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Formulation of *Viburnum erubescens* root asava and its physico-chemical standardization

K. Ponnudurai[1*], K. Prabhu[1], M. Srinivasa Murthy[2], S. Shobha Rani[3] and V. Anitha Kumari[4]

[1*]*Department of Pharmacology, School of Pharmacy, Vivekananda Group of Institutions, Batasingaram, Ranga Reddy District, Telangana, India*
[1]*Department of Pharmacognosy and Phytochemistry, School of Pharmacy, Vivekananda Group of Institutions, Batasingaram, Ranga Reddy District, Telangana, India*
[2]*Department of Pharmaceutical Chemistry, Vignan Institute of Pharmaceutical Sciences, Deshmukhi, Nalgonda District, Telangana, India*
[3]*Centre for Pharmaceutical Sciences, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, Telangana, India*
[4]*Department of Pharmaceutical Chemistry, Nova College of Pharmacy, Jangareddygudem, West Godavari District, Andhra Pradesh, India*

ABSTRACT

The roots of Viburnum erubescens (Adoxaceae) are collected from Nilgiri hills of Tamil Nadu and authentificated. Following a successive extraction and a primary organic analysis, it has been known to contain phenolic compounds as its principal phyto-constituents in their hydroalcoholic fraction. . 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. This study can help performing quality control of ayurvedic formulations such as asava and arista excluding only some conventional methods of analysis any more.

Keywords: *Viburnum;* Dhataki pushpa; freezing point; Refractive index; free sugar content

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INTRODUCTION

The *viburnum* Linn.belonging to Adoxaceae family is a well known genus to most of the pharmacognosists and other researchers in the world[1.2]. The *Viburnum* species grow in India (Himalaya, Khasi hills, Arunachal Pradesh, Tamil Nadu (Nilgiri and Coimbatore hills), Nepal, South America and Java, Korea, China and Japan; Flavonoids were isolated of from the barks of *Viburnum prunifolium*[3]; β-sitosterol ($C_{29}H_{30}O$), m.p. 136 – 137°C was isolated from *Viburnum prunifolium* and *Viburnum opulus* [4]. Procyanidins such as: B_{1-5} and C_1were isolated from the stem barks of *Viburnum burkwoodii*; Catechin, m.p. 177 ^oC, was isolated from *Viburnum burkwoodii* Leaves; (-) Epicatechin, m.p.240 - 242 ^oC, was isolated from the stem barks of *Viburnum* burkwoodi[5]; Bergenin, a sesquiterpenoid was isolated from roots of *Viburnum nervosum* Hook[6]; Pharmacognostical studies were carried

out on the roots of *Viburnum nervosum* Hook[7]; Amentoflavone and apigenin were isolated from the leaf extracts of *Viburnum coriaceum* in pure form and their structures were elucidated by spectral techniques[8]; Fatty oils extracted from seed of *Viburnum coriaceum* Blume, were tested *in vitro* against five species of bacteria, i.e., *Bacillus pumilus, B.subtilis, Salmonella typhosa, S.typhi, S.paratyphi, Micrococcus pyogens albus* and *Staphylococcus albus* and the investigations revealed that the fatty oils have potent antibacterial activity against *B.subtilis*, *S.typhosa* and *S.paratyphi[9];* Deoiled meal of *Viburnum coriaceum* seeds contained 25.9% protein. Meal contained all essential amino acids except tryptophan. Percentage of leucine, lysine, threonine and valine were higher in *V.coriaceum* than in soybean[10]; Iridoid glycosides were isolated from *Viburnum rhytidophyllum*[11]; Decoction of leaves of *Viburnum foetidum* Wall was used in various uterine disorders[12]; Iridoid glycosides and p-Coumaryl iridoids were isolated from methanolic extract of dried leaves of *Viburnum luzonicum* and their cytotoxic effect were also studied[13]; Change on storage of biological activity of *Viburnum opulus* seed components was studied[14]; Rearranged vibsane type diterpenes was isolated from *Viburnum awabuki* and photochemical reaction of vibsane-B was also carried out[15]; Epicotyl Dormancy in *Viburnum acerifolium* was studied[16]; Iridoid glycosides were isolated from *Viburnum chinshanense*[17]; Antinociceptive and anti-inflammatory activities were studied on *Viburnum lanata*[18]; Antinociceptive and anti-inflammatory activities were studied on *Viburnum opulus*[19].

