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Standardization of Preparation of Snuhi Kshara and the Evaluation of Biocompatibility

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ABSTRACT:

Snuhi kshara has been a long-established component in many of the Ayurvedic drugs. However, owing to its vigour nature, a strong validation of its pharmaceutical potency is needed. Authentication of the preparation of Snuhi kshara and the evaluation of its biocompatibility is very vital. The present study focuses on the standardization of Snuhi kshara and evaluation of its biocompatibility. Snuhi kshara was prepared following Ayurveda Sara Samgraha with appropriate modifications. Organoleptic, physicochemical parameters were recorded and biocompatibility was evaluated. It was observed that the Snuhi kshara prepared was containing phenolic compounds and exhibiting viability above 97%. The acute toxicity studies revealed that the test drug is very safe up to 2000 mg/Kg of body weight.

Keywords: Ayurvedic drugs, Pharmaceutical, Physicochemical, Snuhi kshara, Toxicity

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1. Introduction

Snuhi is a recognized medicinal plant in Ayurveda and is botanically identified as *Euphorbia neriifolia Linn*. It belongs to the Euphorbiaceae family. *Euphorbia neriifolia Linn.* (Indian Spurge tree, Hedge Euphorbia commonly known as *Snuhi*) is one of the different species of Euphorbia genus plants. *Snuhi* is a large branched, erect, glabrous, xerophytic shrub1.8-4.5 m, with the pairs of stipular spines on tubercles or swellings of the branchlets. It occurswild on rocky ground throughout Central India [(1)]. *Snuhi* is used for numerous therapeutic applications. Mainly, the milky white latex of *Snuhi* is used as a purgative in the treatment of fistula[(2)]. Its proven further pharmacological actions include anti-bacterial activity, anti-psychotic activity, anti-diabetic activity, anti-hyperlipidemic activity, hepatoprotective activity, anti-inflammatory activity, immunomodulatory effect and wound healing effect [(3)].
A study by Sharma et al., investigated the anti-carcinogenic potential of hydro-ethanolicextract along with an isolated flavonoid of *Euphorbia neriifolia* leaves against N-Nitrosodiethylamine (DENA)-induced renal carcinogenesis in mice and the study has shown significant anticancer potential of the leaves and the isolated flavonoid [(4)]. The hydro alcoholic extracts *Euphorbia neriifolia* leaves also validated to have anti-inflammatory and analgesic effects due to the presence of flavonoids [(5)]. In addition, in the leaf extract retains wound healing activity along with humoral and cell mediated immunostaining activity [(6)]. Antidiabetic and antihyperlipidemic activity of ethanolic extracts of leaves of *Euphorbia neriifolia* is also well established [(7)]. The rest of the parts of the plants like, bark, root, latex, etc., are also reported to possess therapeutic applications [(8)]. The bark has been used as an effective purgative and the root is having antiseptic, antispasmodic, purgative and rubefacient properties. The latex is proved to have oral efficacy and safety on adjuvant arthritis, warts, and earache [(9)]. Haleshappa et al., reporter for active phytochemicals and antioxidant potential of ethanolic extracts of *Euphorbia milii* thorns [(10)].
Snuhi kshara, prepared by processing the ash of *Snuhi Panchang,* is also studied by researchers for exploring its pharmacological properties. Euphol is the chemical compound present in *Euphorbia neriifolia* [(11)]. *Snuhi kshara* is reported to be useful for the treatment of sthoulya (obesity). The *kshara* helps in removing vitiated *Kapha, Medas* and *Kleda* from the body and their by helping to decrease the weight [(12)]. Recent studies have identified compounds present in the latex of the plant, including a range of bi- and tri-terpenoids that exhibit various bioactivities. The anti-carcinogenic activity of plants of euphorbia species is believed to be due to the presence of several terpenes, anthocyanins, alcohols and steroids in them. Phytochemical studies lead to the isolation of triterpenes like nerifolione, cycloartenol, euphol, euphorbol, nerifoliene, taraxerol, β-amyrin, glut-5(10)-en-1-one and glut-5-en-3β-ol from latex, bark, root, whole plant and leaf. Anthocyanins like delphin and tulipanin, and diterpene like antiquorin, 12-deoxy-4β-phorbol-13-dodecanoate-20-acetate and ingenol triacetate was isolated from bark and root [(13)]. As demonstrated by Sharma et al., hydro-ethanolic extract of *Euphorbia neriifolia* (EN) leaves and an isolated flavonoid have the anti-carcinogenic potential against N-Nitrosodiethylamine (DENA)-induced renal carcinogenesis in mice [(4)]. But the anticancer potential of *Snuhi kshara* is yet to be explored. The present study is aimed at the standardization of water soluble *paneeyakshara* made from the burnt whole plant of *Euphorbia neriifolia*. The standardization of drug is a critical process to ensureits biocompatibility, efficacy, and quality.

