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ANTIULCER ACTIVITY OF ETHANOLIC LEAF EXTRACTS OF THREE *VIBURNUM* LINN. SPECIES – A COMPARATIVE EVALUATION

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ABSTRACT

The genus, *Viburnum* Linn., belonging to the family, Adoxaceae (formerly positioned under the family - Caprifoliaceae), has been surveyed to cover about 200 species, in the world, and about 17 of them in India, especially, at an altitude from 800 – 2500 ft, habitat such as Himalaya and Nilgiri hills. The stem parts of these species claim to contain an appreciable quantity of therapeutically valuable phenolic compounds like anthocyanins, phenolic acids, flavones, flavonols and biflavone, and their glycosides. Based on the above facts on records, the present study has been under taken. With an objective of screening the antiulcer potentials 75% v/v aqueous ethanolic stem extracts of some three species of this genus, namely: *V.punctatum*, *V.coriaceum* and *V.erubescens* by aspirin plus pylorus ligation model in rats, using Ranitidine 50 mg/ kg b.w (p.o) as the positive reference drug. From the findings of ulcer score, histo-pathological features and the status of biochemical parameters of gastric contents, it is concluded that extract of *V.coriaceum* at a dose level of 500 mg/kg.b.w possessed a significant antiulcer activity ($p<0.01$, $p<0.001$). However, the magnitude of antiulcer potential among the species was not far different. This study can be a referential tool for isolation of active constituents which are responsible for the above biological activity and to conduct an advanced scientific investigation on these species in that regard.

KEY WORDS: Total phenolic, Topfer's reagent, Sialic acid, SCMC, Pylorus ligation

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INTRODUCTION

Viburnum Linn. Species contain sterols, sesqui and triterpenoids, phenolic compounds and their glycosides as their common chemical constituents[1-5]. A few species among 17 in India, namely: *Viburnum punctatum* Buch.-Ham.ex D.Don, *Viburnum coriaceum* Blume and *Viburnum erubescens* Wall.ex DC; have been reported in literature to posses uterine sedative, anti-asthmatic, astringent, anti-inflammatory and anti-microbial activities[6,7]. A verbal enquiry to the local community and plant vendors of Ooty and Coimbatore, Tamilnadu, also supported that the above listed pharmacological activities were traditional and were promising with roots, stem barks and leaves of these species[8].

Among the above listed chemical constituents, phenolic compounds, terpenoids and their glycosides may be the cause for biological responses. In addition to this, a qualitative chemical screening and spectrophotometric analysis of extracts were performed to reveal that the stem part of these three species contains an appreciable amount and a wide range of phenolic compounds[9,10].

Radical scavenging activities of phenolic compounds play a key role in ameliorating healing and even preventing several ailments in living being. It is a well known fact that the plants synthesis phenolic compounds for diverse purposes, which may be of protective, functional or as metabolic end products in nature[11]. But, human exploit them as valuable medicines/ phyto-

pharmaceuticals by focusing on their anti-oxidant potential with or without modification.

A quest for a search of herbal phenolic compounds is still a renewed interest in the science of natural products as a source of valuable medicines. The herbal phenolic molecules such as flavonoids, anthocyanins, bioflavones and other phenolic glycosides have, already, been explored and known for their applications against several human ailments-cardiovascular disorders, chronic inflammation and GIT related troubles[12-14]. So, it was decided to experiment the ethanolic root fractions of all three species to screen for anti-inflammatory, analgesic and anti-spasmodic potentials using suitable experimental animals against appropriate standard drugs.

