

Assessment of the Antimicrobial Potency of Leaf Extracts from *Vitex Nugundo* and *Gloriosa Superba*

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ABSTRACT

An increase in the number of antibiotic resistant strains makes the discovery of new therapeutic agents critically important. During present study antimicrobial effects of the leaf extracts of *Vitex nugundo* (VN) and *Gloriosa superba* (GS) in combination with chloramphenicol, on *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans* was investigated. For this purpose, the dilution sensitivity and disc diffusion techniques were respectively applied in determining minimum inhibitory concentration (MIC) of the plant extracts, and the sensitivities of the organisms to the plant extracts and their combinations with chloramphenicol. *V. nugundo* and *G. superba* showed very high antimicrobial activity against all the test organisms. In combination, the effect of VN on *E. coli* and *S. typhi* was completely antagonized by that of GS whereas additive effect on *S. aureus* and *C. albicans* was observed, indicating that the combination of VN and GS might be effective against gram positive pathogenic organisms. The combination of either plant extract with chloramphenicol produced synergistic effect on only *C. albicans*. The smaller MIC of GS indicated greater effectivity than VN. It is concluded that the additive effect produced by the combination of the two plant extracts, and the synergic effect from the combination of any of the extracts with chloramphenicol, offer alternative therapy to gram positive bacterial infections and candidiasis respectively.

Key Words: Antibacterial, *Candida albicans*, Chloramphenicol, Microorganisms.

INTRODUCTION

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms. An increase in the number of antibiotic resistant strains makes the discovery of new therapeutic agents critically important. Many of the currently used anti-infective and antineoplastic agents are natural products, initially isolated from plants^[1,2] and algae.^[3,4] Recently there is an increasing concern and the need to source for locally available drugs because of un-affordability of conventional chemotherapeutic agents and clinical cost. Apparently this situation has

generated a few studies on the phytochemistry and the medicinal potency of some of the medicinal plants known to aborigines. Besides, the current wave of antimicrobial resistance to chemotherapeutic drugs is of global concern.^[5] There is need, therefore, to search for such plants that could be resistance-free.

Vitex negundo (VN) Linn (verbenaceae), a large aromatic shrub with typical five foliate leaf pattern, is found throughout the greater part of India at warmer zones and ascending to an altitude of 1500 m in outer, Western Himalayas. It has been claimed to possess many medicinal properties.^[6] Leaves of VN have been investigated for its anti-inflammatory activity in past,^[8-12] including its mechanism of action.^[7,11] Similarly, fresh leaves of VN have been suggested to possess antiinflammatory and pain suppressing activities mediated via PG synthesis inhibition, antihistamine, membrane stabilization and antioxidant activities.^[11] VN has also been reported to possess anti-ulcer activity against piroxicam induced ulcers, probably by increasing PG levels.^[12]

Gloriosa superba (GS) Linn. (liliaceae), is one of the endangered species among the medicinal plants^[13]

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which is a striking tuberous climbing plant with brilliant wavy edged yellow and red flowers that appears from November to March every year.^[14] *G. superba* is a native of tropical Africa and is now found growing naturally in many countries of tropical Asia including Bangladesh, India, Sri Lanka, Malaysia and Myanmar. It is one of the seven upavishas (semi-poisonous drugs) in the Indian medicine, which cure many ailments but may prove fatal on misuse.^[15] The tuberous root stocks of glory lily, *G. superba* boiled with *Sesamum* oil is applied twice a day on the joints, affected with arthritis reduces pain.^[16] It is also used to treat intestinal worms, bruises, infertility, skin problem and impotence. The sap from the leaf tip is used as a smoothening agent for pimples and skin eruptions. The tuberous roots are useful in curing inflammation, ulcers, scrofula, bleeding piles, white discharge, skin diseases, leprosy, indigestion, helminthiasis, snake bites, baldness, intermittent fever and debility. It is also considered useful in promoting labor and expulsion of placenta. Seeds are used for relieving rheumatic pain and as a muscle relaxant.^[17] The toxins of *G. superba* have an inhibitory action on cellular division resulting in diarrhoea, depressant action on the bone marrow and alopecia. Usually all parts of the plant, especially the tubers are extremely poisonous^[18,19] and causes vomiting, purging, stomach ache and burning sensation.^[20] The glory lily has been used for suicidal purposes in India, Burma and Eastern Africa due to presence of colchicines.^[21,22] The tubers contain colchicines, benzoic and salicylic acid, sterols and resinous substances-colchicines, 3-demethyl colchicine, 1,2-didemethyl colchicine, 2,3-didemethyl colchicine, N-formyl, N-deacetyl colchicines, colchicoid, gloriosine, tannins and superbine.^[23] In the world market they are considered as rich sources of colchicines and gloriosine.^[24,25]

