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A STUDY ON PHYSICO-CHEMICAL STANDARDIZATION OF A FORMULATED TRIPLE *VIBURNUM* STEM ASAVA POSSESSING ANTI-HELMINTIC ACTIVITY

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ABSTRACT

The stems and their branches of *V.punctatum* and *V.coriaceum* and *V.erubescens* were collected from Nilgiri hills, Tamil Nadu, India. A primary organic analysis on these species revealed the presence of bio-active molecules such as tannins, saponins, phenolic compounds (flavonoids) and other phenolic glycosides as their principal phyto-constituents. The crude drug (Patha) was formulated in to an asava using conventional anaerobic fermentation process for about 90 days. Apart from some traditional methods of standardization of asava, a new approach was made to select about thirteen numbers of physical, physic-chemical including organoleptic and primary organic analysis and were attempted with the asava to obtain a reproducible and consistent results and the same were recorded. Since the stem asava contained phenolic compounds as principal constituents, a screening for its anthelmintic activities against piperazine citrate induced paralysis $p<0.01$was significant.

Keywords: *Viburnum*, asava, sugar content, Alcohol content, Anthelmintic

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INTRODUCTION

India has a rich heritage of using ayurvedic system of medicine which dates back to 5000 years or more and hosting several thousands of medicinally valuable plants belonging to the hundreds of families. One can not assure that all of these plants possess a long recorded scientific history, although they have been reported to contain medicinally valuable phyto-pharmaceuticals. For many of them, an authentic protocol that has been derived from multidisciplinary approach is very scant. In particular, the plants, which are growing at an elevated altitude ascending more than 2000 ft and forest dominated hilly areas, which are not easily exposed to plant vendors, botanists, plant collectors and pharmacognosists due to an 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 Adoxaceae. 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 sesquiterpenes[3], triterpenes and phyto-sterols; phenolic compounds and their derivatives such as tannins, flavonoids and anthocyanins and iridoid glycosides in their stems, stems 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, the scientific studies on the genus *Viburnum* Linn. were voluminous[6-8]. However, the number of species subjected for the studies and the areas of investigations were markably narrow. After a couple of decades, a few *Viburnum species* re-emerged to be involved for some extensive phytochemical and pharmacological investigations. The typical examples are: iridoid aldehydes and their glycosides in *Viburnum luzonicum*[9] and their cytotoxic effect; vibsane type

diterpenes from *Viburnum awabuki*[10]; iridoid glycosides from *Viburnum tinos;* antinociceptive and anti-inflammatory activities of *Viburnum*[11] *lanata*and *Viburnum opulus*[12]; iridoid glycoside from *Viburnum rhytidophyllum*[13].

The phenolic compounds of plant origin are versatile in biological activities. Their presence in plants, probably may be due to one or all of the following purposes: feed deterrents against cattles; (pathogenic) defence against microbial attack; as a precursors for bio-synthesis or as metabolic end products of plant metabolism; pH-dependent colouring agents, especially in floral organs and leaves; as the building blocks of polymeric phenolic molecules of heavy molecular weight such as tannins, procyanidins and lignans; and as antioxidants.

Isolation of phenolic compound by virtual solvent extraction process is supposed to be highly a tedious process, because of its high magnitude of reactivity with other co-molecules of the plants such as proteins (astringent effect) and carboxylic acids to form esters during extraction, in addition to their delicate nature of decomposition in presence of heat, acids, alkali and inorganic elements such as heavy metals.

Phenols, cresols, xylenols and halogenated phenolic derivatives are most powerful antimicrobials (often referred to be "Disinfectants" which are unsuitable for oral administration in the living beings). In this context, the phenols of plant origin are remarkably best suiting for application in living system. Hence, use of plant as it is may be highly advantageous in case of obtaining biological activity such as anti-inflammatory effect which is attributed to the presence of phenolic compounds[14,15].

MATERIALS AND METHODS

The stems and their branches of *V.punctatum* and *V.coriaceum* and *V.erubescens* were collected from Nilgiri hills, Tamilnadu, India and authentificated by Dr.V.Chelladurai, Ex. Professor, (Botany), Medicinal plant survey for Siddha, Government of India, as *Viburnum punctatum* Buch.-Ham.ex D.Don (VP), *Viburnum coriaceum* Blume (VC) and *Viburnum erubescens* Wall.ex DC (VE). Herbarium of the specimens (labeled V181, VC131 and VE131 for VP, VC and VE respectively) was submitted at the museum of the department of Pharmacognosy, Nandini Nagar Mahavidyalaya College of Pharmacy. The materials were dried in the sun and then in the shade for about 15 days.

