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Formulation and Evaluation of Neem and Goumutra Cream

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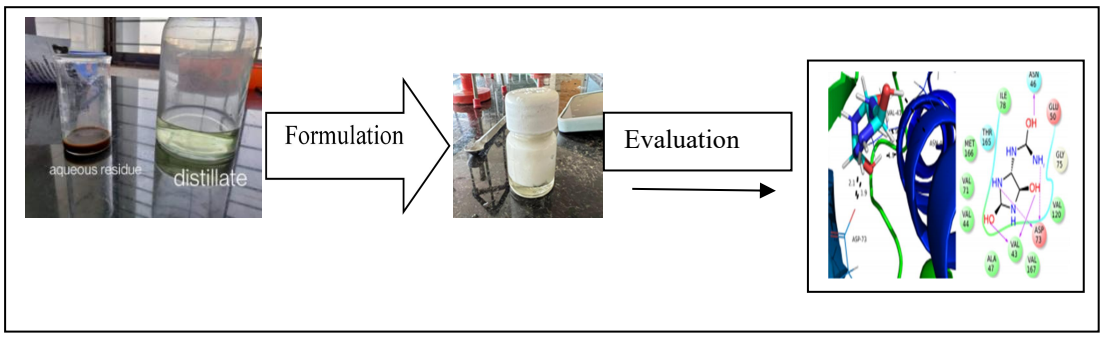
Abstract

Neem (*Azadirachta indica*) and cow urine (*goumutra*) and have been used in Indian culture since centuries together and are reported to have antimicrobial and antifungal activity [S. Vats, 2011]. It has shown beneficial effect in various skin infections. The current study was intended in augmenting antimicrobial activities of neem and cow urine and formulating a skin cream by using stearic acid, triethanolamine, lanolin and mineral oil. Various parameters such as distillation, solubility, selection of cream base were carried out in order to prepare a stable dosage form. The optimized formulation was evaluated for spread ability, dye test, stability test, antimicrobial activity and docking study. Docking study with allantoin revealed that allantoin has the ability to interact with DNA gyrase- II and tend to show anti-bacterial activity. MIC (Minimum Inhibitory Concentration) study of allantoin showed positive effect against both gram positive and gram negative bacteria at 4 µg/ mL for *Bacillus subtilis* and 8 µg/ mL for *Staphylococcus aureus*. It was indicated that, isolated allantoin exhibit equal or better than standard in terms of anti- bacterial activity.

Graphical Abstract



Keywords: Neem, Cow urine, docking analysis, antimicrobial activity

Introduction

Neem consists of almost all the part of the plant which are used as drug of *Azadirachta indica L.* belonging to family *Meliacae*. India is native of Azadirachta. It is also cultivated in Nepal, Pakistan Bangladesh and Sri-Lanka. Different parts of neem have different constituents. Neem leaf contains quercetin, nimbosterol, nimbin, flower contains nimbosterol, kaempferol, bark contains nimbin, nimbidin, nimbosterol and seeds contain azadirachtin,azadiradione, nimbin, vepinin. Neem has been widely used in India for its potent antioxidant, antimalarial, antibacterial antifungal activity. It is used against multi drug resistant pathogenic strains [R. Subapriya *et al*, 2005]. Cow urine is a liquid by-product of metabolism in cows. The bovis cow urine has highest antimicrobial and medicinal property. It is composed of 95% water, 2.5% urea, 2.5% a mixture of salts, hormones, enzymes, and minerals like ammonia nitrogen, Allantoin, Calcium chloride, Creatinine, Magnesium, Potassium, Sodium sulphate, Uric acid. Allantoin which is an anti-inflammatory agent, enhances healing process of wound, antibacterial agent, urea strong anti-microbial property, detoxification of blood and Vitamin C, E, A which are major anti-oxidants and are also immune-stimulators [I Mohanty *et al.,* 2014].

Materials

Neem leaves, cow urine, Stearic acid, Lanolin, Mineral oil, Triethanolamine, water, toluene, methanol, *Bacillus subtilis*, *Staphylococcus aureus,* Tetracycline, allantoin, urea.

