RESEARCH ARTICLE

Synergistic Antifungal Bioassay of Neem Leaves Extract with Essential Oils against Dermatophytes

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ABSTRACT

Introduction: The neem plant is also known as a panacea in dentistry due to its antimicrobial properties and plays vital role in the cosmetic industry as it is extensively used in cosmetic preparations for hair and skin care.

Objective: The present study was carried out to evaluate the synergistic activity of essential oils in combination with neem leaves ethanolic extracts.

Methods: The antifungal activity of neem leaves (*Azadirachta indica*) ethanolic extracts in combination with *A. indica*, *Eucalyptus citriodora* and *Cymbopogon martini* essential oils was conducted by agar well diffusion method followed by M38-A2 broth microdilution method on pathogenic dermatophytes strains; *Microsporum gypseum, Trichophyton mentagrophytes* and *T. rubrum.* Neem leaves extract was obtained by treating the matured green leaves with ethanol. Moreover, HPLC analysis was performed to narrate the terpenoid content having extensively noted antifungal spectrum. The potent antimycotic fluconazole taken as a positive control.

Result: The result exhibited that there was overall growth retardation of the dermatophytes at minimum inhibitory concentration between 125 to 250 µg/mL for leaves extract and between 0.078 to 5.0 µL for selected essential oils. The range of minimum inhibitory concentration (MIC) of fluconazole was found between 0.25 and 0.50 µg/mL.

Conclusion: Neem leaves ethanolic extracts analysis showed the possible existence of terpenoids in extracts that are recognized having extensive biological action. The outcomes of this study showed a novel report on the therapeutic potential of neem leaves extract with *A. indica, E. citriodora* and *C. martini* to control dermatophytosis. The neem leaf extract and antifungal herbal oil in combination have been proven to improve and broad-spectrum antifungal activity and they can be formulated in suitable formulations for the effective treatment of mycosis.

Keywords: High-performance liquid chromatography, Neem, Antifungal spectrum, Minimum inhibitory concentration, Terpenoids.

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INTRODUCTION

Dermatophytosis is one of the most common reasons for dermatology consultations worldwide. Nails and skin fungal infections hits 20 to 25% of world population, with prevalence as high as 40% in tropical regions and subtropical regions due to prevailing temperature and humidity condition.[1] Fungi from the genus *Epidermophyton*, *Microsporum* and *Trichophyton* are prime sources of dermal infections in or on body regions, some studies states that fungus species *Trichophyton rubrum* mostly isolated from feet, groin and hands, followed by *T. mentagrophytes* which is abundant in nails and feet. *Epidermophyton floccosum* was less common than the fungus mentioned above, although it could also be found in the feet, nails, and groin. *M. canis* was the most common fungus in the scalp followed by *T. rubrum*. There was rise in the prevalence of *T. rubrum* in the twentieth century[2] in some parts of the tropical region of the world. The isolation of naturally occurring substances with antifungal action is gaining popularity. Of these, terpenoids have been shown to have either fungistatic or fungicidal effects on a variety of pathogenic fungi.

Due to the significant rise of immunocompromised hosts in recent years, dermatomycoses have become more common. Contemporary antifungal medications exhibit restricted effectiveness and result in significant side effects, including hepatotoxicity, gastrointestinal problems, cutaneous responses, and leucopenia in certain patients under treatment. The modern antifungal agents such as azole derivatives, allyl amines and morpholines widely used in the treatment of dermatomycoses become ineffective as a result of the development of fungal resistance extended duration and cost of the treatment.

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The research about neem has been concentrated on its known antifungal potential and antifeedant activity on insects.[3] There is a progressively greater interest in the separation of components having antifungal action, from where terpenoids have been discovered with fungicidal or fungistatic action on numerous disease-causing fungal species.

