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Development, characterization and *in vitro* antifungal evaluation of topical formulation of *Tridax procumbens* L. Leaf extract

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Abstract
Background: Fungal infections have become major concern and are caused by various fungi. The primary target of these fungi is skin which leads to numerous skin disorders like eczema, dermatitis, candidiasis, athlete's foot, ringworm etc.
Objective: To develop a safe and effective antifungal topical cream formulation of *Tridax procumbens* L. leaf extract.
Method: The ethanolic extract was prepared using soxhlet apparatus. The phytochemical screening and molecular docking studies of extract was performed. The cream was prepared by oil in water emulsion method. MIC studies was carried out by Agar dilution method and antifungal activity of cream was carried out by Agar diffusion method against *Candida albicans*.
Results: Phytochemical screening of extract revealed the presence of various phytoconstituents. Docking results indicated benzamide derivative showing best protein-ligand interactions. The extract of *Tridax procumbens* L. showed potent antifungal activity with MIC value 0.88 mg/ml. The cream also exhibited significant antifungal activity.

Keywords: *Tridax procumbens* L., eczema, dermatitis, candidiasis, athlete's foot, ringworm, molecular docking, agar dilution, agar diffusion, benzamide, sterol demethylase, antifungal activity

1. Introduction
Fungal infections have been a major concern across the globe. The primary target of infection is skin. Most of the fungi causes skin diseases like candidiasis, eczema, dermatitis, etc. Fungal infections are caused by fungus. It is also called as mycosis. Symptoms of fungal infection include itching, redness, swelling, irritation, etc. Different types of formulations like ointments, creams, gels, emulsions etc. have been developed for treating fungal infections. Anti-fungal drugs are those which selectively acts against fungal organism. Polyenes are the class of drugs which interacts with ergosterol and causes loss of membrane selective permeability and of cytoplasmic components. Amphotericin B and nystatin are examples of polyenes. Amphotericin B is commonly used in the treatment of fungal diseases such as histoplasmosis, cryptococcal meningitis. Nystatin is used for topical treatment of fungal infections which are caused by *Candida albicans*. Azoles are another class of antifungal agents. Examples are econazole, miconazole, clotrimazole. These are topical agents which are used for the treatment of skin, oral and vaginal infections. Synthetic antifungal agent's terbinafine and naftifine are administered topically and orally for the treatment of fungal infections caused by *dermatophytes*. Flucytosine is used for Candida infections, systemic cryptococcal and chronomycosis. Griseofulvin is orally administered for treating superficial fungal infections [1].

Table 1: Commercial synthetic drugs used in the treatment of fungal diseases [1]

Chemical Class	Synthetic Antifungals	Uses
Azoles	Clotrimazole, Econazole, Isoconazole, Ketoconazole	Candidiasis, vaginal yeast infection, topical fungal infections
Echinocandins	Caspofungin, Micafungin	Esophageal candidiasis, Salvage therapy
Polyenes	Amphotericin B, Nystatin	Systemic mycosis, Superficial mycosis
Phenolic Cyclohexane	Griseofulvin	Dermatophytic infections
Pyrimidines	Flucytosine	Cryptococcosis, invasive aspergillosis
Phthalimides	Captan	Candida infections, invasive dermatophytic conditions
Morpholines	Amorolfine	Topical fungal infections

Since, many fungi species have developed resistance towards synthetic drugs, it has become a major challenge to develop new drugs and combat resistance. Resistance occurs when the organism no longer responds to antifungal drugs. Due to antifungal resistance, currently only three types of drugs are available for the treatment. Antifungal resistance can develop through many ways. Some strains of fungi are resistant towards the treatment using certain anti-fungal drugs. It can also develop when the fungi are exposed to an antifungal drug for over a period of time, when it is improperly administered to the patient. *Aspergillus* and some strains of *Candida* have shown resistance to antifungal drugs [2, 3, 4].

