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Original Research Article

Evaluation of in vitro anti-acne activity of methanolic extracts of *Cyperus rotundus* and *Nymphaea nouchali*

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ABSTRACT

Background: The objective of this study was to evaluate in vitro anti-acne activity against *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Escherichia coli* (*E. coli*) using the agar well diffusion method.
Methods: Air-dried and ground, matured rhizomes of *Cyperus rotundus* (CR), petals and pollen of *Nymphaea nouchali* (NN) were macerated in methanol. The resultant extracts were concentrated using a rotary evaporator. The anti-bacterial activity was performed against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC12228), and *Escherichia coli* (ATCC 25922) using the agar well diffusion method, and gentamicin was used as a positive control. The whole experiment was done in triplicates.
Results: The data obtained from dose-response curves, methanolic extracts of *Cyperus rotundus* rhizomes against *staphylococcus aureus* (IC50=819.2 µg/ml), *Nymphaea nouchali* pollens against *Staphylococcus epidermidis* (IC50=787.7 µg/ml) and combination against *Escherichia coli* (IC50=813.6 µg/ml) were exhibited the highest potency. A strong positive statistically significant correlation was exhibited between the zone of inhibition and the concentrations of all plant extracts. The p value was less than 0.05 ($p<0.05$) and all the R^2 values were around 1.
Conclusions: The maximum anti-bacterial potency against gentamicin equivalent was observed in *S. aureus*, *S. epidermidis*, and *E. coli* at the concentration of 15625 µg/ml in combination. The present study might bring progress in the treatment of acne using herbs and in developing herbal formulations for safe and effective management of the disease.

Keywords: *Cyperus rotundus*, *Nymphaea nouchali*, Anti-bacterial activity, Agar well diffusion method, *Escherichia coli*, *Staphylococcus aureus*

INTRODUCTION

Acne vulgaris is simply known as acne, and it is a common skin disease mostly among teenagers in the world.[1] It is a multifactorial disease condition in the pilosebaceous unit.[2] The pilosebaceous unit is a small structure consisting of hair follicles, hair shaft, sebaceous glands (oil glands), and arrector pill muscles. The most commonly acne-affected body areas are the face, shoulder area, and trunk because these areas contain large sebaceous glands.[3] Sebaceous glands produce an oily product called sebum. Sebum is a lipid-rich substance secreted by sebocytes and keratinocytes of the sebaceous gland.[4]

The inflammation of acne occurs due to various bacterium types like *Propionibacterium acne*, *Staphylococcus epidermidis*, *Staphylococcus aureus*,

Streptococcus agalactiae, *Klebsiella pneumoniae*, etc. *S. epidermidis* is a gram-positive, aerobic pus-forming bacteria responsible for superficial infection of the sebaceous unit. It produces cholesterol via fatty acid esterification. *S. aureus* is a gram-positive rod-shaped bacterium that has the ability to attach to the human skin. It produces various extracellular enzymes like lipases, proteases, hyaluronidases, and collagenase. Surrounding tissues are injured by these enzymes and spread the disease to the deeper tissues also.[5]

Gentamicin is an antibiotic that belongs to the aminoglycoside group which commonly used for skin and subcutaneous infections. It is effective against acne causative bacteria species such as *Staphylococcus* and *Klebsiella* infections. Therefore, gentamicin can be used in combination with benzoyl peroxide or beta-lactam antibiotics for *S. aureus* infection disease. For this reason, gentamicin was recommended to use as a topical treatment for acne.[6,7]

C. rotundus is a weed plant belonging to the family Cyperaceae. It is a perennial weed with slender scaly creeping rhizomes bulbous at the base and arising singly from the tubers at about 01-03 cm long and may reach a height of up to 100 cm. The tubers measured around 01 to 3.5 cm in length and are blackish and the inner is a reddish-white color with a characteristic odor.[8] The stem is smooth and erect and about 30 to 40 cm in height. The leaves are smooth, shiny, and dark green, 20 to 30 cm long and 0.2 to 01 cm in width with grooved on the upper surface, and a sharp tip on the edge. It consists of tiny flowers with a red-brown husk, bisexual, and has three stamina, three stigma carpel, and three to eight unequal rays on the flower head. The nut is appearing as triangular oblong in its cross-section.[9]

