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A TRAILBLAZING ENDEAVOUR TO EXPLORE THE ROLE OF *TULASI PUSHPA* AS *SANDHANA DRAVYA* (FERMENTING AGENT) IN *SANDHANA KALPANA*

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Keywords:

Fermentation process, Alcoholic fermentation, *Sandhāna kalpanā*, *Tulasi* flowers, *Sandhāna dravyā,* Fermenting agents

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ABSTRACT: *Sandhāna kalpanā* (Fermentation process) is a unique procedure implemented in Ayurveda for the preparation of fermented alcoholic and acidic medicinal formulations. *Sandhāna dravyās* (fermenting agents) act as fermentation initiators in them. The commonly used *Sandhāna dravyās* are *Dhātakipuṣpā* (flowers of *Woodfordia fruticosa*), *madhūkapuṣpā* (flowers of *Madhuka indica*) and yeast. Almost in all *Sandhāna dravyās*, the presence of microflora plays a significant role in fermentation. In recent studies, it was proved that the dried flowers of *Dhātaki* and *Madhūkapuṣpā* have yeast colonies in it. Total aerobic microbial count testing of *Tulasi puṣpā* (*Ocimum tenuiflorum* - flowers) done in our study also revealed the presence of yeast. **Objectives:** To determine whether *tulasi* is having any fermenting activity with respect to the microbial, pharmaceutical and analytical matter. **Methods:** Total aerobic microbial count testing of fresh and dry flowers of *Tulasi* and dry flowers of *Madhūka* was performed. Pharmaceutico-comparative analytical study of 3 samples of *Ariṣṭā* (a fermented medicinal preparation) named *Drākṣāriṣṭā* prepared using them was also done. **Results:** Flowers of *Tulasi* (fresh and dry ones) had shown better microbial count and analytical parameters. Also, it displayed proper features of fermentation in the samples. **Conclusion:** *Tulasipuṣpā* can be used as a *Sandhāna dravyā* for the preparation of ariṣṭā and āsava.

INTRODUCTION: *Sandhāna kalpanā* (Fermentation process) is a unique procedure implemented in Ayurveda, where the drava-dravya (medicinal drug decoction - *kvātha* / drug juice - *svarasa*), *madhura dravyā* (sweetening sources like jaggery or sugar) and *prakṣepaka dravyā* (finely powdered fragrant drugs) along with the sandhāna dravyā (fermenting agents like *dhātakipuṣpā* – flowers of *Woodfordia fruticosa*) are put together in mud pot /porcelain jars and are kept closed for a stipulated duration in order to enable the fermentation process. These formulations have a longer shelf life, excellent therapeutic efficacy, and palatability. *Sandhāna dravyās* act as fermentation initiators.

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The commonly used *sandhāna dravyās* (fermenting agents) are *dhātakipuṣpā* (flowers of *Woodfordia fruticosa*), *madhūkapuṣpā* (flowers of *Madhuka indica*), and yeast. The use of *dhātakipuṣpā* as a fermentation initiator is documented for the first time in *aṣṭāṅgahṛdaya*.

Tulasi Ocimum tenuiflorum) or holy basil is an aromatic plant in the family *Lamiaceae*. The plant is an innate to the Indian subcontinent and is found abundantly in the Southeast Asian tropics. It has religious, traditional and medicinal importance. Traditionally, the purplish flowers of *tulasi* (*Ocimum tenuiflorum*) are used for preparing wines in certain regions of Kerala. Almost in all *sandhāna dravyās*, the presence of microflora plays a significant role in fermentation [1]. In a study conducted recently, it was found that the dried flowers of *dhātaki* (*Woodfordia fruticosa*) had yeast colonies in it [2].

Madhūkapuṣpā (flowers of *Madhuka indica*) also exposed the same in another study conducted[1]. Total aerobic microbial count testing of *Tulasi puṣpā* (*Ocimum tenuiflorum* - flowers) done in our study also revealed the presence of yeast. Hence an attempt was made to prepare 3 samples of *ariṣṭā* (a fermented medicinal preparation) named '*drākṣāriṣṭā*', using fresh as well as dry *tulasi* flowers and dried m*adhūka* flowers as *sandhāna dravyās* (fermenting agent) in order to determine whether *Tulasi puṣpā* is having any fermenting activity. The current study is intended to explicate the role of *tulasipuṣpā* (*Ocimum tenuiflorum*) in *sandhāna kalpanā* with respect to the microbial, pharmaceutical, and analytical matter.

