboratory conditions one week prior to the test, during which time they were fed with their standard diet and received water ad libithum and weighed at the beginning and at the end of the experimentation.

The animals were divided into 3 groups of 10 animals each (one test group, one negative control group and one normal control group) and acclimatized for one week. The test area of the thirty healthy guinea

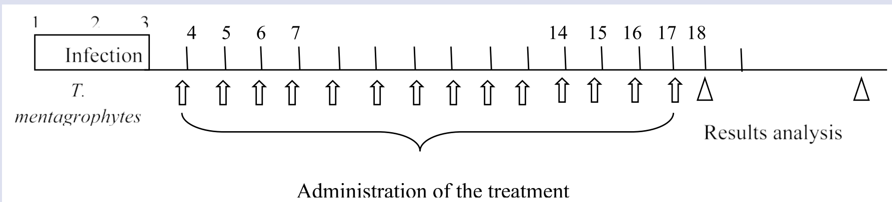

Figure 1: Experimental study of the treatment efficacy on guinea pigs.

Figure 2: Consistency of formulations F1 to F3.

Table 1: Composition (%) of each of Formulations F1 to F3.

Ingredients	Concentration (%)		
	F1	F2	F3
Shea butter	58.5	58.5	58.5
Cetyl alcohol	2.5	2.5	2.5
Stearic acid	1.5	1.5	1.5
Beeswax	8	9	10
Borax	0	1.5	1.5
Polysorbate 60	2.5	2.5	2.5
Lactic acid	0	1 drop	2 drops
Water (solvent)	100	100	100

Table 2: Results of pH, homogeneity test, appearance and stability of formulations.

Properties	Formulations		
	F1	F2	F3
Miscibility Score	+++	+++	+++
Physical stability after zero months	Stable	Stable	Stable
Physical stability after one months	Absence of coalescence	Stable	Stable
Physical stability after three months	Absence of coalescence	Stable	Stable
pH at zero month	~7	~6	~6
pH at three months	~7 *	~6	~6
Appearance	Fluid, soft	Fluid, soft	Thick, soft consistency

* The symbol ~ means that the pH is close to the indicated value; +++ Excellence.

pigs was mowed using an electric clipper, avoiding any skin lesions that could lead to a change in its permeability. This procedure was repeated weekly during the experimentation period.1 ml of cream at 1000 mg / kg body weight was uniformly applied to the shorn dorsal area of the guinea pigs and maintained by means of a dressing porous gauze and plaster 24 hours a day, 7 days a week, for 21 days and observed for any change. The vehicle control group received the negative (cream without extract), while the normal control group received no treatment except shaving.

The volume of the applied cream was determined according the following formula:

$$\mathbf{V}\ (\text{ml}) = \frac{\mathbf{D} \times \mathbf{P}}{\mathbf{C}}$$

V (ml) = Volume of the cream to be applied

P (kg) = Weight of the animal

D (mg / kg) = Dose of the extract

C (mg / mL) = Concentration of the solution to be applied

At the end of the experimentation, the guinea-pigs were observed, weighed and then sacrificed by decapitation. The blood was collected in the dry and heparinized tubes. The liver, kidneys and skin were also collected; weighed and stored in 10% formalin for histo-pathological analyses.

Evaluation of haematological parameters

The blood collected in the heparin tubes served for the assessment of hematological parameters such as leukocytes, lymphocytes, granulocytes, erythrocytes, hemoglobin, hematocrit and platelets were investigated by an automated analyser, Globular counter HYCEL Diagnostics (Celly, type CA 4001 series N °: CA40D 1975) in the laboratory of the Central Hospital of Yaoundé (Labo HDJ).

Evaluation of biochemical parameters

The blood collected in the dry tubes was centrifuged at 5000 rpm during 5 min and the serum recovered used for biochemical assays. The activity of alanine aminotransferase (ASAT) and aspartate aminotransferase (ALAT) in serum was carried out using the SGM Italia GOT-AST and SGM Italia GPT-ALT kits respectively.

