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In vitro and in vivo anti dermatophytes activity of *Lawsonia inermis* L. (henna) leaves against ringworm and its etiological agents

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Abstract: The study was carried out to identify the etiological agents causing ringworm, evaluate the in vitro and in vivo antifungal activity of *Lawsonia inermis* L. *In vitro* study was carried out using agar dilution method. In a total of 50 clinical samples, 4 different species were identified namely; *Microsporum canis, Trichophyton tonsurans, Trichophyton rubrum,* and *Trichophyton mentagrophytes*. However, the results showed that hexane extract exerted a strong antifungal activity against all the identified etiological agents, with a minimum inhibitory concentration of 625µg/ml except *Microsporum canis* which resisted the minimum concentration but susceptible to the higher concentrations. The *in vivo* study was carried out using 15 naturally infected goats. First, second and third group of animals were treated with henna paste, aqueous and ethanolic extract respectively, fourth group were treated with clotrimazole as a positive control while negative control (fifth) group were left untreated with neither henna nor clotrimazole. The treatments were compared and the results showed that henna paste had the highest efficacy against all the types of ringworm tested compared to the remaining treatments. Disappearance of lesion and complete repair of the hair was observed at 30 days after treatment. Significantly similar result was observed in the group of animals treated with aqueous extract, ethanol extract, and clotrimazole in all the parameters. Significant different was only observed between groups treated and negative control.

Keywords: *Lawsonia Inermis*, Antifungal, *Trichophyton, Microsporum*, Clotrimazole

1. Introduction

Henna (*Lawsonia inermis* L.) (Lythraceae) is a tall flowering shrub or tree about 5 m in height, native to tropical and subtropical regions of Africa, southern Asia, and northern Australia in semi-arid zone and oases in the Sahara [1]. Several researchers like [2] have reported the different biological actions of *L. inermis* in various *in-vitro* and *in-vivo* test models. Henna leaves, flowers, seeds, stem bark, roots have been found to exhibit antioxidant, antidiabetic, hepatoprotective, hypoglycemic, antimicrobial, anticancer and wound healing properties [3].Traditional medicinal practice has been known for centuries in many parts of the world for the treatment of various human ailments [2]. However, medicinal plants are part and parcel of human society to combat from the dawn of civilization. According to the report of World Health Organization (WHO) 80% of the world population depend mainly on traditional therapies which involve the use of plant extracts or their active substances [4].

Human and animals infections, especially those associated with skin and mucosal surface constitute a serious problem, especially in tropical and subtropical developing countries [5]. Ringworm is a common contagious disease caused by

fungi known as dermatophytes, which belong to a group of organisms that are able to break down the keratin in tissues such as the epidermis, hair, nails, feathers, horns and hooves [6]. Most of these fungi reside in the soil and are involved in decomposition; however, the dermatophytes can infect living hosts. Some dermatophytes (anthropophilic species) are adapted to humans, and are usually transmitted from person to person. Others (zoophilic species) are adapted to animals. A few (geophilic) species normally live in the environment, but occasionally act as parasites [7].

Therefore, the aim of this research is to evaluate the in vitro and in vivo antifungal activity of *L. inermis*.

2. Materials and Methods

2.1. Collection Identification and Preparation of Plant Material

Henna plant was collected from Wagini town 60km east of Katsina in Batsari Local Government Area, Nigeria and transported to the Department of Biology, Umaru Musa Yar'adua University (UMYU) where it was identified as *Lawsonia inermis*, L. using available information in the laboratory.

2.2. Extraction and Isolation of Henna

100g each of the henna powdered was suspended in to 500ml of ethanol, and extracted using soxhlet extractor for 4 hours [8]. The dried extract were weighed and kept in a freezer until required for further analysis [9].

2.3. Microorganisms

50 samples from skin scrapping, hooves and hair were collected from clinically suspected cases of dermatophytosis. The method of samples collection was that prescribed by [6], [10] and [2].