MATERIALS AND METHODS: The study was a self-financed one and was conducted as a part of a project given as per the decision of the department of *Rasaśāstra and bhaiṣajya kalpana*, Amrita School of Ayurveda. The study comprised of:

- Microbial study- total aerobic microbial count testing (Total Aerobic Bacterial Count & Total Yeast and Mold Count) of fresh and dry flowers of *tulasi* and dry flowers of *madhūka*.
- Pharmaceutical study -3 samples of *drākṣāriṣṭā* preparation.
- Comparative analytical study of 3 samples.

Tulasi (*Ocimum tenuiflorum*) flowers were collected from herbal garden of Amrita School of Ayurveda and cleaned from superfluous matter. *Madhūka* flowers and other ingredients of *drākṣāriṣṭā* were purchased from the market and cleaned. The samples of *Tulasi* and *madhūka* were then given to CARe Keralam LTD, Thrissur for total aerobic bacterial count and total yeast and mold count testing. Fresh and dry flowers of *Tulasi* and dried *madhūka* flowers were used for the purpose.

Microbial Study: The microbial study was designed based on previous research conducted[1] and after consulting the opinion of Amrita Centre for Advanced Research in Ayurveda (ACARA). The medium used for studying total count and the method was also decided based on the previous study mentioned above. The reference followed was API, Part - 2, Volume - 3, first edition 2010.

Aim: To compare the effect of *tulasi* with *madhūka* in terms of total aerobic bacterial count & total yeast and mold count.

Total Aerobic Bacterial Count:

Materials and Medias Required: Soya bean casein digest agar, buffered sodium chloride peptone solution, petri plates, micropipettes, conical flasks, biosafety cabinet, incubator, colony counter, water bath, thermometer, pH meter.

Procedure: 10 g of the sample was weighed and added to 90 ml buffered peptone water w/NaCl. It was serially diluted up to the required dilution. 1ml liquid of each sample homogenate was then dispensed into duplicates of appropriately labelled petri plates. After that 15 to 20 ml of soybean casein digest agar, cooled to 45℃ was added to the petri dishes. The sample homogenate/dilutions and agar were then immediately mixed thoroughly by alternate rotation and rocking to and fro of the plates on a level surface.

The agar was then allowed to solidify, and the solidified agar plates were then incubated in the incubator (inverted position) at 30 - 35 ℃ for 5 days. Negative control for the session of testing samples was done by using sterile distilled water in the place of sample homogenate. The colonies on the plate were then counted with the highest number of colonies but not more than 250 per plate as the maximum, consistent with good evaluation by using the colony counter. The results in colony-forming units based on the average count of duplicate set was reported.

If no growth of colonies on all plates at less than one time, the corresponding lowest dilution was used.

Total Yeast and Mold Count:

Materials and Medias Required: petri plates, conical flasks, micropipettes, BOD incubator, bio-safety cabinet, buffered sodium chloride peptone solution, sabouraud's dextrose agar with chloramphenicol, water bath, thermometer, colony counter, pH meter.

Procedure: 10 g sample was weighed and added to 90 ml buffered peptone water w/NaCl. It was serially diluted up to the required dilution. 1ml liquid of each sample homogenate was then dispensed into duplicates of appropriately labelled petri plates. After that 15 to 20 ml of sabouraud's dextrose agar, cooled to 45℃ was added into the petri dishes. The sample homogenate/dilutions and agar were then immediately mixed thoroughly by alternate rotation and rocking to and fro of the plates on a level surface. The agar was then allowed to solidify and the solidified agar plates was then incubated in the BOD incubator (upright position) at 20 - 25 ℃ for 5 days. Negative control for the session of testing samples was done by using sterile distilled water in the place of sample homogenate. The colonies on the plate were then counted with the highest number of colonies but not more than 100 per plate as the maximum consistent with good evaluation by using the colony counter. The results in colony-forming units based on the average count of the duplicate set were reported. If no growth of colonies on all plates at less than one time the corresponding lowest dilution was used. 500ppm difenoconazole (25.0% m/m) was added to the sabouraud's dextrose agar plates to inhibit the growth of mold in order to get the yeast count separately.