2. Materials and Methods

2.1 Sample Collection

The *Snuhi Panchanga (Euphorbia neriifolia)* was procured from the PG Department of

Dravyaguna Vigyan, Mannam Ayurveda Co-operative Medical College, Pandalam, Pathanamthitta, Kerala. The plant was collected on 03.10. 2021 and dried for 55 days starting from 27.11.2021. The plant was botanically authenticated by a qualified botanist from Tropical Botanical Garden Research Institute, Thiruvananthapuram. The samples collected were not having fruits and flowers as it was in the month of October.

Fig. (1): *Euphorbia neriifolia* morphology and its fruits and flowers collected for the studies

2.2 Preparation of Snuhi Kshara

Snuhi Kshara has been prepared by following method mentioned in the text *Ayurveda Sara Samgraha* with modification in soaking duration at the Postgraduate Department of *Dravyaguna Vigyan*, Mannam Ayurveda Co-operative Medical College, Pandalam, Pathanamthitta, Kerala.

2.3 Preparation of Ash

The plant was cut into small parts and dried in sunlight. Dried plant was subjected to closed-burning (*anthardhuma*) in a big iron pan till it was converted into total ash. After self-cooling, greyish white ash was collected.

2.4 Preparation of Ksharajala

The prepared ash and R.O. (Reverse osmosis) water was taken in cylindrical S.S. vessel with ratio of 1:8 (v/v). The mixture was macerated thoroughly with hands and kept undisturbed for overnight soaking. The next morning, clear supernatant liquid was decanted carefully and filtered seven times through four folded cotton cloth to ensure that all dissolvable alkaline particles were fully extracted. The supernatant particles of charcoal and the solid residue were eliminated.

2.5 Preparation of Kshara

The obtained filtrate (*Ksharajala*) was subjected to heat at 98 ℃ to evaporate the watercontent. After complete evaporation of water portion, the obtained *Kshara* was collected from the

inner surface of S.S. vessel by scraping and stored in an air tight glass container. Care should be taken that the *kshara* obtained was not contaminated or exposed to moisture, as it ismore liable to liquidate. The presence of alkaline salts makes *kshara* more hygroscopic.

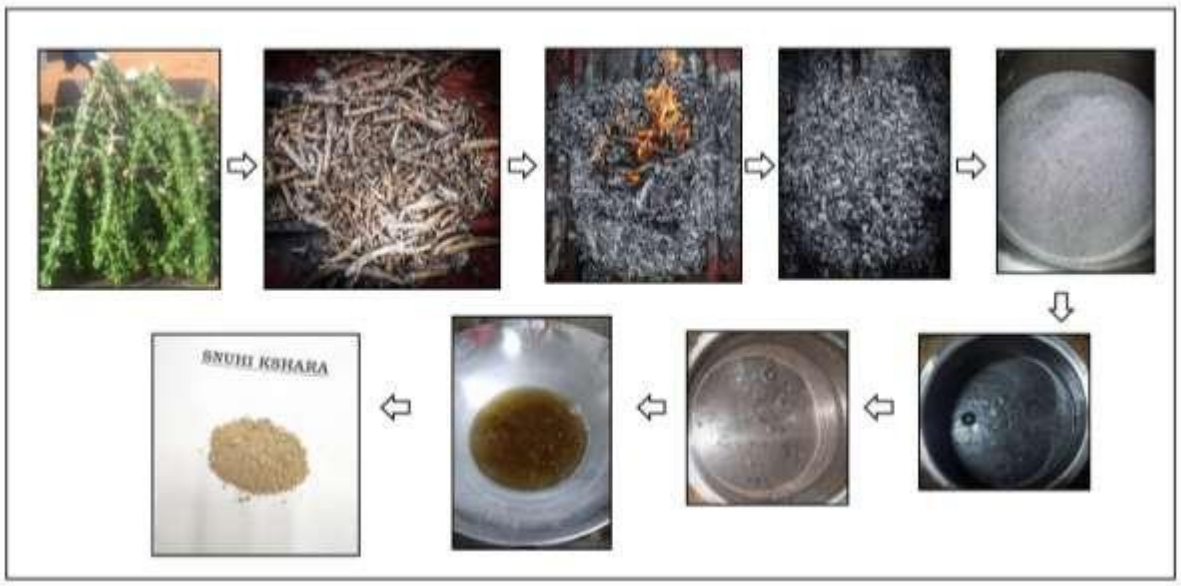

Fig. (2): Preparation of Snuhi Kshara

2.6 Phytochemical Analysis of Kshara
2.6.1 Qualitative Analysis of Phytochemicals
2.6.1.1 Phenolic Compounds
2 ml of the sample was taken in a test tube and added 1% lead acetate solution. Formation of white precipitate will indicate the presence of phenolic compounds.