2.3.1. Microscopic Observation

A drop of 20% KOH (in case of skin and hair) and 40 % (in case of hooves clipping) was kept on a clean, grease free glass slide. Then the sample (Skin scrap and hair) was mixed gently with the KOH drop and the slide passed through a burner flame to hasten keratolysis (keratolysis softened the sample). This preparation was covered by a clean glass cover slip without trapping any air bubbles. After that the mount was observed under high power objective. In case of nail sample, the nail clippings in KOH were kept for overnight. Then the mount was observed under high power objective [10].

2.3.2. Primary Culture of the Samples

The technique followed by [6] was imitated. Samples collected were inoculated in a dermatophyte test media (DTM) specially prepared for the culture of ringworm (40.7 g of the medium was suspended in one liter of purified water, heated with frequent agitation and boiled for one minute to completely dissolve the medium. autoclaved at 121°C for 15 minutes, and cooled to 50°C. The two antibiotics Gentamicin (0.1 g/L) and Chlortetracycline (0.1 g/L) were also added to prevent the unwanted growth of bacterial contaminants).The plates were incubated for 7 days and growth was noted and recorded. Another plate was left un-inoculated as control and this was considered as primary culture. The portions of the colonies were subjected to microscopic examination.

2.3.3. Lacto-Phenol Cotton Blue Mount

A drop of lacto-phenol cotton blue was placed on the clean grease free glass slide, and a tuff of fungal filament was picked up from the culture plate using teasing needle. Then the filaments were transferred to the lacto phenol stain and gently teased and a clean cover glass was carefully placed over the preparation without any air bubbles. This preparation was examined under high power objective. The hyphae, spore structure and their arrangement were observed [3].

2.4. Preparation of Pure Culture of the Fungal Isolates

A pure culture of *Microsporum canis, Trichophyton tonsurans, Trichophyton rubrum,* and *Trichophyton mentagrophytes* were prepared by careful subculture of the portion from the primary culture to center of the surface of fresh DTM plates, slants and incubated at room temperature [11].

2.5. Preparation of Inoculums

A sterile wire loop was used to transfer a portion of the colony of each isolate onto Triphton Soy Yeast broth (T.S.B) that is incubated at room temperature overnight. 0.1ml of the overnight broth culture is diluted with 1ml of the distilled water in a ratio of 1: 100 to form the standard inoculums [6].

2.6. In Vitro Sensitivity Test by Broth Dilution Method

2.6.1. Preparation of Test Plant Extract

To prepare ethanol soluble fractions of the plant, 100mg of the extract was dissolved in 1ml of dimethyl sulfoxide ($DMSO_4$) to make a concentration of 100mg/ml referred to as solution (a). 0.5ml of solution (a) was further dissolved in 0.5ml $DMSO_4$ making solution (b) from which from which 0.5ml was dissolved in another 0.5ml $DMSO_4$ to make solution (c). By dissolving 0.5ml standard solution (c) into 0.5ml $DMSO_4$, solution (d) was obtained. Therefore, the concentrations of standard solutions b, c and d were 50, 25 and 12.5mg/ml respectively.

However, by incorporating 1.0ml of solution b, c and d into 9.0ml of Sabourrauds agar, a final concentrations of 5000, 2500, 1250 and 625µg/ml of the media containing the test extract was obtained respectively.

2.6.2. Bioassay Procedure

Sabouraud dextrose agar (SDA) containing 5000µg/ml, 2500µg/ml, and 1250µg/ml, 625µg/ml and clotrimazole were prepared. This prepared SDA of various concentrations of the extract, clotrimazole and another media with no extract

neither clotrimazole were inoculated with each of the etiological agent and incubated in triplicates at room temperature for 48 hours. Incubation continued up to the next thirty days. Control plates containing no extracts were also inoculated and incubated in each case. Presence or absence of fungal hyphae on the plate at the end of the incubation period were examined and recorded as active (+) when the extracts inhibited a single colony to grow out, otherwise, not active (-) was recorded [11].