100 µl of single reagent previously prepared were introduced into a microplate. To this was added 100 µl of serum samples. The mixture was incubated at 37 ° C. for one minute. The spectrophotometer reading of absorbance at 340 nm from each blood serum sample was performed at 1 min interval for three minutes against white (reactive mixture + distilled water). The ALT activity was calculated from the mean absorbance variation ΔE / min according to the formula below:

ALAT (U / l) = ΔE / min × 1746

ASAT (U / I) = ΔE / min × 1746

Histo-pathological analysis

Histological examinations were carried out at the Laboratory of Histology, Anatomy and Pathology of the Faculty of Medicine and Biomedical Sciences and the Laboratory of Animal Physiology, University of Yaounde 1. The organs viz. liver, kidney and skin were fixed in 10% formalin for histopathological analysis.[17] The sections were cut at 6 mm thickness and stained with hematoxylin and eosin for light microscopic examination of the tissues histoarchitecture.

Evaluation of the effectiveness of the *S. aromaticum*-based cream on guinea pigs infected with T. mentagrophytes

The skin infection model of guinea pigs with *T. mentagrophytes* described by Iwaka *et al.*[18] was used.

Maintenance of dermatophytes and inoculum preparation

Trichophyton mentagrophytes ATCC 4439 from the Laboratory of Biochemistry of the University of Dschang were used. They were maintained in culture by successive sub-culturing every 14 days in the Sabouraud Dextrose Agar medium (SDA) supplemented with chloramphenicol and actidione on slant and incubated at 25 °C.

A spore suspension was prepared by adding 5 ml of physiological solution to a 90 mm diameter petri dish containing five weeks old culture of *T. mentagrophytes*. The obtained suspension was filtered with a No. 120 filter paper which retained debris and allowed the spores to pass. The spore suspension was counted under a microscope; diluted and then adjusted to 10^6 spores / ml.[18] The inoculum thus prepared was used for the infection of animals.

Guinea pig infection

On average, 9 cm^2 of the fur on the left flank of the dorsal region of 60 guinea pigs after being shaved with an electric clipper was rubbed with No. 120 green paper to cause light lesions, and cleaned with the alcohol at 90 ° C. 0.05 ml of previously prepared spore suspension was deposited on the rubbed area and kept in contact with the skin for 24 hours using a non-irritating porous adhesive tape and pad. Macroscopic observations and cultures of Guinea pigs-inoculated areas were carried out to confirm the successful infection three days post-inoculation.

Guinea pig treatment

The infected guinea pigs were divided into 6 groups of 10 animals per group. Groups 1, 2 and 3 were daily treated respectively with 0.2 g of 2.5%, 5% and 10% *S. aromaticum* methanolic extract-based cream respectively. The negative control group 4 received cream formulation without extract; while the normal control, received no treatment. The positive control was treated with Lamisil cream 1%. The treatment was carried out over 14 days during which the animals were observed daily. The effectiveness of the cream was evaluated on two bases: (a) clinical basis by observation of changes at the site of infection every three days from the beginning to the end of treatment. The observed lesion was scored from 0 (absence of lesion) to 4+ (severe lesion equivalent to negative control) according to the Weinstein *et al.*[19] criteria and the mean was calculated as follows:

$$\text{Average Score of lesion} = \frac{\sum_{i=0}^{n} n \times i}{N}$$

N = Number of animals per lesion score

I = Score of injury

N = Total number of animals tested

And (b) on the mycological basis, crusts and cigarette butts taken at the site of infection at the end of treatment and cultured on the SDA medium supplemented with chloramphenicol and actidione allowed us to look for the presence of dermatophytes at the site of infection.[20]

Statistical analyses

The results were analysed by ANOVA, expressed as mean ± SEM (Standard Error of Mean), using the Turkey test at the 5% probability threshold using SPSS 17.0 software.