Researchers analyzed the synergistic effects of *C. martini* and *C. ambrosioides* oils on dermatophytes in male Guinea, and they came to the conclusion that the essential oils derived from these two species, when used in combination might be a future alternative to synthetic antifungal drugs to treat tinea corporis (ring worm) and another superficial mycosis in humans.[4] Antifungal activity of *E. citriodora oil* was proven highest than that of *E. globulus* and standard drugs against zoophillic microbes; *C. albicans* (yeast), *T. mentagrophytes* and *M. gypsum*[5,6] (filamentous forms) while some authors estimated that oil of *E. citriodora* was effective against *T. rubrum.*[7,8] *E. citriodora* oil as a potential antifungal against medically important dermatophytes and bacteria was also investigated,[9,10] while broad-spectrum antimicrobial potency of *C. martini* (Roxb.) W. Watson essential oil in topical formulation was tested against organisms from clinical isolates; *C. albicans, Trichophyton mentagrophytes, T. rubrum*, *M. canis* and *T. verrucosum,*[11] Literature is also available demonstrating the potency of essential oils of *E. citriodora*, *C. martini* and *A. indica* (Neem) against mycosis.

Neem leaves organic extract and their constituents also hinder the growth of numerous pathogenic fungi was proven by SDB dilution method and reported that neem seed extract was effective to inhibit *T. mentagrophytes*, *M. nanum* and *T. rubrum* at 31 µg/mL.[12] On stated dermatophyte the neem leaves extract shown the Minimum Inhibitory Concentration (MICs) in between 150 to 500 µg/mL. After assessing the rising pattern of all dermatophytes, the authors detected that in thirty days the inhibition zone reduced, while mixing the media with neem seed extract. The active antimycotic region in neem oil is a combination of terpenoids resulting from methanol partitioning, when determined against *Drechslera oryzae*, *Fusarium oxysporum* and *Alternaria tenuis* fungus.[13] The earlier studies also support the additive or synergistic effect of terpenoids in neem extract.

Neem plant is also known as panacea in dentistry due to antimicrobial properties and plays vital role in cosmetic industry as it is extensively used in cosmetic preparations for hair and skin care. Active phytochemical compounds found in neem plant includes glycosides, dihydrochalcone, tannins, coumarin, triterpenoids such azadirachtin, nimbin, diterpenoids, Nimbidine, proteins, carbohydrates, sulphurous compounds, polyphenolics[14] etc. Neem oil, also synonymed as margosa oil, an oil obtained from the fruits and seeds of the neem plant; *A. indica* of family Meliaceae contains azadirachtin as the main triterpenoid along with nimbin.

Main components of *E. citriodora* consist of citronella, dl-isopulegol and citronellol with additional important constituents such as limonene, geraniol P-cymene, alpha-pinene, and camphene. The eucalyptus oil is generally used to cure body pains, fever, headache, chronic bowel complaints and dysentery however its antimicrobial activity has been proven against diversity of bacterial and fungal species.[15] The oil of *C. martini* contains trans-geraniol, b-elemene,linalool, geranyl acetate and e-citral. α-bisabolol, α-terpinene, nerolidol and terpinen-4-ol etc.[4] Antimicrobial activity was effectively proven, in the stated study, *A. indica* essential oil was formulated with different essential oils and assessed for antifungal against poultry isolated *Campylobacter jejuni.*[16]

From earlier some studies, the improvement in antimicrobial effectiveness of some essential oils seen when merged together, hence the fusion of essential oils of *A. indica*, *E. citriodora* and *C. martini* was prepared with neem leaves ethanolic extract and subjected to further investigation against opportunistic dermatophytes.

MATERIAL AND METHODS

Neem plant leaves were collected from vicinity of Hanuman Gardh Wardha, Maharashtra and Voucher specimens were authenticated from Bajaj Science College Herbarium situated in Wardha, Maharashtra. freshly collected mature leaves were cleaned, shade dried for 14 days and powdered. 25 gm neem leaves powder was treated with 100 mL of ethanol and allowed to soak overnight with proper caps. It was then centrifuged for 20 minutes at 5000 rpm, the suspension was filtered by using Whatman filter paper no.1. The supernatant fluid was dried in sterile glass petri dishes under germicidal tube light. Completely dried powder was collected by scraping and stored at -4°C and the weight of extract was determined using a Denver Instrument SI- 234 digital balance. Neem leaves extract was further studied for phytochemicals content.

Selected herbal oils such as *A. indica oil, E. citriodora* oil and *C. martini* oil were purchased from local herbal oil vendor. Three strains of dermatophytes used in study included *M. gypseum*, *T. mentagrophytes, T. rubrum* were procured from MTCC Chandigardh, India. Potato dextrose agar (Hi-Media), Saborauds dextrose agar (Hi-Media), Saborauds dextrose broth (Hi-Media), FD035-5VL CC-Supplement(Hi-Media), DMSO(Merck), fluconazole as standard antimycotic drug was used in the study.

