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Antifungal Effectiveness Of 1– (2, 4 – Dinitrophenyl) -2 (2,2,2 –Triphenyl-1-Phenyl Ethylidene) Hydrazine On Epidermophyton Floccosum : Wistar Rats As Case Study

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ABSTRACT

Fungal attack is a major concern in the health status of the human skin. There exist a number of standard drugs for the treatment of skin fungal infections. However, there are increasing reports of resistance by pathogens to current antifungal treatment regimes, hence the need to seek new compounds that are effective and safe antifungal agen Hydrazones have been reported to have biological and pharmacological activities. In this study, we have investigated the antifungal activity of a new hydrazone, 1-(2,4-dinitrophenyl)-2-(2,2,2-triphenyl-1-phenylethylidene) hydrazine using *Epidermophyton floccossum* infected Wistar rats skin, as a model. A sub-cultured clinical isolate of *Epidermophyton floccossum* was used in inoculating the rats. Different ointments containing 0.5%, 1.0% and 2.0% w/w of the synthesized compound in Blue Seal Vaseline alongside 1% Clotrimazole(standard drug) and the vehicle (Blue Seal Vaseline) were used to treat the inoculated rats in order to test the antifungal activity of the compound. Skin sensitivity tests were carried out with the various drugs. Histopathological analysis of the fungal hyphae, hair follicles, sebaceous glands, inflammation and tissue destruction rates of the skin of infected and hydrazone treated Wistar rats, when compared with the standard antifungal drug was carried out to confirm the effectiveness of the formulated creams. The efficacy of the compound showed that it was concentration dependent in the order; 2% > 1% > 0.5%. The 1% and 2% formulations also seemed to be more effective than the standard drug. In conclusion, the synthesized compound appeared to be a potential anti-fungal drug for the management of *Epidermophyton floccossum* related infections.

Keywords: Anti-fungal efficacy, *Epidermophyton floccossum,* Hydrazone, Skin sensitivity.

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1. INTRODUCTION

The interaction of man with his environment often leads to the development of various diseases in man. Human beings have sought relief from these medical conditions by using chemicals as drugs. A group of compounds that have been found to have many biological activities are produced by a condensation reaction between ketones or aldehydes with hydrazines. Hydrazones and their derivatives have been shown to possess diverse biological and pharmacological properties suchas antimicrobial, anti-inflammatory, analgesic, antifungal, anti-tuberculosis, antiviral, anticancer, antiplatelet, antimalarial, anticonvulsant, cardio-protective, anthelmintic, antiprozoal, anti-trypanosomal, anti- schistosomiasis and so on (1-17). However, with time, many fungi causing infections develop resistance to available drugs and hence the constant need to seek new drugs that are safe and effective against such infections. It is in the search for new effective chemicals that we synthesized a new hydrazone, 1-(2,4 -dinitrophenyl) -2- (2, 2, 2 – triphenyl - 1- phenyl ethylidene) hydrazine (18). Computational studies including molecular docking (19,20) have shown that these hydrazones may be effective antibacterial (19), anti tubecular (20) and antifungal (19) agents.

A major disease of the skin is fungal infestations. There are increasing reports of resistance by pathogens to current antifungal treatment regimes (21). In this study, we have investigated the antifungal effectiveness of the synthesized by hydrazine by means of animal study using Blue Seal Vaseline as a vehicle for delivering the chemical. Clotrimazole, was used as a standard drug

2. MATERIALS AND METHODS

Synthesis of the hydrazone

The hydrazone was synthesized using Brady's reagent as previously reported (18) and characterised. The reagents used were analytical grade. 2,2,2-triphenylacetophenone, 2,4-dinitrophenylhydrazine (Sigma-Aldrich chemical company), concentrated sulphuric acid (98% assay), 95% ethanol and distilled methanol.

Preparation of Cream

Samples of cream containing 0.5%, 1%, and 2% w/w active ingredient (hydrazone) in Blue Seal Vaseline respectively were prepared and commercial Ytcan cream containing 1% clotrimazole was used as standard drug.

