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In-vivo anti-ulcer and *in-vitro* anti-oxidant studies on the leaves of *Vitex negundo* from South India

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ABSTRACT

The present scientific investigation deals with the in-vitro and in-vivo pharmacological activities of the leaves of vitex negundo. DPPH assay was used for the evaluation. The preliminary screening of the plant extract revealed the presence of the class of compounds in the plant. The works revealed the biological activity potential of the plant.

INTRODUCTION

Herbal Medicine sometimes referred to as Herbalism or Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. An herb is a plant or plant part valued for its medicinal, aromatic or savory qualities. Herb plants produce and contain a variety of chemical substances that act upon the body. (1)

Herbalists use the leaves, flowers, stems, berries, and roots of plants to prevent, relieve, and treat illness. From a "scientific" perspective, many herbal treatments are considered experimental (2). The reality is, however, that herbal medicine has a long and respected history. Many familiar medications of the twentieth century were developed from ancient healing traditions that treated health problems with specific plants(3). Today, science has isolated the medicinal properties of a large number of botanicals, and their healing components have been extracted and analyzed. Many plant components are now synthesized in large laboratories for use in pharmaceutical preparations. For example, vincristine (an antitumor drug), digitalis (a heart regulator), and ephedrine (a bronchodilator used to decrease respiratory congestion) were all originally discovered through research on plants (4,5).

MATERIALS AND METHODS

PLANT PROFILE

Biological source:
Dried leaves of *vitex negundo*(linn)

PREPARATION OF PLANT EXTRACT AND PHYTOCHEMICAL SCREENING (6)

300gms of crude dry powder of leaf of *vitex negundo*(linn) was extracted by soxhlet extraction (continous hot percolation) by methanol solvent extracted for 40 hours. After completion of extraction the extract was taken and

was distilled by distillation process for removing the solvents. Concentrate extract of drug by keeping in room for removing remaining solvents. Then yielded dark green extract of leafs. It was used for further experiments.

PRELIMINARY PHYTOCHEMICAL SCREENING OF VITEX NEGUNDO

Detection of carbohydrates

Small amount of the extract/fraction was dissolved in 5 ml of distilled water and filtered. The filtrate was subjected to the following tests:

Molisch's Test:

To 2 ml of filtrate, two drops of alcoholic solution of alpha naphthol are added. The mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of test tube. Cooling the test tube in ice water and allow to stand. A violet ring at junction indicates the presence of carbohydrates.

Fehling'sTest:

1 ml of filtrate was boiled on a water bath with 1ml of each Fehliag's solution A anb B formation of red precipitate indicates the presence of sugar.

Barfoed's Test:

To 1ml of the filtrate, 1 ml of Barfoed' s reagent was added and heated on a boiling water bath for 2 minutes. Red precipitate indicates the presence of sugar.

Benedict's Test:

To 0.5 ml of filtrate 0.5ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugars.

Detection of proteins and amino acids

Small amount of extract was dissolved in 10 ml of distilled water and filterated through Whatmann no. 1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

Millon's Test:

To 2 ml of filtrate, few drops of Millon's reagent were added. A white precipitate indicates the presence of proteins.

Biuret Test:
An alliquot of 2 ml of filtrate was treated with one drop of 20/0 copper sulphate solution. To this 1 ml of 950/0 ethanol was added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicates the presence of proteins.

Ninhydrin Test:
About 2 drops of ninhydrin solution were added to two ml of aqueous fitoate. A characteristic purple colour indicates the presence of amino acids.

Xantho protic reaction
Extract usually produce a yellow colour when warmed concentrated nitric acid and the colour becomes orange when the solution is made alkaline.

Detection of alkaloids
Small amount of solvent - free extract/fraction was stirred with little quantity of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

Mayer'stest:
To a few ml of filtrate, two drops of Mayor's reagent were added along 1sides of the test tube. If the test is positive, it gives white or creamy precipitate.