Preparation of Fungal Inoculum

The freeze-dried fungi were maintained on suitable culture media as suggested by MTCC Chandigarh, India. They were sub cultured and further incubated at around 27°C for 7 to 12 days approximately. The preparation of the fungal inoculum was done with fresh fungi broth cultures and adjusted to 0.5 McFarland turbidity. Its standardization was done on Agilent Cary 100 UV visible spectrophotometer as previously described.[17]

Determination of Antifungal Activity

The *A. indica* leaf extract was dissolved in DMSO at a concentration of 1-g/mL, and then sieved using Whatman filter paper. The dissolved extract was further diluted to attain desired 50 to 200 µg/mL concentration. By agar well diffusion method, the sensitivity of test fungi to leaves extracts was

determined on Saborauds dextrose agar plates. For this fresh broth cultures of test fungi were taken and spread evenly using a sterile glass spreader on agar plates. In 4 wells (6 mm holes each) were made onto agar using sterile cork borer. wells were loaded with the leaves extract concentrations from 50 to 200 µg/mL. Fluconazole (25 µg/mL) and DMSO were pipetted into the two wells to serve as standard and control. Agar plates were then incubated for 7 days at 37ºC. Similarly essential oils of *A. indica*, *E. citriodora,* and *C. martini* were tested for each dermatophyte, the diameter of zone was measured after examining the inhibition zone. It was carried out in triplicates and average of three performed independent experiments were measured as results.

Two Combinations of leaves extract and essential oils were made according to the previous result data interpretation and bioassay was carried out in triplicate.

Determination of Minimum Inhibitory Concentration

It was performed as per process described earlier.[18,19] MIC was determined by incorporating various concentrations of extracts (2000 to 15.625 µg/mL) and (40 to 0.075 µl/mL) in Sabouraud dextrose broth. Standardized fungal inoculum was added to broth and suitable dilutions of neem leaves extract and selected essential oils were made seperately. Sabouraud dextrose broth alone worked as control. The Sabouraud Dextrose broth mixed with extract and oil dilutions, standard and Control were added in 96 well microtiter plate and incubated at 37°C in a Biochemical oxygen demand (BOD) incubator (REMI, Cl 16) and turbidity was observed for 7 days. The minimum concentration of the extract/essential oil that hasn't showed any viable growth in between 7 days of incubation is recorded as the MIC. Six combinations of leaves extract and essential oils were made by MIC data interpretation and MIC of combination was recorded.

RESULTS

The diverse levels of growth inhibition among test dermatophytes with neem extract and selected oil combinations were shown in antifungal bioassays. Tables 1, 3 and 5 display notable variation in fungal inhibition between concentration of essential oils and leaves extracts, regarding their moderate fungal growth suppression when used singly against *M. gypseum, T. mentagrophytes* and *T. rubrum* respectively. When comparing the result from each well as per Tables 2, 4 and 6, it was found maximal when the Neem extract was combined with the selected essential oil combinations; highest inhibition zone was obtained in combination with low doses (where zone was no or minimum)of each selected essential oils and neem leaves extract as compared to that of the zones for neem extracts and selected essential oils tested separately. The MIC of combinations within MIC range above and below of each were assessed and thus found promising which therefore reflects the activity range of neem leaves extract and essential oils. Tables 7, 8 and 9 for MIC determination shows complete growth inhibition for selected dermatophytes at the minimum inhibitory concentrations (125 to 250 µg/mL) for neem leaves and (0.078 to

Table 1: Zone of inhibition in mm for *A. indica* leaves extract with essential oil concentrations against *Microsporum gypseum*

S. No	*Name of plant part/ Essential oil*	*Concentration* NLE (µg/ml) EO (µl)	*Zone of inhibition in mm*			*Mean ZOI*
1	*A. indica* leaves extract (NLE)	50	-	-	-	-
		100	7.4	7.5	7.9	7.6
		150	8.8	8.6	8.5	8.63
		200	11.2	11.0	11.6	11.26
2	*A. indica* oil (NO)	2.0	-	-	-	-
		4.0	7.2	7.3	7.3	7.26
		6.0	7.8	7.6	7.7	7.7
		8.0	8.2	8.6	8.4	8.4
3	*E. citriodora* oil (ECO)	2.0	-	-	-	-
		4.0	-	-	-	-
		6.0	7.5	7.4	7.5	7.46
		8.0	8.6	8.5	8.6	8.56
4	*C. martini* oil (CMO)	0.1	-	-	-	-
		0.2	6.6	6.2	7.0	6.6
		0.3	8.5	8.2	8.3	8.3
		0.4	13.3	13.5	13.0	13.26