Tannins: To 2 ml of the sample, added few drops of 0.1% ferric chloride solution. Formation ofbrownish green will indicate the presence of tannins.

Flavonoids: Two ml of the sample was treated with 2 ml of 10% Lead acetate solution. Appearanceof yellowish green colour will indicate the presence of flavonoid.

Saponins: To about 1 ml of the sample was added 2 ml of distilled water in a test tube and shaken vigorously with few drops of olive oil. Foam which persisted will be taken as anevidence for the presence of saponins.

Terpenoids: 2 ml of the samples was mixed with 2 ml of chloroform. Then allowed to evaporate andadded 2 ml of concentrated sulphuric acid and heated for 2 min. Greyish colour will indicate the presence of terpenoids.

2.7 Qualitative Estimation of Ph of Snuhi Kshara
2.7.1 Estimation of Ph of the Powder of *Snuhi Kshara* Using Litmus Paper: Dissolved 0.2 g powder of *Snuhi kshara (Euphorbia neriifolia* L) in water and diluted with 20 ml of distilled water. The test solution was dropped on the strips of red and blue litmus paper. Noted the color change of litmus paper.
2.7.2 Estimation of Ph Using a Ph Meter: Immersed the electrode of the pH meter into distilled water and set the reading shown on the display as 7 by adjusting the calibration knob. Washedthe bulb and wiped it out with soft tissue paper. Then immersed the electrode into the

solutionof *Snuhi kshara (Euphorbia neriifolia* L) and waited for the reading to become stable and notedthe reading.

2.8 Column-Chromatography

A suitable size long cylindrical glass column should stand firm on a column- chromatography stand. Completely dried extract mixed with silica gel to make a fine powdered form for easy distribution of sample in already packed silica gel column. Sample powdered mass should be placed on the top of the pre-packed silica column and sample should be coveredwith a layer of cotton. Then solvents of different polarities were passed through column at uniform rate under gravity to fractionate the sample extract. Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layerchromatography. Thin layer chromatography (TLC) will provide partial separation of both organic and inorganic materials using thin-layered chromatographic plates especially useful forchecking the purity of fractions.

Table (1): Gradient solvent system to be used in the column chromatography

Solvent system	**Ratio**	**Volume (ml)**
Hexane	100%	50
Hexane : Ethyl Acetate	1:1	50
Ethyl Acetate	100%	50
Ethyl Acetate : Methanol	1:1	50
Methanol	100%	50

Rf = Distance travelled by the sample (cm)/Distance travelled by the solvent (cm)

The fractions were collected and TLC was done.

2.9 Thin Layer Chromatography

The chromatographic developments were carried out in a rectangular chamber. TLC- plates made of Merck silica gel 60 were used in all experiments. The percentage composition of the solvent system was Toluene: Ethyl acetate: formic acid (4.5:4.5:1). Each sample was spotted 5 mm from one end with the standard organic acid solutions. Spray plate with 1% ferric chloride indicator reagent, then heat (hottest setting of a conventional hair dryer, or incubate at 100 °C) for colour development. The actual amount spotted varied with each individual solution. The spotted TLC plate was then placed in the bottom of the chromatographic chamber to ensure a sufficient supply of solvent vapour and the chamber was closed. The development of the chromatogram was allowed to proceed until the solvent had travelled 6–7 cm beyond the starting line 20 min at room temperature. The TLC plates were then removed from the chamber and allowed to dry in air. The dried TLC plates were sprayed with the indicator solution, and the colour was developed by brief heating 1–3 min in a hot dry oven. Gallic acid was used as standard.

The Phytoconstituents Were Further Analysed by FTIR And NMR Spectroscopy.

2.10 Evaluation of Biocompatibility of Crude Drug Extracts using L6 cells

L6 cells (2500 cells/well) were seeded on 96 well plates and allowed to acclimatize to the culture conditions such as 37 ˚C and 5% CO_2 environment in the incubator for 24 h. The test samples were prepared in DMEM media (100 mg/mL) and filter sterilized using 0.2 µm Millipore syringe filter. The samples were further diluted in DMEM media and added to the wells containing cultured cells at final concentrations of 6.25, 12.5, 25, 50, 100 µg/mL respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken in order to minimize errors. After treatment with the test samples the plates were further incubated for 24 h. After incubation period, the media from the wells were aspirated and discarded. 100 µL of 0.5 mg/mL MTT solution in PBS was added to the wells. The plates were further incubated for 2 h for the development of formazan crystals. The supernatant was removed and 100 µL DMSO (100%) were added per well.