In carrying out this study, the aim was to evaluate in vitro the antimicrobial potency of *V. nugundo* and *G. superba* by investigating the sensitivities of known pathogenic bacteria, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans*, to the plant leaf extracts, individually and in combination with themselves and with a known antibiotic.

MATERIALS AND METHODS

Sources of test organisms and plants

Known cultures of *E. coli*, *S. typhi*, *S. aureus* and *C. albicans* were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The cultures were preserved in agar slants until they were used. The test plants were collected from Udaipur District of Rajasthan. They were carried to the herbarium in the Botanical Survey of India, Jodhpur, for identification as *V. nugundo* Linn. and *G. superba* Linn.

Preparation of the plants extracts

After the leaves of the plants were air-dried and grounded in a mortar,^[26] the crude extracts of the leaves were prepared using standard procedures.^[27,28] This involved soaking 50 g of the powdered extract in 95% ethanol for 48 h at room temperature to allow for maximum extraction of the components. This was followed by evaporation of the filtrate using a rotary evaporator. The residue was retained as the crude extract for each of the test plants and stored in reagent bottles and maintained in the freezer until it was used.

Preparation of extract concentrations for the determination of zones of inhibition

The crude extract (10 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to obtain a concentration of 10 mg/mL. When 0.1 mL of this solution was dissolved in 9.9 mL DMSO, a solution of concentration 1 mg/mL was obtained. By incorporating 1 mL of this final solution into 9 mL of DMSO, a final concentration of 100 µg/mL was obtained. To test the combined extracts, equal volumes (0.1:0.1) were mixed and the mixture was tested along with the individual extracts separately.

Preparation of extract concentrations for minimum inhibitory concentration (MIC) test

The crude extract (100 mg) was dissolved in one mL of DMSO to make a concentration of 100 mg/mL and labeled solution 1.^[29] When solution 1 was dissolved in 0.5 mL of DMSO, a concentration of 50 mg/mL was obtained, and this was labeled solution 2. Further, solution 2 was dissolved in 0.5 mL of DMSO to obtain a concentration of 25 mg/mL referred to as solution 3. Solution 4 was obtained by dissolving solution 3 in 0.5 mL of DMSO to give a concentration of 12.5 mg/mL. This process was continued to obtain further concentrations 6.25, 3.12, 1.56, 0.78, 0.39 and 0 mg/mL corresponding to solutions 4 to 9 respectively. By incorporating 1.0 mL of each of solutions 1 to 9 into 9 mL Mueller-Hinton broth, final concentrations of 5000, 2500, 1250, 625, 312, 156, 78, 39 and 0 mg/mL were obtained for minimum inhibitory concentration (MIC) test.

Preparation of the concentration of a broad-spectrum antibiotic, chloramphenicol, used for test in combination with test plants

The drug used was chloramphenicol 250 mg (Sigma, USA). Chloramphenicol was selected because it is a drug of choice against *S. typhi* and other gram negative enteric bacterial pathogens, and *S. aureus*, a gram positive bacterium.^[30] Since *C. albicans* also forms part of the microbial flora of the gastrointestinal tract, it was desirable to test the effect of the combination of chloramphenicol with the local herbs on the bacterial pathogens alongside *C. albicans*. 250 mg of the powdered chloramphenicol was dissolved in deionized water and DMSO as solubilizing agent and

made up to a volume of 25.0 mL at room temperature.^[28] This gave a concentration of 10 mg/mL. Further dilutions as with the extracts were made to obtain a solution with a concentration of 1 g/mL. By mixing 1.0 mL of the solution with 9.0 mL of DMSO, a final concentration of 100 µg/mL was obtained. To test the extracts combined with chloramphenicol, equal volumes of extracts and chloramphenicol (0.1:0.1) were mixed and the mixture was tested along with the individual extracts and chloramphenicol separately.