Dragendroffs test:
To a few ml of filtrate, 1 or 2 ml of Dragendroffs reagent were added. prominent reddish brown precipitate indicates positive test.

Detection of phytosterols
Libbermann - Burchard's test:
The extract/fraction was dissolved In acetic anhydride, heated to boling, cooled and then 1 ml of concentrated sulphuric acid was added along the tube. Red, pink or violet colour at the junction of the liquids indicates the presen steroids/triterpenoids and their glycosides.

Salkowski test:
When concentrated sulphuric acid is added to a solution of extract in chloroform a red colour is produced in the chloroform layer.

Detection of phenolic compounds and tannins
Ferric chloride test:
Small amount of extract/fraction was dissolved in distilled water and to this few drops of neutral 50/0 ferric chloride solution was added. Formation of blue, green and violet colour indicates the presence of phenolic compounds.

Gelatin test:
A little quantity of extract/fraction was dissolved in distilled water and 2 ml of 1 % of gelatin containing 10% sodium chloride was added to it. Development of white precipitate indicates the presence of phenolic compounds.

Lead acetate test:
A small quantity of extract was dissolved in distilled water and to this, 3ml of 10% lead acetate solution was added. A bulky white. precipitate indicates the presence of phenolic compounds.

Alkaline reagents:
An aqueous solution of extract was treated with 100/0 ammonium hydroxide solution-yellow fluorescence indicates the presence of flavonoids

Glycosides:
For detection of glycosides, about 50 mg of extract was concentrated hydrochloric acid for 2 hrs on a water bath, filtered and the hydrolysate was subjected to the following tests.

Borntrager's Test:
To 2 ml of filtrate hydrolysate, 3 ml of chloroform was shaken, chloroform layer was separated, and 10% ammonia solution was added to it. Formatlon of Pink colour indicates the presence of glycosides.

Legal'sTest:
About 50 mg of the extract was dissolved m pyridine. Sodium nitro prusside solution was added and made alkaline using 10% sodium hydroxide solution. presence of glycoside is indicated by a characteristic pink colour.

Flavonoids
Ferric Chloride test
Test solution with few drops of ferric chloride solution shows intense green colour.

Lead Acetate solution test
Test solution with few drops of lead acetate solution (10%) gives yellow precipitates.

Detection of Saponins
Foam or Froth Test:
A small quantity of extract/ fraction was diluted with distilled water to 20 ml. the suspension was shaken in a graduated cylinder for 15 minutes. A two centimeter layer of foam or froth which is stable for 10 minutes indicates the presence of saponins.

EVALUATION OF IN-VITRO ANTI-OXIDANT ACTIVITY (7)
DPPH ASSAY
DPPH radical scavenging activity was measured according to the method. An aliquot of 3ml of 0.004% DPPH solution in ethanol and 0.1ml of plant extract at various concentrations were mixed and incubated at 37˚c for 30 min. and absorbance of the test mixture was read at 517nm.The percentage of inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the formula.

STUDY OF ANTI-ULCER ACTIVITY (8)
ETHANOL INDUCED ULCER
Male Albino rats were divided in to six groups of six animals per group and animals were fasted for 24 hrs prior to the experiment in perforated steel cages to avoid coprophagy. Six groups were made as below.
Group I - received 1 % Acacia (1 .0ml/kg p.o) as normal control.
Group II - received 1% Acacia (l.0ml/kg p.o) as vehicle control.
Group III - received (l00mg/kg, P.o) methanol extract of *vitex negundo*
Group IV - received (200mg/kg, p.o) methanol extract of *vitex negundo*
Group V - received (20mg/kg, p.o) omeprazole as standard

One hour after the drug treatment the animals were treated with absolute ethanol [5ml/kg] to induce ulcers. The animals were sacrificed after 1 hr and stomach was opened and percentage inhibition of ulcer was determined .(mozafar khazaei.et.al)

