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PHARMACOGNOSTICAL EVALUATION OF A TRIPLE VIBURNUM CHURNA HAVING ANTI-INFLAMMATORY POTENTIALS

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ABSTRACT

AYURVEDA – one of the noblest and holiest traditional systems of medicine to prevent and heal human ailments. Ayurvedic system of medicine originated from India and spread over several developing and developed countries. Although an artificialization in the modern system of therapy is currently very dominant, a very usage and exploitation of traditional systems of medicine are unavoidable in health practice. The modern systems of drug analysis, with or without the aid of sophisticated in-puts, is capable of formulate and standardize all existing system of medicine adopting modern scientific principles.

Based on this fact it has been decided to prepare a *Triple Viburnum* churna containing the stems of three *Viburnum* Linn. species and then to standardize the same by pharmacognostical and biological means to supplement some scientific informations which can be correlated with existing data base of the crude drugs.

So, the current study undertaken is to cast some scientific information on formulation based pharmacological screening of a crude drug for its anti-inflammatory activity, using a combined churna prepared from the stems of three species – *Viburnum punctatum* (VP), *Viburnum coriaceum* (VC) and *Viburnum erubescens* (VE) – in suitable animal models. Firstly, the churna was evaluated pharmacognostically to obtain reliable and reproducible parameters. Then, the biological screening was progressed with determination of LD_{50} value to ensure a safety of the animals used in experimentation.

In carrageenan induced paw oedema (in rats), (a mixed churna of stem of VP, VC and VE of 500 mg/ kg b.w) showed a significant reduction in paw oedema volume ($p<0.001$) and was comparable to that of the standard (Indomethacin 10mg/kg b.w).

In cotton pellet induced granulation method in wistar rats, a significant reduction in dry weight and wet weight of cotton pellets was observed with the churna treated animals ($p<0.001$) which was comparable to that of the standard (Indomethacin 20mg/kg b.w).

KEY WORDS: *Viburnum*, Churna, Jaggery, Micrometrics, Phenolic compounds, Anti-inflammatory

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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 cannot 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 irridoid glycosides in their stems, roots 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 lanata*[11] 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 stem 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. About 500 g of stems of each VP, VC and VE were powdered separately using a mechanical grinder in to a moderately coarse powder. The powdered specimens were extracted, in a soxhlet apparatus for about 15 – 18 h successively with petroleum ether (60 – 80°C), chloroform and 75% aqueous ethanol. The yield was about-5.2, 6.3 and 15.5 g in case of *V.punctatum*; 5.55, 6.55 and 21.22 g for *V.coriaceum;* and in case of *V.erubescens* 6.10, 6.25 and 19.20 g. All the extracts were phytochemically screened for the presence of phyto-constituents before formulating a churna using their powder.

Prepapration of Triple *Viburnum* churna

The authenticated samples were, carefully examined with the aid of a dissection microscope for the presence of foreign matter, which yielded not more than 0.252% of contaminants.

About 50 g each of stems of VP, VC and VC were dried in the sun for 15 days and powdered using a mortar and pestle after mechanically ground in to coarse powder. The powders (1:1:1 ratio) were passed through muslin cloth. And added were 150 g of Jaggery (1:1 ratio) and triturated well and stored in an air tight container to prevent the entry of moisture and other contaminants[16].

Microscopic examination

With the aid of a compound microscope (Focus (ISI), JPM-1, India) and an eye-piece micrometer which was calibrated with a stage micrometer, microscopic components of churna were randomly studied under both

low- (10x × 10x) and high-power (10x × 45x) magnification.

Powder microscopy

The churna was screened through sieves with aperture size of 180 µm and 125 µm separately to obtain fine and very fine powders, respectively, and then subjected to microscopic examination. The specimens were treated with the following reagents in order to evaluate components of diagnostic value: 50 % glycerin as temporary mountant; 2 % phloroglucinol in a mixture of 90 % ethanol and conc. HCl (1:1) for lignin; 5 % alcoholic ferric chloride for phenolic compounds; 2 % iodine solution for starch grains; and 0.08 % ruthenium red in 10 % lead acetate for mucilage[17]. These reagents helped locating distribution of ergastic cell contents.