Preparation of the Dermatophyte (*Epidermophyton floccossum*)

Clinical isolates of *Epidermophyton floccossum* were obtained from Spectralab Medical Diagnostic Services, Sagamu in Ogun State, Nigeria.

Preparation of the sub-culture

A loop of well grown nutrient alga microorganism of clinical isolates of *Epidermophyton* Epidermophyton were suspended in 30 ml of normal saline water in a plain bottle and kept at a temperature of 25oC in the laboratory fridge fir 48 hours. The culture was observed regularly for the growth of organisms and also for any contamination. The well grown fresh sub- cultured organisms were used for inoculation during experimental investigation

Preparation of the sub-culture of micro-organism

A loop of well grown nutrient agar micro-organism of clinical isolates of *Epidermophyton floccosum* was suspended in 30ml of normal saline water in a plain bottle and kept at a

temperature of 25^{o}C in the laboratory fridge for 48 hours. This was observed regularly for the growth of organisms and also for any contamination. The well grown fresh sub-cultured organisms were used for inoculation during experimental investigation.

Laboratory animal

Albino rats weighing between 120-150g were purchased from the Experimental Animal Unit of the Faculty of Veterinary Medicine of the University of Ibadan. The rats were kept in cages at controlled room temperature 29^{o}C-30^{o}C. Well accessible ceramic feeding bowls were used to provide food and water for the animals. The weights of the animals were taken for each of the stipulated time and these were juxtaposed with their feed intake. Each rat hair was shaved clean at the side of the belly in a dimension approximately 2cm^2 by 2cm^2 and left for 24 hours after which inoculation carried out. The inoculation was done by injecting 2ml of sub-cultured *Epidermophyton floccosum* followed by routine feeding and cleaning. Seven days after inoculation, treatment with the formulated active ingredients of different concentrations, standard drug, and vehicle started. At the end of seven days of treatment, the animals were euthanized by cervical dislocation. Their skins were harvested and preserved for histopathology study.

Experimental selection and grouping of animals

Forty two (47) animals were used and were grouped into 7 groups of 6 rats and a group consisting of 5 animals which were used for skin sensitivity test.

- Group Zero – Non inoculated animals used for skin sensitivity test.
- Group one - Non inoculated animals
- Group two – Inoculated animals but not treated (negative control).
- Group three-Inoculated animals treated with Ytacan cream containing 1% Clotrimazole (Standard drug).The positive control.
- Group four - Inoculated animals treated with Blue Seal Vaseline alone (Vehicle).
- Group five - Inoculated animals treated with 0.5% drug formulation alone.
- Group six - Inoculated animals treated with 1% drug formulation alone.
- Group seven - Inoculated animals treated with 2% drug formulation alone.

Skin sensitivity tests

All the formulated creams containing the synthesized compound in the Blue Seal Vaseline were tested on the shaved skin of group-zero Wistar rats. The test samples were applied in the morning on the shaved sites and allowed to remain there until the following morning. The treated surfaces were then rinsed with water, and patted dry with absorbent paper. The site was examined 24hrs later for signs of irritation like rashes, swelling and redness.

Histopathological Studies

4cm^2 skin area were cut and the skin tissues were fixed in 10% formalin before castifying into 30% phenol acid for 24hours. The tissues were dehydrated and were processed gradually through graded ethanol starting from 70, 80, 90, 95%, and absolute ethanol. The time frame for the tissues in each grade of alcohol was 1 hour while it was kept for 2 hours in absolute ethanol. Xylene was then added to remove the ethanol and at the same time render the tissue transparent. The skin tissues were then impregnated with paraffin wax which had been heated in the oven for 1 hour at 50^{o}C after which the tissues went through sectioning at 4 micrometer with microtome in water bath.

The section was placed on micro slide and then put in oven for dewaxing. It was then stained with haematoxylin for 15minutes and excess acid washed off and then counter stained in eosin for 3 minutes. The tissues was then dehydrated using ethanol and the slide remain in the xylene until it was mounted using DPX. Skin biopsy samples were examined for the presence of fungal hyphae, hair follicles, sebaceous gland, inflammation and tissue destruction using light microscope.