N. nouchali is belonging to the family Nymphaeaceae. It is a day blooming plant that immersed roots and stems. Leaves are floating and round green in color and posttrial is darker in color. They have undulating edges that give a crenelated appearance and about 20-23 cm and spread is 0.9 to 1.8 m. The fruit is globosely containing round flak-shaped seeds. Flowers have an angular appearance to look like a star shape and petals are violet blue in reddish color edges with 04 sepals and held up to 30 cm above the water. They have pale blue with pale yellow stamens and anthers. Fruits are globular in shape with numerous seeds.[10]

Herbs have been used as pure, crude, or in combination with other herbs from ancient times. Manufactures also tend to produce natural products due to the availability of ingredients conveniently. Therefore, these herbal products are more popular as cosmetics. Herbal cosmetic products enhance skin cleansing, lighting, and brightening, reduce pigmentations and heal blemishe.[11] Traditional herbal remedies are more acceptable in most ethnic societies as compared to allopathic medicines because they are considered to be the safest approach to treating diseases with the least side effects on human health. There are many ways to prevent and cure conditions that diminish the quality of life of individuals.[12]

Herbal formulations contain therapeutic properties derived from natural plants and are concern as the best form of skin care. They reduce or prevent wrinkles, age spots, and acne breakouts. Since they are completely natural, free from side effects, and safe for application for a prolonged duration. Hence, this study aims to open a new pathway for the evaluation of anti-acne activity from selected plant parts of *Cyprus rotundas (C. rotundas)* and *Nymphet nuchal (N. nuchal)*.

METHODS

Study design

The study was carried out to evaluate in vitro anti-acne activity of selected medicinal plant; *Nymphaea nouchali* (NN) and *Cyperus rotundus* (CR) based on agar well diffusion method.

Study setting and time period

The study was carried out in the chemistry laboratory and research laboratory, General Sir John Kotelawala Defence University, Rathmalana, Sri Lanka and Pharmaceutical Laboratory, Faculty of Allied Health Sciences in General Sir John Kotelawala Defence University, Werahera, Sri Lanka. This study took place from April 2020 to March 2021.

Plant material collection, identification, and authentication

Matured rhizomes of CR and petals pollens of NN were collected in the Avissawella area (6.95430 N, 80.20460 E), Sri Lanka. Properly dried and pressed specimens were authenticated at the Bandaranayake Memorial Ayurvedic Research Institute, Navinna, Maharagama by Pushpa Jeewandara (Scientific officer/ Pharmacognosy).

Preparation of methanol extracts of CR rhizomes, NN petals, and pollens

Using running tap water, selected plant materials were thoroughly washed and air dried until a constant weight was obtained. The dried plant parts were ground using a grinder to obtain a fine powdered material. For the extraction process, well dried and blended powder samples of each plant's materials were taken. The extraction was obtained using a cold maceration method. Using an electronic balance, 50 gm of the fine powder was weighed and added to 200 ml of solvent (methanol) using a measuring cylinder. This was kept for cold maceration for 7 days. The filtrates were concentrated by using a rotary evaporator. The concentrated extracts were further evaporated by using a water bath at 65°C. The methanolic extracts obtained as solid masses were

weighed and stored at 4±2℃ in well-sealed containers until further studies.[13]

Preparation of plant extracts standard concentrations and positive control

Serial dilutions yielding concentrations of 244.14 µg/ml, 488.28 µg/ml, 976.56 µg/ml, 1953.125 µg/ml, 3906.25 µg/ml, 7812.5 µg/ml and 15625 µg/ml were prepared from the concentrated extracts. Concentrated extracts from the powder form were first dissolved in DMSO and later used as normal saline for the preparation of serial dilutions from the stock. Gentamicin was used as the positive control in the study. It was prepared by using a commercially available 40 mg/ml intravenous injection vial. Six concentrations were prepared for the antibacterial assay (250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, 4000 µg/ml and 8000 µg/ml).