MATERIALS AND METHODS

Plant Material

The research specimens for the present study was collected from Nilgiri hills and taxonomically authenticated by Dr. Chelladurai, (Ex. Professor) Medicinal plants supply for siddha, Govt. of India, Tamilnadu as *Viburnum punctatum* Buch.-Ham.ex D.Don, *Viburnum coriaceum* Blume. and *Viburnum erubescens* Wall.ex DC. Herbarium of the specimens (labelled V181, VC131 and VE131 respectively) was submitted to the museum of the department of Pharmacognosy, Nandini Nagar Mahavidyalaya College of Pharmacy. The specimens were dried in the shade for a couple of weeks and separately ground in a mechanical grinder to obtain moderately coarse powder. About 1 kg of stem powder of each species was soxhleted for 15 – 18 h successively with petroleum ether (60 - 80ºC), benzene and 75 % v/v aqueous ethanol followed by determination of percentage ethanolic extractives. The ethanolic leaves extracts of *V. punctatum*, *V. coriaceum* and *V. erubescens* were labelled to be VPEE, VCEE and VEEE respectively, and the extracts were screened for their chemical fractions with aid of suitable reagents and methods and then subjected to biological screening.

Animals

The animals used throughout the study were housed under standard laboratory conditions in polyacrylic cages, and were provided with pelleted food and water *ad libitum*. Animal studies were approved by Institutional Animal Ethics Committee (IAEC) of DOABA College of Pharmacy, Mohali, Punjab, India, and carried out in accordance with the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

The statistical analysis of study was carried out using one way analysis of variance (ANOVA) followed by Dunnett's 't' test and all calculations were performed using Graph-pad Prism software, $p<0.01$ was accounted significant.

Aspirin plus pylorus ligation model

Wister rats of either sex weighing 180 to 250 g were divided into five groups of six animals each. Animals were placed in cages with grating floor to avoid coprophagy in fasting period.

Group I-Received 1% SCMC 10 ml/kg b.w. (p.o)
Group II-Received Aspirin 200 mg/kg b.w. (p.o) suspended in 1% SCMC
Group III-Received VCEE 500 mg/kg b.w. (p.o) suspended in 1% SCMC
Group IV-Received VEEE 500 mg/kg b.w. (p.o) suspended in 1% SCMC
Group V-Received VPEE 500 mg/kg b.w. (p.o) suspended in 1% SCMC
Group VI-Received Ranitidine 50 mg/kg b.w. (p.o) suspended in 1%SCMC

Aspirin (200 mg/kg) was administered in non fasted rats once daily for five days. Ranitidine (50 mg/kg) and, VCEE, VEEE and VPEE (500 mg/kg) were administered orally to the respective treatment groups 30 min before each aspirin treatment whereas the control group received only 1% SCMC, and was fasted for 36 hours. On sixth day, immediately after Aspirin treatment pylorus ligation was done under ether anaesthesia. Four hours after pylorus ligation, the animals were sacrificed by giving overdose of ether. The stomach was dissected out after tying the oesophageal end. The stomach was cut open along the greater curvature and the contents drained into a small beaker, centrifuged then subjected to analysis for following acid secretary and biochemical parameter[15].

The mucosa was flushed with saline and the stomach was pinned on a frog board. The ulcer score was calculated. Based on their intensity, the ulcer was given scores as follows:

0 – no ulcer,
1 – superficial mucosal erosion,
2 – deep ulcer or transmural necrosis,
3 – perforated or penetrated ulcer

Status of Biochemical Parameter

Determination of pH of the gastric content

1 ml of the gastric juice was collected, and pH was directly measured by using a pH meter[16].

Determination of gastric volume

After sacrificing the rat, the stomach portion was removed, the gastric contents are transferred into a centrifuge tube, and centrifuged at 1000 rpm for 10

minutes, the supernatant liquid was then transferred to a measuring cylinder, and volume was measured[17].

Determination of free acidity and total acidity

1 ml of gastric juice was pipetted into a 100 ml conical flask. 2 to 3 drops of Topfer's reagent was added and titrated with 0.01 N sodium hydroxide (which was previously standardized with 0.01 N of oxalic acid) until all traces of the red colour disappears and the colour of solution was yellowish orange. The volume of alkali added was noted. This volume corresponds to free acidity, then 2 or 3 drops of phenolphthalein solution was added and titration was continued until a definite red tinge reappears. Again the total volume of alkali added was noted[18]. This volume corresponds to total acidity. Acidity was calculated by using the formula:

$$\text{Acidity} = \frac{\text{Vol.of NaOH} \times \text{Actual normality of NaOH} \times 100}{0.1} \text{ mEq/l/100g}$$