From the above observation, it may be stated that *Viburnum* Linn species have biologically potential phyto-constituents and their exploitation in scientific studies necessitates either isolation of phyto-constituents or formulation in crude form to explore their biological value.

From the above, before concluding that the asava formulated contains phenolic compounds of therapeutical potential (confirmed by a primary organic analysis on the various solvent extracts of crude drug (Patha) as well as with the asava itself in the current study) and establishing the therapeutical potential of this asava, it is essential to find out how the formulation can be standardized to obtain reproducible parameters for its identity and recognization. Hence, the current study centres mainly on the standardization of the formulated asava using some physical and physic-chemical methods.

MATERIALS AND METHODS

The roots of *Viburnum erubescens* were collected from Nilgiri hills, Tamil Nadu, India and authentificated by Dr.V.Chelladurai, Ex. Professor, (Botany), Medicinal plant survey for Siddha, Government of India as *Viburnum erubescens* Wall.ex DC. Herbarium of the specimens (labelled VE131) was submitted at the museum of the department of Pharmacognosy, Vivekananda Group of Institutions, Hyderabad. The crude drugs were dried in the sun for a couple of week and subjected to research studies. Preparation of *V.erubescens* root asava by anaerobic fermentation method (An Ayurvedic formulation)[20-22]

Approximately 1.244 seers (40 g) of the roots of *V.erubescens* (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.

Standardization of asava

Determination of total solids[23]

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 desiccators.

Determination of boiling range (Distilling range)[23]
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[23]
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[23]
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[23]
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[24]
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[25]
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[25]
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[25]
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[26]
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.

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[26]
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.

Fluorescence analysis of asava[27]
The arista 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.

Primary organic analysis[28,29]
About 100 g of the barks of *V.coriaceum* (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.

Determination of total free sugar content in asava[30]
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.

RESULTS AND DISCUSSION

The results of physical and physic-chemical analysis of *V.erubescens* root 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).

Table 1. Standardization of asava by physical and physic-chemical methods

S.No.	Parameters	Report
1.	Total solids	45.6±0.021% w/ml
2.	Boiling range	73±0.02 – 106±0.08° C
3.	Congealing point	63±0.08 – 65±0.04° C
4.	Content of ethanol	22% v/v at 32° C
5.	Freezing point	8±0.06° C
6.	Loss on drying	21.42±0.50% w/w
7.	Loss on Ignition	2.5±0.33% w/v
8.	pH	4.5
9.	Refractive Index against water (1.332)	1.588
10.	Viscosity against water (0.9982)	1.7772 poise at 32° C
11.	Weight per ml	1.055 g/ml
12.	Total free sugar content	25 g % w/ml
13.	Fluorescence analysis (Long UV) a. Asava	Dark 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

The *V.erubescens* root 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.

Table 2. 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 pronounced*

Table 3. Organoleptic analysis of asava

S.No.	Parameters/Characters	Results
1.	Colour	Dark Brownish
2.	Odour	Aromatic and alcoholic
3.	Taste	Aromatic and sweet
4.	Texture	Sticky after evaporation
5.	Nature	Pourable, slightly sticky
6.	Colour change at room temperature	darkening when volume reduced on evaporation
7.	Odour upon heating	Ethanolic and agreeable

The asava was brownish black in colour; aromatic in odour; aromatic and sweet in taste; sticky after minutes in texture between fingers; pourable and slightlysticky 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.

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 46.6±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 evaporate from the lowest point in the distillation flask, as far as distilling range of the asava is concerned. In this event, the asava showed 72±0.02° C to 106±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 asavta was determined to be 8±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 21% 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 22.42±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 2.9±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 1.499.

By employing an Oswald - type viscometer, viscosity was determined against water to be 1.8772 poise at 32°C. Since asava is a liquid formulation, by using a calibrated Pygnometer, the weight per ml of the asava was determined to be 1.055 g/ml at room temperature. The total free sugar content using Benedict's reagent for quantitative analysis was determined to be 25 g %.

CONCLUSION

The current study of formulation and its standardization of asava by some physical and physic-chemical methods is a new approach and an attempt to add on the number of analytical methods by which an ayurvedic formulation such as asava could be conveniently subjected for its quality control, unlike adopting a few processes such as "determination of alcohol and sugar content" alone. This study will, definitely, be useful to recommend further advanced investigations on the species as well as on this formulation.

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