Methodology

1. Preformulation Studies

Compatibility of Neem and Cow Urine: When equilibrated amount of cow urine is used with *Azadirachta indica* it shows remarkable synergistic effects against *C. tropicalis, C. glabrata, P. aeruginosa, S. aureofaciens*. Alone, extract of Azadirachta *indica* showed antimicrobial activity towards *C. albicans, C. glabrata, E. coli, P. aeruginosa* and *S. aureofaciens*. In case of cow urine, strong anti-microbial activity is noted towards *E. coli, S. aureofaciens* and *C. albicans* [Chen *et al., 2014*].

Cow urine have antimicrobial activity against disease like eczema, acne, vulgaris, scabies and other hypersensitivity reaction.

Choice of Solvent: Initially fresh neem leaves were taken and weighed. Further they were crushed using a blender or mixer. The neem leaves and the crushed mass was weighed with the help of

digital weighing balance. Out of total 9.597 gm neem leaves, 9.007gm of crushed neem leaves paste was obtained. Half of the quantity of crushed neem leaves was soaked into 500ml water and half in 500ml cow urine in a glass beaker for a while so, and boiled on the same day. Total amount of extract was found to be 300ml after boiling. Later it was cooled and kept standing at a cool and dry place covered with a lid for around 48 hours. After 48 hours the mixture was observed [Adhikari *et al.*, 2020].

Solubility of Extract: The extract was partitioned with chloroform and cow urine and upper layer was of cow urine and lower layer was chloroform. The layers were separated and organic layer was spread on the sodium sulphate layer, it absorbs all impurities and hence it was checked that it is soluble in organic solvents [Adhikari *et al.*, 2020].

2. Preparation of Cream: The neem leaves were weighed (9.597gm). Then they were crushed to form a paste (9.007gm). Half of the neem paste was then soaked in 500 ml water and half in 500 ml cow urine and then boiled. Neem and cow urine extract was filtered with double-layer filter paper and again filtered for the second time with a cotton pad. After double filtration, 200ml filtrate was observed [Adhikari *et al.*, 2020].

Distillation Process: The extract was then used for distillation at 70-80 degree Celsius with the help of vacuum. Residue and distillate were collected [Adhikari *et al.*, 2020].

Thin Layer Chromatography: Silica powder was added in water and a paste of optimum consistency was made. The paste was evenly spread on the glass slide with the help of a glass rod. Prepared plates were allowed to air dry. 4.5 ml of toluene was mixed with 0.5 ml of methanol for solvent (mobile phase) preparation. On the prepared TLC plate 2 spots were put [one of aqueous (neem and water) extract and another of organic (neem and cow urine) extract. The prepared mobile phase solvent was poured in a beaker. Silica plate with organic and aqueous extract spots was dipped in the mobile phase. The beaker was properly sealed with plastic foil. The spots were observed [Adhikari *et al.*, 2020].

Formulation of Cream: The quantities of stearic acid, lanolin, and medicated extract dissolved in mineral oil were weighed and heated in a water bath until all the ingredients melted. This mixture was kept warm while heating the water, triethanolamine and rose water mixture in another beaker. After the water solution had reached a temperature between 80° and 90°C, it was removed from the heat and slowly poured over the melted stearic acid-lanolin-mineral oil mixture little at a time,

stirring constantly. Perfume was then added and the mixture was stirred well to blend [Adhikari *et al.,* 2020].

