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ANTINOCICEPTIVE AND ANTI-INFLAMMATORY POTENTIALS OF 80 PERCENT METHANOL SOLUBLE FRACTIONS OF AQUEOUS ROOT EXTRACTS OF THREE *VIBURNUM* LINN. SPECIES

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ABSTRACT

The genus, *Viburnum* Linn., belonging to the family Caprifoliaceae includes about 200 species throughout the world and some 17 species growing in India at an altitude from 1800 – 2500 ft. Many of these species have been investigated for their pharmacognostic features and phytochemical elaboration lacking information on biological potentials of them. So, the current study undertaken casts some scientific information on phytochemical based pharmacological screening such as anti-inflammatory and analgesic activities, using Methanol soluble fractions of aqueous root extracts of three species – *Viburnum punctatum* Buch.-Ham. ex D. Don (VPMF), *Viburnum coriaceum* Blume (VCMF) and *Viburnum erubescens* Wall. ex DC (VEMF) – in suitable animal models. The acute oral toxicity study was carried out according to the OECD (Organization for Economic Co-operation and Development) guidelines 423 (Acute toxic class method). In acetic acid induced writhing in mice, a significant reduction in writhing was observed with all the fractions than the control group ($p<0.001$) which received 1% CMC. However, reduction in number of writhings was not proximal to the standard (Diclofenac sodium 15mg/kg b.w, p.o). In cotton pellet induced granulation method in wistar rats, a significant reduction in dry weight and wet weight of

cotton pellets was observed with all the three fractions (500mg/kg b.wt) ($p<0.001$) which was comparable to that of the standard (Indomethacin 20mg/kg b.w, p.o).

KEYWORDS: *Viburnum*, phenolic compounds, $ED_{50,}$ OECD, anti-inflammatory.

INTRODUCTION

India has a rich heritage of using medicinal plants and hosting several thousands of medicinally valuable plants belonging to hundreds of families. One cannot assure that all of these plants possess a long recorded history of medicinal values in the current world of science, although they have been reported to contain medicinally valuable phyto-pharmaceuticals. For many of them, an authentic protocol derived from multidisciplinary approach is very scant. In particular, the plants, which are growing at elevated altitude ascending more than 2000 ft and forest dominated hilly areas, are not exposed to plant vendors, botanists, plant collectors and pharmacognosists due to inaccessibility and climatic conditions of the locations.

The genus *Viburnum* Linn. is a typical example of such a kind, which is dwelling at a high altitude, belonging to the family Caprifoliaceae. The genus *Viburnum* Linn. includes about 17 species in India and about 200 species distributed throughout the world.[1,2] *Viburnum* Linn. Species have been reported to contain sesquiterpenoids.[3] triterpenoids and sterols; phenolic compounds and their glycosides such as tannins, flavonoids and anthocyanins and irridoid glycosides in their stem, root and leaves, and investigated to posses uterine sedative, diuretic, cardiovascular stimulant, antimicrobial, anti-inflammatory, anti-nociceptive, antispasmodic, anti-asthmatic and astringent activities.[4,5] In the late 1960s and early 1980s, scientific studies on the genus *Viburnum* Linn. were voluminous.[6,7,8] However, the number of species subjected for studies have and areas of investigations were narrow. On the basis of the above facts, some scientific studies have been undertaken very recently on three of these species as follows: Pharmacognostic investigations on the leaves of *Viburnum coriaceum* Blume.[9] and *Viburnum punctatum* Buch.-Ham. ex D. Don.[10] Stem of Two *Viburnum* Linn. species.[11] Leaves and Stems of *Viburnum erubescens* Wall.ex DC.[12] Total Phenolic Content and *in vitro* Antioxidant Potentials of Ethanolic Stem Extracts of Three *Viburnum* Linn. Species.[13] Anti-inflammatory, analgesic and anti-spasmodic activities of three *Viburnum* Linn. Species.[14] Antiulcer Activity of Ethanolic Stem Extracts of three *Viburnum* Linn. Species – A Comparative Evaluation.[15] Morphological, Microscopical and Physico-chemical Investigations on the roots of *Viburnum punctatum* Buch.-Ham. ex D. Don.[16] Histo-