The absorbance at 570 nm was measured with micro plate reader. Two wells per plate without cells served as blank. All experiments were done in triplicates.

2.11 Acute Toxicity Study in Experimental Animals

Healthy Wistar Albino female rats, weighing about 150-200 gm were procured from animal house. The entire study was approved by the Institutional Animal Ethical Committee (IAEC) which is certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals [14, 15].

The animals were kept in clean and dry polycarbonate cages and maintained in a well-ventilated animal house with 12 hrs. light – 12 hrs. dark cycle. The animals were fed for 7 days with standard pellet diet and water was given ad libitum. For experimental purpose the animals were kept fasting overnight but allowed free access to water. Following the period of fasting, the animals were treated with the test drug at the dose of 2000 mg/kg body weight, orally. As most of the test drug possess LD_{50} value more than 2000 mg/kg body weight and this was used as starting dose. After oral administration, the rats were observed on hourly basis for 24 hours to access mortality and to detect any changes in the autonomic or behavioural responses viz. alertness, spontaneous activity, salvation, respiration, urination, aggressiveness, irritability, convulsion and corneal reflex etc. The rats were observed regularly for 14 days to note the mortality or toxic symptoms. Since there was no death as per the guidelines, the study was repeated with the same dose to confirm the results [15-17].

3. Results and Discussion

3.1 Macroscopic Evaluation of Snuhi Panchang (*Euphorbia neriifolia*)

E. neriifolia is a large glabrous erect much branched succulent shrub or small tree 2-6 feet high. *Snuhi* stem was cylindrical, succulent, and glabrous. The internodes were located at a distance of 4-10 cm and the diameter was about 2-6 cm. A pair of strong stipular spine of length 5-8 mm was located on tubercles with a small circular gland at the base. The branches were more or less obtusely 5-gonous in section and more or less confluent in vertical or slightly spirally reticulated bark. Leaves were short living, fleshy, alternate, glabrous, deciduous, nearly sessile, obovate, oblong 6 to 12 cm in length and tapered at the base. In fresh condition the colour was dark green and on indentation white latex oozes out. The odour was disagreeable and possessed astringent taste.

Upon drying the stem became tough, shrivelled, longitudinally ridged, furrowed and wrinkled. The stem became brown in colour and easily breakable at the nodes exposing hollow pith attached with white parenchymatous papery scales. Odour and taste were same as that of

the fresh stem.

3.2 Microscopic evaluation of Snuhi Panchang (*Euphorbia neriifolia*)

The transverse section (TS) of the external and internal portion of the stem of *Snuhi* isshown below. The TS of the external portion shows a layer of epidermis shielded externally with a well-developed layer of cuticle. The hypodermis lying beneath the epidermis represents a wider zone comprising of vertically placed chlorenchymatous and parenchymatous cells. Intermittently, elongated cavities are formed throughout the parenchymatous band. The innermost region of cortex region is parenchymatous, and embedded with many starch granules. The pith is also very wide. The stelar region displays a narrow ring of angular xylem in continuation with phloem and a distinct cambium.

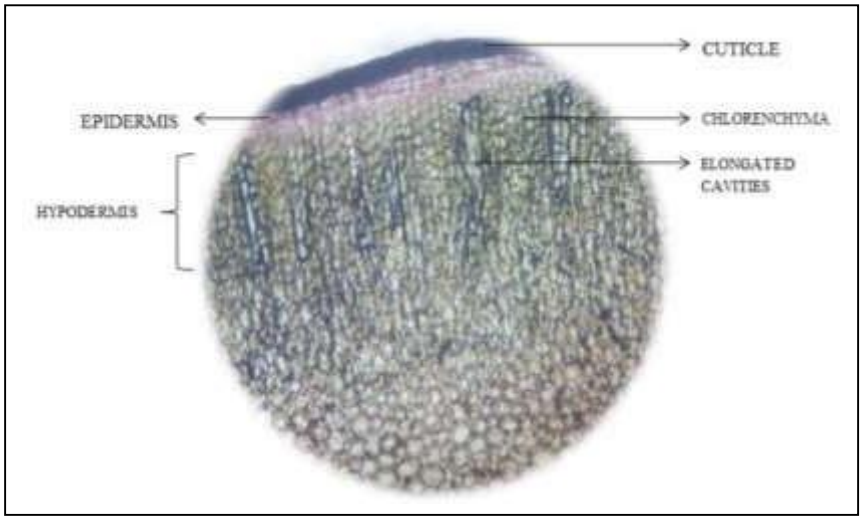

Fig. (3): Transverse section of the external portion of the stem of *Euphorbia neriifolia*