Table 2: Zone of inhibition for *A. indica* leaves extract in combination with essential oil concentrations against *M. gypseum*

S. No	*Name of combination*	*NLE*	*NO*	*ECO*	*CMO*	*Zone of inhibition*			*Mean ZOI*
1	Combination 1	50	2.0	4.0	0.2	14.9	15.0	15.2	15.03
2	Combination 2	50	4.0	6.0	0.3	16.2	16.8	17.1	16.7

Table 3: Zone of inhibition in mm for *A. indica* leaves extract with essential oil concentrations against *T. mentagrophytes*

S. No	*Name of plant part/ Essential oil*	*Concentration* NLE (µg/mL) EO (µl)	*Zone of inhibition in mm*			*Mean ZOI*
1	*A. indica* leaves extract (NLE)	50	-	-	-	-
		100	6.5	6.5	6.7	6.56
		150	7.6	7.5	7.7	7.6
		200	7.0	7.5	7.4	7.3
2	*A. indica* oil (NO)	0.5	-	-	-	-
		1.0	-	-	-	-
		1.5	6.3	6.5	6.7	6.5
		3.0	7.8	8.2	7.0	7.6
3	*E. citriodora* oil (ECO)	0.1	6.2	6.7	6.4	6.43
		0.3	7.5	7.8	8.0	7.76
		0.5	10.8	11	11.4	11.06
		1.0	11.5	11.2	10.9	10.93
4	*C. martini* oil (CMO)	0.2	6.8	7.0	7.3	7.03
		0.4	7.3	7.5	7.8	7.53
		0.6	7.8	8.2	8.4	8.13
		0.8	9.0	8.6	8.9	8.83

Table 4: Zone of inhibition for *A. indica* leaves extract in combination with essential oil concentrations against *T. mentagrophytes.*

S. No	*Name of combination*	*NLE (*µg/mL)	*NO*	*ECO*	*CMO*	*Zone of inhibition*			*Mean ZOI*
1	Combination 1	50	1.0	0.1	0.2	16.6	17.0	17.8	17.13
2	Combination 2	50	2.0	0.3	0.4	17.2	17.5	18.0	17.56

Table 5: Zone of inhibition in mm for *A. indica* leaves extract with essential oil concentrations against *T. rubrum*

S. No	*Name of plant part/Essential oil*	*Concentration* NLE (µg/mL) and EO (µl)	*Zone of inhibition in mm*			*Mean ZOI*
1	*A. indica* leaves extract(NLE)	50	-	-	-	-
		100	-	-	-	-
		150	6.9	7.2	7.0	7.03
		200	7.8	8.2	8.5	8.16
2	*A. indica* oil (NO)	0.5	-	-	-	-
		1.0	-	-	-	-
		1.5	7.0	7.2	7.5	7.23
		3.0	17.2	20	21.4	19.53
3	*E. citriodora* oil (ECO)	0.1	13.8	15	16.3	15.03
		0.3	15.7	16.1	16.8	16.20
		0.5	14.6	16.3	18.1	16.33
		0.7	No growth	No growth	No growth	No growth
4	*C. martini* oil (CMO)	0.1	No growth	No growth	No growth	No growth
		0.2	No growth	No growth	No growth	No growth
		0.3	No growth	No growth	No growth	No growth
		0.4	No growth	No growth	No growth	No growth

Table 6: Zone of inhibition for *A. indica* leaves extract in combination with essential oil concentrations against *T. rubrum*

S. No	*Name of Combination*	*NLE* µg/ml	*NO* µl	*ECO* µl	*CMO* µl	*Zone Of Inhibition*			*Mean ZOI*
1	Combination 1	50	0.5	0.1	0.1	16.8	17.2	17.0	17.0
2	Combination 2	50	1.0	0.3	0.2	20.2	22.6	22.1	21.63