2.7. In-Vivo Sensitivity Testing Using Goats

Fifteen goats, naturally infected by ringworm of between 10 to 12 months old were identified and obtained from market. They were randomly distributed into 5 groups, with 3 goats in each group. Before conducting the research, the ethical approval of the use of animal was obtained from the National Union of Medical Herbal Practitioners, Nigeria. During the research animals were caged separately in the same yard and supplied with food and water. The experimental protocol following the ethical guidelines on the proper care and use of animals was maintained and adapted throughout the research. The method followed by Al Hamdani et al., [12] was imitated.

Henna paste was prepared by soaking 100g of powdered plant in 900mL of water [13]. The mixture was stirred until it had a thick, pasty texture. The first group of animal was treated with the paste at the lesions twice daily as prescribed by [14] and [2]. The second group of animals was treated with the aqueous extract. 10s0g each of the powdered leaves was suspended in to 900ml distilled water and extracted using soxhlet extractor for 4 hours. The extract was filtered using a Whatman's No. 1 filter paper and the filtrate was dried using water bath. Concentration of 5000µg/ml of the dried extract was prepared using distilled water as solvent and applied topically on the lesions of the second group of animals twice daily. To the third group of animals the same extraction procedure as in previous was repeated but in this case ethanol was used as an extraction solvent in place of distilled water. Clotrimazole cream was obtained from pharmacy and applied topically twice daily on the lesions of the fourth group of animal as prescribed by the manufacturer and this was referred as a positive control. Clotrimazole is used to treat fungal infections of the skin. Plant paste was applied topically on the ringworm lesion created on artificially infected rabbit to evaluate it [12]. [10] Prescribed the topical treatment of the whole-body or individual lesions with clotrimazole or miconazole preparations as one of the best way of treating ringworm. The fifth group of animals was left untreated and considered as a negative control. The samples were collected from the treated lesions and diagnosed for the visibility and observation of viability of the etiological agents at two days interval from the first day of treatment until no viable sample was viewed or obtained. Finally, initial and complete hair regrowth was measured using ruler and the result was recorded. The same process of diagnosis of the lesions was repeated to all other remaining group of animals.

3. Results and Discussion

3.1. Isolation and Identification of Dermophytes

In this study, a total of 50 clinical samples (skin, hair and hooves) were collected from a total of 23 goats and all the collected samples were plated on dermatophytes test medium (DTM). Most of the dermatophytic fungi were isolated from skin samples than hair and hooves. Based on the colony morphology and microscopic observations, 4 different species were identified and presented in Table (1). They are; *Microsporum canis* invaded hairs show an ectothrix infection and fluoresce a bright greenish-yellow under Wood's ultra-violet light. Colonies are flat, spreading, white to cream-coloured, with a dense cottony surface and usually have a bright golden yellow to brownish yellow reverse pigment, but non-pigmented strains may also occur. Macroconidia are typically spindle-shaped with 5-15 cells, verrucose, thickwalled and often have a terminal knob. *Trichophyton mentagrophytes*, colonies that appeared flat, white to cream color with powdery granular, under microscope, a numerous subspherical to pyriform micro conidia, spiral hyphae and spherical chlamydoconidia confirmed to be *T. mentagrophytes. Trichophyton rubrum*, Flat to slightly raised velvet, yellow brown to red pigment on reverse side of the medium and tear-drop macro conidia, abundant micro conidia as small pyriform observed under microscope and *T. tonsurans* appear suede-like to powdery, flat with a raised center or folded, often with radial grooves, hyphae are relatively broad, irregular, much branched with numerous septa and numerous characteristic microconidia varying in size and shape from long clavate to broad pyriform, are borne at right angles to the hyphae, which often remain unstained by lactophenol cotton blue. Shahitha et al., [3] reported that a total of 6 species of dermatophytes viz., *Trichophyton rubrum, Trichophyton verrucosum, Trichophyton tonsurans, Trichophyton equinum, Microsporum canis* and *Microsporum gypsum* were isolated and identified from 50 samples collected from clinically suspected cases of dermatophytosis.

