

Trividha Snehapaka of Panchagavya Ghrita: A critical comparative evaluation

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ABSTRACT

Background: The three stages of Snehapaka formulations namely *Mridu*, *Madhyama* and *Khara Paka* have been characteristically advocated for different routes of administration—*Nasya*, *Pana/Basti* and *Abhyanga*, respectively. Guidelines or established method for post-formulation characterization for the same is hardly available. **Objective:** The present communication is the comparative study of *Mridu*, *Madhyama* and *Khara Paka* of *Panchagavya ghrita* (PGG). **Materials and Methods:** Laboratory prepared samples of PGG following classical method were analyzed for different physicochemical, spectroscopic, chromatographic parameters, and antioxidant activity. **Results:** No significant difference was found among *Mridu*, *Madhyama* and *Khara Paka* in physicochemical parameters as well as chromatographic profiles. The ratio of absorbance at 240 and 294 nm showed steady increase from *Mridu* to *Madhyama* to *Khara Paka* in the ultraviolet (UV)-visible spectra of unsaponifiable matter. The high performance thin layer chromatography (HPTLC)-2,2 Diphenyl-1-picryl hydrazil (DPPH) bioautography assay revealed presence of two antioxidant compounds in low concentration in all the samples. This was further supported by estimation of total reducing power and DPPH assay. No significant difference was found among the three samples. **Conclusion:** Comparison of various physicochemical parameters, chromatographic profiles, and *in vitro* antioxidant activity determination is of little help in establishing any significant difference among the samples. However, spectrophotometric analysis of unsaponifiable matter reveals some encouraging characteristic findings which will be useful in establishing difference among the three stages of processing of PGG as well as Snehapaka in general.

Key words: Antioxidant, High performance thin layer chromatography (HPTLC), HPTLC-2 Diphenyl-1-picryl hydrazil (DPPH) bioautography, *Panchagavya ghrita*, *Mridu/Madhyama/Khara Paka*

INTRODUCTION

Panchagavya ghrita (PGG) is a widely used Ayurvedic formulation mentioned in various treatises of Ayurveda and has wide therapeutic indications. It is indicated in conditions like *Apasmara* (~ epilepsy), *Jvara* (~ pyrexia), *Unmada* (~ psychosis), *Kamala* (~ jaundice), etc.^[1] Its anti-convulsant^[2] and hepatoprotective^[3] activities have also been evaluated and proven experimentally. PGG is

a kind of *Snehapaka* (~ medicated oil/ghee) prepared by processing ghee with milk, curd, urine and dung extract (all from cow source). The ingredients are mixed in designated proportions and are processed as per the standard method for preparation of *Snehapaka* formulations. Classically, *Snehapaka* (*Sneha* = fat and *Paka* = processing) procedure is subdivided into three stages (which are of medicinal utility) namely, *Mridu Paka*, *Madhyama Paka* and *Khara Paka*.^[4] Preparation of any formulation under *Snehapaka* category can be terminated at any of these three *Paka* levels at discretion of the person preparing it. Each of these *Paka* is intended for different but specified medicinal use viz. *Mridu Paka* for *Nasya* (~ administration through nose), *Madhyama* for *Pana* (systemic use) or *Basti* (~ per rectal) and *Khara* for *Abhyanga* (topical use).^[5]

PGG has been described in the Ayurvedic Formulary of India and also included in the Ayurvedic Pharmacopoeia of India (API).^[6] The general monograph for *Ghrita* (medicated ghee) formulations in API^[6] also describes the three stages of *Snehapaka*. However, there is no *Paka* differentiation for any specific formulation. In case of PGG for instance, only *Madhyama Paka* has been described from both preparatory

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and analytical points of view. Our extensive literature review did not reveal any scientific study on the objective differentiation of three *Paka* levels. As it is in practice to use PGG for *Nasya*, *Pana* and also *Abhyanga*, the comparative study of PGG—*Mridu Paka*, *Madhyama Paka* and *Khara Paka* has been carried out with an aim to look for the difference, if any, among the three *Paka* levels. The samples were evaluated by comparative physicochemical analysis, ultraviolet (UV)-Visible spectroscopy, high performance thin layer chromatography (HPTLC) profiling as well as antioxidant activity.

MATERIALS AND METHODS

Chemicals and solvents

2,2 Diphenyl-1-picryl hydrazil (DPPH) from Sigma-Aldrich, Germany and all other chemicals from Merck India of pure or guaranteed reagent grade.