Table 7: Minimum Inhibitory concentration for *A. indica* leaves extract with essential oil concentrations against *M. gypseum*

Minimum inhibitory concentration (MIC) in µg/mL for neem leaves extract and µl/mL for essential oils										
S. No	*NLE*	*Remark*	*NO*	*Remark*	*ECO*	*Remark*	*CMO*	*Remark*	*Comb*	*Remark*
1	2000	---	80	---	80	---	80	---	C 1	---
2	1000	---	40	---	40	---	40	---	C 2	---
3	500	---	20	---	20	---	20	---	C 3	---
4	250	---	10	---	10	---	10	---	C 4	---
5	125	---	05	---	05	---	05	---	C 5	+++
6	62.5	+++	2.5	+++	2.5	+++	2.5	---	C 6	+++
7	31.25	+++	1.25	+++	1.25	+++	1.25	---		
8	15.625	+++	0.625	+++	0.625	+++	0.625	---		
9			0.312	+++	0.312	+++	0.312	---		
10			0.156	+++	0.156	+++	0.156	+++		
11			0.078	+++	0.078	+++	0.078	+++		

No Growth :- --- Growth :- +++

Table 8: Minimum inhibitory concentration for *A. indica* leaves extract with essential oil concentrations against *T. mentagrophytes*

S. No	*MIC in µg/mL for neem leaves extract and µL/mL for essential oils*									
	NLE	*Remark*	*NO*	*Remark*	*ECO*	*Remark*	*CMO*	*Remark*	*Comb*	*Remark*
1	2000	---	80	---	80	---	80	---	C 1	---
2	1000	---	40	---	40	---	40	---	C 2	---
3	500	---	20	---	20	---	20	---	C 3	---
4	250	---	10	---	10	---	10	---	C 4	+++
5	125	---	05	---	05	---	05	---	C 5	+++
6	62.5	+++	2.5	---	2.5	---	2.5	---	C 6	+++
7	31.25	+++	1.25	+++	1.25	---	1.25	---		
8	15.625	+++	0.625	+++	0.625	+++	0.625	---		
9			0.312	+++	0.312	+++	0.312	---		
10			0.156	+++	0.156	+++	0.156	+++		
11			0.078	+++	0.078	+++	0.078	+++		

No Growth :- --- Growth :- +++

Table 9: Minimum inhibitory concentration for *A. indica* leaves extract with essential oil concentrations against *T. rubrum*

MIC in µg/mL for neem leaves extract and µL/mL for essential oils										
S. No	*NLE*	*Remark*	*NO*	*Remark*	*ECO*	*Remark*	*CMO*	*Remark*	*Comb*	*Remark*
1	2000	----	80	---	80	---	80	---	C 1	---
2	1000	----	40	---	40	---	40	---	C 2	---
3	500	----	20	---	20	---	20	---	C 3	---
4	250	----	10	---	10	---	10	---	C 4	---
5	125	+++	05	---	05	---	05	---	C 5	---
6	62.5	+++	2.5	---	2.5	---	2.5	---	C 6	+++
7	31.25	+++	1.25	+++	1.25	---	1.25	---		
8	15.62	+++	0.625	+++	0.625	---	0.625	---		
9			0.312		0.312	+++	0.312	---		
10			0.156		0.156	+++	0.156	---		
11			0.078		0.078	+++	0.078	---		
12							0.039	+++		

