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Process Standardisation of *Ghrita Avartana* with special reference to *Guduchi Ghrita* and its Immunomodulatory activity

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ABSTRACT:
Siddha ghrita and taila(medicated lipids) are the most widely used Ayurvedic dosage forms that posea great challenge of palatability owing to strong odor, greasiness, and higher dose in the present era. A major need for standardisation of these dosage forms, arises due to the crude methods of manufacture. Avartana, mentioned in context with snehakalpana means potentiation of medicated lipids by repeated heating cycles using the same ingredients which may offer dose reduction. This study was intended to manufacture and developSOP for the process of ghritaavartana, using Guduchighrita from Chakradatta Vataraktachikitsaadhyayaas an experimental drug. Comparative immunomodulatory activity of Guduchi Ghrita was assessed with its three and seven times avartita versions. Randomised controlled pre-clinical study using cell mediated immunity model in Wistar strain Albino rats of both the sex. The first step in the standardization of process ofghritaavartana was the preparation of Guduchighrita(GG). Later this Guduchighrita was subjected to 3 and 7 repetitions of heating cycles. Three batches of test drugs were prepared to get total of 9 samples. Later three samples were randomly selected and subjected to experimental study. Paired t-test was applied to arrive at the possible conclusion. The mean of the samples complied with parameters as per API confirming adherence to SOP. In the present study 3 times potentiated Guduchighrita showed significant inhibition of paw edema suggestive of effect and limit of potentiation. Experimental evaluation of the test drugs indicated that three times avartitaghrita has significant immunomodulatory activity when compared to the Guduchighrita and seven times avartitaghrita, which may be suggestive of the increased therapeutic effect of avartana as well as limit of potentiation.
***Keywords**:Avartana, Siddha ghrita, Guduchighrita, Immunomodulatory activity.*

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INTRODUCTION

Sneha kalpana comprising *siddha ghrita* (medicated ghee) is one of the most popular among clinicians in day-to-day Ayurveda practice. Cow ghee is the best among the lipids, as it incorporates the properties of ingredients with which it is processed without losing own properties [1]. *Siddha ghrita* are multi-purpose formulations which can be delivered through oral, nasal and topical routes for treating several diseases. These lipid dosage forms transfer aqueous herbal extracts into a lipid base there by enhancing the absorption and therapeutic potential of the formulation.

Ayurveda pharmaceutics adheres to the principles of *sanyoga* and *vishlesha. Avartana* is one such technique explained by ancient intellectuals of Ayurveda where the principle of *sanyoga* is followed. In this procedure, Cowghee is repeatedly processed with same ingredients to enhance the therapeutic efficacy of the medication. A*vartita*ghee formulation is predominantly mentioned for *rasayana* purpose [2].

Here an attempt has been made to standardize the process of *avartana* of *ghrita*with *guduchighrita* as the model drug. *Guduchighrita* is mentioned in the treatment of *Vatarakta*in the textbook of *Chakradatta Vatarakta Chikitsa* [3]. *Guduchi*, the drug of choice is described as best drug for the treatment of *Vatarakta.* (*Asht.Hr.Ut.40/59*). It means *Guduchi* can be used as rasayana for disease *Vatarakta*. The alcoholic and aqueous extracts of *T.cordifolia*are reported to have beneficial effects on the immune system. Hence, immunomodulatory activity using the model of CMI (Cell Mediated Immunity) of one-time processed *guduchighrita* has been compared with three and seven times processed i.e., *avartitaguduchighrita*.
A comprehensive review on *guduchighrita* has revealeda few of its important pharmacological actions like anti-pyretic [4], immunomodulatory [5], adaptogenic activities [6].
Four different publications on Avartana were studied by authors wherein, Biswajit Patgiri et.al. (2008) observed a gradual shift of *taila pakasiddhilakshana* (*Phenodgama)* to *ghritapaka siddhi lakshana (Phenashanti*) by 19th*avartana* of 1-7-50- *avartitaksheerabalataila* [7]. C. Roshy Joseph and R. Illanchezhian (2010) studied that the *saptavartitahingusauvarchaladighrita* has shown better anticonvulsant activity in comparison to plain *hingusauvarchaladi ghrita* in PTZ induced mice model [8]. Zala et.al (2012) observed that Avartita Panchatikta Ghrita has significant anti-inflammatory and analgaesic potential whereas Murchita Panchatikta Ghrita showed only analgaesic action [9]. Also, Gadgil S. and Wele A. (2020) have reviewed the process of *avartana*~potentiation and further established that it results in increasing the amount of bio actives into the ghee [10].
In this study, an attempt has been made to standardize the process of *avartana* and its reflection in effect on experimental animals through immunomodulatory activity.