Preparation of Triple *Viburnum* stem asava by anaerobic fermentation method (An Ayurvedic formulation)

Approximately 1.5 seers (60 g) of the stems (each 20g) (patha) were coarsely powdered and added with 32 seers (1024 ml of water) and kept for about 3 – 5 h to prepare a mixture. The mixture was taken in wooden vats of 2 litre capacity, to which dissolved were 12.25 seers (400 g) of jaggery and boiled for an hour and cooled well.

Dravyas and Dhataki pushpa (*Woodfordia fructicosa*) were then added to the mixture kept in the wooden vats. The vessel was closed with a clean lid followed by wrapping around the lid with seven consecutive layers of clay smeared cloth. The vessel was buried in cellar (basement) for about three months towards the completion of fermentation process (sandhana).

After the stipulated period (90 days), the vessel was withdrawn to examine the preparation which showed a brownish black fluid with a frothing and aromatic odour and a slight alcoholic taste. The final fluid was decanted and filtered through a cotton cloth to obtain a clean transparent asava. Then the asava was bottled and labelled and subjected to some modern methods of standardization[16-18].

Physico-chemical Standardization of asava

Determination of total solids

A shallow, flat bottomed flanged dish, about 75 mm in diameter and about 25 mm deep, made of nickel was used for this analysis. Accurately 5 ml of asava was pipetted out and placed in the dish and evaporated at as low temperature as possible on a water bath until the solvent was removed and the residue is apparently dry. Then the dish was placed in an oven and dried to constant weight at 105°C. After the dish was provided with well-fitting cover, it was cooled in a desiccator[19,20].

Determination of boiling range (Distilling range)

A distillation unit fit with a thermometer was employed to determine the boiling range of the asava. The apparatus consisted of a distilling flask of 200 ml capacity; a condenser of 60 cm long; a receiver of 100 ml capacity which was graduated with 1 ml division; and a thermometer showing 0°C - 240° C.

The thermometer was positioned in the centre of the neck and the entire assembly was shield after dropping about 100 ml of asava to the distilling flask. With the aid of metallic stand and clamps, the entire assembly was placed on an electric heater having a thermostat, so that adjustment in temperature could be done conveniently. Distillation was switched on and the recorded was the temperature of first drop of the distillate. Then the temperature was increased in such a way the receiver could collect 4 – 5 ml per min. The process was

continued until 25% (25 ml) of the distillate reached the receiver and the temperature of the last drop of the distillate to the receiver was also noted.

Necessary correction was employed observing the temperature readings from any variation in the parametric pressure from the normal (101.3 kPa) using following expression.

$$t_1 = t_2 + K\,(a - b)$$

t_1 – corrected temperature; t_2 – the observed temperature; a= 101.3; b – the barometric pressure of the time of the determination; K – the correction factor.

Determination of congealing range or temperature

The congealing temperature is that point at which there exists a mixture of the liquid phase of a substance and a larger proportion of the solid phase. This experimentation required 1 litre beaker in which two test tubes were placed in such a way one was inserted in to another test tube. The inner test tube contained 15 ml of asava and stopperd with a cork attached with a stirrer and a thermometer with 0.2° C graduation. The beaker was filled with water and the test tubes were clamped in such a way they were immersed in water and distance of 18 mm be maintained between the bottoms of the beaker and test tube. The temperature at which a substance solidifies upon cooling is a useful index of purity.

Preparation of reference substance

Since asava is a liquid, the process of determination of congealing point was carried out in the same way of raising temperature, while stirring, about the room temperature using the apparatus for congealing point determination and noted down as a reference value.

Preparation of test substance of asava

The temperature of the bath was maintained near 15° C using addition of ice cubes and placed on a heating mantle which was kept turned off. Then the sample was stirred constantly to a rate of 20 cycles per min with simultaneous observation of rise in temperature with the thermometer. The congealing point was still hidden up to the room temperature. Hence, a slow rise of temperature was aided to the bath using the heating mantle until the congealing point appeared which was comparable to that of the standard. The process was repeated three times and the average was tabulated.

Determination of ethanol

25 ml of asava were accurately measured and mixed with 100 ml of double distilled water and poured in to a separating funnel. The mixture was saturated with sodium chloride and added was 100 ml of hexane, shaken vigorously 2 – 3 min. The mixture was allowed to stand for half an hour. The lower layer was run in to a distillation flask. The hexane layer was washed with 25 ml of concentrated sodium chloride solution in a separating funnel then the NaCl layer was added to the distillation flask. The whole mixture was made alkaline with 1 M sodium hydroxide solution using solid phenolphthalein as indicator. To this added were a little pumice powder and 100 ml of water.