Photomicrography

Photomicrographs were taken in addition to microscopic examination, whereever necessary, at different magnifications using a Nikon Labphot 2 microscope. For normal observations, a bright field was used while for the study of calcium oxalate crystals, starch grains and lignified cells, polarized light was employed. Since these structures have bi-refringent properties under polarized light, they appear bright against dark background. Magnifications of the figures were indicated by scale-bars[18,19] or degree of magnification.

Numerical and physical standards

Determination of extractives

Approximately 5 g of the churna was macerated with 100 ml of the solvents for 24 h. After filtration, 25 ml of the filtrate was evaporated to dryness and the percentage of extractives was calculated with reference to the air-dried drug.

Determination of ash values

Approximately 3 g of the churna was incinerated in a tarred silica crucible (Vitrosil, India) in a furnace at a temperature not exceeding 450 °C until free from carbon. It was washed in hot water to exhaust the charred mass, and the residue was incinerated on an ash less filter paper and weighed. Further treatment was carried out to derive water soluble, acid insoluble and sulphated ash values[20].

Microchemical studies

Histochemical analysis was carried out on the specimens using, separately, dilute iodine solution, Lacto-phenol, Dragendorff's reagent, dilute ferric chloride solution and phloroglucinol-HCL (1:1). The reagent treated hand sections as well as powder of the plant tissue were observed under microscope to detect the presence of histo-chemical components[21].

Fluorescence studies

Fluorescence analysis[22] was carried out in an ultraviolet cabinet (MAC, MSW-508, Long UV, India) at 365 nm.

Micrometric studies

Micrometric evaluation was carried out with the aid of a compound microscope fitted with a camera lucida (Swift Ive's) as described elsewhere[23,24].

Preliminary screening for foreign matter

The shade-dried stems were ground to a moderately coarse powder in a mechanical grinder. Approximately 100 g of the powder was extracted successively with petroleum ether (60 – 80 ºC), benzene, chloroform and ethanol (95 %) using a Soxhlet apparatus. Each solvent extraction was carried out for 24 h. The extracts were taken in a tarred porcelain dishes, evaporated to dryness over a water bath and dried in an oven at 105 ºC to a constant weight. The extractives (%) were calculated with reference to the air-dried drug[25].

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 various studies were carried out using analysis of variance (ANOVA) followed by Dunnett's 't' test and standard deviation, $p<0.01$ was accounted significant.

The acute oral toxicity[26] 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 b.w p.o of the churna 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 ethanolic extract of the churna 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 selected as therapeutic dose of the experiment.

Evaluation of anti-inflammatory activity by carrageenan induced paw oedema model

Wistar rats of either sex weighing 180 to 250 g were divided into three groups of six animals each. Group I - Received 1% SCMC 10 ml/kg b.w (p.o), Group II- Received Churna 500 mg/kg b.w (p.o) suspended in 1% SCMC and Group III-Received Indomethacin 10 mg/kg b.w (p.o) suspended in 1% SCMC.

The paw oedema was induced by injection of 0.1ml of 1% carrageenan in 0.9% saline into sub-plantar region of the left hind paw of the rats. The churna treated, standard (Indomethacin 10 mg/kg) and control (1% SCMC) were administered 60 minutes before carrageenan injection. The volume of injected paw was measured at 1st, 2nd, 3rd, 4th and 5th hour after the carrageenan injection, using a plethysmometer and the oedema was expressed by changes in paw volumes[27].

Cotton pellet induced granuloma formation in rats

Wistar rats of either sex weighing 180 – 250 g were divided into three groups of six animals each. Group I - Received 1% SCMC 10 ml/kg b.w.(p.o), Group II- Received Churna 500 mg/kg b.w (p.o) suspended in 1% SCMC and Group III-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 h. 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 h and their dry weight determined[28,29]. The inhibition of granuloma formation was determined by comparing with the control.

RESULT AND DISCUSSION

Organoleptic features: The churna was yellowish brown to brown in colour (Table 1) with woody characteristic odour, strongly astringent and slightly bitter in taste; rough and fibrous and non-mucilagenenous with water, but treatment with dilute ferric chloride solution and a mixture of phloroglucinol-HCl (1:1) turned the churna blackish and pinkish respectively.

The churna (**Table 1**) under microscope showed the following components very frequently: xylem vessels with a dimension of 50 – 85 µm in diameter and most of them represented annular thickening. The starch grains were abundant, circular, oval and semicircular single and rarely compound; they were concentric with eccentric hilum, ranging from 6 – 15 µm in size.

