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INVESTIGATION OF THERAPEUTIC ACTIVITY OF *VITEX NEGUNDO* NOCHI

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ABSTRACT

Phytochemicals are ecologically derived plant secondary metabolites produced by the plants to protect them against environmental stress and invasions against pathogenic microbes. These phyto ingredients are known to elicit beneficial as well as pharmacological actions against human ailments. It is an established fact that the active principles present in the medicinal plants act synergistically to alleviate the primary and secondary complications of several diseases. *Vitex negundo L.* (Verbenaceae) is widely used as a therapeutic agent as well as dietary supplement for the treatment of Covid-19. In the present study, the ability of the aqueous extract VN in scavenging free radicals was assessed by using DPPH, ABTS, NO, FRAP and super oxide dismutase assays, *in vitro* anti-inflammatory activity by protein denaturation assay and antidiabetic activity against alpha amylase. VN also investigated for antibacterial activity against *S.aureus* and *P.aeruginosa* and Brine shrimp lethality test. The results obtained evidenced that the aqueous extract(VN-A) ethanolic extract(VN ET) possesses significant antioxidant and anti-inflammatory activity properties, moderate anti- diabetic activity the data presented provide scientific evidence for the antioxidant and other therapeutic efficacy of the medicinal plant, which in turn may be due to the presence of biologically active molecules present in the herbal.

KEYWORDS: Vitex negundo, *in vitro* antioxidant assays, Anti-inflammatory, antidiabetic.

INTRODUCTION

Vitex negundo L. (Verbenaceae) *Vitex negundo* (VN) is widely used as a therapeutic agent as

well as prophylactic supplement in indian system of Medicine. Excellent pharmacological and therapeutic benefits of VN is due their enriched phytochemical constituents and micronutrients[1-2] VN reported for broad spectrum pharmacological activity due their chemical compounds.[3-4] VN recommended for treatment of Covid-19 as immunomodulatory due to their therapeutic benefits and approved by AYUSH Ministry for Covid-19.

In the present study, the ability of the aqueous extract of VN A and ethanol extract (VN ET) in scavenging free radicals was assessed by using DPPH, ABTS, NO and FRAP assays, *in vitro* anti-inflammatory activity by protein denaturation assay. Preliminary antidiabetic activity also performed by inhibition of alpha amylase enzymatic activity. *In vitro* antibacterial activity also tested against human pathogenic bacteria such as (gram positive and gram negative) *S. aureus* and *P. aeruginosa* by well plate methods. Brine shrimp lethality test for assessment of toxicity.

A. MATERIAL AND METHODS

Collection of the sample

Vitex negundo collected from Aravindh herbal laboratory, Rajapalayam, Tamilnadu for this investigation.

Method of Preparation of Sample

10 gram of *Vitex negundo* is heated with 100 ml of water and ethanol separately for 5 hours under reflux condenser in water bath, cool and filter. The filtrate is evaporated under vaccum to get aqueous (VN-A) and ethanolic extract (VN-ET).

Pharmacological Evaluation

In vitro Antioxidant Activity

Vitex negundo aqueous extract [VN] investigated for *in vitro* antioxidant activity by DPPH, ABTS, FRAP and NO for the estimation of anti-oxidant potential of Rhumatigo aqueous extract.[5]

Determination of *DPPH Radical Scavenging Activity*

Antioxidant activity in the sample VN were estimated for their free radical scavenging activity by using DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals (George *et al.*, 1996).[6]

100µL of VN extract was taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was

added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively and read the plate on Elisa plate reader at 490nm Standard ascorbic acid was used as reference. All the analysis was performed in triplicates and average values were taken.

Radical scavenging activity was calculated by the following equation.

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) / (Absorbance ofcontrol)] x 100

ABTS assay

ABTS decolourization assay involves the generation of the ABTS+ chromophore by the oxidation of ABTS with ammonium persulphate. The scavenging activity of the aqueous extract of Aero Pile in ABTS radical cation was measured at 734nm. ABTS radical scavenging activity of aqueous extract of VN was determined according to the method of Re et al., 1999.[7] Briefly ABTS radical cation was produced by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Then, ABTS•+ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. To 3.0 ml of diluted ABTS•+ solution, different concentrations (200-1000 µg/ml) of leaves extract in ethanol was added and after 1 min, the decrease in absorbance was measured at 734 nm spectrophotometrically. All the analysis was performed in triplicates and average values were taken.

Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated spectrophotometrically at 546nm. Sodium nitroprusside (5 mM) in phosphate buffer pH 7.7 was incubated with 200-1000 µg/ml concentrations of aqueous extract of VN was dissolved and the tubes were incubated at 25°C for 120 minutes. At intervals, 0.5ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent N-napthylethylenediamine was measured at 546 nm.[8] All the analysis was performed in triplicates and average values were taken.

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of leaves extract was determined using the ferric reducing ability of plasma FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593nm owing to the formation of a blue coloured Fe II-tripyridyltriazine compound from colourless oxidized Fe III by the action of electron donating antioxidants. The ability to reduce ferric ions was measured using the method[9] described by Benzie and Strain (1996). The FRAP reagent was produced just before use by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ (tripyridyltriazine) solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Aqueous extract of VN was at different concentrations (200-1000 µg/ ml) were then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37 C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of leaves extract was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of leaves extract. The optical density was read at 593 nm. All the analysis was performed in triplicates and average values were taken.

***In vitro* anti-inflammatory activity-Inhibition of albumin denaturation**

Reaction mixture 0.5ml aqueous extract of VN contains drug 0.05ml in the concentration (1mg/ml) with 0.45 ml aqueous solution of bovine albumin fraction (5%). The pH (6.3) of solution was adjusted using small amount of 0.1N HCl at 37 ^{0}C for 20 min, then heat to 57 ^{0}C for 30 min. Cool the solution and transfer to the 96 well plate and measure the absorbance at 660nm. Standard was used as Diclofenac sodium (1000µg/ml) and control contain 0.05ml distilled water.[10]

The percentage of inhibition of albumin denaturation was calculated by the following formula, Percentage of inhibition (%) =[(A control – A sample) / A control] x 100

Where A control - Absorbance above all mixture except drug.A sample - absorbance reaction mixture with Sample).

***In vitro* Antidiabetic Activity**

In vitro amylase inhibition was studied by the method of Bernfeld.[11] In brief, 100 µL (100 µg/ml) of the test extract was allowed to react with 200 µLof α-amylase enzyme (fungal diastage) and 100 µL of 2 mM of phosphate buffer (pH-6.9). After 20-minute incubation, 100

µL of 1% starch solution was added. The same was performed for the controls where 200 µLof the enzyme was replaced by buffer. After incubation for 5 minutes, 500 µL of dinitrosalicylic acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula.

Inhibition (%) = Abs 540 (control) – Abs 540 (extract) * 100 Abs 540(control)

Suitable reagent blank and inhibitor controls were simultaneously carried out.

Antibacterial activity

The antibacterial activity of evaluation of plant extracts Performed by well diffusion methods.[12] The inoculum of the microorganism was prepared from the bacterial cultures. 15ml of nutrient agar (Hi media) medium was poured in clean sterilized Petri plates and allowed to cool and solidify. 100 µl of broth of bacterial strain was pipette out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 6mm in diameter were bored using a sterile cork borer. Solutions of all the extracts (1mg/ml) in DMSO were prepared. 100µl of VN extracts solutions was added to the wells. The petri plates incubated at 37^0C for 24 h. streptomycin (1mg/ml) was prepared as a positive control DMSO was taken as negative control. Antibacterial activity was evaluated by measuring the diameters of the zone of inhibitions (ZI) all the determination were performed in triplicates.

Brine shrimp lethality test

The brine shrimp eggs (*Artemiasalina*) were hatched in artificial sea water and used after 48 h, providing large numbers of larvae. These tiny shrimp larvae have been extensivelyused as a tool to monitor the cytotoxicity of samples under study.[13-16] This is a rabid, inexpensive and general bioassay which has been developed for screening. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample is sufficient. Furthermore, it does not require animal serum, as it is needed for determination of cytotoxicity.

RESULTS AND DISCUSSION

Vitex Negundo extracts VN A and VN ET investigated for *in vitro* antioxidant activity by DPPH, ABTS, NO and FRAP method using ascorbic acid as standard. Both VN extract had significant antioxidant activity when compared with ascorbic acid under similar conditions.(Table 1-3)

Evaluation of antioxidant properties of the given samples assayed by *in vitro* methods using ascorbic acid as standard.