COLD RESTRAINT STRESS INDUCED ULCER (9)
Various physical and psychological stressors cause gastric ulceration in humans , and rat models have been developed to mimic the disease condition in humans. This model employs the restraint technique developed by Brodie and Hanson coupled with the cold-water or ordinary-water immersion method by Levine . The combination of these methods is reported to be synergistic in inducing acute stress lesion in rats , arising mainly from physiological discomfort. Gastric ulcers induced by cold-water-restraint stress (CWRS) or cold-restraint stress (CRS) or water-immersion stress (WIS) in rats or mice are known to resemble human peptic ulcers, both grossly and histopathologically . The model is widely used and is reported to be useful for assessing or studying the effects of agents/medicines on the healing of ulcers in rats.

PROCEDURE
Albino Wistar rats of either sex were divided into five groups with six animals in each group as follows:
Group I: Control (untreated) group.
Group II: Stress control group

Group III: Standard treatment group (ranitidine 20mg mg/kg)
Group IV: Test treatment group (*vitex negundo* 100 mg/kg)
Group V: Test treatment group *(vitex negundo* 200 mg/kg)

Stress was induced by immobilizing the animals in a cylindrical cage (19.5cm length, 6.5cm diameter), at 4˚C for 1 hour daily for 7 days. On the 7th day animals were humanely sacrificed using ether and the stomachs were excised. Ulcers were observed under magnifying glass for measuring the area of ulcers and the ulcer index was calculated.

NSAID'S (ASPIRIN) INDUCED ULCER

Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, aspirin and ibuprofen are known to cause gastric ulcers, especially when abused. This phenomenon has been employed in the development of NSAIDs-induced gastric ulcer models in rats. The model is important in investigating the potential usefulness of anti-secretory and cytoprotective agents since the underlying pathophysiology involves gastric acid secretion and mucosal prostaglandin synthesis. It is the most commonly used ulcer model in antiulcer studies. The frequency of usage could be attributed to the fact that NSAID induced peptic ulcers are the second most common etiology of peptic ulcers aside those caused by Helicobaceter pylori

PROCEDURE

Albino Wistar rats of either sex were divided into five groups with six animals in each group as
Group I: Control (untreated) group
Group II: Toxicant group (aspirin 200 mg/kg)
Group III: Standard treatment group (omeprazole 20 mg/kg)
Group IV: Test treatment group (*vitex negundo* 100 mg/kg)
Group V: Test treatment group (vitex negundo 200 mg/kg)

All rats were fasted for 24 hours but excess water was allowed. The standard drug (omeprazole 20 mg/kg) and the test drugs (*vitex negundo*100 and 200 mg/kg) were administered orally to the respective groups. One hour after their pretreatment, all animals were gavaged with aspirin(200 mg/kg). After 4 hours, they were humanely sacrificed by using diethyl ether. The numbers of ulcer spots in the glandular portion of the stomach were counted in both normal control and drug treated animals and the ulcer index was calculated

Ulcer index (UI)

The mucosa was Hushed with saline and stomach was pinned on frog board. The lesion in glandular portion was examined under a 10x magnifying glass and as measured using a divider and scale and gastric ulcer was scored. Ulcer was calculated by adding the values and their mean values were determined˙ (Malirajan, p.2006 .)

- 0 - Normal coloured stomach
- 0.5- Red colouration
- 1- Spot ulceration
- 1.5- Haemorrhagic streak
- 2-ulcers
- 3-Perforations

Percentage inhibition

Percentage inhibition was calculated using the following formula (Malirajan, p. 2006)

$$\%\text{inhibition} = \frac{UI_{\text{ulcer control}} - UI_{\text{ulcer treated}}}{UI_{\text{ulcer control}}} \times 100$$