Sensitivity test

To determine the effect of the extracts individually, combined with themselves and with chloramphenicol on the test organisms, a disc diffusion technique using the Kirby-Bauer method was applied in testing pure cultures of the test organisms for their antimicrobial sensitivities based on zones of inhibition on agar plates.^[31] In this method, punched circular discs from filter paper (Whatman No.1) that were sterilized in a hot air oven for 1 h were impregnated with 0.1 mL of each of the plant extracts. They were air-dried for a few minutes, and transferred aseptically onto the surface of previously prepared Mueller-Hinton agar plates. This followed incubation at 37°C for 24 h, following which the plates were observed for zones of inhibition.

To test the combined extracts, or extracts combined with chloramphenicol, individual concentrations of the extracts or chloramphenicol were both mixed in equal volumes as earlier described, before impregnating the discs with the combinations. The combinations were tested along with the individual extracts or chloramphenicol separately.

To determine the minimum inhibitory concentration (MIC), a standard inoculum was first prepared by transferring a portion of the pure culture of each isolate into tryptone soya broth (Oxoid CM129) that was incubated at room temperature overnight. The overnight broth culture (0.1 mL) was diluted with 1 mL of distilled water in the ratio of 1:100 to form the standard inoculums^[29] following which the dilution susceptibility test technique^[30] was applied. This involved inoculating the previously prepared Mueller-Hinton broth containing various concentrations of the extracts of the plants with the standard inoculum.

This was done for each of the test organisms followed by incubation at 37°C for 16 to 20 h. At the end of incubation, the presence or absence of growth for each concentration was recorded. The lowest concentration of the extracts resulting in no growth after 16 to 20 h of incubation was taken as the minimum inhibitory concentration (MIC). The same treatment was given to the combination of the extracts of the two plants and those of the individual extracts and chloramphenicol in the appropriate volume ratio.

Statistical analysis

Differences, if any, in the effectivities of the test plants, singly, in combination with each other and with chloramphenicol, were determined using the statistical method, analysis of variance (ANOVA).^[32,33]

RESULTS AND DISCUSSIONS

The effect of the extracts of *V. nugundo* (VN) and *G. superba* (GS) on the test organisms are shown in Table 1. Both VN and GS showed appreciable zones of inhibition (≥ 15 mm) indicating reasonably good effectivity of each of the plants on test organisms. In the combination of VN and GS, the effect of VN on *E. coli* and *S. typhi* was completely antagonized or masked by that of GS which was not disturbed and remained the same as it was when uncombined. However, the combination of VN and GS produced an enhanced or additive effect on *S. aureus* and *C. albicans*.

The combination of VN and CAF or GS and CAF appeared to produce little additive or enhanced effect on all organisms except *C. albicans* for which there was synergistic effect on the organisms as shown in Table 2. However, there was significant difference ($p < 0.05$) between the plants extracts and their combinations with chloramphenicol, and between the test organisms with respect to their sensitivities to the extracts singly and in combination.

Table 3 shows the minimum inhibitory concentrations (MIC) *V. nugundo* (VN) and *G. superba* (GS), singly and in combination on the test organisms. There was significant difference ($p < 0.05$) between the plants extracts (VN, GS and VN + GS) with respect to minimum inhibitory concentrations (MIC). GS showed advantage over VN (which had higher MIC) whereas there was no significant difference ($p > 0.05$) between GS and VN + GS. There was significant difference ($p < 0.05$) between the test organisms. Both *E. coli* and *S. typhi* were more susceptible to VN and GS than *S. aureus* and *C. albicans*.

Results obtained in this study indicate that *V. nugundo* (VN) and *G. superba* (GS) have very high antimicrobial activity

Table 1: Effect of ethanolic extracts of *V. nugundo* (VN) and *G. superba* (GS) on the test organisms

Test organisms	Zone Size (mm)		
	VN	GS	VN + GS
<i>Escherichia coli</i>	16.6 ± 0.42	20.9 ± 0.43	20.8 ± 0.43
<i>Salmonella typhi</i>	20.3 ± 0.33	25.4 ± 0.35	25.2 ± 0.29
<i>Staphylococcus aureus</i>	15.9 ± 0.28	20.4 ± 0.13	30.5 ± 0.50
<i>Candida albicans</i>	18.2 ± 0.19	21.4 ± 0.15	26.8 ± 0.16

The Figures represents means and standard deviation of triplicate zones of inhibition obtained from 100 µg/mL of extracts.