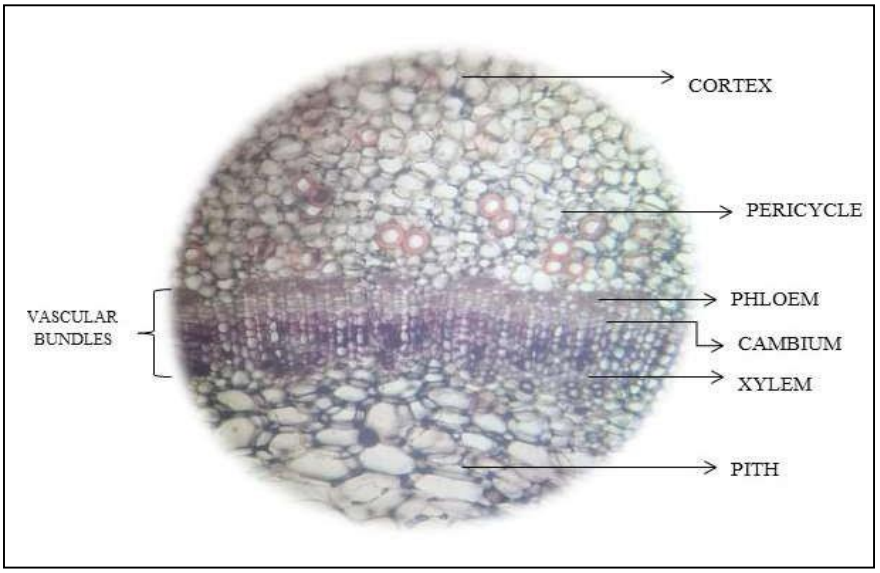

Fig. (4): Transverse section of the internal portion of the stem of *Euphorbia neriifolia*

Table (2): Details of Ash of Dry Snuhi Panchanga

Parameters	**Results**
Weight of fresh *Snuhi Panchanga* (Kg)	10
Weight of dry *Snuhi Panchanga* (Kg)	2.9
Weight loss of *Snuhi Panchanga* after drying(Kg)	7.1
% Loss of *Snuhi Panchanga* after drying	71
Weight of ash obtained (g)	228
% of Ash obtained (Dry *Snuhi Panchanga*)	7.86

3.3 Preparation of Kshara

The results tabulated in the table 2 show that out of 10 Kg fresh *Snuhi Panchanga* collected, 228 g of ash was obtained. The yield % was about 7.86%. About 71% of weight loss was observed after drying. The ash collected was dissolved in six times by weight of water and the soluble portion alone was separated. The process was repeated using the same ash three times to assure that all dissolvable alkaline particles were fully extracted. The supernatant particles of charcoal and the solid residue were eliminated. The fluid thus obtained is called *Ksharajala*.

Table (3): Details of Ksharajala

Parameters	**Results**
Weight of Ash taken (g)	228
Volume of Ash taken (L)	0.75
Volume of R.O. water taken (L)	6
Ksharajala obtained after filtration (L)	5.2
% of *Ksharajala* obtained in v/v	86.66

About 228 g of ash was taken and mixed with 6 L of R O water to obtain a final volumeof 5.2 L. The % yield of *Ksharajala* was 86.66%. *Ksharajala* was then heated at 98 ℃ to evaporate the water content. After removing all the water content, the powder obtained was scrapped off and stored. This powder called, *Snuhi Kshara,* was analysed further for its anticancer properties. As shown in the table given below, the % yield of *kshara* was 20.88%.

Table (4): Details of Snuhi kshara

Parameters	**Results**
Volume of *Ksharajala* taken (L)	5.2
Kshara obtained in w/w (g)	47.625
Weight of Ash taken (g)	228
% of *Kshara* obtained in w/w (in comparison to dry *Snuhi* ash)	20.88

3.4 Phytochemical Analysis of *Kshara*

The qualitative analysis of the sample revealed the presence of phenols, tannins, saponins and alkaloids (figure 6). But other phytoconstituents tested were absent (figure 6). The various studies from the plant extracts show that phytochemicals like alkaloids, terpenoids and polyphenols are the molecules that are analgesic and can be used to relieve the pain and also, they exhibit antioxidant potential. The phytochemical analysis of the seeds of *Simarouba glauca* reported for the presence of alkaloids, saponins and tannins [18, 19] and also in various leaf extracts by researchers [20, 21].