3. RESULTS AND DISCUSSION

Skin sensitivity

No irritation or inflammation was noticed on the skin of the animals when each of the different formulations and the control drug and vehicle was applied on the shaved portions. This showed that the formulated drugs and the control drug and vehicle could be used jn the experiment.

General discussion

The first sign of the breaching of the protective mantle of the skin is shown by presence of itchy areas, rashes and inflammation of the skin which can be measured by the swelling of the epidermal tissues in a gradual manner as the concentration of the cause of inflammation increases within the tissue. Whether the breaching is caused by allergens or microorganisms. In many cases, the keratinocytes in the hair follicles become infected, causing de-keratinization which eventually lead to tissue destruction. There are chemicals that can ameliorate this reactions and thus reverse the effect as can be seen in this study(Table 1& 2 and Figures 1&2). In this study, epidermal thickness varies in the order Group 2 > Group 4 > Group 5 > Group 3 > Group 6 > Group 7 > Group 1.

The mist irritated rat skin belong to the untreated group, while the least irritated were seen among rats that were not inoculated with the fungi. Showing that the infection causes this type of irritation. The drugs were seen to ameliorate the inflammation and in a concentration dependent manner. The highest dose of the prepared ointment was able to completely stop skin inflammation (Figure1) The results of keratinization studies also showed similar trend (Figure 2). De- keratinization can be used as a measure of tissue destruction. The histopathological studies have used these results along with others to explain the effect of the concentration of treatment drug on the of the study rats(Table 3 & plates 1 -7)

Animal Studies

Group 1 (uninfected rats/ Normal Control)

There is no observable lesion. No inflammation, but mild tissue destruction was observed in the group (Table 3). There was absence of fungal hyphae, showing that the animals were in the uninfected state. There was marked presence of hair follicles and sebaceous gland which is associated with healthy rat skin. Inflammation and discontinuity/tissue destruction was mild (Plate 1).

Table 3: Histopathological study results across study groups☐

Group	Fungal hyphae	Hair follicles	Sebaceous gland	Inflammation	Continuity/Tissue destruction
1	_	+++	+++	_	+
2	+++	_	-	+++	+++
3	_	+++	+++	_	_
4	+++	++	+	++	+++
5	+	++	++	+	+
6	_	++	++	_	_
7	_	+++	+++	_	_