MATERIAL AND METHODS

Pharmaceutical study

Materials

Guduchi [*Tinospora cordifolia* (*Willd*) Miers] stem in the fresh form was identified and collected [11]. Authentication and analysis were done from the Botany Department of authorized institute. It was used for the preparation of *kalka*(paste)and *kwath* (decoction). Cow-ghee was procured from an authentic source. Ghee was prepared from the milk obtained from Gir cow (native Indian breed) using traditional method practiced in Indian household [12]. Ghee was analytically tested in an ISO certified lab and was used further as the base for preparation of *Guduchighrita.* Cow milk of the FDA approved company was collected, and physico-chemical analysis was done at the ISO certified lab and was used for preparation of test drugs.

Methodology

Pharmaceutics of the study samples.

The first step in the standardization of process of *avartana* was the preparation of *Guduchighrita*(GG). Later this *Guduchighrita* was subjected to 3 and 7 repetitions of heating cycles. Three batches of test drugs were prepared to get total 9 samples. The following steps are involved in the process of preparation.

Preparation of *guduchikalka* and *kwatha.*

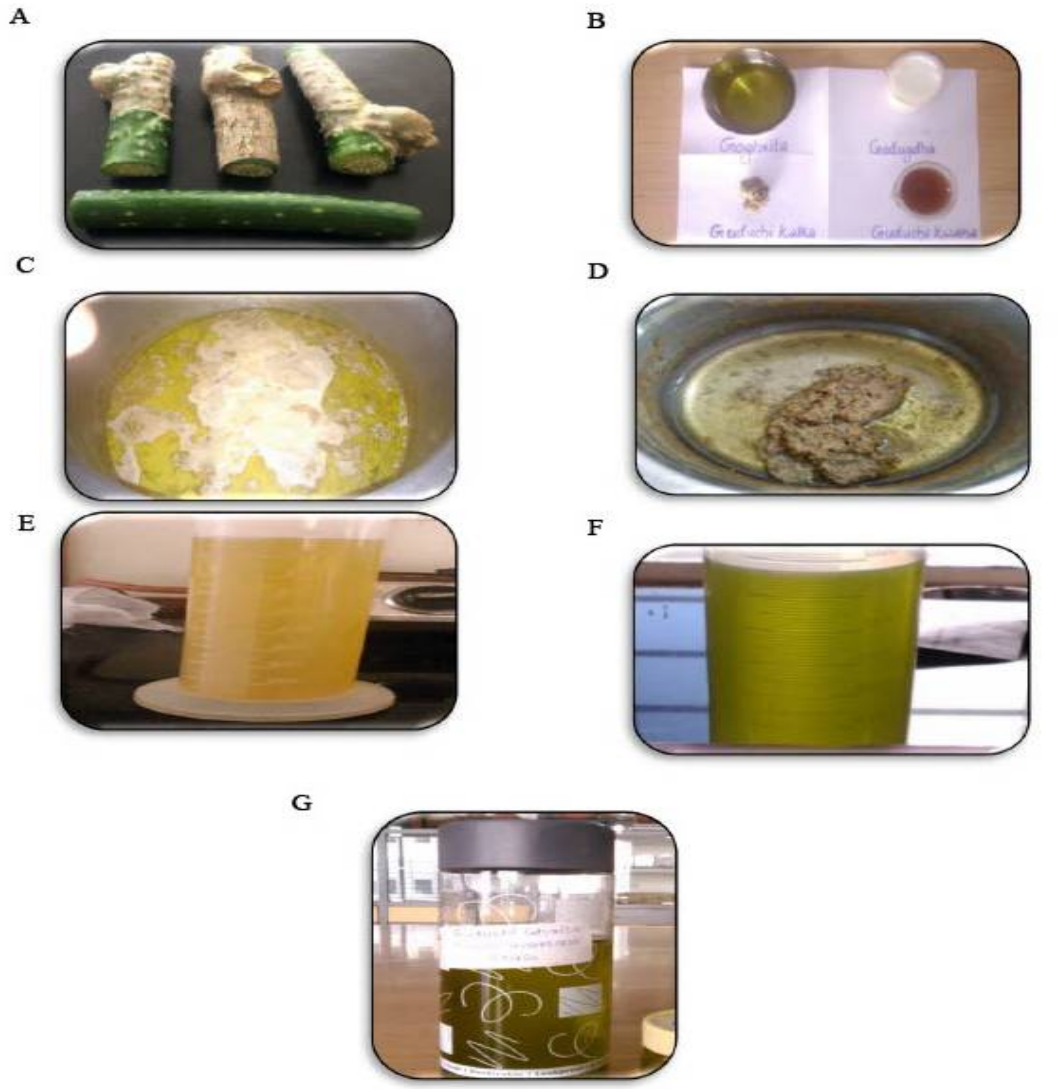
167 gm fresh *guduchi* stem was pounded until it forms a soft semisolid mass or *kalka* which was used as the *kalkadravya.*
For the preparation of *kwatha,* 1 kg of fresh *guduchi* stem cut into pieces of equal size (1-2 cms) was taken. It was cleaned thoroughly with water and wiped well. It was further pounded, 8-liter water was added, soaked overnight, and subjected to heating until it gets reduced to 2 liters. 2-litre *kwatha* was taken as the first *dravadravya.*