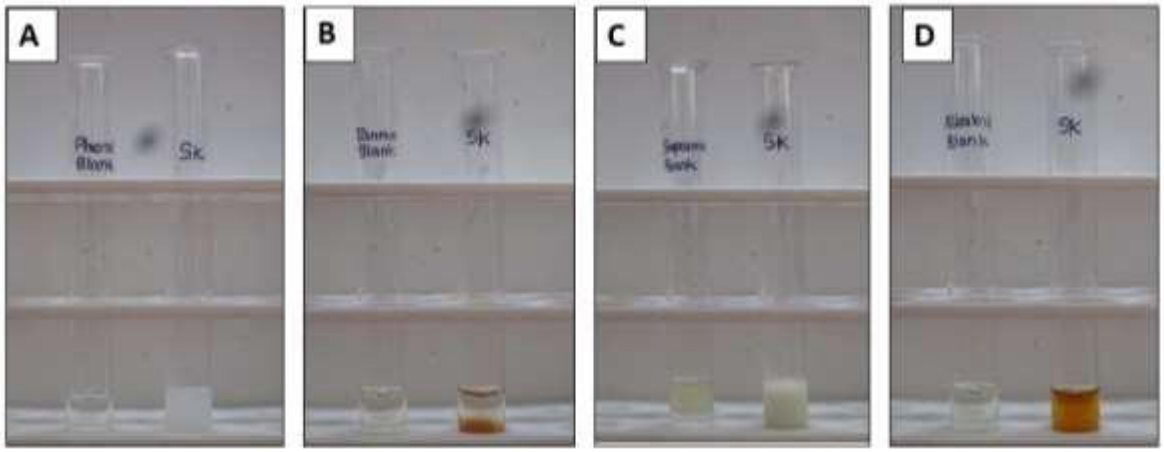

Fig. (5): Qualitative analysis confirming the presence of (A) Phenols (B)Tannins (C)Saponins and (D) Alkaloids

Table (5): Qualitative Analysis of Snuhi Kshara where, '+' denotes positive and '-'denotes negative

Name of Test	Inference	Observation
Phenols	+++	Formation of white precipitate
Tannins	++	Formation of brownish precipitate
Flavonoids	-	Formation of yellowish green color
Saponins	+++	Persistent foam
Terpenoids	-	Formation of greenish color
Alkaloids	+	Appearance of reddish-brown precipitate
Glycosides	-	Formation of brown ring
Quinones	-	Appearance of yellow precipitate
Fatty acids	-	Appearance of transparency in filter paper
Steroids	-	Upper reddish layer and greenish yellowacid layer

Some of the plant derived phenols are reported to have the anti-cancer properties. The analysis of crude drug has shown the presence of flavonoids, but they were absent in the *kshara* sample. The presence of phenols needs to be further confirmed by spectroscopic studies and same has been reported by many researchers [21, 22]

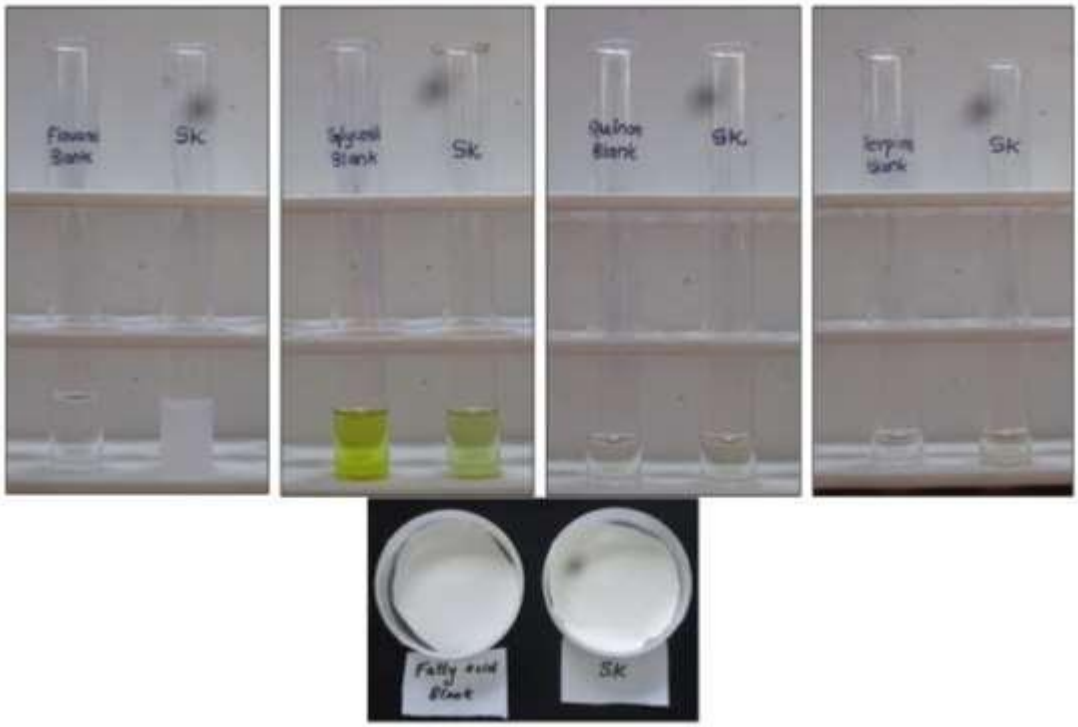

Fig. (6): Qualitative Analysis showing the absence of flavonoids, glycosides,quinones and terpenoids

The presence of phenol was further confirmed by column chromatography and thin layer chromatography.