Table 1: Formula for cream

Ingredients	Quantity
Stearic acid	2.5gm
Lanolin	3.5gm
Mineral oil	2.5gm
Triethanolamine	0.5ml
Water	12ml
Medicated Extract	2 gm
Rose water	q.s
Perfume	q.s

3. Evaluation of Cream

Organoleptic Characteristics:

The cream was evaluated for its organoleptic characters like colour, odour, and state. The appearance was tested for physical appearance, colour, texture, phase separation and homogeneity. These characteristics were evaluated by visual observation. The consistency of the formulations and presence of coarse particles were used to evaluate the texture and homogeneity of the formulations. Immediate skin feel (including stiffness, grittiness, and greasiness) was also evaluated. [Avish *et al.*, 2018]

Dye Test: The cream was observed under the microscope after treating with dye (methyl red). [Avish *et al*, 2018]

Stability Test: In the mechanical test cream samples were inserted into centrifuge tube at a speed of 3750 RPM for half an hour of 5000 to 10,000 RPM for 15 minutes then observed whether a separation exist or not, to determine phase separation. [kishore *et al.*, 2014]

Spreadability: Spreadability refers to the area covered by a fixed amount of cream sample after the uniform spread of sample on the glass slide. The spreadability of test samples was determined using the following technique: 0.5 g test formulation was placed within a circle of 1 cm diameter

pre-marked on a glass plate, above which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to spreading of the test formulation was noted. Average of three determinations was noted. [Wadher,2009]

Anti-Microbial Activity: The anti-bacterial activity of the isolated compound allantoin was tested against the *Staphylococcus aureus* and *Bacillus subtilis*, determined by well diffusion method. The bacterial culture (107/ mL) was swabbed uniformly on using sterile cotton swab. The different concentration of cream (10- 40 µg/ mL) was added into each well, having concentration of allantoin and uric acid as 1,2,3,4 % and then was incubated at 37°C for 18 hours. After incubation, the appeared zone was measured. Standard anti- biotic, tetracycline was used as a positive control. Minimum Inhibitory Concentration (MIC) of allantoin was determined against above mentioned pathogenic bacteria by Broth dilution method. In brief, different concentrations of allantoin (128, 64, 32, 16, 08, 04, 02, 01, 0.5, 0.25 µg/ mL) was suspended into 50 µL of the MHB of medium and taken into the 96 well plate followed by 20 µL of bacterial suspension (1 x 10 CFU/mL) were added to each well and then it was incubated for 18 hours, at 37°C. Tetracycline hydrochloride was taken as a positive control. After incubation 30 µL of resazurin (0.015 %) was added to each well and incubation period was extended for another 3 hours. Based on the visible colour changes the inhibitory concentration was calculated. [Lakshmanan *et al.*, 2019]

Docking Analysis: -Molecular docking analysis was performed on Centos 6 Linux Work station using Maestro. GLIDE 6.0 searches were performed to understand docking interactions between natural compound (allantoin) and DNA gyrase- II. The three- dimensional crystal structure of DNA gyrase- II (PDB- 5L3J) was downloaded from the PDB (http://www.rcsb.org. Protein preparation wizard of Schrodinger was used for DNA gyrase preparation. No hydrogen atoms were minimized until the average root mean square deviation reached a default value of 0.3 Å. Sitemap 2.3 was used to understand binding site in the ligand- binding domain (LBD) of the DNA gyrase- II. Induced fit docking was performed to compounds binding modes and structural movements in the LBD region of DNA gyrase- II using Glide and Prime modules. The prepared protein was loaded on the work station and the grid values were calculated about 20 Å to cover the entire active site amino acids. About 20 conformational images were created and analysed for the best conformation pose based on the docking score and glide energy [kothawade, 2022].

Results and Discussion

Preformulation Studies: Neem extract in water was found to have fungal growth on its surface whereas, crushed neem in cow urine was found clear liquid without any fungal growth. Therefore, neem leaves in cow urine extract were used in this formulation. In the thin layer chromatography, the layers got separated immediately.

Figure 1: Fermentation of pulverised neem leaves and cream urine

Figure 2: Distillate and aqueous residue are separate filtration and distillation

Figure 3: Cream formulation

Preparation of Cream: The cream was found to be lump free and homogenous.

Evaluation of Cream: The formulated cream was white, with characteristic odour, with pH 5.2, was easily removed by water. The cream was an o/w emulsion with emollient after feel. The cream was stable with no phase separation.