Table 1: List of fungal infections with corresponding causative organism [2, 3, 4]

Fungal Infections	Causative Organism
Athlete's foot	*Trichophyton rubrum*
Jock itch	*Dermatophytes*
Ring worm	*Dermatophytes*
Yeast infection	*Candida albicans*
Toenail fungus	*Trichophyton rubrum*

Tridax procumbens L. is a widely spread hispid, procumbent herb, usually found as a weed and is perennial in nature with flowering-fruiting throughout the year. The plant is commonly known as coat buttons or tridax daisy and is a species of daisy family. It is native to the tropical America. Its flowers are white or yellow with three – toothed ray florets. Leaves are toothed; arrowhead shaped and fruits are hard; achene covered with stiff hairs. Feathery, plume-like white pappus at an end. The chemical constituents present are flavonoid procumbenetin, alkyl esters, sterols, pentacyclic triterpenes, fatty acids and polysaccharides. The plant is rich in minerals such as iron, copper, manganese, sodium and zinc and other trace minerals such as magnesium, phosphorous, potassium, selenium and calcium. The aqueous extract contains phytochemicals such as alkaloids, steroids, carotenoids, flavonoids, saponins and tannins. While organic solvent extraction with ethyl acetate has flavonoids and bergenin. Some of the 2° metabolites presents are fatty acid derivatives, sterols, lipid constituents, luteolin, glucoluteolin, quercetin, isoquercetin and fumaric acid [5].

Traditionally, *Tridax procumbens* L. has been in use in India for wound healing and as an anticoagulant, antifungal, and insect repellent. Its leaf extracts were used for infectious skin diseases in folk medicines. It is used in Ayurvedic medicine for liver disorders, as a hepatoprotective, for gastritis, and heartburn. It is also used in the treatment for boils, blisters, and cuts. In regions of Central America, it is used to treat inflammation, cold and anemia. The juice of leaves is used for treating wounds and also as a mild anti-coagulant. It is also used for treating viral, bacterial infections and fungal infections such as vaginitis and skin infections and also for mucosal inflammation, diarrhea, stomach pain. A study has reported that use of this plant reduced the symptoms of anemia in lactating women. It is also useful for treating conditions such as respiratory and GI infections, diabetes and hypertension. In some regions, the plant is used for treating protozoal infections such as dysentery, leishmaniasis. The use of this entire plant for treating epilepsy, typhoid fever, etc. has also been reported. The aqueous extract is found to be showing anti-plasmodial action whereas ethanolic extract shows activity against *S. aureus.* It is also rich with minerals such as Magnesium, Calcium, Sodium and Potassium. It is also showing cytotoxic activity against malignant tumor cells [6].

2. Materials and Methods

2.1 Materials

The chemicals, media, and solvents used in this study were absolute ethanol, cetyl alcohol, dimethyl sulfoxide, liquid paraffin, methyl paraben, propyl paraben, propylene glycol, stearic acid, stearyl alcohol, triethanolamine and white bees wax and Sabouraud dextrose agar were purchased from HiMedia Laboratories (India).

2.2 Methods

2.2.1 Collection of Plant Material and Extraction

T. procumbens (leaf part) was collected from the forests of Ramnagara district, Karnataka, India. The identity of the plant was authenticated by Dr. Noorunnisha Begum, lead researcher botanist in Foundation for Revitalization of Local Health Traditions, Bangalore, Karnataka, India. Following collection and identification, fresh leaves of *T. procumbens* (100 g) were washed and dried in air for 2 hrs followed by drying in hot air oven for 7 hrs at 50 °C. The dried leaves were crushed into coarse powder and placed in a 1 L soxhlet cylinder attached with 1 L round bottomed flask. The plant was wetted with 750 ml of absolute ethanol. The leaf extract was obtained by soxhlet extraction using soxhlet-type apparatus for 3 days. The extract was collected and concentrated on water bath at 50 °C till 75% solvent is lost. Then it was further dried in desiccator [7].

2.2.2 Phytochemical Screening of Extract

Standard chemical tests were used for the qualitative screening of phytochemical ingredients of ethanolic extract. The presence of chemicals including Alkaloids, Carbohydrates, Fixed oils and fats, Flavonoids, Glycosides, Phenolic compounds, Proteins, Steroids, Tannins, Terpenes and Terpenoids were tested according to standard tests and confirmed [7].