Pharmaceutical Study: As per the reference of AFI, Part 1[3] and API, Part 2[4], 3 samples of *drākṣāriṣṭā* (2 L each) were prepared. As per the opinion of the faculties of the department, only 1 sample of *drākṣāriṣṭā* was run for each flower. Sample 1 with dry flowers of tulasi, sample 2 with fresh flowers of tulasi and sample 3 with dry flowers of *madhūka*. All the ingredients taken were the same as per the reference and the only difference was there in the *Sandhāna dravyā* taken. The ingredients of *drākṣāriṣṭā* (samples 1, 2 & 3) along with the quantity used are exhibited in **Table 1.**

TABLE 1: INGREDIENTS OF DRĀKṢĀRIṢṬĀ (SAMPLES 1, 2 AND 3)

S. no.	Ingredients	Latin name	Part used	Quantity used
1	sandhāna dravyā			
	a. tulasipuṣpā-dry(sample 1)	a. Ocimum tenuiflorum	Flower	64g
	b. tulasipuṣpā-fresh(sample 2)	b. Ocimum tenuiflorum	Flower	128g
	c. madhūkapuṣpā-dry (sample 3)	c. Madhuka indica	Flower	64g
2	drākṣā	Vitis vinifera	Dried fruit	0.4kg
3	Jala	Water		8.1920 L reduced to 2.0480 L
4	Guda	Jaggery		1.6kg
5	Tvak	Cinnamomum zeylanicum	Stem bark	8g
6	elā	Elettaria cardamomum	Seed	8g
7	Patrā	Cinnamomum tamala	Leaf	8g
8	Kesarā	Mesua ferrea	Stamen	8g
9	priyaṅgu	Callicarpa macrophylla	Flower	8g
10	Marica	Piper nigrum	Fruit	8g
11	kṛṣṇā	Piper longum	Fruit	8g
12	viḍaṅga	Embelia ribes	Fruit	8g

Method of Preparation: *Drākṣā* (*Vitis vinifera*), of appropriate quantity, washed in hot water and crushed was boiled for making the decoction in the prescribed volume of water taken after measuring accurately using an electronic weighing balance with a capacity of 500 kg present in the attached IP pharmacy of our college. The decoction, after getting reduced to the prescribed limit mentioned in the table, was filtered using a muslin cloth and mechanical squeezing apparatus. The obtained filtrate was measured again in an electronic weighing balance and 1.6 kg *guda* (jaggery) was added into it after pounding. *Guda* (jaggery) was dissolved properly in the decoction and was filtered once again to remove the impurities present in the jaggery. This solution was then transferred into

mud jars which were washed previously using hot water, dried, smeared with ghee and fumigated using drugs like .*agaru* (*Aquilaria agallocha*), *karpūra* (*Cinnamomum camphora*), *guggulu* (*Commiphora mukul*), *sarja rasa* (*Vateria indica*) and *uśīra* (*Vetiveria zizanioides*) [5]. The ingredients numbered from 5 - 12 in the table, which were dried and cleaned earlier, were powdered individually, sieved, and measured properly. This was then added to the filtrate solution, followed by the addition of *sandhāna dravyā*. Double quantities of Tulasi fresh flowers were used since, in the classical references, it is told that wet drugs should be taken in double quantity as the dry ones. The jar was then closed, sealed, and kept undisturbed for the process of fermentation. **Fig. 1, 2,** and **3** show the images of the samples of *drākṣāriṣṭā*.

FIG. 1: SAMPLE 1

FIG. 2: SAMPLE 2

FIG. 3: SAMPLE 3

Analytical Study: As per the standard methodology (API, Part 1, Volume 6), a comparative study of the three samples of *drākṣāriṣṭā* was done. Organoleptic characters along with pH, specific gravity, total solids, alcohol content, acid value, total sugar, and reducing sugars were carried out in the quality control laboratory of Amrita School of Ayurveda. Since the study was a self-financed one performed as a part of the project given, only a basic level of analytical tests was done.