Fig. (7): Column chromatographic Assessment of Snuhi Kshara

As of column chromatography, 20 fractions of 10 ml were collected and TLC was donefor the fractions and the fractions with similar spots were pooled.

Table (6): TLC Profile of Fractions eluted in Column Chromatography

Sample code	Solvent front	Sample front	Rf value	Colour
Gallic acid	6 cm	3.44	0.57	Blue
Fraction 7,8,9		3.51	0.59	Blue

Thin Layer Chromatography profile is an additional analysis of *Snuhi Kshara*. The fractions 7, 8, 9 pooled together showed single band at Rf 0.59. The bright blue bands correspond to phenols.

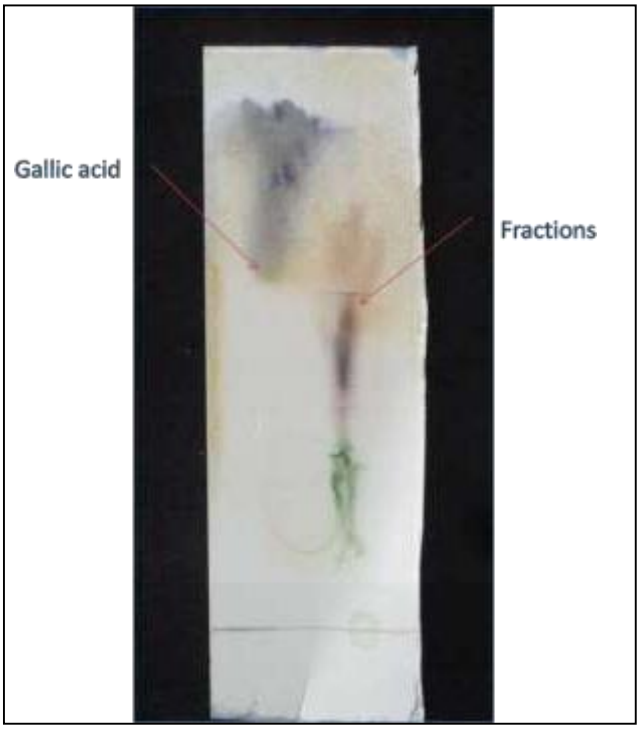

Fig. (8): Chromatographic Assessment of Snuhi kshara by Thin Layer Chromatography

The sample was further analysed by NMR and FTIR. In FTIR spectra, the characteristicpeaks of vinyl, carbonyl and hydroxyl groups were observed.

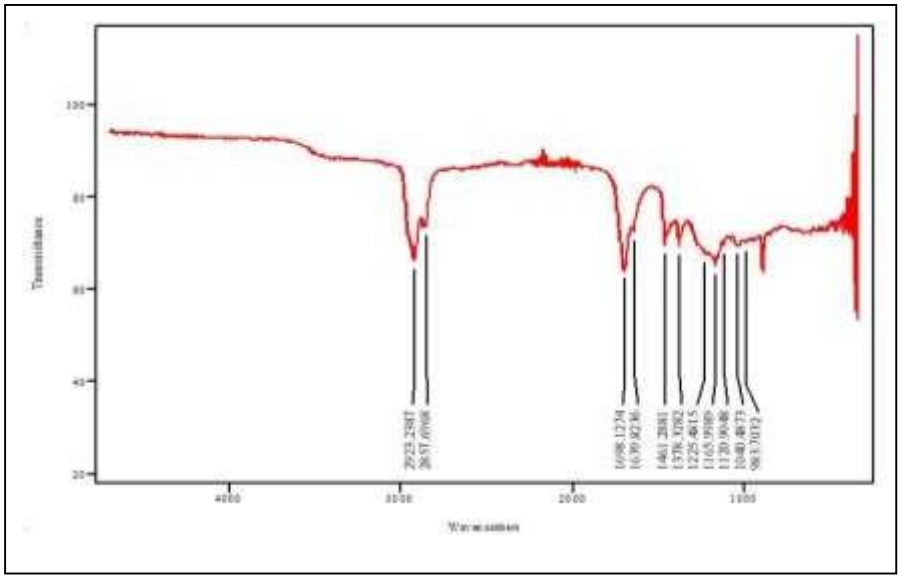

Fig. (9): FTIR Spectra of Snuhi Kshara

Table (7): Peak Characteristics of FTIR Spectra

Peak At	Peak Height	Functional Group
983.7032	70.1883	C=C (Vinyl)
1040.4873	69.5976	C-O (Carbonyl)
1120.9048	69.8722	
1165.9989	65.0314	
1225.4815	67.6446	
1378.3282	69.4145	
1461.2881	69.8928	
1639.8236	72.5591	C=C
1698.1274	64.1880	C=O
2857.6968	73.4716	C-H
2923.2387	66.2237	OH (Alcohol)

NMR spectra of *Snuhi kshara* again confirmed the presence of alcohols, ethers, halides,amino groups and aliphatic carbon.