Preparation of Panchagavya ghrita

PGG was prepared in our laboratory by following the classical method described in *Charaka Samhita*.^[7] The raw materials namely dung, urine and milk were obtained from *Geer* variety of indigenous cow, that is, *Bos indicus*, reared in-house in the *Gaushala* run by Shree Prabhav Hem Kamdhenu Girivuhar Trust, Waghaldhara, Valsad, Gujarat. The curd and ghee were prepared by processing milk from the same source. Five hundred grams of freshly collected cow dung was mixed homogeneously with 500 mL of water and the mixture was strained through a cloth. This dung extract (about 500 mL), along with 500 mL each of freshly collected cow urine, cow milk (boiled and cooled), and curd from cow milk (night old) were mixed with 500 mL of molten cow ghee and heated on mild heat with continuous stirring. The process was continued uninterruptedly for about 3-3.5 h till separation of phases occurred and then allowed to cool. After keeping it overnight, next day, the processing was continued on mild heat till *Siddhi Lakshana*.^[8] (~ process termination signs) were obtained for *Mridu Paka*. A part of *Ghrita* along with roughly proportionate *Kalka* (~ residue in ghee) was separated at this stage and the process was continued. Similarly, *Madhyama Paka* sample was also collected and the rest was processed till *Khara Paka*.

Analytical study

All the samples were analysed by using different organoleptic, physicochemical parameters, UV-visible spectroscopy as well as thin layer chromatography (TLC) and HPTLC.

1. Physicochemical parameters

Various physicochemical parameters like specific gravity, refractive index, acid value, saponification value, unsaponifiable matter, iodine value, and peroxide value were employed following the method as described in API.^[6]

- i. Specific gravity
Specific gravity at 40°C was determined using a pycnometer.
- ii. Refractive index
Refractive index was measured at 40°C using Abbe's refractometer.
- iii. Acid value
About 10 g PGG, accurately weighed, was taken with 50 mL of a mixture of equal volumes of methanol and diethyl ether. The mixture was titrated with 0.1 N potassium hydroxide (number of mL required: a).

Acid value was calculated using the formula:

$$\text{Acid value} = \frac{a \times 0.00561 \times 1000}{W}$$

Where W is the weight in grams (g) of the substance taken.

- iv. Saponification value
To about 2 g of PGG, accurately weighed, 25 mL of 0.5 N alcoholic potassium hydroxide solution was added and refluxed for 1 h. The excess alkali was titrated with 0.5 N hydrochloric acid using phenolphthalein as indicator (number of mL required: a). The experiment was repeated with the same quantities of the same reagents omitting the substance (number of mL required: b).

Saponification value was calculated using the formula:

$$\text{Saponification value} = \frac{(b-a) \times 0.02805 \times 1.000}{W}$$

Where W is the weight in grams (g) of the substance taken.

- v. Unsaponifiable matter
About 5 g PGG, accurately weighed, was taken with 40 mL of 1 N alcoholic potassium hydroxide solution and refluxed on a water-bath for 1 h, frequently rotating the contents. To this, 100 mL of water was added and extracted repeatedly with diethyl ether. The combined ether extract was washed with water. Ether was distilled off and the residue, that is, the unsaponifiable matter, was weighed and expressed as %w/w with respect to the sample taken.
- vi. Iodine value
About 0.5 g PGG, accurately weighed, was

dissolved in 10 mL of carbon tetrachloride in a dry iodine flask. To it, 20 mL of iodine monochloride solution was added and the stoppered flask was kept in dark for 30 min. Further 15 mL of solution of potassium iodide and 100 mL water was added to it; mixed and titrated with 0.1 N sodium thiosulphate, using starch solution as indicator (number of mL required: a). The experiment was repeated with the same quantities of the same reagents omitting the substance (number of mL required: b).

Iodine value was calculated using the formula

$$\text{Iodine value} = \frac{(b-a) \times 0.01269 \times 100}{W}$$

Where W is the weight in grams (g) of the substance taken.

vii. Peroxide value:

To 5 g PGG, accurately weighed, 30 mL of a mixture of three volumes of glacial acetic acid and two volumes of chloroform was added, mixed until dissolved and 0.5 mL of saturated potassium iodide solution was further added. After exactly 1 min, with occasional shaking, 30 mL of water was added and titrated gradually, with continuous and vigorous shaking, with 0.01 M sodium thiosulphate using starch solution as indicator (number of mL required: a). The experiment was repeated with the same quantities of the same reagents omitting the substance (number of mL required: b).