RESULTS AND DISCUSSION

Formulation and evaluation of the physico-chemical stability of the cream vehicle

Formulations F1; F2 and F3 were made Figure 2 and Table 2 below show the results obtained on the quality and physico-chemical stability of the three formulations. Figure 4 shows that F1 and F2 have fluid appearances whereas F3 has a thicker consistency. By increasing the amount of beeswax, the consistency of the cream is increased[14] and could justify the consistency of F3.

As shown in Table 2 below, the first results show that all three formulations have excellent miscibility.

The pH of F1 approaches 7 and the pH of F2 and F3 approaches 6. Formulations F1 and F2 pHs were adjusted with lactic acid. This pH difference is related to the presence of this acid, according to Moghimipour *et al.*, 2009. After three months of storage, no significant difference in pH was observed, indicating chemical stability [14, 21, 22]. In addition, one month of storage revealed a phase separation of the F1 formulation and no significant change of F2 and F3. This could be explained by the presence of borax in F2 and F3 which plays an important role in increasing the stability of a cream [22]. From the results of these various tests, F3 shows a good appearance and has been stable during the three months of storage; therefore, has the best characteristics. It was chosen as the basic formulation for the incorporation of the methanolic extract of *S. aromaticum* having the best activity. As a prelude to its antidermatophyte activity on guinea-pigs, the cutaneous safety of this extract cream on guinea-pigs was carried out and the results obtained below.

Toxicity and effectiveness of the cream based on *S. aromaticum* on the animal model

Dermal toxicity of the cream based on S. aromaticum

Summary of some clinical parameters observed: Behavioural analysis

The results in Table 3 show no deaths recorded throughout the trial.

Behavioural analysis shows no change in mobility and sensitivity to sound and touch. On the other hand, an increase in the weight of guinea pigs was observed in each group. Guinea pigs have normal weight growth, suggesting that the cream might not have any impact on carbohydrate, fat and protein metabolism.[23]

Table 3: Summary of parameters observed at the end of the trial period.

Groups	Number of animals per group	Number of deaths	Average weight on day zero	Average weight on day 21	Mobility	Sound Sensitivity	Touch sensitivity
Negative controls	10	0	400 ± 31.62	436.33 ± 31.62	Normal	Normal	Normal
Assay	10	0	396.16 ± 33.28	436 ± 33.28	Normal	Normal	Normal
Vehicle control	10	0	400 ± 36.87	445.33 ± 36.87	Normal	Normal	Normal

Table 4: Results of anatomo-pathology analysis.

Organ	Average relative weight			Histo-pathological findings		
	Normal control	Assay	Negative control	Normal control	Assay	Negative control
Liver	11.99 ± 1.11	11.92 ± 1.18	12.02 ± 1.01	1/10 Vascular congestion	1/10 Vascular congestion	10/10 Normale
Kidney	2.82 ± 0.58	2.77 ± 0.53	2.91 ± 0.39	10/10 Normale	10/10 Normale	10/10 Normale
Skin	**ND***	**ND***	ND*	10/10 Normale	10/10 Normale	10/10 Normale