2.2.3 Molecular Docking Studies

Two protein targets were selected namely Dihydrofolate reductase and 14-α Sterol demethylase of *Candida albicans.* The X-Ray Diffraction structures of proteins were taken from RCSB Protein Data Bank in PDB ID: 3QLS and 5FSA in PDB format. The ligands were the phytoconstituents of ethanolic extract of tridax leaves which were obtained through GC-MS analysis report [8] referred from the literature. The proteins were prepared using Discovery Studio 2021 Client by removing water molecules, unwanted residues and other inhibitors (ligands) already present in protein. The structures of phytoconstituents were taken from Pubchem in 3D conformer as SDF file format. All the ligands were then converted to PDB file format using Discovery Studio 2021 Client. Two standard antifungal drugs namely Ketoconazole and Methotrexate for sterol demethylase and DHFR were selected for comparison of docking scores. The structure of protein was loaded in PyRx software where the Kollmann and Gasteiger charges were assigned. The proteins were then converted to PDBQT file format. Then the ligands were loaded into PyRx, energy minimized and converted to PDBQT file format. Then protein and the ligands were selected for docking and grid box appeared in the protein structure. The position of grid box was adjusted based on the dimensions noted for the binding site as center x = 2.9778, center y = -0.5747 and center z = 27.6016 for DHFR and

center x = 194.671260608, center y = -3.66085036537 and center z = 38.2285334129 for Sterol demethylase. The grid size was 45 x 45 x 45 for both proteins. Molecular docking was performed using Vina in PyRx software after assigning grid dimensions. The process was allowed to run and all the ligands were docked each giving 9 poses with corresponding docking scores. The ligand which gave best score compared to standard drugs against the two proteins was chosen and its docking interactions were visualized in 2D conformation using Discovery Studio 2021 Client.

Table 3: Molecular Docking Studies - Selected Target Proteins

Target proteins	PDB ID
Dihydrofolate Reductase	3QLS
14-α Sterol Demethylase	5FSA

Table 4: Standard Drugs used for Molecular Docking

Standard Drugs (Ligands)	Target protein	Activity
Ketoconazole	5FSA	Antifungal Activity
Methotrexate	3QLS	Antifungal Activity

2.2.4 Preparation of Topical Formulation
The formulation base was prepared by oil in water emulsion method in accordance with the formula given in table 5. Different concentration (5% and 10% w/w) of the leaf extract topical formulations were prepared by incorporating the extract into the soft mass of the formulation base. The base, without the extract, was used as negative control in the assay [9, 10].

Table 5: Composition of formulation base used to prepare leaf extract topical formulations.

Oily Phase		Aqueous Phase	
Ingredients	Quantity (% w/v)	Ingredients	Quantity (%w/v)
White Bees Wax	1	Methyl Paraben	0.018
Stearic Acid	2	Propyl Paraben	0.04
Stearyl Alcohol	4	Triethanolamine	2
Liquid Paraffin	4	Propylene Glycol	6
Cetyl Alcohol	6	Water	100

2.2.5 Physical Evaluation of Topical Formulation
2.2.5.1 Organoleptic Examination
The prepared formulations were inspected visually for their physical appearance, color, texture, phase separation, and homogeneity. Homogeneity and texture were tested by pressing small quantity of the formulated product between thumb and index finger. The consistency of the formulation and presence of coarse particles were used to evaluate the texture and homogeneity of the formulation [11].

2.2.5.2 Determination of Viscosity
The viscosity of prepared formulations was determined using Brookfield Viscometer using Spindle S-04 at 20 RPM.

2.2.5.3 Centrifugation Test
A 10 g portion of formulation was placed in a centrifuge tube (1 cm diameter) and centrifuged at 2000 rpm for 5, 15, 30, and 60 min. Then the phase separation and solid sedimentation of the formulations were inspected [11].

2.2.5.4 Stability Test
The stability was checked by keeping formulations in Environmental stability chamber at 25° – 27 °C for 14 days. The formulations were inspected for creaming or coalescence [11].