The Peroxide value was calculated using formula

$$\text{Peroxide value} = \frac{10 (a - b)}{W}$$

Where W is the weight in grams (g) of the substance taken.

2. UV-visible spectra

The UV-visible spectra were recorded in a PerkinElmer Lambda 25 UV-visible spectrophotometer with preloaded UV-Winlab software. The unsaponifiable matter obtained from the sample of 5 g was dissolved in 20 mL of diethyl ether and the spectrum was recorded (200 nm-600 nm).

3. TLC profile of unsaponifiable matter

Experiments were carried out by using different chromatographic conditions to evolve suitable TLC

profile of the unsaponifiable matter of the samples. Various mobile phases, single solvents as well as various blends of solvent mixtures in varying proportions were tried to achieve optimum resolution. Varied detection techniques employing different spraying reagents as well as UV radiations were also tried. Following optimum conditions which showed better resolutions, were used for evolving the TLC profile:

Stationary phase: Silica gel 60F₂₅₄ (precoated plates, Merck, Germany),

Mobile phase: Toluene - Ethyl acetate (93:7)

Detection: Spraying with Anisaldehyde-H₂SO₄ reagent^[9] followed by heating at 110°C for 5-10 min

4. HPTLC profile

Two gram of PGG was extracted with 20 mL methanol at about 40°C for 3 h, cooled, allowed to separate the layers. The methanol layer (PGGM: Methanol fraction of Panchagavya ghrita) was used for the study. HPTLC was performed on 10 cm × 10 cm aluminium backed plates coated with 0.2 mm layers of silica gel 60F₂₅₄ (Merck, Germany). Concentrated PGGM was applied in a band with Linomat V applicator (CAMAG, Switzerland), equipped with a 100 µL syringe. Plates were developed vertically, in a CAMAG twin trough chamber previously saturated with mobile phase vapour for 20 min at room temperature. Various mobile phases were tried to obtain better resolution. Toluene - ethyl acetate - glacial acetic acid (7:3:0.2), giving optimum resolution, was used as mobile phase. Densitometric scanning was performed with CAMAG TLC Scanner 4 at 254 nm, operated by Wincats software version 1.4.6. The source of radiation utilized was deuterium and tungsten lamp emitting a continuous UV spectrum between 200 and 700 nm.

The plate was also sprayed with anisaldehyde-H₂SO₄ reagent followed by heating at 110°C for 5-10 min and details were noted.

Evaluation for *in vitro* antioxidant activity

1. Rapid screening for *in vitro* antioxidant activity by HPTLC-DPPH bioautography method:

Preliminary screening for antioxidant activity of the samples was carried out by using HPTLC-DPPH Bioautography method.^[10,11] After development, as mentioned above, the plate was dried at room temperature, sprayed with 0.2% w/v methanolic DPPH solution and observed after keeping in dark for 30 min.

2. Estimation of total reducing power

The total reducing power was estimated with a reported method.^[12] Different concentrations of PGGM, diluted with 1 mL distilled water, were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium hexacyanoferrate solution (1% w/v). The mixture was incubated at 50°C for 20 min, 1.5 mL of trichloroacetic acid (10% w/v) was added to the mixture and filtered. 2.5 mL of filtrate was mixed with equal volume of distilled water and 0.5 mL of ferric chloride solution (0.1% w/v) and the absorbance was measured at 700 nm after 40 min. Ascorbic acid solution (100 µg/mL) was taken as the known standard. Increased absorbance of the reaction mixture indicates stronger reducing power. A calibration curve was plotted for absorbance of ascorbic acid standard against its concentration in micrograms and the equivalent values for samples were extrapolated from the slope equation of line against their respective absorbances. The results were expressed in microgram equivalents of ascorbic acid with respect to the original sample (PGG).

Further, PGG taken in petroleum ether (60-80°C) was also subjected to the same assay in a similar manner. For this, 0.1 mL from 10% w/v solution of PGG in petroleum ether (PGGP) was utilized.

3. Assay for *in vitro* DPPH-free radical scavenging activity (DPPH assay):

DPPH radical gives strong absorbance at 517 nm (deep violet colour) due to its unpaired electron. When this radical pairs off in presence of a free radical scavenger, the absorption diminishes and the resulting discoloration is stoichiometric with respect to the number of electrons taken up.