Preparation of Guduchighrita

Guduchighrita was prepared with, 1 kg cow ghee, 167g *guduchikalka*, 2litre *Guduchikwatha*, and 2litre *cow milk.* Cow ghee was taken in a thick bottom stainless-steel vessel. It was subjected to heating over *low flame* (50-54^{0}c) and then the guduchi*kalka* was added. *Kalka* was fried until a good aroma of *kalkadravya*came. Then the *Guduchikwatha* was added. When the temperature of mixture reached 70^{0}c- 75^{0}c, boiled cow milk of temperature 70^{0}c was added to the above mixture. According to the guideline from *Sharangdhara Samhita,* whenever cow milk is used in *snehapaka,* it is imperative to add water for the optimum extraction of active principles and appropriate boiling of *Sneha* [13], 4 litres of water was added to the above mixture and *snehapaka* was carried out on constant medium flame (96-98^{0}c). It was stirred continuously until the *sneha siddhi lakshana* were obtained [14].

Preparation of Avartita Guduchi Ghrita

Figure-1: Pharmaceutics of *Guduchi Ghrita* and its *Avartana*.



A. Fresh *Guduchi* stem; B. Ingredients for *Guduchi ghrita* preparation; C. *Snehapaka* in process; D.*Sneha* gets seperated from the *kalka*; E. *Guduchi ghrita* (GG); F. Three times *avartita Guduchi ghrita* (A3GG); G. Seven times *avartita Guduchi ghrita* (A7GG)

The filtered ghee after the first paka was used as the base material for the preparation of further samples. In this way every time siddha ghrita from preceding paka was used as the base material for succeeding snehapaka until 7thavartana. The process was repeated for the preparation of the next two batches, to develop the SOP for the process. Every time freshly prepared Guduchikwatha, Guduchikalka and Cow milk were added. For all the three batches, the paka or heating was carried out for 2 days and nights and Sneha was filtered on the third day to comply with the ushitapaka method explained by acharya Sharangdhara [15].

Experimental Study

Experimental study was carried out after obtaining permission from institutional animal ethics committee, (Research project No: 265) of National Toxicology centre, Pune.

Materials

Wistar Strain Albino rats of either sex weighing around 200-250 gm, Test Drugs (GG, A3GG, and A7GG), Triple antigen solution (triple antigen 1 ml, Normal saline 0.9% 4ml, Potash alum 10% 1ml), Vernier calliper.

Methodology

Husbandry conditions: Animals were exposed to natural day and night cycles with ideal laboratory condition in terms of ambient temperature (22 ± 2ºC) and humidity (50 to 60%).

Dose fixation: The dose of *siddha ghrita* as mentioned in API (Ayurvedic Pharmacopoeia of India) is 10 gm. Based on this, dose for the experimental study was calculated as per the table of Paget's and Barnes 1964, by extrapolating the human dose to animal dose based on the body surface area ratio.[16] This was calculated as 0.960g/kg body weight for rats.

Animal grouping: The selected animals were assigned to different groups randomly, each group comprised of six animals with three males and three females. Grouping of animals was done according to test drugs, ***table 1.***

Table 1: Grouping of animals according to the test drugs.