2.2.5.5 Determination of pH
A suspension of formulation in 1% potassium nitrate solution was prepared and its pH was determined using Digital pH meter. A magnetic stirrer was used to produce homogeneity [11].

2.2.5.6 Spread ability
The spread ability was determined by applying 1g of each formulation on a glass plate over which the second plate was placed. The sample was pressed between two plates. A weight of 100 g was allowed to rest on the plate for 5 mins. The increased in diameter due to spreading of formulation was noted [11].

2.2.6 Test Organism
Standard fungal strain of *Candida albicans* (grown cultures) [12].

2.2.7 Commercial Topical Antifungal Formulations
Candid cream (1% Clotrimazole I.P, Batch No.: 11212062, Glenmark Pharmaceuticals Ltd., India), Fungicros cream (0.25% Amorolfine I.P, Sun Pharmaceutical Industries, India) and Ketoconazole cream (2% Ketoconazole I.P, Batch No: HH161, Hegde and Hegde Pharmaceutica LLP, India) were obtained from local drug retail outlets in Bengaluru, India. The commercial topical drugs were used as positive control in the assay.

2.2.8 Antifungal Assays
2.2.8.1 Minimum Inhibitory Concentration (MIC)
The MIC value of *T. procumbens* extract was determined using agar dilution assay. Two-fold serial dilutions of leaf extract in dimethyl sulfoxide having a final concentration of extract ranging from 0.17% (w/v) to 14% (w/v). Control plates, containing no extract, were run simultaneously. The agar plates mixed with the dilutions of extract and the control plate were inoculated by standard inoculum (having optical density of 0.08 - 01 at 625 nm wavelength) of five-day (C. albicans) grown cultures. The plates were incubated at 25 °C for seven days. After incubation, the end-points for the extract were determined by placing plates on a dark background and observing the lowest concentration that inhibits visible growth, which is recorded as the MIC [12].

2.2.8.2 Antifungal Activity of Topical Formulation
Antifungal activity of topical agents was performed using the method adopted in our laboratory. 30 ml of Sabouraud dextrose agar (for fungi) was added to three sterile Petri dishes and allowed to solidify. After solidification, three holes with 10 mm diameter was made by cutting out plug of agar at equal distance in each plate using sterile cork-borer. The plates were inoculated by standard inoculum of C. albicans using cotton swab. About 1 ml of designated topical formulations were added to two wells followed by adding 1 ml of designated commercial topical formulation in third well of each plate. After the plate was solidified for 2-3 minutes, it was inverted and allowed to stand for 1 hour at room temperature and incubated at 25 °C for 7 days. After incubation, the diameter of zones of inhibition was measured [13, 14].

3. Results

3.1 Phytochemical Screening of Extract

The standard chemical tests indicated the presence of range of phytoconstituents in ethanolic extract of *T. procumbens* leaves which are summarized in Table 6.

Table 6: Phytoconstituents present in ethanolic extract of *Tridax procumbens* L

Phytoconstituents	Plant Extract
Alkaloids	+
Glycosides	-
Steroids	+
Carbohydrates	+
Flavonoids	+
Phenolic Compounds	+
Terpenes and Terpenoids	+
Fixed Oils and Fats	+
Tannins	+
Proteins	-

3.2 Molecular Docking Studies

The docking of sixteen compounds of ethanolic leaf extract as per GC-MS report was done using PyRx software. Among all the compounds, benzamide derivative showed best docking scores compared to standard drugs which are summarized in Table 7 and the interactions of the derivative with the target proteins is shown in Figure 1-2.