DPPH-free radical scavenging activity assay of PGG was carried out using a reported method^[13,14] with suitable modifications.

Reagent/solutions: DPPH solution - 0.3 mM in methanol (freshly prepared), standard ascorbic acid solution-1 mg/mL in methanol.

Different concentrations of PGGM/standard were taken in a set of test tubes and methanol was added to make the volume to 3 mL. To this, 1 mL of DPPH reagent was added, mixed thoroughly and absorbance was recorded at 517 nm after 30 min of incubation in dark at room temperature (A_s). One milliliter of DPPH reagent diluted to 4 mL with methanol was taken as reagent blank (A_0). Percent scavenging activity was calculated as:

$$\% \text{ Scavenging} = \frac{A_0 - A_s}{A_0} \times 100$$

Different concentrations of PGGP were also subjected to the same assay in similar manner, where DPPH reagent was prepared in chloroform base and mixture of chloroform-methanol (1:1) was used as diluent.

RESULTS

Organoleptic characters

All samples were semisolid and of pale yellow colour at room temperature, and of moderate red colour with pleasant smell on melting.

Physicochemical parameters

The comparative data of physicochemical analysis is presented in Table 1. No significant difference was found among *Mridu*, *Madhyama* and *Khara Paka* in these parameters.

UV-visible spectroscopy

The UV-visible spectra of all three samples revealed two absorption peaks around 240 nm (A) and 294 nm (B) [Figure 1]. The absorption of both the peaks increases from *Mridu paka* (0.71 and 1.09) to *Madhyama paka* (1.15 and 1.50) but it again decreases in *Khara paka* sample (1.00 and 1.01).

It is interesting to note that, though all the samples showed similar pattern, there is difference in the ratio of absorbance at A and B. A/B for *Mridu* and *Madhyama paka* are 0.65 and 0.76, respectively whereas that for *Khara paka* is 0.99.

TLC profile of unsaponifiable matter

The TLC profile of unsaponifiable matter of the sample [Figure 2] showed five prominent spots at R_f 0.09 (brown), 0.21, 0.34, 0.45, and 0.49 (all purple). There is no notable difference among the samples.

Table 1: Physicochemical data of PGG

Parameter	Mridu Paka	Madhyama Paka	Khara Paka
Specific gravity (at 40° C)	0.9109	0.9112	0.9428
Refractive Index (at 40° C)	1.46	1.46	1.46
Saponification value	209.48	211.81	211.35
Acid value	8.3	8.57	8.97
Unsaponifiable matter (%w/w)	0.260	0.271	0.210
Iodine value	32.208	33.160	35.610
Peroxide value	3.070	3.000	3.360

PGG = Panchagavya Ghrita

HPTLC profile

The HPTLC profile revealed some minor difference between three samples. Densitogram of the samples has been presented in Figure 3, which reveals a major compound at R_f 0.87 in all the samples. In addition to this spot, two more minor spots at R_f 0.25 and 0.59 are also present in the samples with slight variation in their concentrations. The comparative 3D-graph of the samples is presented in Figure 4.

The spectra of the major spot at R_f 0.87 was recorded and the spectral data of the samples were compared. The comparative spectra [Figure 5] shows similar pattern indicating the presence of same compound. The data reveals that there is a marginal difference in the HPTLC profile of the three stages of PGG.

The plate after spraying with anisaldehyde- H_2SO_4 reagent, revealed four prominent spots at R_f 0.26 (brownish), 0.60 (violet), 0.79, and 0.90 (both brownish) in all the samples [Figure 6] indicating similar chemical profile.

In vitro antioxidant activity

i. HPTLC-DPPH bioautography method:

The HPTLC-DPPH bioautography method is used for rapid screening of natural products for antioxidant activity. In this method, the presence of antioxidant compounds are detected by yellowish spots against a purple background. All the samples showed two minor yellowish spots indicating presence of antioxidant compounds in low concentration [Figure 7].

ii. Estimation of total reducing power:

The estimated total reducing power of both PGGM and PGGP samples is presented in Table 2. The values are expressed in ascorbic acid microgram equivalents with respect to original sample (PGG). The data shows that PGG has a moderate antioxidant activity as far as this *in vitro* method is concerned. The difference in the activity of the samples is also insignificant.

iii. Assay for *in vitro* DPPH- Free Radical Scavenging Activity (DPPH assay):

On DPPH assay, none of the PGGP samples showed any recordable DPPH-free radical scavenging activity. However, moderate activity was recorded in PGGM fraction [Table 3].