The sclereids were extremely thickened by lignifications leaving a narrow lumen at the centre. Very rarely seen were the elongated sclereids. The brachio-sclereids measured from 25 – 60 µm in diameter. The fibres were libriform in nature appeared with either thick walled with a thin lumen or thin walled with thick lumen. They were as long as 1 mm in length. Druses are abundant in the churna which were up to 25 µm in diameter (**Figure 2**).

By employing lycopodium spore method the number of starch gains and sclereids were enumerated per mg of churna to be 850±25 and 580±55. The percentage extractability of water and ethanol 95% v/v was determined to be 5.52±0.067 and 7.78±0.48; while crude fibre content and a loss on drying showed 8.25±0.05 and 4.85±0.035 respectively.

The percentage total ash value was determined about 7.88, being 11.52, the sulphated ash value.

A notable colour change was observed under UV for alcoholic extract, treatment of powder with an acid and base, which resulted formation of reddish brown and reddish green colour respectively under long UV (**Table 2**).

In addition to observation of 80% methanolic extract under UV showing 285 nm and 235 nm as peak maxima and sub maxima, an organic analysis was carried out on the successive solvent extracts of the churna employing suitable qualitative chemical reagents. It revealed the presence of triterpenes and phytosterols in non-polar fractions and the presence of glycosides, saponins, free sugars flavonoids and amino acids with alcoholic and aqueous fractions. A spectroscopic analysis (Folin Ciocalteau, UV 650 nm) revealed the churna to possess 12.70±0.45 mg/g of total phenolic content.

The churna itself, when qualitative chemical analysis was carried with suitable chemical reagents, it gave a positive test for phenolic compounds, carbohydrates, proteins, saponins and glycosides (General). However, a chemo-microscopy of the churna was carried out under microscope revealing the presence of tanniniferous content in parenchyma of phloem; suberin in periderm cells; starch grains in medullary parenchyma; druses in cortical parenchyma and ray cells; and lignin in vascular tissue such as vessels and xylenm fibres.

The churna of *Viburnum* was subjected to preliminary phytochemical and thin layer chromatographic screenings to explore the nature of phenolics. All extracts gave a positive test for diverse classes of phenolic compounds such as tannins (Gold beater's test), chlorogenic acid (ammonia vapour treatment with ethyl

acetate fraction of ethanol), flavonoids (Shinoda test), phenolic glycosides (test for reducing sugar and phenolic compounds upon hydrolysis, after exhausting free sugar) and condensed tannins (paper chromatography).

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 calculated for *in vivo* studies. The maximum effect of oedema inhibition (**Table 3**) was obtained after 3rd and 5th hour of treatment at dose, the churna of 500mg/kg (37.5% and 67.56% respectively) while standard showed 40.62 % and 70.27% ($p<0.001$). Both wet and dry cotton pellets weight showed a marked reduction for the churna treated group which is significant and comparable to the standard $p<0.001$ (57.31% inhibition in case of churna treated while the standard showing 68.29%) (**Table 4**)

Probable mechanism of anti-inflammatory potentials of *Viburnum* churna

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 living being.

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 indefinite number of components, which on administration in a living system may target a wide range of receptors or their relevant factors at a single time point, or every single component of the extract may target not more than one receptor type at a single time point, which leads to an increase in magnitude of the drug activity. Considering this phenomenon in to account, a probable mechanisms of action of churna is possibly unfolded in this study which deserves a mention and can be useful to progress advanced pharmacological studies.

The mechanism of biological activity of extracts *in vivo* may be probably through one or all of the following ways: Phospholipid metabolism is catalysed by enzymes such as phospholipase A_2 (PLA_2), cyclooxygenase ($COX_{1,\ 2\ and\ 3}$), lipooxygenase (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), thromboxanes (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, flavonones of flavonoids classes, both in free and glycoside form, possess a potent anti-inflammatory activity by targeting COX, LO and AT by forming phenol-proteins complex leading to blockade of their action thereby preventing conversion of metabolites from their precursors to form the inflammatory mediators. The enzyme deactivation may be either reversible or irreversible is a matter of speculation, which can be proven only over some advanced studies involving the receptors.