Table 2: Effect of extracts of *V. nugundo* (VN) and *G. superba* (GS) and chloramphenicol (CAF) on the test organisms

Test organisms	Zone of inhibition (mm)				
	VN	GS	CAF	VN+CAF	GS + CAF
<i>E. coli</i>	16.6 ± 0.42	20.9 ± 0.43	28.3 ± 0.21	31.3 ± 0.27	31.80 ± 0.27
<i>S. typhi</i>	20.3 ± 0.33	25.4 ± 0.35	36.6 ± 0.06	36.6 ± 0.12	39.70 ± 0.18
<i>S. aureus</i>	15.9 ± 0.28	20.4 ± 0.13	21.4 ± 0.04	26.6 ± 0.14	26.60 ± 0.22
<i>C. albicans</i>	18.2 ± 0.19	21.4 ± 0.15	0.00	20.3 ± 0.07	23.50 ± 0.04

The Figures represents means and standard deviation of triplicate zones of inhibition obtained from 100 µg/mL of extracts and chloramphenicol.

(zone of inhibition ≥ 15 mm) against the test organisms, *E. coli*, *S. typhi*, *S. aureus* and *Candida albicans*. Of course, it has been reported that *V. nugundo* (VN) and *G. superba* (GS) have high levels of alkaloids and cardiac glycosides besides anthranoids, anthraquinones, saponins and tannins which are mainly associated with antimicrobial activity.^[34,35] The combination of the extracts of the two plants revealed that the effect of *V. nugundo* (VN) on *E. coli* and *S. typhi* was completely antagonized (or masked) by that of *G. superba* (GS), the effect of which remained the same as it was when uncombined. However, the combination produced an enhanced (or additive) effect on *S. aureus* and *C. albicans*. This implies that the combination of the extracts of the two plants may be effective for the treatment of infections from gram positive organisms and may not be effective for the treatment of infections from gram negative organisms.

Little additive effects on all the organisms were observed when *V. nugundo* and chloramphenicol, or and *G. superba* and chloramphenicol were combined. Synergism was observed on *C. albicans*. The factors responsible for the synergistic effect were not known during the study especially as *C. albicans* is a fungus. However, since *C. albicans* is gram positive,^[31] the mode of action of the plant extracts might be similar to that of chloramphenicol on gram positive bacteria. Elsewhere, synergistic effect was observed for the combination of garlic and omeprazole against *Helicobacter pylori* and none or even antagonistic effect was observed between garlic and amoxicillin, clarithromycin or metronidazole.^[36] This indicates that synergism between a medical plant and a chemotherapeutic agent may be selective. That is, there may be synergism between one plant and a particular chemotherapeutic agent, and may not be so in another combination.

There was antagonism observed between the two test plants in their combined effect on *E. coli* and *S. typhi* as exhibited by the MIC of the plant extracts. The combined MIC of the plant extracts showed neither antagonism nor synergism on *S. aureus* and *C. albicans*. Significant differences ($P < 0.05$) in the MIC of the plant extracts (VN, GS and VN + GS) was observed. Least significant difference (LSD) test showed that there was significant difference ($P < 0.05, 0.01$) between VN and GS and between VN and VN + GS, while there

Table 3: The minimum inhibitory concentration (MIC) of *V. nugundo* (VN) and *G. superba* (GS) singly and in combination on the test organisms

Test organisms	MIC (µg)		
	VN	GS	VN + GS
<i>Escherichia coli</i>	6.15	4.22	4.22
<i>Salmonella typhi</i>	11.40	5.15	5.15
<i>Staphylococcus aureus</i>	50	5.25	3.12
<i>Candida albicans</i>	50	50	25

was no significant difference ($p > 0.05$) between GS and VN + GS. Thus, *G. superba* (GS) with smaller MIC against the test organisms was more active than *V. nugundo* (VN).

We conclude that *V. nugundo* (VN) and *G. superba* (GS) might individually be very effective against *C. albicans* infections. Chloramphenicol alone has no effect on *C. albicans*, but its combination with either VN or GS might produce a synergistic effect on *C. albicans* infections and on gram positive bacterial infections. There is need for further research in this aspect. Antagonism among herbs should be further studied to assist traditional herbalists who always combine them in treatment.

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