No Growth :- --- Growth :- +++

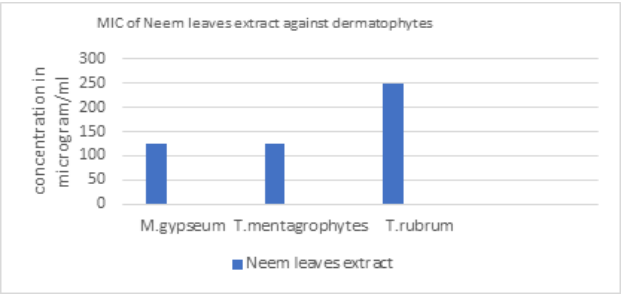

Figure 1: MIC of neem leaves extract against dermatophytes

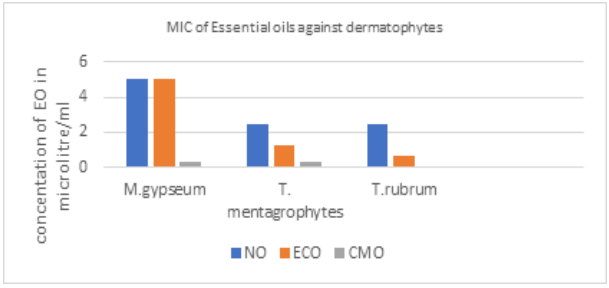

Figure 2: MIC of selected essential oils against dermatophytes

5.0 µL/mL) for selected essential oils. Figure 1 shows minimum inhibitory concentration of neem leaves extract and Figure 2 reflects the MIC of neem leaves extract against *M. gypseum, T. mentagrophytes* and *T. rubrum,* respectively.

DISCUSSION

Numerous authors established the potential of "neem" as potent antifungal agent. The use of herbal preparations to treat innumerable ailments involving skin disease was retrospective

to age of ancient times.[20] Even in same generation of medical progress and development, herbs use in aromatherapy was pretty popular.[21] This has increased interest in creating essential oil-based antimicrobial treatments to treat known infections, which may eventually replace synthetic antimicrobials to which the organisms have developed resistance. Therefore, the goal of this work is to quantify and assess the antibacterial efficacy of essential oils in combination with neem leaves extract against microorganisms that cause skin diseases. Many studies have acknowledged the significance of neem leaves extract, selected essential oils under study and their components as antimycotic agents. It is noteworthy that the MICs of leaves (125–250 µg/mL) and essential oils (0.078–5.0 µL/mL) was able to inhibit the dermatophytes under study. Hence it may be inferred that the antifungal properties of extracts and essential oils against dermatophytes is related to their chemical composition; terpenoids nimbin and nimbidin present in neem leaves and seed oil of *A. indica* (NO), similarly two major oxygenate monoterpenes Citronellal and Citronellol present abundantly in *E. citriodora* oil (ECO) and most abundantly found trans-geraniol upto 60.9% in *C. martini* essential oil (CMO).

CONCLUSION

In an effort to find novel phytochemicals with possible medicinal applications, ethnobotanical study has grown once more in recent years. Specifically, a number of studies on the in vitro antidermatophytic activity of plant essential oils have been published, but not much research has been done on the synergistic effects of essential oils with neem leaves extract. Comparing our MIC findings with those from earlier publications can be challenging, though, as the composition of essential oils can differ significantly based on a plant's chemotype, the presence of specific endophytic microbes, the location, the age of the plant, and the method of oil extraction. The selected essential oils demonstrated notable inhibitory effects against the studied dermatophytes, when combined with neem leaf extract.

The proliferation of *M. gypseum, T. mentagrophytes* and *T. rubrum* is reciprocally slowed down by the combination of Neem leaves and selected essential oils. Neem extract had the outstanding antifungal property in all but shown at quite higher concentration furthermore *Eucalyptus citriodora* is effective against *T. mentagrophytes and M. gypseum* dermatophytes at very low to moderate concentration while*, T. rubrum* is susceptible to *C. martini* at very low doses. *A. indica* essential oil has shown the effect at higher concentration but was negligible as compared to the other essential oils under study. Although *A. indica* leaves extract and essential oil has been proven more effective on *Candida albicans* and other dermatophytes by several authors. The result shows overall dermatophytes growth inhibition showed at MIC between 125 to 250 µg/mL for Neem leaves extract, and 0.078 to 5.0 µL/ml range for the selected essential oils. When the selected essential oils and Neem extract were studied together in a combination below their MIC values, it was demonstrated that the combination entity that had demonstrated the prominent zone of inhibition at a dose much lower than that of individuals effect that could have been the origin of the synergistic phenomena. Positive control MIC range was found within the range of 2.5 to 12.5 µg/mL. Even though MICs of essential oils and Neem extracts has different magnitudes above than the standard Fluconazole, it must account for the noteworthy adverse effects of azoles like synthetic drugs. Outcomes of this study showed a novel search on curative ability of neem with *C. martini* and *E. Citriodora* essential oil to control and manage dermatophytosis. The extract of neem leaves and antimycotic herbal oils in combination have been proved for improved and broad-spectrum antifungal action and it is imperative to blend the extract of neem leaves with selected essential oils in suitable topical dosage form that can effectively manage the opportunistic fungal skin disorders that are on the rise these after COVID 19 era.

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