RESULTS: Every stage of fermentation was evaluated as per the classical methods (*i.e.*, initial onset, after the onset, and completion of fermentation) in the pharmaceutical study. Effervescence and hissing sound was observed in all the 3 samples during the 15th day while it was absent on the 30th and 45th day. *Prakṣepaka dravyās* got settled down, and the sweet smell present in the beginning changed to a mild alcoholic smell by the 30th day. A burning candle test was performed on the 15th and 30th days and found that the candle turned off. On the 45th day, while doing the burning candle test, the burning candle continued to burn, and an alcoholic odour was present in all the 3 samples. The images of samples 1, 2, and 3 of *drākṣāriṣṭā* are given in **Fig. 1, 2,** and **3**. The results of the microbial count study and analytical test reports are displayed in **Table 2** & **Table 3.**

TABLE 2: RESULTS OF MICROBIAL COUNT STUDY

Name of the sample	Total Plate Count for Bacteria (CFU/g)	Total Yeast and Mold Count (CFU/g)
Tulasi dry flowers	$>300\times10^4$	Total yeast count: 31×10^2 Total mold count: $>150\times10^3$
Tulasi fresh flowers	$>300\times10^5$	Total yeast count: 39×10^2 Total mold count: $>150\times10^3$
Madhūkadry flowers	172×10^2	8×10^2

TABLE 3: RESULTS OF ANALYTICAL STUDY

Parameters	Analytical Test Results		
	Sample – 1	Sample – 2	Sample – 3
pH	3.57	3.57	3.40
Specific Gravity	1.185	1.185	1.180
Brix	44.0%	44.0%	44.2%
Alcohol content	5%	6%	3%
Acid value	5.755 mg NaOH / ml	5.925 mg NaOH / ml	5.742 mg NaOH / ml
Total Sugar	44.609 %	44.994 %	43.054 %
Reducing Sugar	42.67 % w/v	42.73 % w/v	40.38 % w/v

As per the microbial count study results, *Tulasi* flowers are comparable with *madhūka* flowers according to the microbial load. The following images show the total aerobic bacterial count report of the samples of flowers in soya bean casein digest agar media incubated at 30 - 35 ℃ for 5 days and the total yeast and mold count report of the samples of flowers in SDA media incubated at 20 - 25°C for 5 days. **Fig 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8, Fig 9, Fig. 10,** and **Fig. 11** show total aerobic bacterial count and total yeast and mold count reports of *Tulasi* dry, *Tulasi* wet, and *madhūka* dry flower samples incubated for 5 days respectively. **Fig. 12, Fig. 13, Fig. 14** and **Fig. 15** show the report of negative control plates incubated for 5 days.