Table 1. *Growth screening of ringworms species on the Dermatophyte Test Medium (DTM).*

Ringworm	*M. canis*	*T.mentagrophytes*	*T. rubrum*	*T. tonsurans*
scalp	+	+	+	+
face	+	-	+	-
body	+	+	+	-

3.2. In Vitro Antidermatophytic Assay

In this study, anti dermatophytic activity of *Lawsonia inermis* (henna) against the isolates of *Microsporum canis, Trichophyton rubrum, Trichophyton mentagrophytes,* and *Trichophyton tonsurans*, were obtained frequently in all the detected samples. The ethanol extract in Table (2) showed strong antifungal activity against *Microsporum canis, T.*

mentagrophytes, and *T. rubrum* at all the concentrations; at concentration of 625µg/ml and 1250µg/ml T. tonsurans was found to be resistant to the test plant. This proved that the higher the concentration the better the fungicidal effect. Mansour et al., [16] reported in their research that *L. inermis* leaves extract have developed a fungicidal effect against *T. mentagrophytes* and *Candida albicans*. Padron-Marquez et al., [5] reported that compared to control, the best antifungal activity found in their investigation was observed with the hexane extract, which inhibited all the tested dermatophytes. [16] Reported in their research that *L. inermis* leaves extract have developed a fungicidal effect against *T. mentagrophytes* and *Candida albicans*. Nasreen et al., [15] reported that during screening of barks of 30 plant species against dermatophytes, only *Lawsonia inermis* (Henna) extract exhibited absolute toxicity. The extract showed broad fungitoxic spectrum when tested against 13 ring worm fungi. *Lawsonia inermis* exhibited absolute toxicity against ringworm causing fungal species such as *Microsporum gypseum and Trichophyton mentagrophytes* [1].

Table 2. *Sensitivity test of the ethanol L. inermis leaf extract on the growth of individual species of dermatophytes.*

Dermatophyte species	Conc. µg/ml 5000	Conc. µg/ml 2500	Conc. µg/ml 1250	Conc. µg/ml 625
Microsporum canis	+	+	+	+
T. mentagrophytes	+	+	+	+
T. tonsurans	+	+	–	–
T. rubrum	+	+	+	+

Key + =sensitive - =not sensitive

3.3. In Vivo Antidermatophytic Assay

Hair resume growing after the microorganism became susceptible to the treatments and initial regrowth was noticed and recorded in Table (3), per Days After Treatment (DAT). However, hair regrowth was fist noticed at 5DAT in groups of henna paste, ethanol extract, and clotrimazole cream, while respectably 6DAT and 39DAT in aqueous extract and negative control groups. Likewise, the sample of etiological agents collected from the treated region of infection was only viable at 3DAT, but the progress of inhibition of the growth and development of the dermatophytes was noticed at 4 days after treatment in which no visible growth of fungal was seen from the cultured samples. Regrowth of hair in the treated lesions was first noticed at 13 days after treatment. Subsequently, disappearance of lesion and complete regrowth of the hair was observed at 30 days after treatment. Figure (1, 2, 3 and 4) shows image of the lesions of the ringworm before and after treatment with henna paste, ethanolic extract, aqueous extract and clotrimazole cream respectively, while figure (5) shows the image of one of the control animal at the beginning of treatment and at the time where all the treated groups completely recovered. Significantly similar result was observed in the group of animals treated with aqueous extract, ethanol extract, and clotrimazole in all the parameters. Significant different was observed between groups treated with all the previously listed extracts, clotrimazole (positive control) and Control (negative control) in each case. Lawsone, 2-hydroxy-1,4-naphthoquinone was considered by [17] to be responsible for henna's fungicidal activity. The result of [22] in 2014 supports the traditional usage of the *L. inermis* and suggests that the henna extracts possess compounds used in traditional medicine. While the findings of [8] demonstrated that henna extracts have antifungal activity *in vitro* against the fungal which causes *Pityriasis versicolor, Pityrosporum foliculities* and dandruff. They added that aqueous extracts are more effective on Malassezia than methanolic and chloroformic extracts. However, in most cases, the biological activity of henna against ringworm and its etiological agents is related to the ability of quinones to accept one or two electrons from microorganisms to form highly reactive radical anion intermediates, which are responsible for the oxidative stress observed in the microbial cells [18]. In fact, some clinically active antimicrobial drugs, such as marinone debromomarinone, contain the quinone moiety as a relevant part of their structures [19]. Quinonoid compounds by virtue of their easy redox cycling capacity are known to possess wide-ranging antimicrobial as well as anti-cancer features. Therefore Lawsone, 2-hydroxy-1, 4-naphthoquinone is responsible for henna's fungicidal activity [17].