Absent = , Mild = +, Moderate = ++ Marked = +++

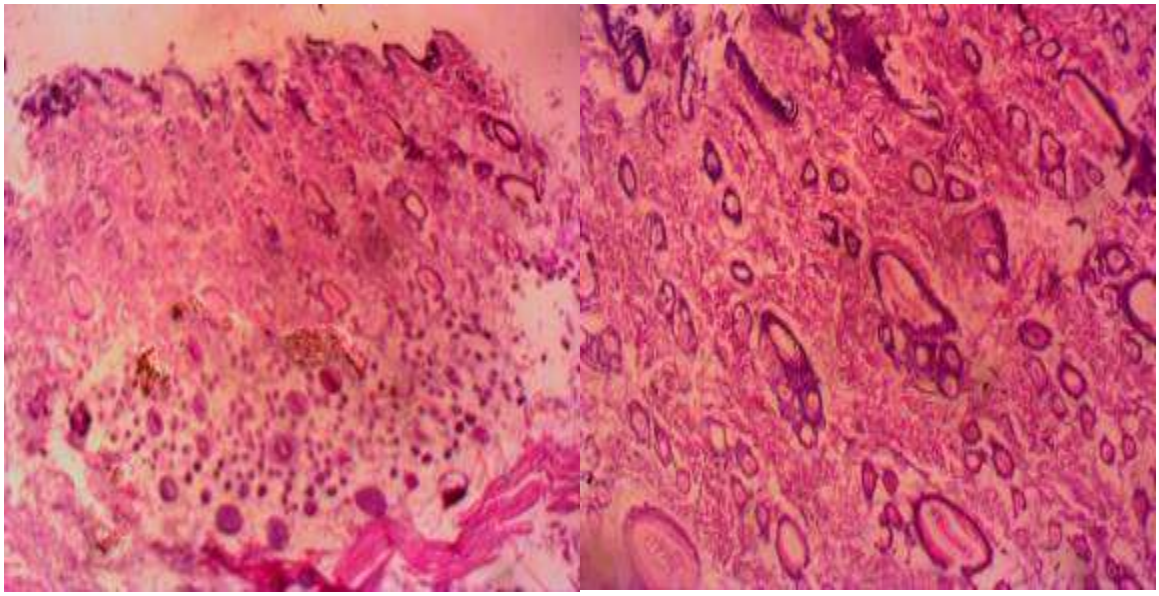

Plate 1: Skin of uninfected albino rat(normal control)

Group 2 (Infected but not treated rats)
There was severe dermatisis with obliterated adnexia. Marked inflammation of the skin and foci of discontinuity of the epidermis were observed, which indicated successful infection. There was a marked presence of fungal hyphae . There were no hair follicles and nor sebaceous glands as they had been destroyed. Marked tissue destruction was observed This indicated that the fungal infections was deeply rooted in comparison to the other groups (Plate 2).

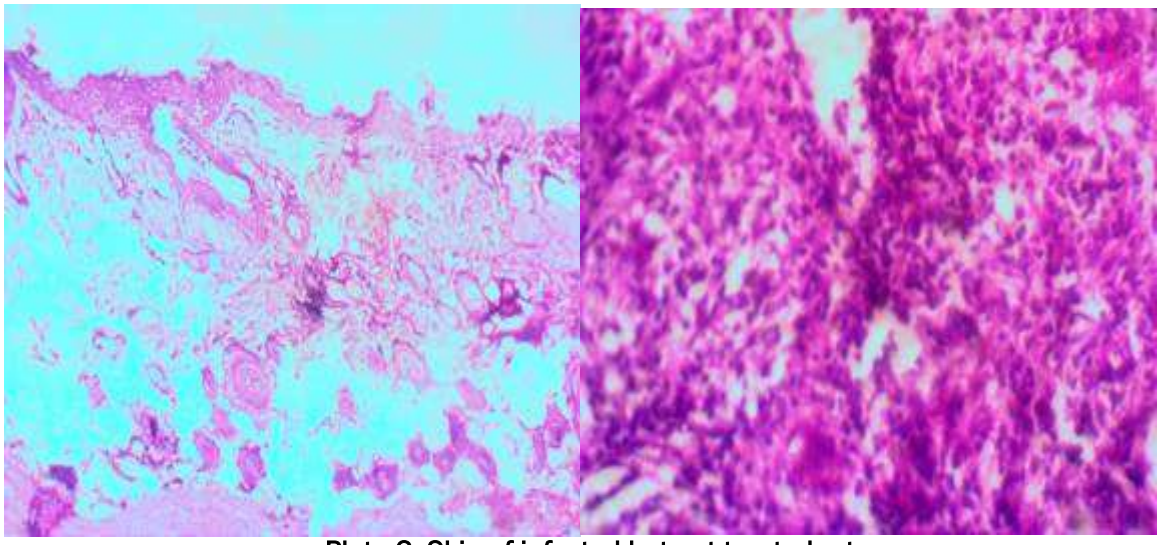

Plate 2: Skin of infected but not treated rat

Group 3 (Standard drug)

There was moderate dermatisis with obliterated adnexia. There were no fungal hyphae seen. Numerous hair follicles and sebaceous glands were observed. There was no inflammation nor tissue destruction, indicating control of fungal growth and treatment without harm to the rat skin (Plate 3).