Anti-bacterial activity screening using the agar well diffusion method

The anti-bacterial activity was performed against Gram-positive *S. aureus, S. epidermidis,* and Gram-negative *E. coli* bacteria by using the agar well diffusion method.[14,15] The bacterial suspension was prepared according to 0.5 McFarland turbidity. Agar plates were prepared by using double agar layers. The upper layer was inoculated with microorganisms, but the lower layer was free of microorganisms. Aluminium cylinders were placed on the lower layer after solidifying the lower layer. The upper layer was poured on top of the lower layer. After solidifying, the aluminium cylinders were removed using sterile forceps.

Each prepared well was filled with 100 µl of the volume of each methanolic plant extract, positive control, and negative control. Gentamicin was used as the positive control and DMSO was used as the negative control. Each step of the experiment was repeated in triplicate. All the plates were incubated at 37°C in an incubator for 24 hours.[16]

After incubation, the diameters of the inhibition zones were measured using a ruler. Measurements were obtained from the triplicates to obtain the average zone of inhibition.

Statistical analysis

The statistical work for this research was done by using the software Graph pad prism 8 (version 8. 4. 2. 679). All the assays were done three times and results were expressed in average zone of inhibition±standard error method (SEM). Significant level was setup at $p<0.05$.

RESULTS

According to Figure 1, the combination was found to be the most effective and have a high anti-bacterial potency (89.82%) with a concentration of 15625 µg/ml and the average zone of inhibition was 20.66±0.10 mm against *S. aureus*. The lowest anti-bacterial potency (79.69 %) and average zone of inhibition (18.33±0.11) were observed from methanolic extract of CR rhizomes to the highest concentration of 15625 µg/ml. No zone of inhibition was observed for concentrations of 244.12 µg/ml and 488.28 µg/ml. Therefore, the minimum inhibitory concentration was 976.56 µg/ml for *S. aureus.*

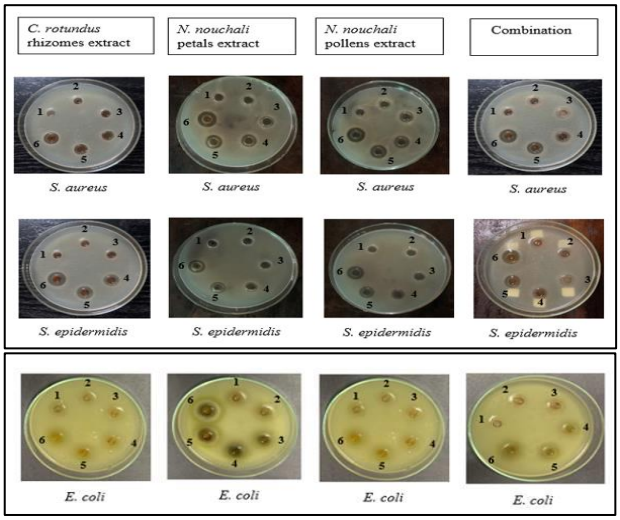

Figure 1: Average zone of inhibition of methanolic extract of CR rhizomes and NN petals, pollens and combination against *S. aureus*, *S. epidermidis* and *E. coli*; 1) 488.28 µg/ml, 2) 976.56 µg/ml, 3) 1953.12 µg/ml, 4) 3906.25 µg/ml, 5) 7812.50 µg/ml, 6) 15625 µg/ml.

The highest average zone of inhibition (19.66±0.10 mm) and anti-bacterial potency (85.47 %) was observed in combination with the concentration of 15625 µg/ml *against S. epidermidis.* The lowest average zone of inhibition (17.00±0.00 mm) and anti-bacterial potency (73.91%) was shown in NN petals with a concentration of 15625 µg/ml. And the minimum inhibitory concentration was 976.56 µg/ml for *S. epidermidis*.