The whole mixture was distilled to obtain 90 ml of distillate. The distillate was poured in to a 100 ml volumetric flask and made the volume to 100 ml with double distilled water. Using this mixture relative density was determined to calculate the percentage v/v alcohol of the asava.

Determination of freezing point of asava

Freezing point is the maximum temperature occurring during the solidification of a super-cooled liquid. The apparatus for its determination was designed as that of the apparatus used in the determination of congealing point of asava.

About 5 ml of asava was placed in the inner test tube, which was immersed in a 500 ml capacited beaker containing water, fitted with a thermometer and a stirrer. The stirring was carried out at a rate of 25 cycles per min with simultaneous reduction in temperature by keep on adding ice cubes. When the temperature of the asava was observed to be 5° C or below, the beaker was filled with saturated NaCl solution to stabilize or maintain temperature. The process was continued until some seed crystals of asava were present. The process was repeated 3 times at least to get the average freezing point of asava.

Loss on Drying

About 10 ml (10.75 g) of the asava under study were accurately pipetted out and transferred to a tarred china dish which was known for its weight and kept in a hot air oven at 100 – 105º C for an hour. Then, the sample was weighed along with china dish to deduct the actual weight of tarred china dish. The weight of the content was noted to calculate the percentage loss on drying with reference to the asava.

Loss on Ignition

A silica crucible was heated for about 30 min to red hot and cooled in a desiccator to note down its weight. About 10 ml of the asava was pipette out and then dried at 100 – 105º C for 1 h and ignited to constant weight in a muffle furnace at 600 - 625º C, until a carbon free ash formed. The crucible was allowed to cool in a desiccator after each ignition and care was taken to avoid catching fire. The weight of the carbon free ash was determined. The procedure was repeated to obtain a standard deviation to ensure consistency and then tabulate.

Determination of pH of asava

To determine the acidity or alkalinity of the asava at room temperature, potentiometric method was employed. The buffer solutions A – H were prepared using carbon

dioxide free water as solvent as given in Indian Pharmacopoeia-1996 (A-95) which helped to detect the pH of asava whose range may be from 1.7 – 10.12.

Determination of Refractive index

The refractive index (n) of a substance with reference to air is the ratio of the sine of angle of incidence to the sine of the angle of refraction of beam of passing from air in to the substance. The refractive index was conveniently measured using the Abbe refractometer at 25º C employing the wavelength of the D line of sodium (λ=589.3 nm), after calibrating the apparatus against distilled water whose nD^{20} at 25º C was 1.3225.

Determination of viscosity of asava

The determination of viscosity of asava was carried out by means of capillary viscometer at room temperature. The viscometer was washed and dried completely. Then the viscometer was filled and examined through L tube to slightly above the mark G using a long pipette to minimise wetting the tube above the mark. The tube was placed vertically in a water bath maintained a temperature of 35º C and allowed to stand for half an hour to reach equilibrium. The volume of asava was adjusted so that the bottom of the meniscus settled at the mark G. The liquid was sucked to the point about 5 mm above the mark E and the pressure was revealed[21].

The time taken was measured for the bottom of the meniscus to fall from the top of mark E to the top edge of mark F. Then, the kinematic viscosity (V) in square mm per sec (mm^2s^{-1}) using the expression

$$V=Kt$$

The constant (K) of the instrument was determined on a liquid of known viscosity (Dextran injection or saline).

Determination of weight per ml of asava

The weight per ml of a liquid is the weight, in g, of 1 ml of the liquid when weighed in air at room temperature. A thoroughly clean and dry Pycnometer was selected and filled with asava and weighed in air at room temperature. The procedure was repeated 3 times and average value of the weight of 1 ml of asava was calculated.

Determination of total free sugar content in asava

The total free sugar content of asava was estimated using Benedict's reagent for quantitative analysis and reported in terms of percentage w/ml as per the reference[22].

Fluorescence analysis of asava

The asava as it is and added with water; methanol and ethyl-acetate were shaken well and kept under a long UV light aided chamber to note down the colour change[23].

Primary organic analysis

About 100 g of the stems (Patha) were powdered in a mechanical grinder, after a screening for the presence of foreign bodies, in to a moderately coarse powder were soxhleted successively with solvents of increasing polarity such as petroleum ether, benzene, chloroform and 75% ethanol (15 – 19 h) and a part of the extracts and the asava were subjected for the determination of and a primary organic analysis.