Table 3. *Comparative Anti fungal susceptibility testing of ringworm infected goats treated with the henna paste, aqueous extract, ethanolic extract, clotrimazole and control.*

Treatment type	Response of treatments/days					LSD 0.05
	Improvement	Negative culture of hair	Initial re growth of hair	Disappearance of lesions	Total	
Henna paste	5	4	13	30	39	4.03
Aqueous extract	6	4	13	30	40	4.03
Ethanol extract	5	5	11	30	40	4.03
Clotrimazole cream	5	4	13	27	36	3.99
Control	39	48	44	70	157	8.33

(a) (b)

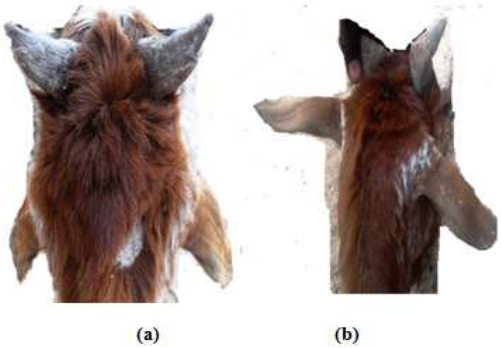
Figure 1. *Before and after the In vivo sensitivity test of henna paste on goat naturally infected by ringworm.*

(a) (b)

Figure 2. *Before and after the In vivo sensitivity test of Lawsonia inermis ethanolic extract on goat naturally infected by ringworm.*

(a) (b)

Figure 3. *Before and after the In vivo sensitivity test of Lawsonia inermis aqueous extract on goat naturally infected by ringworm.*



(a) (b)

Figure 4. *Before and after the In vivo sensitivity test of Lawsonia inermis clotrimazole on goat naturally infected by ringworm.*

(a) (b)

Figure 5. *The negative control animals which was left untreated before and after the research in which left at the beginning of the research while right at the end of the research.*

3.4. Percentage Cure and Regrowth of Hair

The percentage hair growth results and graphical representation of both treatments and types of ringworm are given in Tables (4, 5 and 6) and Figure (6, 7 and 8). Moreover, measurement of percentage of hair length begin when the hair reaches 5% of its original length. However,, the highest initial percentage (11.50±1.23) regrowth of hair against ringworm of scalp during treatment was first observed at 15DAT in group treated with clotrimazole cream (positive control, followed by group treated with aqueous extract (7.50±1.77) and henna paste respectively (7.40±1.56) and the lowest percentage (5.32±1.17) was observed in group treated with ethanol extract both at 16DAT. How ever, reasonable initial percentage in control was first observed at 45DAT. The result is summarized in table 4 and Figure (6). Likewise, full hair regrowth to 100% was initially observed in group treated with clotrimazole cream at 25DAT, followed by groups treated with henna paste, ethanol extract and aqueous extract at 30DAT each. Finally, control reaches its 100% regrowth at 70 DAT. Consequently, the data presented in Table 3 and Table 6 indicates Percentage regrowth of hair against ringworm of face and ringworm of body respectably during treatment. Hence, initial and 100% regrowth was observed at 15DAT and 25DAT respectively in group treated with clotrimazaole, while, initial and 100% regrowth was observed at 15DAT and 30DAT in the remaining treatments and finally initial and 100% regrowth was observed at 45DAT and 70DAT in controlled groups. However, in animals ringworm infection generally runs a course within a few weeks or months and disappears even without treatment. On contrary, to the ringworm of scalp in children which if not treated remain active on their hosts until they reaches puberty. Therefore, treatment; speed up recovery time, minimizes further spread of the disease to different part of the body or to the available susceptible host and or to the surrounding area. Furthermore, it prevents possible damage such as permanent alopecia on the affected area if allowed to heal on its own. *Tinea capitis* patient treated with *Euphorbia paralias* matrix has completely recovered with hair growth after a month of starting the treatment [2]. The findings by Fariba et al. [5] demonstrated that henna