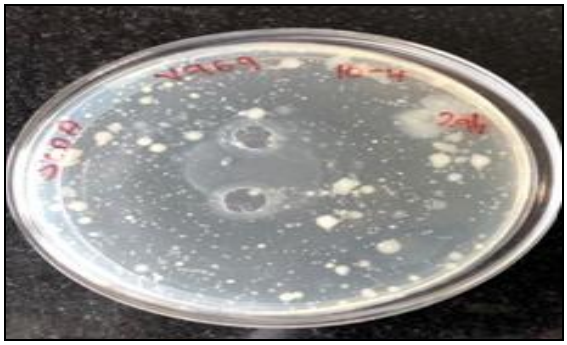

FIG. 4: TULASI DRY BACTERIAL PLATE

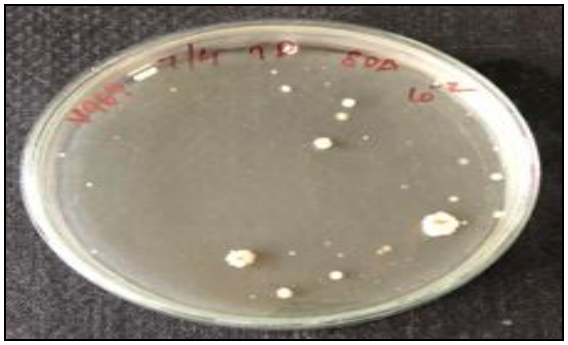

FIG. 5: TULASI DRY YEAST PLATE

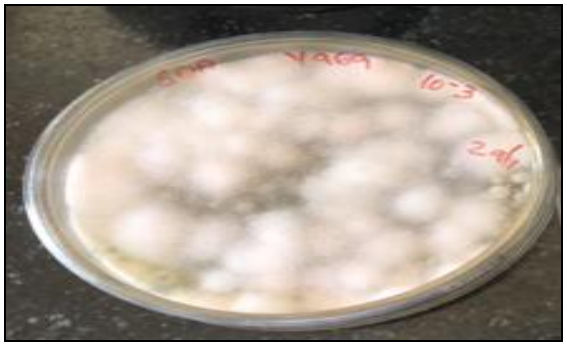

FIG. 6: TULASI DRY MOLD PLATE

FIG. 7: TULASI WET BACTERIAL PLATE

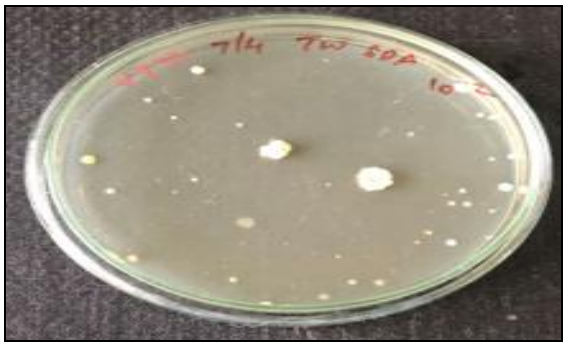

FIG. 8: TULASI WET YEAST PLATE

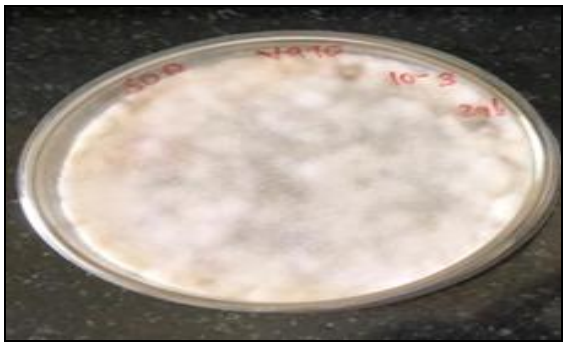

FIG. 9: TULASI WET MOLD PLATE

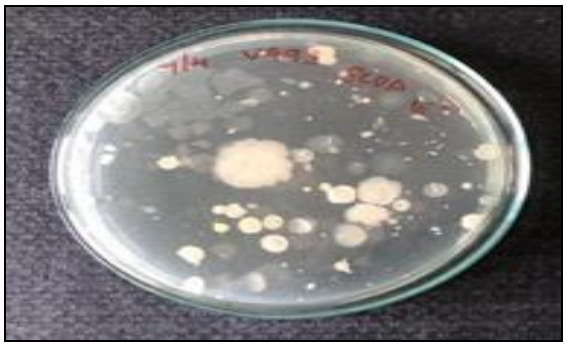

FIG. 10: MADHUKA DRY BACTERIAL PLATE

FIG. 11: MADHUKA DRY TOTAL YEAST & MOLD PLATE

FIG. 12: MEDIA CONTROL SCDA PLATE

FIG. 13: TIP CONTROL SCDA PLATE

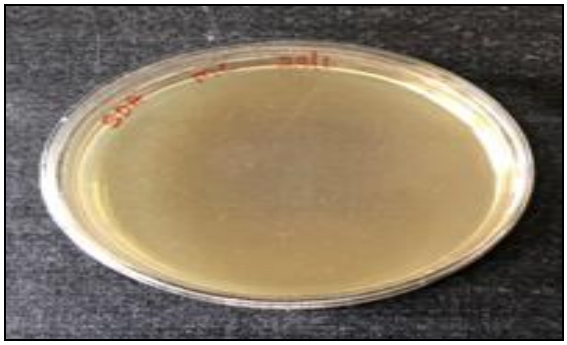

FIG. 14: MEDIA CONTROL SDA PLATE

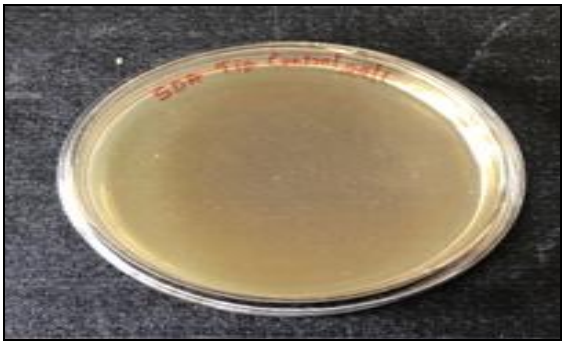

FIG. 15: TIP CONTROL SDA PLATE

DISCUSSION: To discover the precise role of *Tulasi pushpa* (*Ocimum tenuiflorum*) in *sandhāna kalpanā* (Fermentation process), this study was conducted. The microbial load of the fresh and dry flowers was carried out using standard methods. *Drākṣāriṣṭā* is a wonderful preparation mentioned in Ayurveda, holding incredible effects. According to the anticipated work, *drākṣāriṣṭā* was prepared in three ways. Sample1with dry flowers of *Tulasi*, Sample 2 adding fresh flowers of *Tulasi,* and Sample 3 by adding dry flowers of *madhūka*. On the forty-fifth day, when the coverings were opened, samples 1, 2, and 3 exhibited proper features of fermentation. The sample1 exhibited a fragrant and alcoholic odour, dark brown colour and sourness with sweet taste. Samples 2 and 3 also exposed the same. *Prakṣepaka dravyās* (fine powder of aromatic drugs) were seen settled at the bottom of all the 3 samples.

Analytical parameters aid to comprehend the efficacy of *tulasipuṣpā* (*Ocimum tenuiflorum*) in the fermentation process. The analytical values of all the three samples of *drākṣāriṣṭā* have provided us with esteemed results. In the current study, the P^H of all the three samples have not shown considerable alterations, and all were in a P^H range of 3.40 – 3.57. Specific gravity is the ratio of the density of a substance to the density of given reference material. S1, S2, and S3 have not shown much variation among these values. Brix is commonly used for determining the dissolved solids in samples. No dissimilarities were seen in the Brix values also. Ariṣṭās are fermented preparations having self-generated alcohol [6]. The alcohol content of the samples was under the limit with only a minimal difference. The acid value indicates the total acids present in the product which are formed during the fermentation process and storage. These acids are responsible for the sour taste of those preparations. The acid value of