chemical analysis on the Leaves, Stem and Roots of three *Viburnum* Linn. Species.[17] Pharmacognostical Evaluation of a *Triple Viburnum* churna having anti-inflammatory potentials.[18] Formulation and physico-chemical standardization of *Viburnum coriaceum* bark arista.[19] Total Phenolic Content and *In vitro* Anti-oxidant Potentials of Ethanolic Root Extracts of Three *Viburnum* Linn. species – A Comparative Study.[20] Formulation and standardization of a triple *Viburnum* churna Having Anti diarrhoeal Activity.[21] A study on physico-chemical standardization of a formulated Triple *Viburnum* root Asava possessing Anti-helmintic activity.[22,23] Isolation of astragalin and amentoflavone from the roots of *Viburnum erubescens* Wall. ex DC and their effect against human pathogenic bacteria.[24] A study on physical and physico-chemical standardization of a formulated asava possessing anti-diarrhoeal potential.[25] A Preliminary chromatographic detection of phenolic compounds from ethanolic stem extracts of *Viburnum* Linn. species by TLC and PC.[26] A Comparative preliminary phytochemical screening on the leaves, stems and the roots of three *Viburnum* Linn. Species.[27] Formulation of *Viburnum erubescens* root Asava and its Physico-chemical Standardization.[28] A comparative study on proximate analysis conducted on three *Viburnum* Linn. Species.[29] Preliminary detection, isolation and characterization of astragalin and amentoflavone from *Viburnum* Linn. Species.[30] Phytochemical Investigations on the Hydro-alcoholic Stem Fractions of *Viburnum* Linn. Species Possessing Antibacterial Potentials.[31] Antiulcer activity of Ethanolic leaf extracts of three *Viburnum* Linn. species – A Comparative Evaluation.[32] Isolation and spectral identification of Arbutin from the roots of *Viburnum erubescens* Wall. ex DC.[33] Pharmacognostical evaluation of a triple *Viburnum* root churna possessing anti-inflammatory potentials.[34] Formulation and Pharmacognostical evaluation of *Viburnum erubescens* churna.[35] Formulation of *Viburnum coriaceum* root Arista and Evaluation of its Antiulcer potential.[36] Formulation and biological activities of *Viburnum coriaceum* root arista.[37] A comparative micro-morphological and micrometric investigations amoung the stems of three *Viburnum* Linn. Species.[38] Formulation and Pharmacognostical evaluation of *Viburnum punctatum* churna.[39] Isolation of chlorogenic acid from the stems of *Viburnum coriaceum* Blume.[40] Fomulation and physico-chemical standardization of *Viburnum coriaceum* arista.[41] Unities and Diversities among Powder characteristics of Three *Viburnum* Linn. species – An evaluation.[42] A study on physico-chemical standardization of a Formulated Triple *Viburnum* stem Asava possessing anti-helmintic activity.[43] Preparation and physico-chemical standardization of *Viburnum punctatum* arista.[44]

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.[45] 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. Hence, it was decided to fractionate aqueous root extracts of some three *Viburnum* Linn. Species with 80% methanol and to screen the fraction for its antinociceptive and anti-inflammatory activities. [46-48]

MATERIALS AND METHODS

Plant Material

The research specimens for the present study was collected from Nilgiri hills at an altitude from 1500 – 1800 ft 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 and sample specimens were submitted to the museum of 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 root powder of each species was cold macerated with double ditilled water for a couple of days and then filtered. The extracts were evaporated under controlled temperature to obtain brownish residues, after determining their percentage yield being 5.65±0.212, 4.55±0.122 and7.11±0.055 respectively. The residues were added to sufficient quantity of 80 percent methanol, stirred well and filtered to get 3.55, 2.43 and 6.33 percent of respective methanolic fractions. The methanolic fractions of *V. punctatum*, *V. coriaceum* and *V. erubescens* were labelled to be VPMF, VCMF and VEMF respectively, and the extracts were screened for their chemical fractions with aid of suitable reagents and methods.[49,50]

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 Nova College of pharmacy, Jangareddygudem, Andhra Pradesh, 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 various studies were carried out using analysis of variance (ANOVA) followed by Dunnett's 't' test and standard deviation, $p<0.05$ was accounted significant.