Estimation of total proteins

The dissolved protein in gastric juice was estimated in the alcoholic precipitate obtained by adding 90% of alcohol with gastric juice in 9:1 ratio respectively. Then 0.1ml of alcoholic precipitate of gastric juice was dissolved in 1 ml of 0.1 N NaOH and from this 0.05ml was taken in another test tube. To this 4 ml of alkaline mixture was added and kept for 10minutes. Then 0.4 ml of phenol reagent was added and again 10 minutes was allowed for colour development. Reading was taken against blank prepared with distilled water at 610 nm. The protein content was calculated from standard curve prepared with bovine albumin and has been expressed in terms of µg/ml of gastric juice[19].

Estimation of total hexoses

To 0.4 ml of hydrolysate, 3.4 ml of orcinal reagent was added. The mixture was then heated in the boiling water bath for 15 minutes. This was then cooled under running tap water and intensity of the colour was read at 540 nm against blank by using distilled water instead of hydrolysate. Total hexoses content was determined from the standard curve of D (+) galactose – mannose and has been expressed in µg /ml of gastric juice[20].

Estimation of Hexosamine

0.5 ml of hydrolysate fraction was taken. To this 0.5 ml of acetyl acetone reagent was added. The mixture was heated in boiling water bath for 20 minutes and then cooled under running tap water. 1.5 ml of 90% alcohol was then added followed by an addition of 0.5 ml of Ehrlich's reagent. The reaction was allowed to take place for 30 minutes. The colour intensity was measured at 530 nm against blank prepared by using distilled water instead of hydrolysate. Hexosamine content of the sample was determined from the standard curve prepared by using D (+) glucosamine hydrolchloride and concentration has been expressed in µg /ml of gastric juice[21].

Estimation of fucose

The method was carried out by using three test tubes. In one test tube 0.4 ml of distilled water was taken to serve as control and in each of the other two test tubes 0.4 ml of hydrolysates was taken. To all three test tubes 1.8 ml of sulphuric acid: water (6:1) was added by keeping the tubes in the ice-cold water bath to prevent breakage due to strong exothermic reaction. This mixture was then heated in boiling water bath for exactly 3 minutes. The tubes were taken out and cooled. To the blank and to one of the hydrolysate containing tube (unknown) 0.1 ml of cysteine reagent was added while cysteine reagent was not added to the last tube containing the hydrolysate (unknown blank). It was then allowed to react for 40 minutes for completion of the reaction. The reading was then measured at 396 and 430 nm. This was read against standard curve prepared with D (+) fucose. The fucose content is expressed in terms of µg /ml of gastric juice[21].

Estimation of Total carbohydrates

The dissolved mucosubstance in gastric juice were estimated in alcoholic precipitate obtained by adding 1ml of gastric juice to 9 ml of 90% alcohol and the mixture was kept for 10 minutes and the supernatant was discarded. The precipitate separated was dissolved in 0.5 ml of 0.1N sodium hydroxide. To this 1.8 ml of 6 N HCl was added. This mixture was hydrolysed in the boiling water bath for 2 hours. The hydrolysate was neutralized by 5 N sodium hydroxide using phenolphthalein as indicator and used for the estimation of total hexoses and hexosamine[21].

Estimation of Sialic Acid

To 0.5 ml of the hydrolysate in 0.1 N sulphuric acid, 0.2 ml of sodium periodate was added and mixed thoroughly by shaking. A time of 20minutes was allowed to elapse before the addition of 1ml of sodium arsenite solution to this mixture. The brown colour produced was disappeared by shaking. Then 3 ml of thiobarbituric acid

was added, 4.5 ml of cyclohexanone was added and thorough shaking was done for 15 seconds, till all the colour was taken up by the cyclohexanone supernatant. The mixture was centrifuged to get a clear pink layer of cyclohexanone. The supernatant was pipetted out and intensity of colour was measured at 550 nm. The sialic acid content of the sample was determined from the standard curve of sialic acid and has been expressed in µg /ml of gastric juice[21].