The combination was found to have the highest average zone of inhibition (22.66±0.09 mm) and anti-bacterial potency (98.52%) with a concentration of 15625 µg/ml against *E. coli*. No zone of inhibition was observed for CR rhizomes against *E. coli.*

All plant extracts individually and in combination have not obtained an average zone of inhibition for concentrations of 244.12 µg/ml and 488.28 µg/ml against all microorganisms used for this study, and CR rhizomes did not exhibit a zone of inhibition against *E. coli* for all concentrations. Therefore, NN petals, pollen, and a combination of plant parts together showed 976.56 µg/ml as the MIC for *S. aureus, S epidermidis,* and *E. coli*. But CR rhizomes showed 976.56 µg/ml as the MIC only for *S. aureus* and *S. epidermidis.*

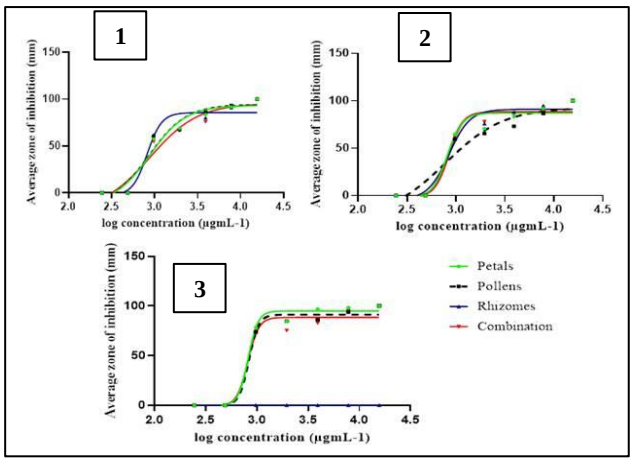

Figure 2: Dose response curves of methanolic extract of CR rhizomes and NN petals, pollens and combination against (1) *S. aureus*, (2) *S. epidermidis* and (3) *E. coli*.

According to the data obtained from dose-response curves (Figure 2), methanolic extracts of CR rhizomes against *S. aureus* (IC50=819.2 µg/ml), NN pollens against *S. epidermidis* (IC50=787.7 µg/ml) and combination against *E. coli* (IC50=813.6 µg/ml) were exhibited the highest potency. A strong positive, statistically significant correlation was exhibited between the zone of inhibition and concentrations of all plant extracts (Table 2). The p value was less than 0.05 ($p<0.05$) and all the R square values were around 1. NN petals showed the highest R square value (R square =0.9574) against *S. aureus*, the combination showed the highest R square value (R square =0.9747) against *S. epidermidis*, and CR rhizomes showed the highest R square value (R square =1.0000) against *E. coli*. This study would be able to get a synergistic effect from the combination of all three plant parts together. But it does not take place that way. However, individual and combinations of plant parts obtained good results against *S. aureus*, *S. epidermidis*, and *E. coli*.

Table 1: IC_{50} values of plants extracts against *S. aureus*, *S. epidermidis* and *E. coli*.

Microorganism		IC50 values for plants extracts (µg/ml)			
		C. rotundus	*N. nouchali*		Combination
		Rhizomes	Petals	Pollens	
S. aureus	IC50	819.2	885.3	866.3	889.1
S. epidermidis	IC50	846.1	816.4	787.7	840.0
E. coli	IC50	-	820.0	831.8	813.6

(-) Absence of IC_{50} values.

Table 2: Correlation study data of anti-bacterial assay.

	S. aureus		*S. epidermidis*		*E. coli*	
	P value	R square	P value	R square	P value	R square
C. rotundus rhizomes	0.0010	0.9483	0.0012	0.9729	-	1.0000
N. nouchali petals	0.0007	0.9594	0.0013	0.9560	0.0049	0.9880
N. nouchali pollens	0.0008	0.9527	0.0007	0.9271	0.0041	0.9870
Combination	0.0005	0.9516	0.0017	0.9747	0.0025	0.9654

(-) Absence of value.