extracts have antifungal activity in vitro against the fungal which causes Pityriasis versicolor, Pityrosporum foliculities and dandruff. They added that aqueous extracts are more effective on Malassezia than methanolic and chloroformic extracts. Al-ani et al., [20] reported that domestic animals of different ages are susceptible to ringworm infection. Their results showed that rapid and effective cure of affected calves occurred with two to three applications of locally prepared ointment at 3-4-day intervals. However, it was reported that irrespective of sex, the extract-oil formulation extract from the stem bark of *Polyscias fulva* Hiern (Araliaceae) at 5% was able to cure ringworm infected animals after fourteen days of treatment while griseofulvin produced the same effect after thirteen days [21].

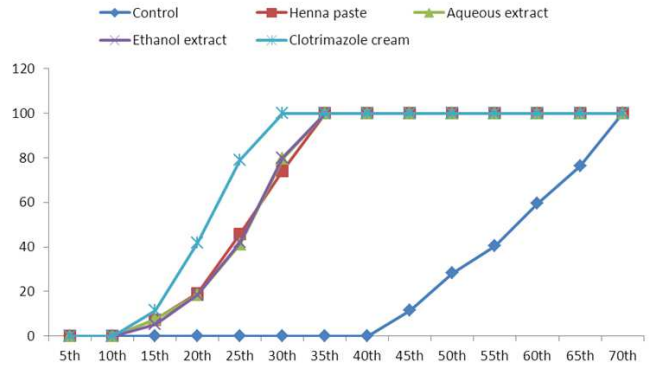


Figure 6. *Percentage regrowth of hair in lesions of the ringworm of scalp per days after treatment.*

Table 4. *Percentage regrowth of hair against ringworm of scalp during treatment.*

Days after treatment	Control	Henna paste	Aqueous extract	Ethanol extract	Clotrimazole cream
5	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
15	0.00	7.40±1.56	7.50±1.77	5.32±1.17	11.50±1.23
20	0.00	19.33±1.13	18.53±0.36	18.57±0.12	42.00±0.74
25	0.00	45.83±0.35	41.33±0.12	42.10±0.74	79.13±0.72
30	0.00	74.23±0.70	79.66±1.22	80.13±0.72	100
35	0.00	100	100	100	100
40	0.00	100	100	100	100
45	11.57±1.04	100	100	100	100
50	28.37±2.14	100	100	100	100
55	40.53±1.22	100	100	100	100
60	59.57±1.16	100	100	100	100
65	76.26±1.12	100	100	100	100
70	100	100	100	100	100

Table 5. *Percentage regrowth of hair against ringworm of face during treatment.*

Days after treatment	Control	Henna paste	Aqueous extract	Ethanol extract	Clotrimazole cream
5	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
15	0.00	18.37±1.14	13.41±1.46	14.42±1.21	28.37±1.24
20	0.00	42.20±0.64	21.19±1.23	23.17±1.14	62.12±0.84
25	0.00	60.10±0.72	42.20±0.74	47.60±0.14	84.23±0.32
30	0.00	80.23±1.11	77.73±0.72	87.73±0.74	100
35	0.00	100	100	100	100
40	0.00	100	100	100	100
45	15.27±1.33	100	100	100	100
50	38.24±1.17	100	100	100	100
55	50.82±1.25	100	100	100	100
60	79.56±1.90	100	100	100	100
65	86.44±1.75	100	100	100	100
70	100	100	100	100	100