The acute oral toxicity.[51] study was carried out according to the OECD (Organization for Economic Co-operation and Development) guidelines 423 (Acute toxic class method) a starting dose of 2000 mg/kg body weight/p.o of VCMF, VEMF and VPMF was administered to each of 3 healthy female rats, and then observed for three days. There was no considerable change in body weight before and after treatment of the extracts and no signs of toxicity were observed. When the experiments were repeated again with the same dose level, 2000 mg/kg p.o of VCMF, VEMF and VPMF for 3 more days, and then observed for about 14 days, no changes were observed from the first set of experiment. LD_{50} cut off mg/kg body weight was observed as X (unclassified) and Globally Harmonized System (GHS) and comes under X (Unclassified). Hence, 5000mg/kg b.w was considered to be LD_{50} value and 500mg/kg b.w ($1/10^{th}$ of the Lethal Dose) was randomly selected as therapeutic dose of the experiment.

Cotton pellet induced granuloma formation in rats

Wistar rats of either sex weighing 180 – 250 g were divided into five groups of six animals each. Group I -Received 1% SCMC 10 ml/kg b.w.(p.o), Group II-Received VCMF 500 mg/kg b.w (p.o) suspended in 1% SCMC, Group III-Received VEMF 500 mg/kg b.w (p.o) suspended in 1% SCMC, Group IV-Received VPMF 500 mg/kg b.w (p.o) suspended in 1% SCMC and Group V-Received Indomethacin 20 mg/kg b.w (p.o) suspended in 1% SCMC.

Adsorbent cotton wool was cut in to pieces weighing 20 ± 1 mg and made up to a pellet and sterilized in a hot air oven at 120 ^{0} C for 2 hrs. Pellets were implanted subcutaneously under light-ether anaesthesia and sterile condition. The test drugs were administered in a once daily dosage regimen for a period of seven days. On the 8th day, rats were sacrificed by a large

dose of pentobarbital sodium, the pellets dissected out, carefully removed from the surrounding tissues and weighed immediately for the wet weight. The pellets were then dried at 60 ^{0} C for 18 hrs and their dry weight determined. The inhibition of granuloma formation was determined by comparing with the control.[52-55]

Acetic acid-induced writhing test in mice

One hour after receiving oral (p.o.) administration of the plant extract, reference substance or solvent to a group of 6 mice (18 – 25 g), each mouse was given intraperitoneally 0.6% aqueous solution of acetic acid (2 ml/kg body weight). Immediately after the algic compound injection, each animal was placed in a transparent observation cage and the number of writhes per mouse was counted for 30 min. The writhing activity consisted of a contraction of the abdominal muscles together with a stretching of the hind limbs.[56]

RESULT AND DISCUSSION

Corroborating the findings of references cited in this article it was decided to undertake a primary organic analysis dependent biological study on the methanol soluble fractions of aqueous root extracts of three *viburnum* species. Firstly, VPMF, VCMF and VEMF were involved in preliminary phytochemical analysis such as thin layer chromatographic screenings and primary organic analysis to explore the nature of phyto-constituents. All fractions gave a positive test for diverse classes of phenolic compounds such as tannins (Gold beaters test), chlorogenic acid (ammonia vapour treatment with ethyl acetate fraction of methanol), flavonoids (Shinodas test, P^{H} dependance colour transition) and phenolic glycosides (test for reducing sugar and phenolic compounds upon hydrolysis, after exhausting free sugar). The 85% methanolic solutions showed their peak maximas at 285, 255 and 235 nm under UV, showing the presence of conjugated carbon chain containing compounds.

The LD_{50} was found to be more than 5000 mg/ kg b.w. p.o. in acute toxicity testing. The therapeutic dose 500mg/ kg b.w. p.o. (ED_{50}) was accounted for *in- vivo* studies. On random selection as well as from the pharmacological studies conducted on these species during the last decade

In acetic acid induced writhing in mice model, percentage reduction in writhing was considered as parameter of magnitude of analgesic activity, VCMF, VEMF, VPMF and the standard (Diclofenac sodium 15 mg/kg) showed a significant analgesic activity when compared to the control ($p<0.01$, $p<0.001$). Analgesic effect of Diclofenac sodium was

significantly more than that of all the test drugs ($p<0.001$).However amoung the species, VCMF represented more pronounced analgesic activity during time period of 21-30min of observation (Table 1).

In cotton pellet induced granuloma formation, there were five groups of animals and a significant reduction in both wet and dry weights of cotton pellets was observed in all the test drug treated groups when compared to that of control group. Activity against wet weight reduction was significant ($P<0.001$) for the groups treated with 500 mg/kg of VCMF, VEMF, VPMF compared to the control group. The dry weight was significantly inhibited ($P<0.001$) in all the drug treated groups as compared to the control group. The indomethacin 20 mg/kg treated group showed significant reduction $P<0.001$) of both wet and dry weights, when compared to the control group and proximal to the test drugs (Table 2).