* Nd = not determined; 1/10 = one guinea pig in 10; 10/10 = all the guinea pigs

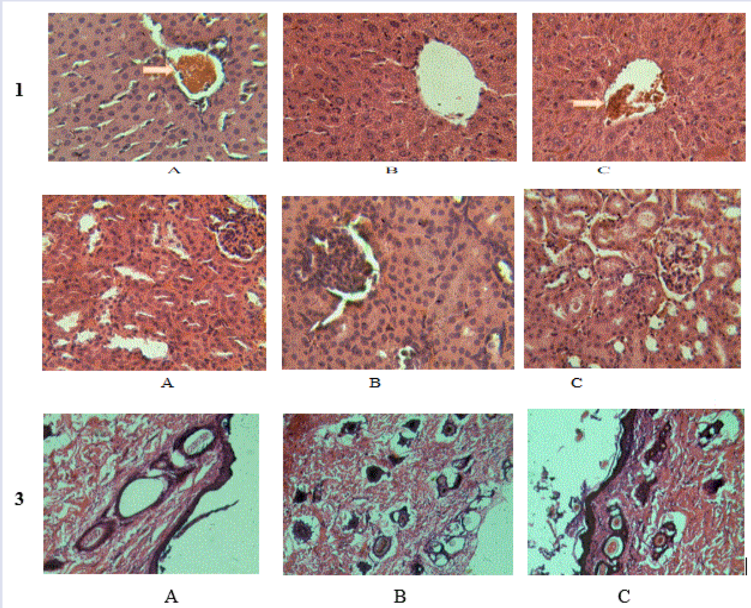

Figure 3: Microphotographs showing sections of tissues of negative control, *S. aromaticum*-based cream treated guinea pig. (× 400): 1 = Liver (× 400), 2 = Kidneys (× 400), 3 = Skin (× 100); A = Negative control, B = *S. aromaticum*-based cream treated guinea pig ; 1000 mg / kg, C = Vehicle controls; VC = Vascular congestion.

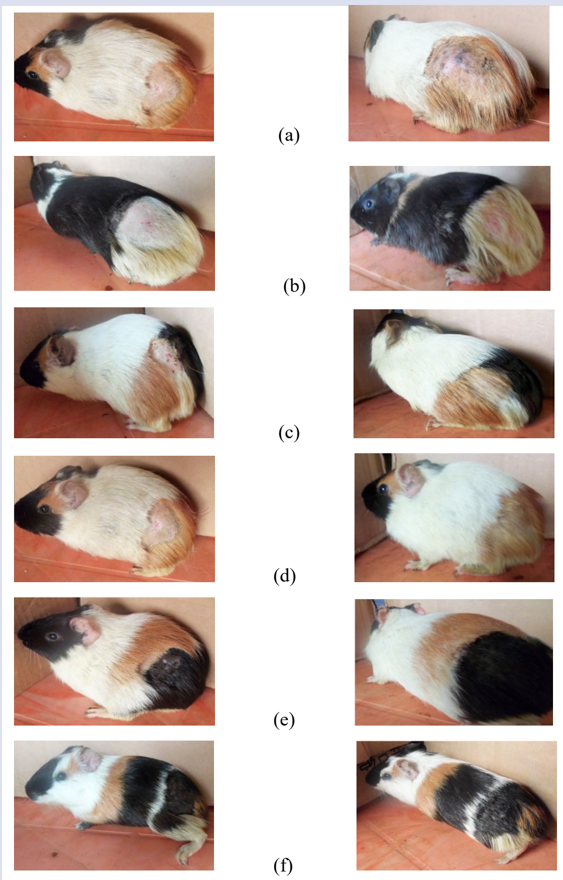

Figure 4: Effect of plant-based cream on *T. mentagrophytes* infected guinea pigs. (A) lesions on the first day of treatment; (B) lesions on day 21, (a) negative control, (b) control vehicle, (c) 2.5% cream treatment, (d) 5% cream treatment, 10% cream treatment, (f) treatment with Lamisil 1% (positive control).

Table 5: Results of biochemical and haematological parameters.

	Negative control	Vehicle control	Assay	Normal Values*
Haematological Parameters				
WBC (x10^3/mm^3)	8 ± 0.00	11.2 ± 7.18	14.15 ± 0.90	7-18
LYM (%)	45.86 ± 2.73	42.35 ± 2.56	51.7 ± 6.25	39-72
RBC (x10^6/mm^3)	4.64 ± 0.48	5.78 ± 0.73	5.41 ± 0.54	4.5-7
HCT (%)	37.96 ± 4.04	47.2 ± 6.78	44.86 ± 4.36	37-48
MCH (pg)	24.73 ± 0.35	23.86 ± 0.50	24.66 ± 0.20	23-27
MCHC (%)	30.20 ± 0.50	29.33 ± 0.66	29.73 ± 0.70	26-39
HGB (g/dl)	11.46 ± 1.19	13.8 ± 1.66	13.33 ± 1.35	11-15
PLT (x10^3 /mm^3)	268 ± 0.00	338.66 ± 71.15	316 ± 6.08	250-850
Biochemical Parameters				
ALAT (U/l)	33.30 ± 3.26	35.90 ± 4.81	31.47 ± 4.23	25-59
ASAT (U/l)	45.01 ± 3.91	48.71 ± 4.13	52.05 ± 5.67	28-68