Primary organic analysis of the both the extracts and the asava were carried out with suitable chemical reagents of research grade which led to a conclusion that the phenolic compounds were well pronounced[24,25].

***In vitro* Anthelmintic screening**

The earthworms of 3-5cm in length and 0.1-0.2cm in width were used for all experimental protocol due to their anatomical and physiological resemblance with the intestinal round worm parasites of human being. The earthworms for the study were authentificated by Dr.P.N.Tripathi, professor, Department of zoology, K.S.Saket P.G.college, Faizabad, Uttar Pradesh, India as *Pheretima posthma.*

The assay was carried out on adult Indian earth worms (*Pheretima posthuma*). About 5 groups of each six worms were subjected for the current study. The group-I and II were released in to double distilled water and piperazine citrate 10 mg/ml in distilled water respectively as the solvent control and the reference.

The group III, IV and V were dropped in to the *Viburnum* stem asava of concentrations 100, 250 and 500 mg equivalent/ml. The total volume of each solution was maintained about 50 ml, so that the worms can be conveniently exposed to the different substances selected.

Observations were made for the time taken to paralysis and death of individual worms. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50°C[26].

RESULT AND DISCUSSION

The results of physical and physic-chemical analysis of *V.coriaceum* bark asava were tabulated and discussed in detail under the section discussion (Table 1). The primary organic analysis on the both ethanolic extract of the crude drug (Patha) as well as the asava itself gave a positive test for carbohydrates (Molisch's test); amino acid (Xanthoproteic test); free sugar (Fehling's' and Benedict's test); tannins (Gold beater's test); general phenolic compounds (dilute ferric chloride test); flavonoid (Shinoda's test and pH dependent colour test by Mg-HCl); saponins (Haemolytic test); general glycosides (by hydrolytic test after exhausting free sugar); phenolic glycoside (by hydrolysis followed by phase separation by non-polar solvent and testing of the same); and the presence of anthocyanins (Blood red

colouration of both alcoholic and aqueous extract) (Table 2). An organoleptic analysis was also carried out on the asava and the results were tabulated (Table 3).

The stem asava itself and the asava added with water, 80% methanol and ethylacetate were observed under UV radiation showing dark brown, yellowish brown, yellowish brown and pale brown colouration respectively.

A primary organic analysis conducted on the asava itself as well as the ethanolic extract of the patha revealed the presence of carbohydrate, amino acid, free sugar, saponins, tannins, phenolic compounds (general), flavonoids, saponins and glycosides (phenolic glycosides). However, presence of phyto-sterols and triterpenes were in the negative.

The asava was brownish black in colour; aromatic in odour; aromatic and sweet in taste; sticky after minutes in texture between fingers; pourable and slightly sticky in nature to view; it showed a darkening after its evaporation, when kept under room temperature; and smelled ethanolic and pleasant while heating on a boiling water bath.

Physico-chemical parameters

The term total solid is applied to the residue obtained where the prescribed amount of the preparation is dried to constant weight. The total solid of the asava were determined to be 48.6±0.2% w/ml. The lower limit of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser and the upper limit is the temperature at which the last drop evaporates from the lowest point in the distillation flask, as far as distilling range of the asava is concerned. In this event, the asava showed 74±0.02° C to 111±0.08° C as its boiling range.

The congealing temperature is that point at which there exists a mixture of the liquid (fused) phase of a substance and increasing proportion of solid phase. The asava, in this case, showed 63±0.08° C to 66±0.04° C as the congealing point. Making no modification in the setting of apparatus the freezing point of the asava was determined to be 9.5±0.06° C.

Since the principle behind the formulation of asava is that conversion of sugar (jaggery) in to ethanol by anaerobic fermentation process, the total alcohol concentration was determined to be 22% v/v at 32° C by distillation cum specific gravity method. Loss on drying is a versatile method of standardization applicable for materials existing in liquid, solid, semisolid state. On the basis of the above principle, loss on drying of the asava was determined to be 32.42±0.60% w/w.

Although loss on ignition is best suiting to standardize formulation such as churna, it cannot be stated that asava may not be standardizable by this method. Because, the principle behind the loss on ignition is to determine the quantity of inorganic elements which could be convertible in to their corresponding oxides, which include both physiological as well as non-physiological ashes.

Hence, the loss on ignition of the asava in percentage w/v as determined to be 3.5±0.33% w/v. To determine the acidity or alkalinity of the asava, pH value was determined to be 4.4 by potentiometric method. Determination of refractive index is one of the best suiting standardizing process for liquid formulation with reference to air; the refractive index of the asava using as Abbe refractometer against water was measured to be 1.422.