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

Table No: 1

SL NO.	TEST FOR	RESULTS		
		A	B	C
1	Carbohydrates:			
	a. Molisch's test	+	+	+
	b. Fehling's test	+	+	+
	c. Barfoed's test	+	+	+
	d. Benedicts test	+	+	+
2	Proteins and amino acids:			
	a. Million's test	-	-	-
	b. Ninhydrin test	-	-	-
	c. Xanthoprotic reaction	-	-	-
3	Alkaloids:			
	a. Mayer's test	+	+	+
	b. Dragendroff's test	+	+	+
4	Phytosterols			
	a. Libbermann-Burchards test	+	+	+
	b. Salkowski test	+	+	+
5	Phenolic compounds and tannins			
	a. Ferric chloride test	-	-	-
	b. Getain test	-	-	-
	c. Lead acetate test	-	-	-
	d. Alkaline reagent	-	-	-
6	Glycosides			
	a. Borntrager's test	-	-	-
	b. Legal's test	-	-	-
7	Flavonoids			
	a. Ferric Chloride test	+	+	+
	b. Lead Acetate solution test	+	+	+
8	Saponins			
	a. Frothing test	-	-	-

A - Petroleum ether extract
B - Chloroform extract
C - Methanol extract

Table No: 2

EXTRACT	Quantity in micrograms(µg),mean±s.e.m					IC_{50} values
	25	50	100	200	400	
AA	26.45±1.11	40.81±0.78	54.98±1.79	72.38±2.33	87.79±2.01	71.50
VNME	10.59±0.68	18.54±0.80	28.71±0.51	34.45±0.80	40.30±0.61	169.28
VNCE	19.44±2.22	31.11±1.20	37.88±1.11	46.50±1.03	49.20±0.80	122.18

AA-Ascorbic acid, VNME-Methanolic extract of vitex negundo, VNCE-Chloroform extract of vitex negundo

ANTI-ULCER SCREENING

Ethanol induced ulcer

Effect of methanol extract of *vitex negundo* on ulcer index induced by ethnol in rats are shown in table.

Ethanol induced gastric damage showed gross mucosal lesion,including long haemorrhage bands and petechial lesion.Animal pretreated with methanol extract of *vitex negundo* and standerd drug omeprazole showed very mild lesions and some times no lesion at all,when compared to ulcer control group.

Vitex negundo showed a dose dependent curative ratio compared to ulcer control groups.the extracts exhibited an inhibition percentage of 74.29and 92.84 at doses of 100 and 200mg/kg doses respectively.the ulcer protective action of extract at different doses was better than that of standard drug,omeprazole,which exhibited an inhibition percentage of 94.5

Percentage inhibition and IC_{50} values of DPPH radical in-vitro by AA, VNME, VNCE