RESULT

VCEE, VEEE and VPEE were screened for their antiulcer potential at a dose level of each 500 mg/kg b.w, p.o and the result of the sudy as follows: There were six groups of animals subjected in aspirin plus pylorus ligation model. The ulcer score was significantly increased ($p<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($p<0.001$) decrease in ulcer score, when compared to group-II . With aid of a pH meter, the pH of the stomach content was monitored; the pH level was significantly increased ($p<0.001$) in the group-II animals as positive control, when compared to that of the solvent control (group-I); and administration of VCEE, VEEE, VPEE to group-III, IV & V respectively, and Ranitidine-dosed (group-VI) animals showed a significant ($p<0.001$) decrease in pH level, when compared to group-II animals.The gastric volume was significantly increased ($P<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($P<0.001$) decrease in gastric volume level (III, IV and V groups), when compared to that of the group-II.The free acidity was significantly increased ($P<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($P<0.001$) decrease in free acidity, when compared to that of the group-II. The total acidity was significantly increased ($P<0.001$) in the group-II animals, when compared to control (group-I). Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($P<0.001$) decrease in total acidity, when compared to that of the group-II. The total protein was significantly increased ($p<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($p<0.001$) decrease in total protein level, when compared to that of the group - II animals (**Table 1.**).The total hexose was significantly decreased ($p<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($p<0.001$) increase in total hexose, when compared to that of the group-II).The hexosamine was significantly decreased ($p<0.001$) in the group-II animals, when compared to control group-I. Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($p<0.001$) increase in hexosamine, when compared to group-II. The fucose was significantly decreased ($p<0.001$) in the group-II animals, when compared to that of the control (group-I). Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($p<0.001$) increase in fucose, when compared to that of the group-II .The total carbohydrate was significantly decreased ($p<0.001$) in the group-II animals, when compared to that of the control (group-I). Administration of VCEE, VEEE, VPEE and Ranitidine showed a significant ($P<0.001$) increase in total carbohydrate level, when compared to group-II.The sialic acid was significantly decreased ($p<0.001$) in the group-II animals, when compared to that of the control (group-I). Administration of VCEE, VEEE, VPEE and Ranitidine level showed a significant ($p<0.001$) increase in sialic acid, when compared to that of the group-II (**Table 2.**).The status of biochemical parameters were comparable to the solvent control as well as the reference drug-treated group which is shown in the glandular portion and its pathological symptoms.

DISCUSSION

Ulcer induction and aggravation in aspirin plus pylorus ligation model and its counteraction by VCEE, VEEE and VPEE

Acetylsalicylic acid, itself, is an acid which reduces a rise in pH of the GIT. Moreover, aspirin can block the harmonious supply of HCO_3^- ions, which are supplied from the blood stream through the vascular bed of the stomach. This is governed by the effect of a prostaglandin. A failure in supply of HCO_3^- ions leads to a rise in acid accumulation.

Probable mechanism of action of VPEE, VCEE and VEEE against aspirin induced and acid accumulation upon ligation of pylorus

All the extracts have, already, been proven to be anti-cholinergic in action (especially, muscarinic blocker).The muscarinic receptors of oxyntic or parietal cells posses tendency to activate mast cells, which are neighbouring receptors to (M_3), to augment the release of histamine which leads to an activation of (H_2) histamine-2 dependent K^+/H^+–ATPase pump.In this event, the constituents of test drugs are supposed to block muscarinic receptors of the oxyntic cells leading to a

partial blockade in the release of histamine followed by a suppression of H_2 dependent acid formation and its release to the lumen of the stomach. Phenolic molecules are efficient reactors prone to bind with proteins and thereby leading to denaturation of protons (often called astringent effect). Among phenolic compounds, the ones, namely: tannins – a powerful astringent. The VPEE, VCEE and VEEE were confirmed to contain tannins of condensed in nature and their relevant polymeric compounds such as procyanidins and anthocyanidins (blood red colour – brownish nature of alcoholic and aqueous etracts of these species on heating).So, it may be a way that the tannins of test drugs can afford an astringent activity on the mucosal and sub-mucosal part and thereby resisting further corrosions of stomach walls by excessive acid and also progress and promote a healing process in the lumen.