At a concentration of 1000µg/ml, the percentage inhibition of the respective free radicals by the samples was presented.

VN tested for *in vitro* anti-inflammatory activity by protein denaturation method using diclofenac sodium as standard. VN A exhibits potent anti-inflammatory activity when compared with standard diclofenac sodium (Table 4).

Antidiabetic activity of VN was measured by inhibition of alpha amylase activity and compared with standard acarbose under similar conditions, VN A had moderate antidiabetic activity when compared standard (Table 5).

VN A also investigated for *in vitro* antibacterial activity against *S aureus* and *P aeruginosa* by well plate method in nutrient agar medium and compared with standard streptomycin under similar condition. VN had significant antibacterial activity when compared with standard under similar conditions (Figure 1(A&B), Table 6 and 7).

Brine shrimp lethality test: The brine shrimp eggs (*Artemiasalina*) were hatched in artificial sea water and used after 48 h, providing large numbers of larvae. These tiny shrimp larvae have been extensively used as a tool to monitor the cytotoxicity of samples under study. This is a rabid, inexpensive and general bioassay which has been developed for screening. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample is sufficient. Furthermore, it does not require animal serum, as it is needed for determination of cytotoxicity.[11-16]

The LC50 of an agent is the concentration, which will kill, or inactive 50% of the test animal. LC50 is inversely proportional to the toxicity of a compound, i.e. the lower is the LC50 and the higher is the cytotoxicity. Moreover, this significant lethality of the crude plant extracts (LC50 values less than 1000 ppm or µg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds (Table 8).

Crude extracts resulting in LC50values less than 250 µg/ml were considered significantly active and had the potential for further investigation.[22]

Enhancing potency of the extracts, Flavonoids, terpenoids, steroid and tannins are some of the plant secondary metabolites found to have different biological activity and the obtained results may attribute to any of these bioactive compounds present in test extracts.

Table 1: *In vitro* anti-oxidant activity of *Vitex Negundo* extract.

Sample No	DPPH in %	ABTS in %	No in %	FRAP in %
VN-ET	41.64	54.49	53.36	49.54
VN-A	81%	74%	86%	82%
VN-A	84%	76%	82%	79%
VN-A	71	64	61	60

Table 2: *In vitro* anti-oxidant activity of *Vitex Negindo* extract.

Treatment	DPPH Scavenging (%)	Superoxide scavenging (%)	Photochemiluminescence (nmoles ascorbic acid/ gm equlivaent)
Ascorbicacid	97.67±1.08	90.26±2.82	NA
VN-ET	62.60±2.7	78.70±1.9	3.05±1.8
VN- A	53.05±1.4	76.37±2.7	2.30±1.7

Table 3: *In vitro* anti-oxidant activity of *Vitex Negundo* extract.

Sample code	DPPH• radical scavenging activity	
	Absorbance at 517nm	% inhibition
Control	0.79	
standard Ascorbic acid (1000 µg/ml)	0.21	73.41
VN A(1000 µg/ ml)	0.29	63.29

Table 4: *In vitro* anti-inflammatory activity of *Vitex Negundo.*

Cocentration (µg/ ml)	OD	% inhibition
Blank	0.66	
Standard Diclofenac Sodium (1000 µg/ ml)	0.13	80.59
VN 1000 µg/ ml)	0.30	54.54

Table 5: The percent inhibition of extracts of plants on alpha amylase inhibitory assay.

Sr. No.	SampleCode	Conc. µg/ml	OD	OD	OD	Mean	% inhibition
1	Blank					1.086	
2	Standard –Acarbose	1000				0.322	70.34
3	VN-A	1000	0.594	0.493	0.383	0.490	54.88