Table 7: Docking Scores of Benzamide Derivative of Tridax Extract against selected proteins

Compound	Dihydrofolate Reductase (PDB ID: 3QLS)	14-α Sterol Demethylase (PDB ID: 5FSA)
N-[3′-Cyano-6′-(3″-methyl-5″-oxo-1″-phenyl-2″-pyrazolin-4″-yl)-4′-phenylpyridin-2′-yl] benzamide	-10.8	-11.7
Ketoconazole	-	-10.6
Methotrexate	-10.4	-

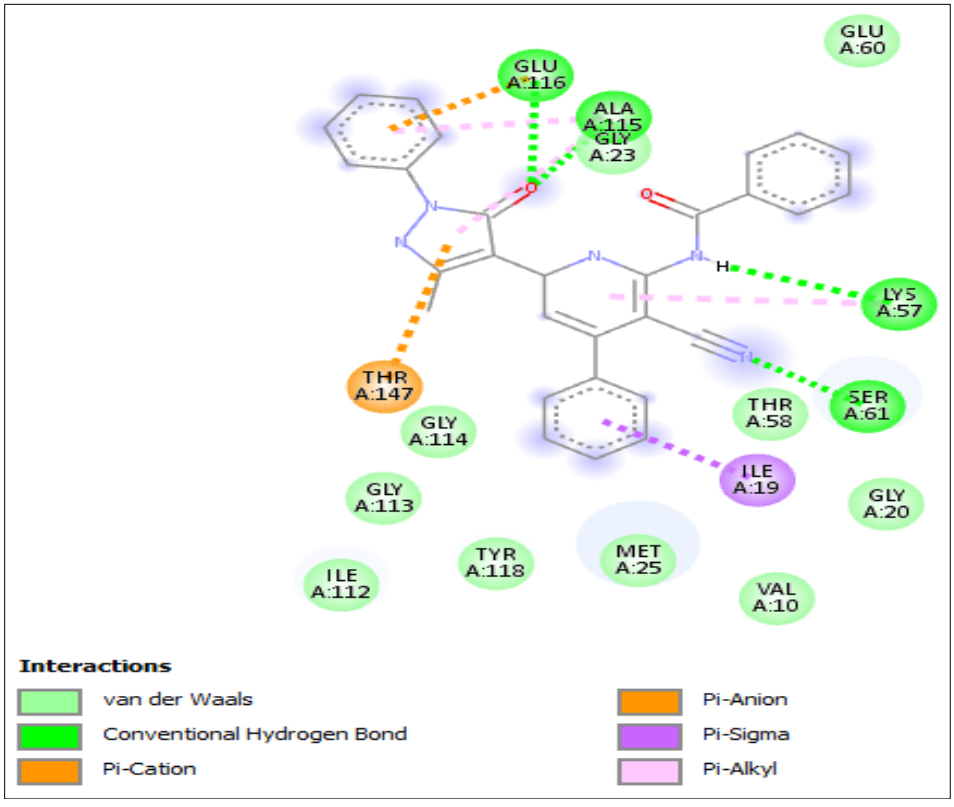

Fig 1: Binding interaction of Benzamide Derivative with Dihydrofolate Reductase of C. albicans (PDB ID: 3QLS)