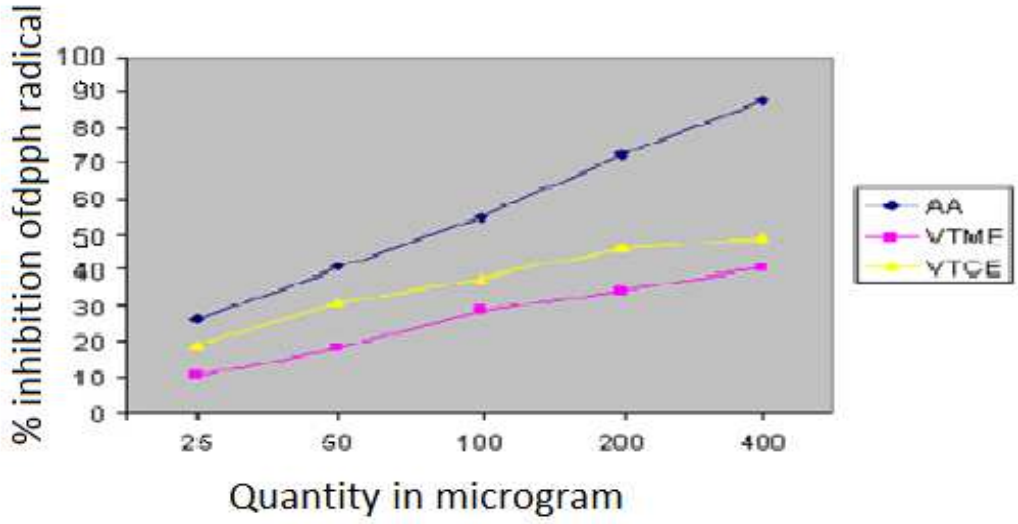

Figure: 1 *In-vitro* concentration dependant percentage inhibition of DPPH radical in-vitro by methanolic and chloroform extract of *vitex negundo* and ascorbic acid

Table No: 3 Effect of *Vitex negundo* on ulcer index in ethanol induced gastric ulcer

GROUP	ULCER INDEX(UI)	PERCENTAGE INHIBITION (%)
Normal control	0	-
Ulcer control	19.66±0.55*	-
Vitex negundo (100mg/kg)	4.66±0.35*	74.29%
Vitex negundo (200mg/kg)	1.8±0.21*	90.84%
Omeprazole (20mg/kg)	1.08±0.15*	94.5%

All values are expressed as mean±S.E.M; (n=6) animals in each group.*p<0.001, ulcer control was compared with Normal control Group. Omeprazole and extract treated Groups were compared with ulcer control group

ASPIRIN INDUCED ULCER

All rats were fasted for 24 hours but excess water was allowed. The standard drug (omeprazole 20 mg/kg) and the test drugs (VN 100 and 200 mg/kg) were administered orally to the respective groups. One hour after their pretreatment, all animals were gavaged with aspirin(200 mg/kg). After 4 hours, they were humanely sacrificed by using diethyl ether. The numbers of ulcer spots in the glandular portion of the stomach were counted in both normal control and drug treated animals and the ulcer index was calculated

Table No: 4 effect of vitex negundo on ulcer index in as pirin induced gastric Ulcer

GROUP	ULCER INDEX(UI)	% PROTECTION
Normal control	0	-
Ulcer control	4.85±0.41	-
Vitex negundo (100mg/kg)	1.75±.30*	57.71%
Vitex negundo (200mg/kg)	1.18±0.30*	70.20%
Omeprazole (20mg/kg)	1.03±0.22*	77.5%

Values are mean ±S.E.M. (n = 6), Significant as compared to control P < 0.001*

COLD RESTRAINT STRESS INDUCED ULCER

Stress was induced by immobilizing the animals in a cylindrical cage (19.5cm length, 6.5cm diameter), at 4˚C for 1 hour daily for 7 days. On the 7th day animals were humanely sacrificed using ether and the stomachs were excised. Ulcers were observed under magnifying glass for measuring the area of ulcers and the ulcer index was calculated.

Table No: 5 effect of vitex negundo on ulcer index in as pirin induced gastriculcer

Treatment group	Mean ulcer score	CRU	
		Ulcer Index	% inhibition
Stress control group	2.917±0.41	0.9848±0.04	---------
Vitex negundo(100mg/kg)	1.617±0.08a	0.7570±0.03a	23.13
Vitex negundo(200mg/kg)	1.33±0.27b	0.5908±0.02b	38.48
Ranitidine (20 mg/kg)	1.050±0.4463b	0.359±0.053b	36.90

Values are mean ±SEM; N = 6 in each group P values : a < 0.05 when experimental groups compared with Stress Control < 0.01 when experimental groups compared with Stress Control

CONCLUSION

From the present investigation we conclude that the leaves of *Vitex negundo* possess significant anti-oxidant activity when screened through the DPPH assay method. The in-vivo biological works also revealed that the alcoholic extracts having very good anti-ulcer activity. The plant is very much used in Ayurvedic system of medicine. So the present work reveals the biological potency of the plant being used in traditional system of medicine. Further works like isolation of potent molecules from the drug can be initiated because the emergence of new active molecules will be a boon for the entire pharmaceutical industry.

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