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

CONCLUSION

From the above observation it has been revealed that the Triple *Viburnum* of stems of all the three species exhibited 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 present study will, surely, be useful to begin with some advanced pharmacological experimentation on these species to address their pharmacological values. The pharmacognostical including preliminary organic analysis may provide useful information to re-evaluate the churna as a referential tool.

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Table 1: Physico-chemical and micrometric parameters of Triple *Viburnum* churna

S.No.	Components/ Parameters	Report in µm/ Number		
		min	mean	max
1.	Dimension of starch grains	d 6	10.5	15
2.	Dimension of druses	d 10	18	25
3.	Fibre dimension	l 210	670	1300
		w 10	20	25
4.	Xylem vessel dimension	d 50	60	85
5.	Sclereids	d 25	40	60
6.	Starch grains/ mg of churna	850±25*		
7.	Sclereids/ mg of churna	580±55*		
8.	Crude fibre content	8.25±0.05**		
9.	Percentage aqueous extractive	5.52±0.67**		
10.	Percentage ethanolic extractive	7.78±0.48**		
11.	Loss on drying	4.85±0.035**		
12.	Total ash value	7.88±0.085**		
13.	Water soluble ash value	5.52±0.045**		
14.	Acid insoluble ash value	3.82±0.035**		
15.	Sulphated ash value	11.52±0.075**		

Values are presented as mean±S.D, n=3, d – diameter, l – length, w – width, * – number, ** –percentage w/w

Table 2: Fluorescence analysis and primary organic analysis of *Viburnum* churna

S.No.	Solvent extract under long UV/Constituents	Colour change/ test (+/-)
1.	Churna itself	Reddish brown
2.	Petroleum ether (60 – 80 °C)	Orange
3.	Benzene	Reddish brown
4.	Chloroform	Yellowish green
5.	Ethanol 95%	Red colour
6.	Methanol 80%	Reddish green
7.	Aqueous extract	Yellowish green
8.	Treatment with 1N NaOH	Reddish brown
9.	Treatment with 1N HCl	Reddish green
10.	λ_{max} under UV (80% methanolic)	285 nm, 235 nm
11.	Phyto-contituents	
	a. Carbohydrate	(+) in aqueous and alcoholic fraction
	b. Amino acid	(+) in aqueous and alcoholic fraction
	c. Phenolic compounds	(+) in aqueous and alcoholic fraction
	d. Triterpenes	(+) in pet.ether and benzene fraction
	e. Sterols	(+) in pet.ether and chloroform fraction
	f. Alkaloids	(-) in pet.ether and chloroform fraction
	g. Glycosides	(+) in aqueous and alcoholic fraction
	h. Volatile oils	(-) in aqueous and alcoholic fraction
	i. Mucilage	(-) in aqueous and alcoholic fraction
	j. Flavonoids	(+) in aqueous and alcoholic fraction
	k. Lignin	(+) in vascular element in section
	l. Total phenolic content (by spectrophotomety)	12.7±0.45 mg/g

Values are presented as mean±S.D, n=3

Table 3: Anti-inflammatory activity of *Viburnum* churna on carrageenan induced paw oedema

Treatment Groups	1st hour	2nd hour	3rd hour	4th hour	5th hour
Control	0.17±0.010	0.26±0.035^{NS}	0.32±0.041^{NS}	0.34±0.068^{NS}	0.37±0.081^{NS}
Triple *Viburnum* Churna	0.18±0.014 (5.88)	0.24±0.017* (7.69)	0.20±0.011** (37.5)	0.18±0.016** (47.05)	0.12±0.009** (67.56)
Indomethacin 10 mg/kg	0.17±0.014 (0)	0.22±0.001* (15.38)	0.19±0.010** (40.62)	0.15±0.014** (55.88)	0.11±0.012** (70.27)

Values are presented as mean ± SEM from 6 animals in each group and values in parenthesis percentage inhibition. Symbols represent statistical significance: *– $p<0.01$, **– $p<0.001$, ^{NS} – non significant, Unit: ml

Table 4: Effect of *Viburnum* churna and Indomethacin on cotton pellet-induced granuloma formation in rats

Group	Treatment	Wet weight (mg)	Dry weight (mg)	% Inhibition
I	1% CMC	190.62 ± 10.57	82. 15 ± 6.35	-
II	Triple *Viburnum* Churna 500 mg/kg	163.07 ± 6.01*	47.20 ± 5.24*	57.31 %
III	Indomethacin 20 mg/kg	148. 24 ± 4.05*	56.61 ± 3.04*	68.29 %