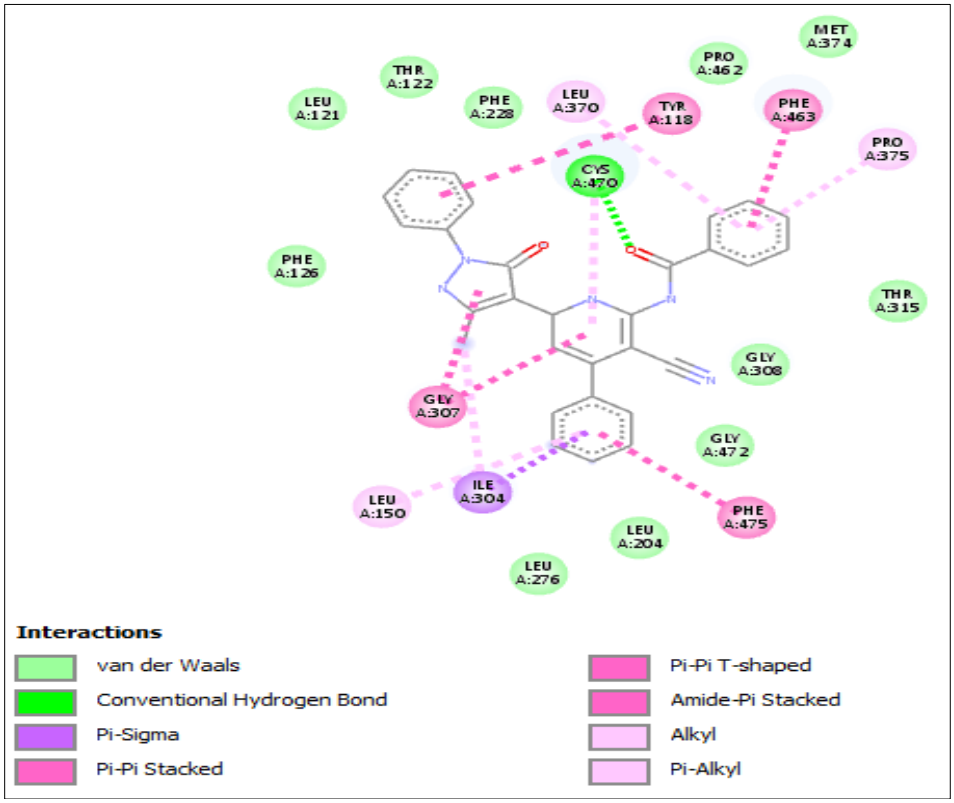

Fig 2: Binding interaction of Benzamide Derivative with 14- α Sterol Demethylase of C. albicans (PDB ID: 5FSA)

3.3 Physical Evaluation of Topical Formulations

Table 8 showed the physical characterization of both the formulations. The result showed that the formulations had good appearance, homogenous in nature, and stable to creaming, coalescence and centrifugation test. 10% Formulation of *T. procumbens* leaf extract has shown better spreadability then 5% formulation. No weight loss was recorded in formulations [11].

Table 8: Physicochemical evaluation of *T. procumbens* leaf extract topical formulation

Evaluation parameters	Plain Formulation	Formulation F1 (5% w/w)	Formulation F2 (10% w/w)
Physical state	Semi-solid	Semi-solid	Semi-solid
Color	White	Light Green	Dark Green
Texture	Smooth	Smooth	Smooth
Homogeneity	Homogenous	Homogenous	Homogenous
Appearance	Soft and shiny	Soft and shiny	Soft and shiny
Fluidity	Viscous	Viscous	Viscous
pH	7.8	8.09	8.32
Viscosity (cP)	365.7	392.8	544.7
Spreadability (cm)	7.0	7.2	8.2
Centrifugation Test	Stable	Stable	Stable
Stability	Stable	Stable	Stable

3.4 *In vitro* Antifungal Activity

3.4.1 Minimum Inhibitory Concentration

The MIC studies for the leaf extract was carried out on C. albicans fungal strain and it was found that the extract showed better antifungal activity against the selected fungal pathogen with MIC value of 0.88 mg/ml.

3.4.2 Antifungal Activity of Topical Formulation

Table 9 shows antifungal activity of both cream formulations containing leaf extract in concentration of 5% w/w and 10% w/w. The 5% cream showed zone of inhibition in the range of 16 – 20 mm & 10% cream has zone of inhibition in range of 20 – 24 mm. Among the two creams, 10% cream showed better zone of inhibition than 5% and was close to the values of standard drugs. The 10% cream showed higher zone of inhibition with 20 mm value than Amorolfine standard having 17 mm value.

Table 9: Antifungal activity of topical formulations containing *T. procumbens* leaf extract using agar well diffusion technique.

Fungal strain	Zone of Inhibition in Diameter (mm)								
	PP 1			PP 2			PP 3		
Candida albicans	F1 5%	F2 10%	Ketoconazole	F1 5%	F2 10%	Clotrimazole	F1 5%	F2 10%	Amorolfine
	18	23	25	20	24	28	16	20	17

4. Discussions

Fungal infections have been a major concern across the globe. The primary target of infection is skin. Most of the fungi causes skin diseases like candidiasis, eczema, dermatitis, etc. Different types of formulations like ointments, creams, gels, emulsions etc. have been developed for treating fungal infections. Since, many fungi species have developed resistance towards synthetic drugs, it has become a major challenge to develop new drugs and combat resistance. Also, synthetic drugs have got side or adverse effects and so the use of these medications is limited. For these reasons, plant products are used for the treatment which has been always a traditional method employed in treating different types of fungal diseases. In the present study, the leaf extract of plant *Tridax procumbens* was used to evaluate its potency against *Candida albicans* and its topical formulation was prepared.