DISCUSSION

Acne is a chronic inflammatory dermatological disorder that affects young adults and adolescents around the world. It directly affects the facial appearance of a person and makes permanent scars even after healing it. Acne patients are frequently exposed to discomfort and emotional stress.[17]

At present topical or systemic antibiotics are playing an important role in the treatment of acne. But there is considerable potential for side effects and increases the risk of antibiotic resistance. According to the previous study, some antibiotics are already found to be resistant and less effective against *P. acnes* and *S. aureus*. Therefore, the discovery of an effective new herbal drug formulation for acne treatment is important because herbal drugs have fewer side effects, and it helps to reduce antibiotic-resistant risk also.[18]

According to the literature reviews, the anti-acne activity of CR and NN was not evaluated in Sri Lanka. Flavonoids, alkaloids, tannins, and phenols are compounds that can exhibit anti-bacterial activity.[19] A phytochemical analysis was conducted for the rhizome of CR. The result of that was the presence of alkaloids, phenols, flavonoids, and terpenes as chemical compounds.[20] Another phytochemical analysis was carried out for the flower of NN and it was proved, that the flower contains phenols, flavonoids, tannins, saponins, alkaloids, fixed oil, and fats.[21] Hence these two plants were selected for this study.

Selection of a solvent for extraction was done, according to the nature and polarity of the plant constituent. Water, ethanol, methanol, propanol, and acetone are mostly used as a polar solvent for extraction.[22]

The extraction process of previous studies related to the selected plants was carried out with ethanol.[23,21] The boiling point of ethanol is 78.37ºC while the boiling point of methanol is 64.7ºC. Therefore, to get the final extract, the solution needs to be heated to its boiling point to evaporate the remaining solvent. In the case of using ethanol, the solution has to be heated to a higher temperature than the methanol solution. Thus, the high temperature can affect the phytochemical constitute of the plant extract. Hence methanol was selected as the solvent for the study. However, methanol is toxic when it is absorbed or inhaled. The above risk was eradicated by evaporating the total amount of methanol present in the solution using a rotary evaporator.[22]

The main conventional extraction methods are hot water baths, maceration, and Soxhlet extraction. Hot water baths and Soxhlet extraction can affect the heat-sensitive compounds of the extracts.[22] Therefore, this study, maceration was used as the ideal extraction method.

Several groups of bacteria are involved in the pathogenesis of acne. In this study, *S. epidermidis* and *S. aureus* were used as acne-causative gram-positive bacteria. They were grown in Mueller-Hinton agar medium and in normal aerobic conditions during incubation. *P. acne* is a gram-positive bacterium that plays an important role in the pathogenesis of acne. But it should be growing in blood ager and should supply anaerobic conditions during incubation at 35ºC for 48 hours. Since *P. acne* was not selected for this study due to fewer available facilities. *E. coli* was used in this study to examine the gram-negative activity of selected plant parts. *E. coli* was grown in Mueller-Hinton agar medium and normal aerobic conditions during incubation.[24]

Due to the time limitation, the freeze drying was unable to perform. Freeze drying of crude plant material extracts should be the best standard, limit chemical changes and formation of chemical artefacts. All the extracts were observed more than 85% gentamicin equivalent potency for highest concentration. Therefore, further fractionation and characterization of the methanolic extracts will be useful to isolate new chemical entity for anti-acne activity.

CONCLUSION

In this study, the combination had the highest anti-bacterial activity and anti-bacterial potency against *S. aureus*, *S. epidermidis*, and *E. coli*. But not as effective as the reference drug (gentamicin). According to the dose-response study, the combination exhibited the highest potency against *E. coli*.

It was concluded that the present study might hopefully bring progress in the treatment of acne using herbs as well as in developing herbal formulations for safe and effective management of the disease.

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