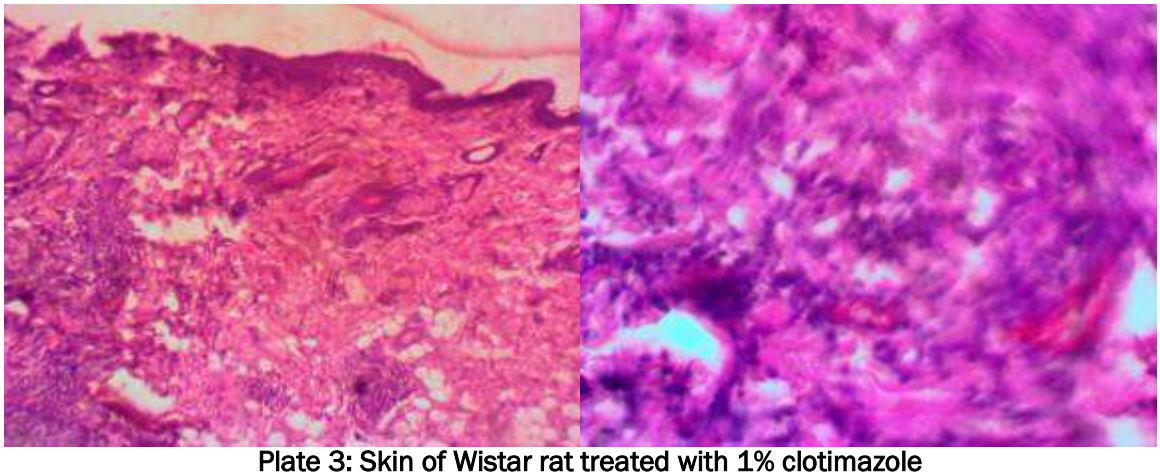

Plate 3: Skin of Wistar rat treated with 1% clotimazole

Group 4 (Rats treated with Vaseline only (Vehicle)

There was hyperkeratosis, acenthosis with hydropic degeneration of keratinocytes. Marked presence of fungal hyphae was observed. There were moderate hair follicle and few sebaceous gland in the dermis. Skin inflammation was moderate while tissue destruction was marked. This showed that Vaseline had little or no effect on the inhibition the growth of fungi, when compared with the groups treated with hydrazone formulations (Plate 4).

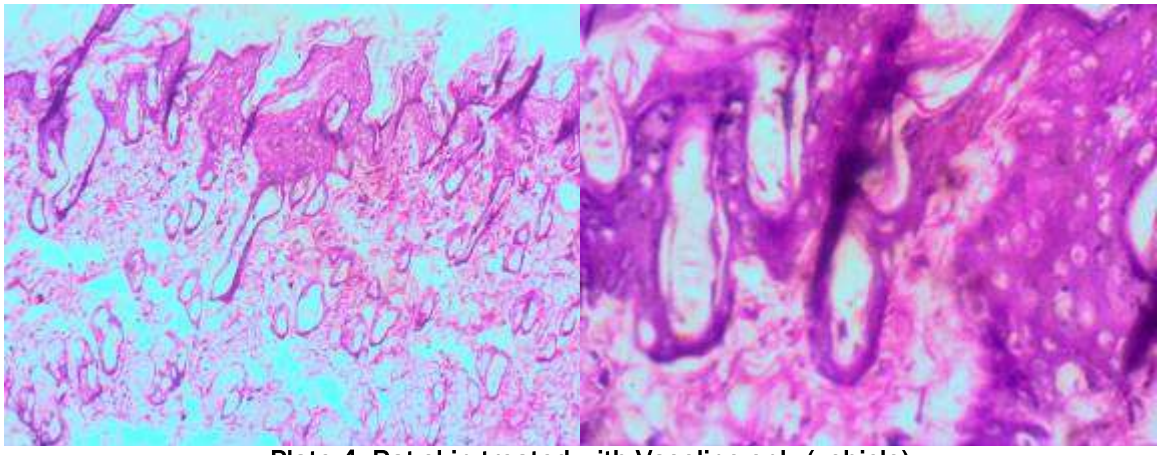

Plate 4: Rat skin treated with Vaseline only (vehicle)

Group 5 (rats treated with 0.5% hydrazone ointment)

There was hyperkeratosis, dermatitis and exocytosis. Mild presence of fungal hyphae in the dermis. The presence of sebaceous glands and hair follicles were mild. There was mild inflammation and tissue destruction. This shows that the formulation partially inhibited the growth of the fungal hyphae, but was not able to stop the destruction of hair follicles and the tissue (Plate 5), when compared with Groups 6 (1% ointment) and group 7 (2% ointment) which prevented inflammation and the destruction of hair follicles in the rat skins Plates 6 and 7).