DISCUSSION

The three stages of *Snehapaka* formulations namely *Mridu*, *Madhyama* and *Khara* have been characteristically advocated for different routes of administration—*Nasya*, *Pana/Basti* and *Abhyanga*, respectively.^[5] Indications to check

the attainment of different stages of *Snehapaka* during processing are available in Ayurvedic classics. However, any guidelines or established method for post-formulation characterization for the same is not available so far. Hence, an attempt has been made to evolve suitable parameters to differentiate between these three stages by employing various modes of evaluation viz. physicochemical, spectrophotometric, chromatographic, and *in vitro* antioxidant activity determination.

None of the samples exhibited significant difference in any of the physicochemical parameters.

All the samples followed a similar pattern in the UV-visible spectra of unsaponifiable matter showing prominent peaks at 240 nm (A) and 294 nm (B). However, marked difference in the ratio of absorbances at these two peaks was exhibited by the samples. Further, a characteristic trend could also be observed that this A/B ratio went on increasing from *Mridu* to *Madhyama* to *Khara Paka*. This observation could prove useful in establishing difference among the three stages of processing of PGG.

Natural products are known to contain multiple compounds of varying chemical natures. Use of versatile techniques like TLC/HPTLC proves to be of importance in identifying and characterizing such complex structures many a times. Hence, TLC of unsaponifiable matter as well as HPTLC of PGGM have also been carried out by using various conditions. However, no significant difference could be observed in any of them.

Free radicals play an important role in degenerative diseases like cancer, cataract, immune system weakness, and brain disorders, collectively called oxidative stress.^[15] PGG has been indicated and even reported to be effective in conditions like epilepsy, jaundice, etc. Panchagavya, another Ayurvedic preparation containing all the ingredients of PGG, but

Table 2: Data on total reducing power of PGG

Sample	Ascorbic acid equivalent ($\mu\text{g}/10 \text{ mg}$ of PGG)	
	PGGP	PGGM
Mridu	0.262	5.984
Madhyama	0.305	6.355
Khara	0.314	6.150

PGG = *Panchagavya Ghrita*, PGGP = PGG in petroleum ether, PGGM = methanol fraction of PGG

Table 3: Data on DPPH free radical scavenging activity of PGGM fraction

Sample	% Scavenging activity/20 mg of PGG
Mridu	18.17
Madhyama	15.39
Khara	16.07

PGG = *Panchagavya Ghrita*, PGGM = methanol fraction of PGG

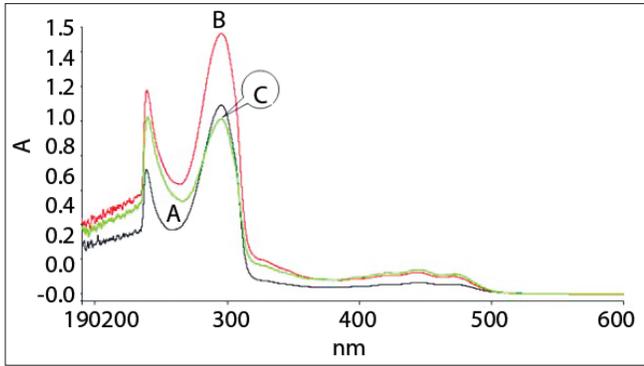


Figure 1: Comparative UV-visible spectra of unsaponifiable matter of PGG samples revealing difference in absorption intensities. A = Mridu Paka, B = Madhyama Paka, C = Khara Paka

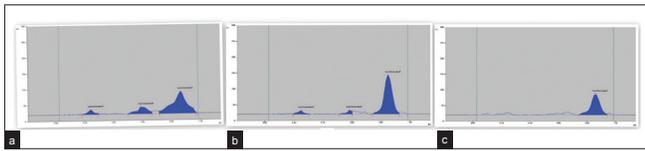


Figure 3: HPTLC densitogram of PGGM. (a) Mridu, (b) Madhyama, and, (c) Khara revealing quantitative difference