*Normal values according to[28, 29]; WBC = White blood cells, LYM = Lymphocytes, GRA = Granulocytes, RBC = Red blood cells, HCT = Haematocrit, MCH = Mean Corpuscular Haemoglobin, MCHC = Mean corpuscular haemoglobin concentration in, HGB = Haemoglobin, PLT = Plalettes

Table 6: Clinical efficacy of the cream against skin infection by *T. mentagrophytes* in guinea pigs.

Treatment	Number of the Day of observation	Number of animals per lesion score					Average lesion score
		0*	1+*	2+*	3+*	4+*	
Negative control	3	0	0	5	5	0	2.5
	6	0	0	2	8	0	2.8
	9	0	0	0	7	3	3.3
	12	0	0	0	5	5	3.5
	15	0	0	0	2	8	
Vehicle Control	3	0	0	5	5	0	2.5
	6	0	0	4	6	0	2.6
	9	0	0	2	6	2	3.0
	12	0	0	1	5	4	3.3
	15	0	0	0	3	7	
2.5% Cream	3	0	0	8	2	0	2.2
	6	0	0	9	1	0	2.1
	9	0	2	8	0	0	1.8
	12	2	2	6	0	0	1.4
	15	6	3	1	0	0	0.5
5% Cream	3	0	0	8	2	0	2.2
	6	0	1	8	1	0	1.8
	9	1	5	4	0	0	1.3
	12	4	4	2	0	0	0.8
	15	8	2	0	0	0	0.2
10% Cream	3	0	1	7	2	0	2.1
	6	0	3	6	1	0	1.8
	9	2	4	4	0	0	1.2
	12	5	3	2	0	0	0.7
	15	9	1	0	0	0	0.1
Control Lamisil 1%	3	0	2	7	1	0	1.9
	6	0	4	6	0	0	1.6
	9	2	5	3	0	0	1.1
	12	5	3	2	0	0	0.7
	15	9	1	0	0	0	0.1

* 0 = absence of lesions; 1 = weak lesions; 2 = moderate lesion; 3 = severe lesions; 4 = severe lesions

Table 7: Mycological efficacy of the cream.

Treatment	Total number of Guinea pig	Number of cured Guinea pig	Healing rate (%)*
Normal Control	6	0	0.00
Vehicle Control	6	0	0.00
Cream 2.5%	6	4	66.66
Cream 5%	6	5	83.33
Cream 10%	6	5	83.33
Lamisil 1%	6	5	83.33

* Healing rate = (number of cured animals / total number of animals) x100; The numbers of cured animals is equivalent to the number of animal sample free from *T. mentagrophytes* after culture.

Anatomo-pathological analyses results

The results of the anatomo-pathological analysis shown in Table 4 indicate that after 21 days, the relative weight of the livers and kidneys of the test and negative controls was not significantly different. This may suggest that no atrophy of these organs was noted during treatment period.

Tan *et al.*[24] and Towatana *et al.*[25] suggested that in the case of a non-significant difference in the relative weight of the organs relative group, the extract would be weakly toxic. As well, a reduction in the relative weight of organs after prolonged exposure to toxicants is considered a toxicity index.[26]

The results of haematological and biochemical analyses are given in Table 5. The activity of ALT and ASAT remain comparable to those of the controls and are in the range of normal values highlighting a lack of hepatic involvement. Indeed, ALT and ASAT are commonly used to assess liver integrity,[1] and any alteration of the liver cells results in a rise in blood levels.[27-29] Moreover, the plant-based cream did not affect haematological parameters indicating a not toxic effect on the parameters.

Analysis of the histological sections of the liver, kidneys and skin of the guinea pigs (Figure 3) shows no abnormalities.