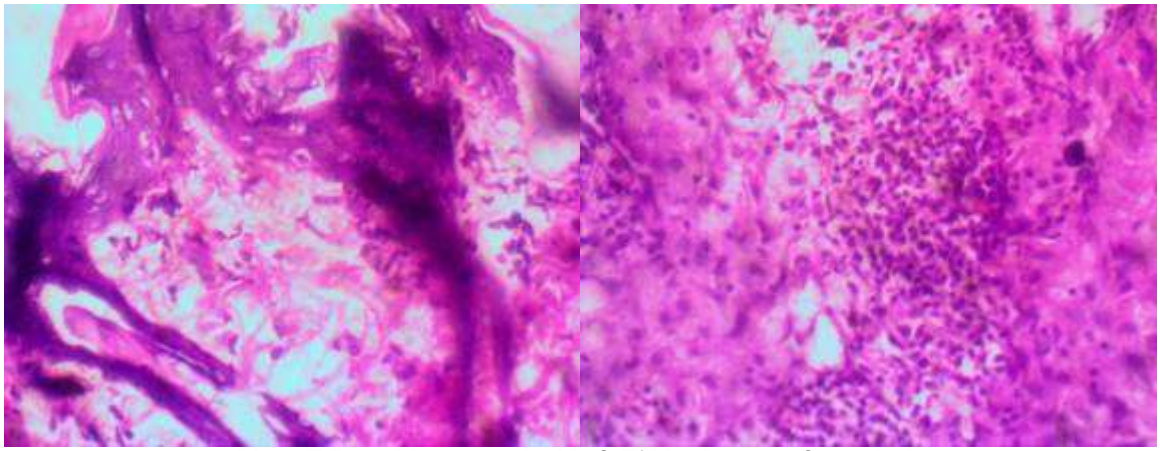

Plate 5:Rat skin treated with 0.5% hydrazone formulation

Group 6 and Group 7 (1% and 2 % hydrazone ointment)

There is no observable lesion in both groups. No inflammation and no tissue destruction were observed. There was absence of fungal hyphae indicating that the drug was able to eradicate the infection caused by the dermatophyte. There was moderate hair follicles in the dermis and numerous sebaceous glands in Group 6 while the hair follicles and sebaceous glands were numerous in Group 7. This suggests that though 1% formulation was effective, as it appeared to have protected the skin from all destructive effects of the fungi. The 2% formulation also appeared to be more effective than 1% clotrimazole (standard drug), (plate 6 and plate 7).