By extensive literature survey *Tridax procumbens* was found to have antimicrobial action and the constituent, present in ethanolic extract of its leaves, Benzamide derivative was predicted to be responsible for this activity as it showed best docking result compared to standard drugs. Hence, the leaf extract in the form of cream formulation was used to study antifungal activity.

Synthetic azole antifungals namely ketoconazole, clotrimazole and Amorolfine were used as the standard drugs for comparison of the results as they are standardized medicine for topical fungal infections.

Phytochemical screening of the extract was performed and indicated the presence of alkaloids, flavonoids, carbohydrates, tannins, phenols, terpenes and terpenoids, fats and oil and steroids. The GC-MS report of phytoconstituents present in ethanolic extract of plant leaves was referred from literature.

Molecular docking was performed using PyRx program (version 11, Schrodinger, LLC, New York, 2016). It is a method used to study the way one molecule orients itself for binding to another molecule in order to form stable complexes. Specifically, ligands are studied for the way they orient themselves to bind to specific receptors or target sites to produce effects. The various constituents of *Tridax procumbens* L. were subjected to docking against the target DHFR and Sterol demethylase of C. albicans. The score of the best constituent i.e., Benzamide derivative was found to be in the range of -10.8 to -11.7 against selected proteins. The docking scores less than -6.0 were considered in the following study for enhanced and more precise results. Among 16 phytoconstituents obtained from GC-MS report of ethanolic extract of plant, the benzamide derivative was found to have significant docking score. This stands out to be major evidence of the interactions between the drug and the target. It was also initiated that all the molecules docked in the present study, exhibited binding affinities towards the various hydrophilic interactions found in the docking studies and were considered to be extensively vital for further analysis. From the results obtained it was found that *Tridax procumbens* L. could be prospectively utilized as probable potential drug in the form of a cream formulation for the effective treatment of fungal diseases.

A homogenous cream formulation of the drug was prepared, evaluated and found to comply with all the parameters tested. The extract was incorporated into formulation at 5 mg/g and 10 mg/g concentrations. Further, the antifungal activity of formulation was compared with the marketed products and the results were promising.

Based on the results obtained through docking studies, MIC studies and antifungal evaluation of cream formulations, it was found that *Tridax procumbens* L. could be efficaciously used in deteriorating the damage caused by candida species.

5. Conclusion

Result of current study indicated that leaf extract of *T. procumbens* had potent antifungal activity against the selected fungal strain C. albicans. Moreover, cream formulation containing leaf extract of *T. procumbens* also showed better antifungal activity against the test organism when compared to their respective commercial topical formulations. Therefore, leaf extract of *T. procumbens* and its cream formulation could be recommended as alternative topical product to synthetic topical drugs for the treatment of skin infection with potent activity, less toxicity, and low risk of emergence of drug resistance due to the presence of substantial number of chemical constituents believed to have different mechanism of action against the targets. However, further studies should be conducted to explore the shelf life, chronic toxicity, and clinical study to evaluate its safety and efficacy on human subject.

6. Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

7. Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

8. Authors' Contributions

Ashok Babu V. L. designed the protocol, coordinated the overall project and advised the formulation work. Merin Sara Mathew, Burhanuddin Madriwala, Jeevitha. L, Abhilash. S and Anjani Singh conducted the collection, processing, and extraction of plant. Phytochemical screening was carried out by all the authors. Burhanuddin Madriwala performed molecular docking studies of the plant extract. Merin Sara Mathew, Burhanuddin Madriwala, Jeevitha. L, Abhilash. S and Anjani Singh worked out the formulation development and its evaluation studies. MIC studies and antifungal evaluation of formulation was supervised by Shwetha. K and assisted by all other authors. Burhanuddin Madriwala and Merin Sara Mathew reviewed the manuscript and all authors approved the final version of the manuscript.

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