Vascular congestion was observed on the microphotographs of the liver of the normal and negative control (Figure 3-1 A&B). This suggest that the cream could not be responsible for these congestions but rather due to the sacrifice of the animals used.[30]

There was no evidence of organ-related modification in guinea pigs, suggesting that the limit test with a single dose level (1000 mg / kg body weight) resulted in no observable toxic effects. From another point of view, flavonoids present in *S. aromaticum* extract might have reduced the extract hepatotoxicity.[31-34]

Effect of *S. aromaticum*-based cream on guinea pigs infected with *T. mentagrophytes*

The effects of the cream on guinea pigs are shown in Figure 4 and Table 6 below:

The lesion score of infected animals receiving no treatment (negative control group) and those receiving the vehicle (vehicle group) increased considerably from the third day to the last day of the follow-up. On the other hand, the lesion score of animals treated with cream Lamsil 1% (control group positive) and those treated with cream at 2.5%, 5% and 10% decreased gradually from the third day to the end treatment. Furthermore, the reduction of lesion score was more rapid in the animals treated with Lamisil 1% and with cream 5% and 10% than with cream 2.5%. Almost no significant difference in treatment with Lamisil 1%, 5% and 10% cream.

The results in Table 7 show that all animals in the control group negative and vehicle showed infection at the end of treatment, while 66.66% of animals were cured with the 2.5% cream extract and 83.33% with 5% and 10% cream and Lamisil 1%Based on clinical and mycological findings, the evolution of the lesions in the animals receiving the cream without extract is similar to that of the negative control, thus demonstrating the neutrality of the vehicle. Treatment with 2.5% extract cream reduces the lesion score but does not completely eliminate the infection after two weeks. No significant difference in treatment with 5% and 10% creams was found from clinical and mycological findings.

CONCLUSION

At the end of this work, which aimed to evaluate the anti-dermatophyte activity of the methanolic extract of *S. aromaticum*-based cream led to no signs of dermal toxicity and efficacy on *T. mentagrophytes*-infected guinea pigs. However, additional studies are mandatory to assess the tolerability and ability of the formulated cream to improve the health of patients with clinical signs of dermatophytosis.

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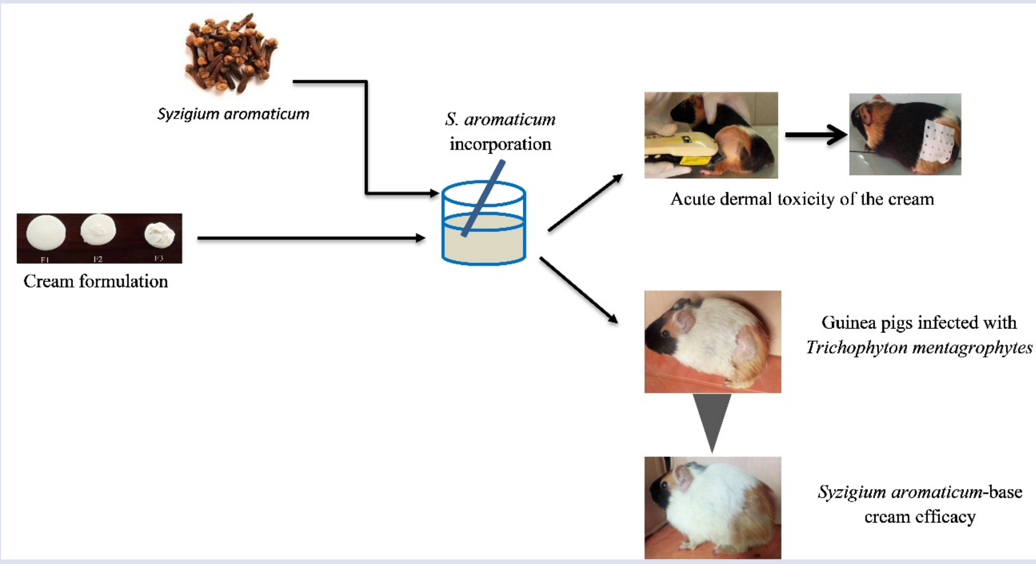
GRAPHICAL ABSTRACT



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