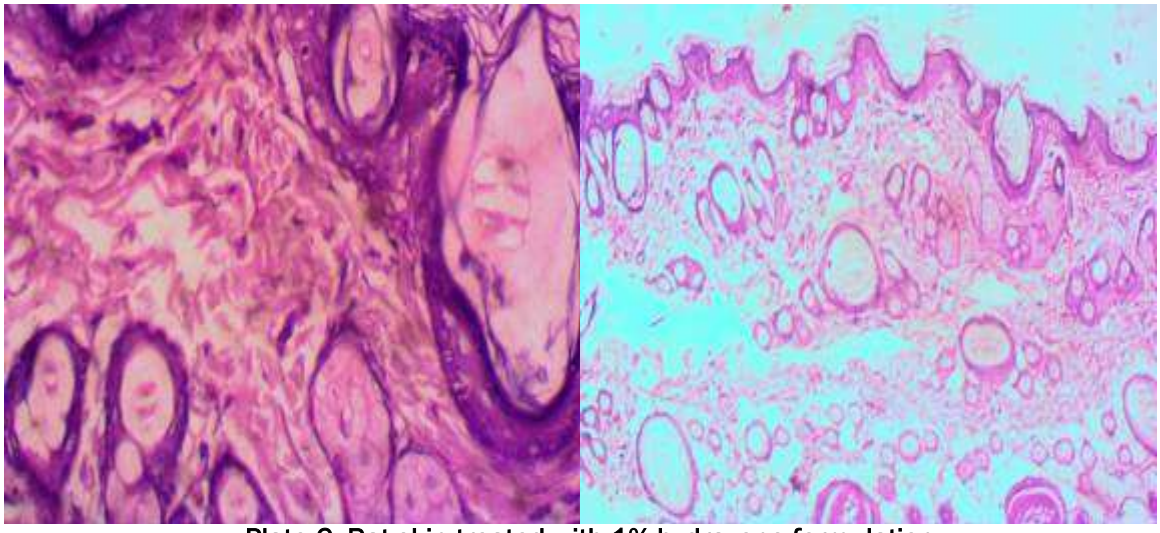

Plate 6: Rat skin treated with 1% hydrazone formulation

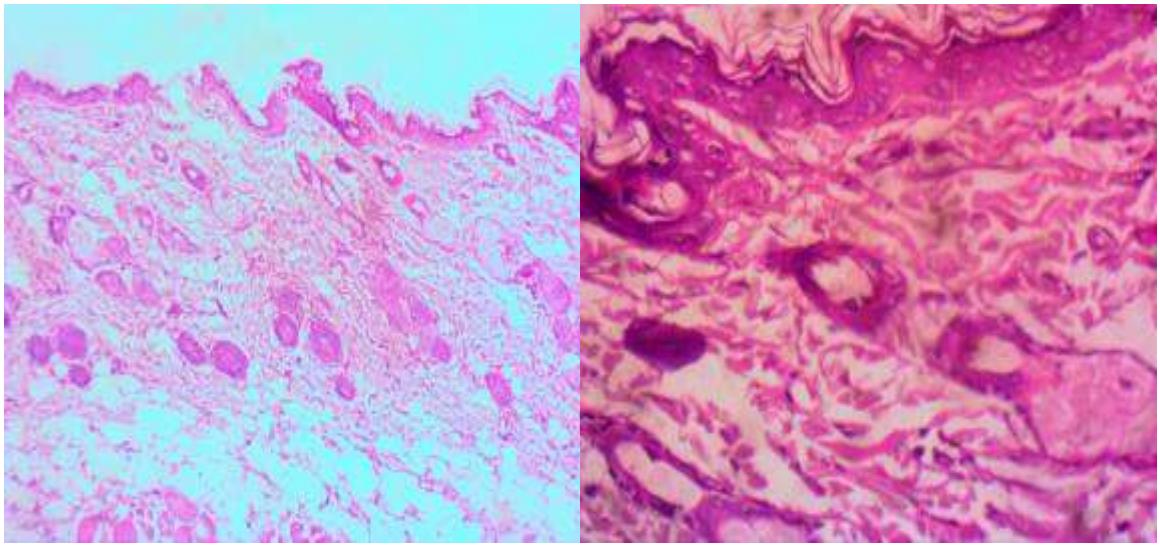

Plate 7: Rat skin treated with 2% hydrazone formulation

5. Conclusion

The efficacy of the formulations were concentration dependent in the order 0.5% <1% <2%. However in order to reduce inflammation of the skin to the very minimum 2% formulation could be used. It was observed that 0.5% formulation was as effective as 1% clotrimazole. This study showed that the compound 1-(2, 4-dinitrophenyl) -2- (2,2,2 triphenyl-1-ethylidene) hydrazine had significant antifungal potentials which was concentration dependent. The 2% ointment of this compound showed similar activity as clotrimazole a standard antifungal drug already in market. Furghet toxicological profile of the compound needs to be determined, in order to predict its safety in use in humans.

EndNote/Acknowledgement

This work was carried out in collaboration between the authors. Author FOO designed the study and performed the statistical analysis and edited the final article.Author TOO wrote the protocol and the first draft, carried out the animal study. Author ABF managed the analysis of the study and the literature searches, while Author OMB supervised the animal study. Author BBA suggested the use of the hydrazone and provided chemicals for its synthesis and proof read the final draft.

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