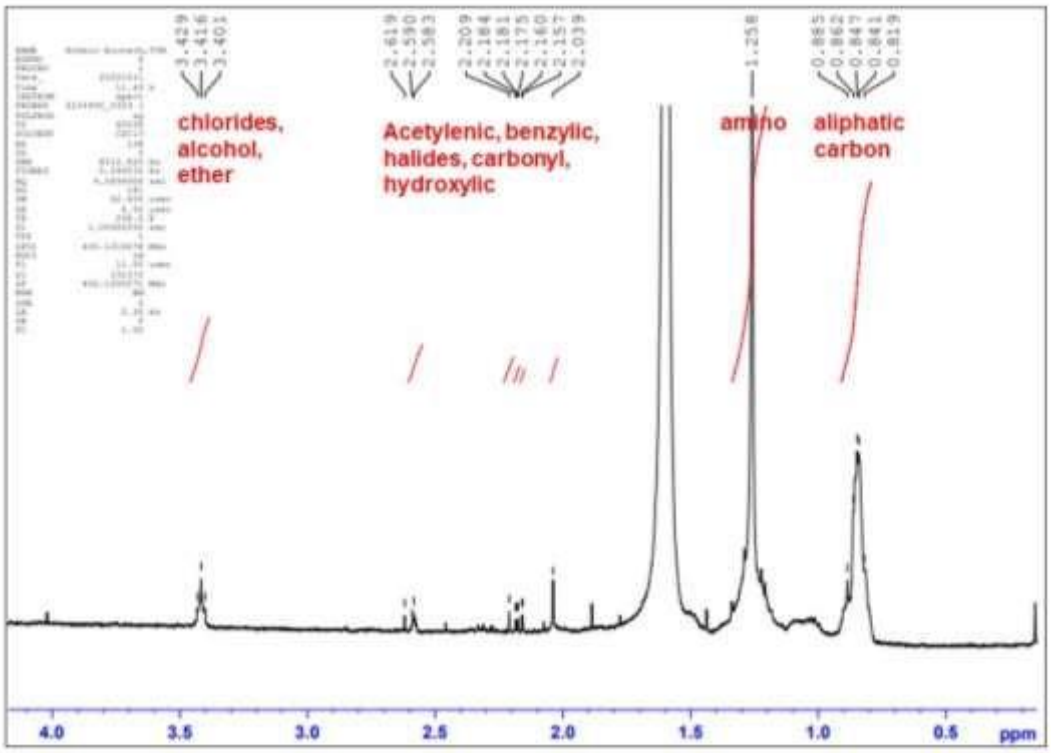

Fig. (10): NMR spectra of Snuhi Kshara

3.5 Biocompatibility Studies of Crude Drug extracts

Biocompatibility of the aqueous and methanol extracts of the crude drug was evaluated by MTT assay in L6 cell lines. MTT assay measure the cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) got reduced to purple formazan crystals by metabolically active cells. The insoluble formazan crystals were dissolved using DMSO and the resulting purple coloured solution was quantified by measuring absorbance at 570 nm using an ELISA plate reader. The biocompatibility assay proved that the L6 cell lines are viable with the crude drug extracts, showing a viability % above 97. Kolgi et al justified similar finding using *Leucas aspera* plant leaves extracts as an antioxidant and anticancer potential in in vivo studies [(21)].

3.6 Acute Toxicity Study

During the acute toxicity study, the test drug was administered orally and animals were observed for mortality, changes in the autonomic nervous system, central nervous system and behavioural responses. There was no mortality observed even at 2000 mg/Kg for the test drug. All the animals were found to be normal and there were no behavioural changes till the end ofthe observation period. This observation revealed that the test drug is very safe up to 2000 mg/Kg of body weight termed as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. To support this work Patil et al., reported similar bioactive constituents in petroleum ether extract in *Citrus medica* seeds [(23)].

4. Conclusion

The present study confirms the preparation of biocompatible *Snuhi kshara,* that is lacking any acute toxicity. The phytochemical analysis of *Snuhi kshara* discloses the presenceof phenolic compounds which in turn throw light into its medicinal properties. The results

demonstrate that *Snuhi kshara* can be further explored for their antioxidant and anticancerproperties.

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