Table. 6: Antibacterial activity against *Pseudomonas aeurogenosa.*

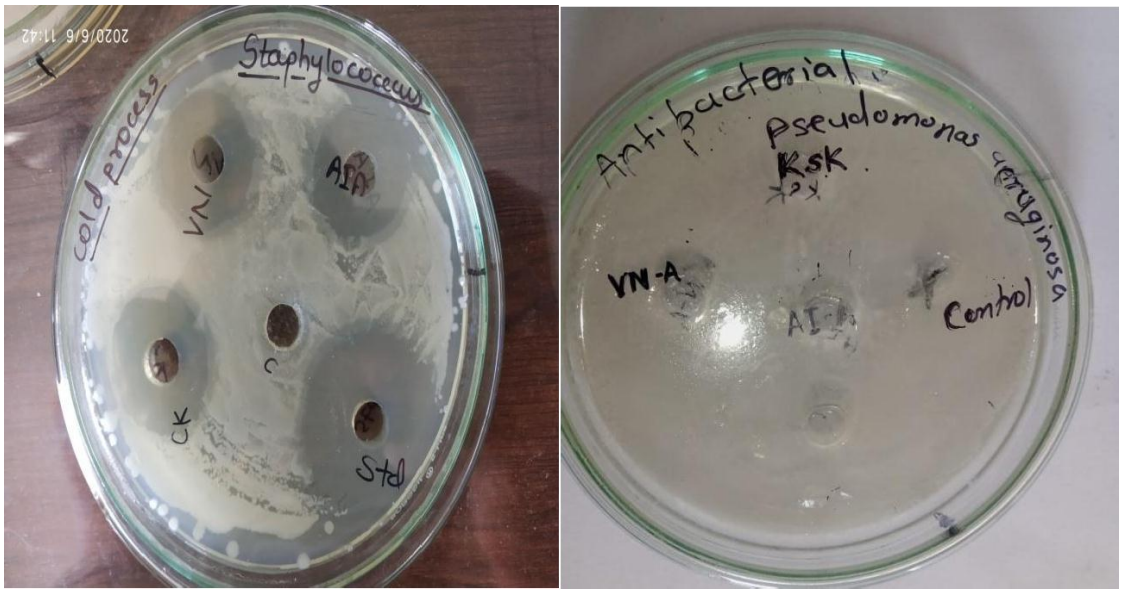
S. No.	Sample	Zone of Inhibition (mm)
1.	Control	--
2	Standard (Streptomycin100µg/ml)	27 mm
3	VN A	17 mm

Table 7: Antibacterial activity against *Staphylococcus auras.*

S. No.	Sample	Zone of Inhibition (mm)
1.	Control	--
2	Standard (Streptomycin100µg/ml)	28mm
5	VN-A	18 mm

Table 8: Brine Shrine Assay.

Sample (1mg/ml)	Total dead naupali (out of 10)		Dead napuli (Out of 20)	% mortality
	T1	T2		
VN A	1	1	2	10
VN ET	0	0	0	0



A B

Figure 1: Antibacterial activity of VN-A. (A) *P.aeurogenosa* (B) *Staphylococcus auras.*

DISCUSSION

In this study *vitex negundo* extract aqueous (VN-A) and ethanolic extracts (VN-ET) tested for *in-vitro* antioxidant activity (Tables 1-3), anti-inflammatory activity (Table 4) anti-diabetic activity (Table 5), antibacterial activity (Table 6,7 and Figures 1, A&B).

In vitro anti-inflammatory activity studied by inhibiting the albumin denaturation property. In

albumin denaturation technique (Table 1).

Evaluation of *in vitro* antioxidant properties studied by DPPH, ABTS, NO and FRAP assay of the given samples was assayed using ascorbic acid as the standard. DPPH Scavenging (%) Superoxide scavenging(%) of both VN A & VN ET extracts shown good activity(Table 1-3) *In vitro* antidiabetic activity was studied by inhibiting alpha amylase enzyme. Acarbose used as a standard. VN– A extract shown good anti diabetic activity. (Table 5)

In vitro antibiotic activity was studied against both *Staphylococcus auras* and *pseudomonas aeruginosa* in Well plate diffusion method. Streptomycin 100µg/ml used as standard. VN A extracts shown good anti-biotic activity (Table 6,7 & Fig 1, A&B))

CONCLUSION

As per the observation indicated in the table, the percent mortality is very less, and the shrimp survival rate is more. Overall, this product at the dose 1000µg/ml showed non-toxic and exhibits good anti-inflammatory, anti-oxidant, anti-diabetic & antibiotic activities.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content andwriting of this article.

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