Table 2: Organoleptic evaluation of cream

Evaluation Parameters	Result
Colour	White
Odour	Characteristic
pH	5.2
Removal	Easily removed by water
Dye test	o/w emulsion
Homogeneity	Homogenous,
After feel	Emollient
Stability	No phase separation occurred
Spread ability	Easily spreadable

Anti-Microbial Activity:

The Minimum Inhibitory Concentration (MIC) of allantoin showed against both gram positive and gram-negative bacteria, even at the lower concentration. It is 4 µg/ mL for *Bacillus subtilis* and 8 µg/ mL for *Staphylococcus aureus*. It was indicated that, isolated allantoin exhibit equal or better than standard in terms of anti- bacterial activity.

Table 3: Antimicrobial activity of the cream

Cream (µg/ mL)	*Bacillus subtilis*	*Staphylococcus aureus*
10	-	-
20	7 ± 0.8	7 ± 0.6
30	8 ± 1.2	8 ± 0.8
40	10 ± 1.5	10 ± 1.2
Standard tetracycline	18 ± 1.4	18 ± 1.6

Docking Analysis:

Natural compound (Allantoin) docked with bacterial drug target for DNA gyrase- II (PDB ID: 5L3J) protein- ligand interactions 3D pymol view 2D maestro view. The DNA gyrase-II (PDB: 5L3J) was used for the molecular docking analysis. The active site of AT bound proteins consists of ILE 78, ASN 46, GLU 50, GLY 75, VAL 120, ASP 73, VAL 167, VAL 43, ALA 47, VAL 44, VAL 71, MET 166, and THR 165. It is very interesting to note that this AT molecule tends to show three hydrogen bonding interactions with ASP 73, VAL 43 and ASN 46 with a distance of 2.1 Å, 2.0 Å and 2.0 Å, respectively. It revealed that this allantoin has the ability to interact with DNA gyrase- II and tend to show anti-bacterial activity.[10]

Table 4: Docking analysis of cream

Drug target	Compound	Docking score (Kcal/mole)	Glide energy (Kcal/mole)
DNA gyrase- II (PDB ID: 5L3J)	Allantoin	-7. 48	-25.18

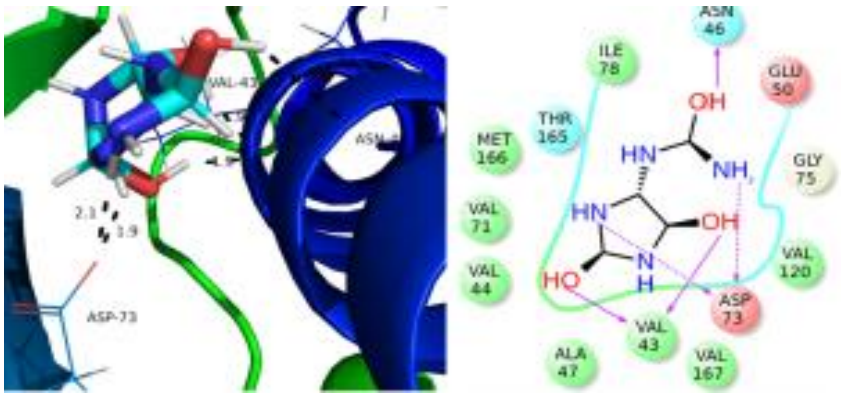


Figure 4: Hydrogen bond interactions in DNA gyrase.

Conclusion

The preparations of neem and cow urine cream have gained significant attention in recent years due to its potential medicinal properties. This research article highlights the various pharmacological activities of neem and cow urine, which have been extensively studied and reported in the literature. The combination of these two natural ingredients in cream form has been found to exhibit numerous health benefits, including antimicrobial, antioxidant, anti-inflammatory, and wound-healing properties. Furthermore, by well-diffusion method it exhibits greater antibacterial activity and molecular docking shows docking score of -7.48 which show a greater affinity on DNA GYRASE 2 receptor and greater antibacterial activity. Overall, the neem and cow urine cream preparation holds promise as a natural and effective alternative to conventional medicines for various health conditions.

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Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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