CONCLUSION

From the findings of ulcer score, histo-pathological features and the status of biochemical parameters of gastric contents, it is concluded that extract of *V.coriaceum* at a dose level of 500 mg/kg.b.w possessed a significant antiulcer activity ($p<0.05$, $p<0.001$). However, the magnitude of antiulcer potential among the species was not far different. This study can be a referential tool for isolation of active constituents which are responsible for the above biological activity and to conduct an advanced scientific investigation on these species in that regard.

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Table 1. Effect of ethanolic extracts on ulcer core, pH, gastric volume, free acidity, total acidity and total acid out put

Groups	Ulcer score	pH	Gastric volume (ml/100g)	Free acidity (mEq/l/100g)	Total acidity (mEq/l/100g)	Total acid out put (mEq/100g)
Normal control	2.01 ± 0.21	4.08 ± 0.21	3. 61 ± 0.18	54.20 ± 1.31	61.81 ± 1.25	219.25 ± 9.20
Positive control	19.48 ± 1.71*	1.48 ± 0.18*	5.86 ± 0.13*	70.11.± 2.83*	81.20± 1.68*	327.24 ± 12.71*
VCEE (500 mg/kg)	10.80 ± 0.03**	2.87 ± 0.08*	2.62 ± 0.21**	48.14± 1.68**	62.13± 1.87*	156.15± 8.94*
VEEE (500 mg/kg)	15.14 ± 1.88*	2.03± 0.17*	3.02 ± 0.15*	52.36± 2.10**	65.26± 1.60*	165.28 ± 13.54*
VPEE (500 mg/kg)	9.37 ± 1.74*	2.56 ± 0.11**	2.44± 0.10**	46.81 ± 1.96*	62.44± 1.33*	161.55± 12.52*
Ranitidine (50 mg/kg)	6.13 ± 0. 0.69**	3.77 ± 0.09**	1.89± 0.21**	40.38 ± 1.24**	55.18± 1.08**	133.05± 11. 26*

Values represented as mean ± SEM of 6 animals, * – p< 0.05, ** – p< 0.01

Table 2. Effect on total protein, hexose, hexosamine, fucose, sialic acid, total protein and total carbohydrate- protein ratio

Groups	Total protein (µg/ml)	Hexose (µg/ml)	Hexosamine (µg/ml)	Fucose (µg/ml)	Sialic acid (µg/ml)	Total carbohydrates (µg/ml)	TC : P ratio
Normal control	273.11± 7.41	413.16± 10.48	238.03± 8.11	61.84± 1.25	70.18 ± 6.04	769.12± 8.37	2.88 ± 0.21
Positive control	410.60± 8.35*	218.71± 6.93*	143.16± 4.54*	103.22±4.83*	34.50± 4.28*	526.14± 6.33*	1.15 ± 0.17*
VCEE (500 mg/kg)	376.01± 10.61*	336.16± 9.24*	178.66± 5.17*	91.36 ±3.18 *	41.86± 3.45*	698.61 ± 8.21*	1.82 ± 0.30*
VEEE (500 mg/kg)	398.13± 11.17*	368.41± 9.63*	159.30± 4.65*	94.51± 3.66*	46.35± 5.02*	710.88 ± 10.24*	1.67 ± 0.16*
VPEE (500 mg/kg)	384.60± 8.66*	345.66± 11.02*	164.83± 7.16*	84.16± 6.01*	40.81± 3.77*	703.41 ± 6.37*	1.74 ± 0.25*
Ranitidine (50 mg/kg)	291.86± 9.41**	389.21± 10.52**	221.32± 5.83**	68.53± 2.16**	56.20± 2.46*	738.01 ± 10.43**	2.26 ± 0.32*

Values represented as mean ± SEM of 6 animals, *p< 0.05, **p< 0.01

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