Many plants, so far, have been screened to possess potent anti-oxidant property due to presence of phenolic compounds (one or more (-OH) group on the benzene moiety of their molecules). Phenolic compounds play a crucial role in counteracting excessive production and accumulation of free radicals which are powerful oxidants leading to several ailments in biological system. Formation of chronic inflammation leading to pain and other implications is a typical example of what the excessive free radicals do with healthy cells.

The receptor/ molecular level theory of a single chemical entity is experimentally predictable at ease, rather than theory of drug mechanism for a crude extract. Plant extract may possess several components, which on administration in a living system may target only one receptor type at a single time point (i.e., all to one) or every single component may target more than one receptor type at a single time point. Considering this phenomenon, only probable mechanisms of action for VPMF, VCMF and VEMF is possibly unfolded in this study which may be useful to progress advanced pharmacological studies.

The mechanism of biological activity of extracts *in vivo* may be probably through the following ways: Phospholipid metabolism is catalysed by enzymes such as phospholipase A_2 (PLA_2), cyclooxygenase ($COX_{1,\ 2\ and\ 3}$), lipooxigenase (LO, 5LO, 12LO and 15LO) and acetyl transferase (AT) that leads to formation of various inflammatory mediators such as prostaglandins (prostanoids) (PGI_2, $PGE_{2\alpha}$, PGD_2 and PGE_2), throboxanes (TXA_2), leukotrienes (LTA_4, LTC_4 and LTB_4) and platelet activating factor (PAF).

The phenolic compounds of the extracts, especially flavon-3-ols, biflavones, flavonols of flavonoids classes, both in free and glycoside form, possess a potent anti-inflammatory activity by targeting COX, LO and AT leading to blockade of their action thereby preventing generation of inflammatory mediators. The enzyme deactivation may be reversible is yet to be known and a matter of speculation through appropriate experimental model.

It is also probable that unlike conventional NSAIDs the extracts contain several phenolic compounds of diverse chemical nature which may target amino acid domains of the COX by hydrogen bonding to subside the functional status of the enzyme.

Table 1. Effects of methanolic root fraction of *Viburnum* Linn. species on acetic acid-induced writhings in mice.

Time (min)	Control (1% Tween 80)	VCMF (500mg/kg)	VEMF (500mg/kg)	VPMF (500mg/kg)	Diclofenac Na^+ (15mg/kg)
5-10	13.51±0.64	10.06±0.47**	12.32±0.34*	11.4±0.52*	6.61±0.37**
11-20	19.03±0.81	14±0.55**	15.40±0.68**	14.32±0.70**	7.22±0.63**
21-30	6.16±0.53	3.35±0.71**	5.01±0.44*	4.60±0.53**	1.8±0.24**
Total	36.80±1.73	25.72±1.45** (26.39)	30.05±2.13* (14.62)	26.31±2.35** (22.07)	12.6±1.04** (61.04)

Values are mean ± SEM. from 6 animals in each group and values in parenthesis percentage inhibition.*p<0.01, **p<0.001 when compared to control.

Table 2. Effects of methanolic root fractions of *Viburnum* Linn. species and Indomethacin on cotton pellet-induced granuloma formation in rats.

Group	Treatment	Wet weight (mg)	Dry weight (mg)	% Inhibition
I	1% CMC	187.45 ± 7.67	77.51 ± 5.46	-
II	VCMF 500 mg/kg	158.42±5.93*	34.20 ±3.61*	55.64 %
III	VEMF 500 mg/kg	166.37±3.84*	40.82±4.31*	48.15 %
IV	VPMF 500 mg/kg	161.08 ±5.43*	37.35 ±4.09*	51.64 %
V	Indomethacin 20 mg/kg	153. 24±4.05*	29.06 ±3.28 *	62.13 %

Values are mean ± SEM from 6 animals in each group. *p<0.001.

CONCLUSION

From the above observation it has been revealed that the methanolic fractions of aqueous extracts of all three species exhibited a moderate analgesic and an appreciable anti-inflammatory (blockade of COXs and LOs enzymes and crippling prostanoids) activities which are comparable to the potential of a conventional NSAID. The findings of the current study will, surely, be useful to begin with isolation of phytoconstituents attributable to these activities and to further some advanced pharmacological experimentation on these species to record their potential medicinal values pertaining to their phenolics and saponins.

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