By employing an Oswald - type viscometer, viscosity was determined against water to be 1.9797 poise at 32°C. Since asava is a liquid formulation, by using a calibrated Pycnometer, the weight per ml of the asava was determined to be 1.125 g/ml at room temperature. The total free sugar content using Benedict's reagent for quantitative analysis was determined to be 24 g % (Table 3).

Anthelmintic potential

The earthworms introduced to distilled water (group-I, control) were very alive and showed neither signs of paralysis nor any death. The group-II, which was treated with reference anthelmintic drug (piperazine citrate 10 mg/ml, showed a significant paralysis of worms, 20.50±1.02), however, no death was evident.

The group III, IV and V, which were introduced to the solution containing the asava equivalent to 100, 250 and 500 mg/ml respectively, resulted both paralysis (33.81±0.49, 21.54±0.55 and 13.41±0.37) as well as death (92.01±2.33, 61.47±0.94 and 29.11±0.42) significantly. Hence, the paralytic capacity of the asava was comparable to that of the reference subjected, but the same time, the formulation is more powerful and effective than the standard in causing a significant morality of the earth worms $p<0.01$ (Table 4).

The phenolic compounds such As tannins and flavonoids bind with intestinal enzymes of earthworms as well as the glycoprotein part of the cuticular layer of the worms thereby, crippling the protein dependent movements of the body (muscles) of the worms leading to an irreversible paralysis and finally to death.

CONCLUSION

Viburnum Linn. species are medicinally valuable population. Hence, an asava from stems of three species was formulated and analysed for its primary organic and sensual features. Considering the nature of the phyto-constituents of the formulation, anthelmintic activities

was screened for the asava to obtain a markable results that the drug possesses a comparable magnitude of such effect, when compared to that of the reference subjected in the present study. This study can be useful as a referential tool to progress some advanced research on this formulation.

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Table 1. Primary organic analysis of asava against patha

S.No.	Phytoconstituents	asava	75% ethanolic extract of patha
1.	Carbohydrate	+++	+++
2.	Free reducing sugasr	+++	++
3.	Amino acid	++	++
4.	Alkaloid	-	-
5.	Saponins	+++	++
6.	Phyto-sterols	-	-
7.	Triterpenoids	-	-
8.	Tannins	+++	+++
9.	Flavonoids	+++	+++
10.	Glycosides (general)	+++	++
11.	Glycoside (specific) (Phenolic glycosides)	+++	++
12.	Anthocyanins	++	+++

$^{+}$ - Test positive, $^{-}$- Test negative, $^{++, +++}$ -Test well pronounce

Table 2. Organoleptic analysis of asava

S.No.	Parameters/Characters	Results
1.	Colour	Brownish black
2.	Odour	Aromatic and alcoholic
3.	Taste	Aromatic and sweet
4.	Texture	Slightly Sticky after evaporation
5.	Nature	Freely Pourable
6.	Colour change at room temperature	Darkening, when volume reduced
7.	Odour upon heating	Alcoholic and agreeable

Table 3. Standardization of asava by physical and physic-chemical methods

S.No.	Parameters	Report
1.	Total solids	48.6±0.021% w/ml
2.	Boiling range	74±0.02 – 110±0.08° C
3.	Congealing point	63±0.08 – 66±0.04° C
4.	Content of ethanol	22% v/v at 32° C
5.	Freezing point	9.6±0.06° C
6.	Loss on drying	32.42±0.50% w/w
7.	Loss on Ignition	3.5±0.33% w/v
8.	pH	4.4
9.	Refractive Index against water (1.332)	1.422
10.	Viscosity against water (0.9982)	1.9797 poise at 32° C
11.	Weight per ml	1.125 g/ml
12.	Total free sugar content	24 g % w/ml
13.	Fluorescence analysis (Long UV) a. Asava	brown
	b. Asava in water	Yellowish brown
	c. Asava with methanol	Yellowish brown
	d. Asava with ethylacetate	Pale brown

Results are presented as mean±Standard Deviation, n=3

Table 4. Anthelmintic activity of Triple*Viburnum* stem asava

S.No.	Test Substance	Concentration (mg/ml)	Time taken for Paralysis (P) and Death (D) of worms in min	
			P	D
1.	Distilled water (control)	-	-	-
2.	Piperazine citrate (Standard)	10	20.50±1.02	-
3.	*Viburnum* stem asava	100	33.81±0.49	92.01±2.33
4.	*Viburnum* stem asava	250	21.54±0.55	61.47±0.94
5.	*Viburnum* stem asava	500	13.41±0.37	29.11±0.42

Values are presented as mean±SEM, n=6

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