the samples was also without ample changes. Reducing sugar is any sugar that is capable of acting as a reducing agent since it has a free aldehyde group or a free ketone group and it includes all monosaccharides, some disaccharides, some oligosaccharides and some polysaccharides. The samples were not at all having markable differences in these values. Thus, *Tulasi* flowers have shown a precise role in proper fermentation, which has been reflected in their analytical study.

While comparing the results of the previous microbiological studies of the flowers of *dhātaki* and *madhūka*, *Tulasi* flowers have almost analogous microbial contents. Microbiological studies of *dhātakipuṣpā* have shown that dry flowers of *dhātaki* possess yeast cells and are best fermentative [2]. Correspondingly, the studies on *madhūkapuṣpā* had found that dry flowers of

madhūka also contain the presence of yeast and have a definite role in *sandhāna kalpanā* (fermentation process) [1].

CONCLUSION: Since the olden days, *dhātakipuṣpā* (flowers of *Woodfordia fruticosa*), *madhūkapuṣpā* (flowers of *Madhuka indica*), and yeast are used as *Sandhāna dravyās* (fermenting agents) in Ayurveda. *Tulasi* (*Ocimum tenuiflorum*) flowers which are freely available and cost-effective, can be used as a *sandhāna dravyā* (fermenting agent) since the following facts have been proved successfully:

- The microbial count estimation of fresh and dry *Tulasi* flowers revealed the presence of yeast.
- Pharmaceutical study established that *tulasi*is having a definite role in *Sandhāna kalpanā*(fermentation process).
- The analytical study of the three samples also revealed supportive proofs for the study. The samples S1 and S2 have given analytical values similar to that of S3.

From all the above results, flowers of *tulasi* (fresh and dry ones) have shown better microbial count and analytical parameters than *madhūkapuṣpā*. The entire study has highlighted the fact that *tulasipushpa* (flowers of *tulasi*) has a definite role in *Sandhāna kalpanā* (fermentation process) and can be used as a *sandhāna dravyā* (fermenting agent) for the preparation of Ariṣṭā and Asava (fermented medicinal preparations). However, further studies need to be conducted after maintaining appropriate controls to prove that the inoculum of fermentation was introduced through the flowers of *tulasi* exclusively. Also, the microbial properties of the final product after completion of fermentation have not been studied which can be of significance.

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CONFLICTS OF INTEREST: We declare no conflicts of interest.

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