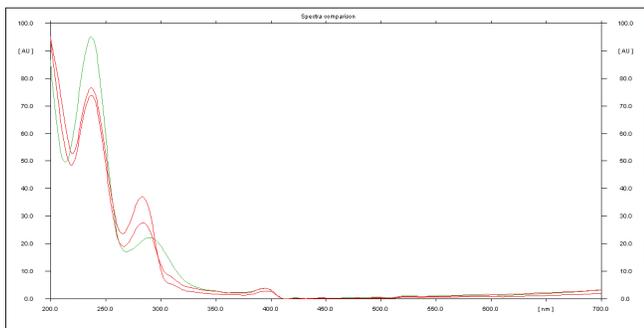


Figure 5: Comparative spectra (Rf 0.87) of PGGM samples exhibiting similar pattern but with quantitative difference

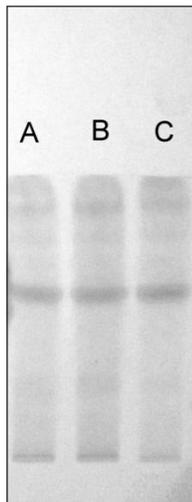


Figure 6: PGGM samples after spraying with anisaldehyde-H₂SO₄ reagent showing almost similar pattern. A = Mridu Paka, B = Madhyama Paka, C = Khara Paka



Figure 2: Comparative TLC profile of unsaponifiable matter with almost similar pattern

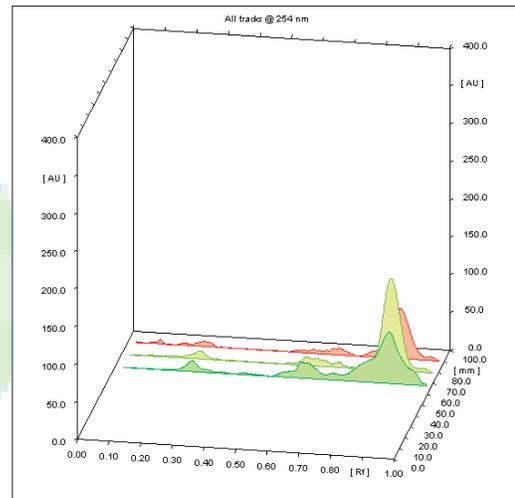


Figure 4: Comparative 3D-graph of PGGM samples revealing quantitative difference



Figure 7: HPTLC-DPPH bioautography of PGGM samples exhibiting mild antioxidant activity. A = Mridu Paka, B = Madhyama Paka, C = Khara Paka

processed in a different manner, has been reported to possess antioxidant activity.^[16] Hence, it was thought worth of evaluating all the samples for their antioxidant activity.

A preliminary HPTLC-DPPH bioautography assay revealed two minor yellowish spots in all the samples indicating presence of antioxidant compounds. Hence, quantitative assessment of *in vitro* antioxidant activity was also carried out by two methods.

Moderate antioxidant activity, without any significant difference, was revealed in all the PGGM fractions in both—total reducing power as well as DPPH assay methods.

Considering the method of preparation of *Snehapaka*, both polar as well as non-polar constituents are expected to be present in PGG. Though methanol will extract considerable amount of both polar and non-polar constituents, but it will not represent the entire sample. Hence, to have an idea about the biological activity of the sample as a whole, antioxidant activity of PGG taken in petroleum ether (since ghee is the base of PGG) was also assessed. The data regarding total reducing power, presented in Table 2, reveals about 20 times more activity in PGGM, which is noteworthy. In DPPH assay, the PGGP did not reveal any activity while PGGM showed moderate activity. In both the assay methods, PGGM showed better antioxidant activity as compared to PGGP i.e. Panchagavya ghrita as a whole indicating partition of active constituents in methanol fraction.

CONCLUSION

Mridu, *Madhyama* and *Khara Paka* of *Panchagavya ghrita* have been comparatively assessed by employing different modes of evaluation to evolve suitable parameters to differentiate among them. Comparison of their various physicochemical parameters, chromatographic profiles, and *in vitro* antioxidant activity determination is of little help in establishing any significant difference. Spectrophotometric analysis of unsaponifiable matter, emerges as a plausible assessment criterion in establishing difference among the three stages of processing of *Panchagavya Ghrita* as well as *Snehapaka* in general.

The present study clearly reveals quantitative difference in the chemo-profiles of *Mridu*, *Madhyama* and *Khara Paka* of *Panchagavya Ghrita*. This first step supports that identification of the chemical components and their quantification will prove very much useful to differentiate them. HPTLC-Mass Spectrometry (MS) for methanolic fraction and unsaponifiable matter as well as Gas Chromatography

(GC)-MS targeting the fatty portion will definitely be of help while extending the study further.

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