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

Figure 1: Churna of *Viburnum* Linn. species (separately photographed)

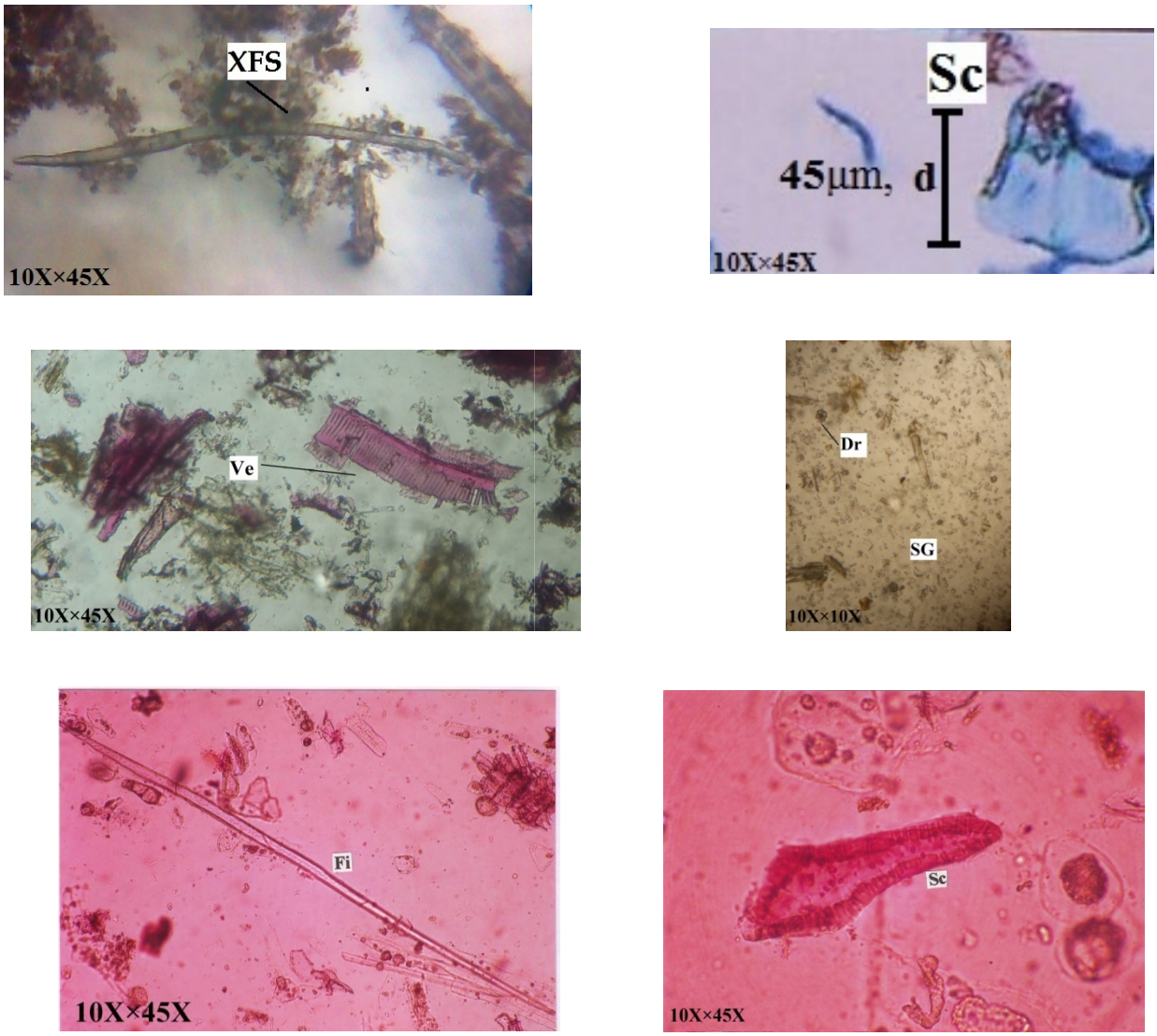

Figure 2: Some Powder Characteristics of *Viburnum* Stem churna
Dr - Druses; Fi - Fibres; Sc – Sclereid; SG – Starch Grains; XP – Xylem. Ve – Vessels annular thickening (magnified); XFS – Xylem fibre.

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