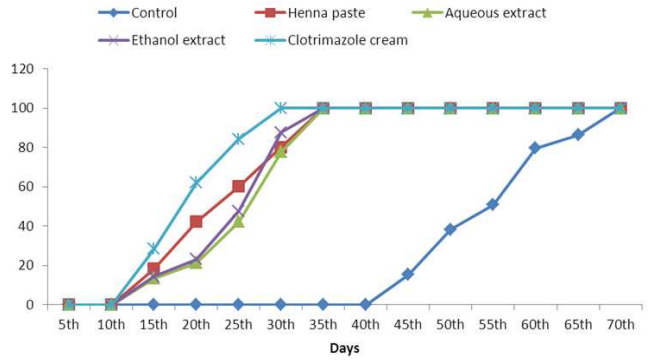


Figure 7. *Percentage regrowth of hair in lesions of the ringworm of face per days after treatment.*

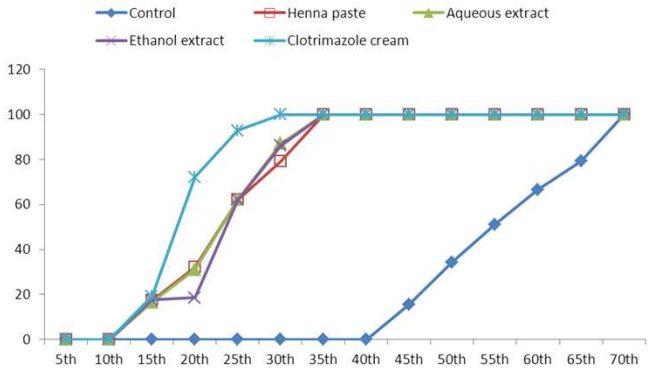


Figure 8. *Percentage regrowth of hair in lesions of the ringworm of body per days after treatment.*

Table 6. *percentage regrowth of hair against ringworm of body during treatment.*

Days after treatment	Control	Henna paste	Aqueous extract	Ethanol extract	Clotrimazole cream
5	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
15	0.00	17.48±1.24	16.70±1.16	17.58±1.17	19.27±0.12
20	0.00	32.20±0.31	31.10±1.23	18.67±1.65	72.12±0.55
25	0.00	62.12±1.12	62.24±0.34	62.10±0.43	92.84±1.12
30	0.00	79.23±0.11	87.13±0.72	86.13±0.55	100
35	0.00	100	100	100	100
40	0.00	100	100	100	100
45	15.57±1.13	100	100	100	100
50	34.34±0.13	100	100	100	100
55	51.12±1.15	100	100	100	100
60	66.56±1.10	100	100	100	100
65	79.24±1.50	100	100	100	100
70	100	100	100	100	100

4. Conclusion

Base on this research varieties of etiological agents were identified to cause infectious dermatoses among domestic animals in Northern part of Nigeria. The *in-vitro* and *in-vivo* sensitivity test of henna provide us with positive antifungal activity against all the etiological agents identified and have the efficacy to cure all the tested animals within a reasonable time. Therefore, the antimicrobial properties of the medicinal plants are reported from all over world and used in the treatment of many diseases such as ringworm and other skin infections. Medicinal plants are the best source to obtain a variety of newer herbal drugs. The result of this study showed that henna paste had the efficacy against all the types of ringworm tested. Therefore, topical application of this natural product (henna) paste is recommended for domestic animals that suffer from all types of ringworms, as it is affordable, available, and highly active and no adverse effect was observed. However, further research to isolate and purify active antifungal agents in henna plant is recommended.

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