Sr. No	Group	Test Drug	No of animals	Dose	Route of administration	Duration
1.	Disease Control (Triple antigen injection)	No test drug given	6	0.1 ml	Injected on the hind paw	On the 7th day.
2.	GG	*GuduchiGhrita*	6	0.960g/kg body weight of rats	Orally through gastric catheter	For 7 days (Followed by injection of triple antigen solution on the hind paw)
3.	A3GG	3 times *avartitaGuduchighrita*	6	0.960g/kg body weight of rats	Orally through gastric catheter	For 7 days (Followed by injection of triple antigen solution on the hind paw)
4.	A7GG	7 times *avartitaGuduchighrita*	6	0.960g/kg body weight of rats	Orally through gastric catheter	For 7 days (Followed by injection of triple antigen solution on the hind paw)

Procedure: The animals were sensitized subcutaneously on the first day of drug administration at the nape of neck with the Triple antigen solution [Triple antigen 1 ml, Normal saline 0.9% 4ml, Potash alum 10% 1ml (0.5 ml/ 100 gm body weight)]. pH of above solution was maintained between 5.6-6.8 using 10% sodium carbonate. Each group was administered test drug for seven consecutive days. The test drugs were administered according to the body weight of the animals by oral route with the help of gastric catheter of suitable size sleeved to a syringe nozzle. On the 7th day, one hour after drug administration, initial volume of hind paw of each animal was noted then 0.1 ml of the Triple antigen solution was injected into the plantar aponeurosis of the same paw. Volume of immunological oedema thus produced was measured using vernier calliper immediately after injection, after 24 hours, and after 48 hours [17].

FIGURE 2: PICTORIAL PRESENTATION OF EXPERIMENTAL STUDY

RESULTS AND DISCUSSION

Raw materials.

Organoleptic characteristics of *guduchi, guduchikwatha*, cow milk and cow ghee have been recorded in ***table 2.***

Table 2: Organoleptic characteristics of ingredients of Guduchighrita

Sr. No	Parameter	*Guduchi*	*Guduchikwatha*	Cow milk	Cow ghee
1.	Colour	Greenish	Light Brown	Creamy white	Yellowish
2.	Odour	Specific (bitter after removing outer skin)	Specific	Characteristic	Characteristic
3.	Taste	Bitter	Bitter	Sweet	Sweet
4.	Consistency	Soft, Slimy	Watery	Watery	Viscous

Physico-chemical characteristics of *guduchi, guduchi kwatha*, cow milk and cow ghee.

Guduchi was devoid of any foreign matter. Its loss on drying at 110^0c was 79.05%, Ash value-1.69, and water-soluble extracts were 16.19%.

For *guduchi kwatha* the ash value was 0.86%, water soluble extracts- 99.11%, pH- 6.09, and specific gravity-1.0102 g/ml.

Cow milk, the pH- 6.83, specific gravity- 1.031g/ml, total fat-6.2%, total solids-15.05% and Solid Not Fats (S.N.F) was 9.05%

For the cow ghee, following parameters were studied. pH- 5.48, specific gravity- 0.9098, viscosity at 40 degrees-1.4606 CPS, acid value- 0.45, saponification value- 217.28, and total fat was 99.8 %.All the values complied with standard parameters of API.

Observation during the preparation of three batches of GG, A3GG and A7GG.

The initial quantity of ghee was 1000 ml each for three batches. An average loss of 4 % was observed after the completion of the first*paka.* With the succession of *paka*, for 3rd avartana an average of 4.4 % gain was observed and for 7th*avartana*, an average gain of 5.6% was there due to the addition of milk fats.150-200ml sample was removed each time after 1st, 3rd, and 7th*avartana* for the purpose of physicochemical analysis and experimental study.

As per *Sharangdhar Samhita*, the *ushitapaka* method was adopted in the present study. Average duration for the completion of 1st*paka* was around 6 h and 30 min (3hrs on 1st day, 2 hrs. on the 2nd day and 1.5 hrs. on the third day before filtration). Duration for 3rd*avartana* was 6 h (3 hrs. for 1st day, 2 hrs. for 2nd day and 1 hr. on the 3rd day.) and for 7th*avartana*, an average of 5 h 30 min (2 hrs. on 1st day, 2 hrs. on 2nd day and 1.5 hrs. on 3rd day).

Observations of final product.

Guduchighrita was yellowish in colour. At the end of 3rd*paka* it became greenish and at the end of 7th*paka*, slight dark green colour was observed. Bitter taste was increased with the progression of *paka.* It became thicker with 3rd*avartana* whereas there was not much change in consistency after 3rd*avartana*till 7th. The samples possessed characteristic odour corresponding to the smell of *guduchi.*

The values of the analytical tests and their means has been quoted in ***table 3***.

Table 3. Physico-chemical parameters of the final products- GG, A3GG and A7GG.

Sr. No	Parameter	Sample	Batch 1	Batch 2	Batch 3	Mean
1	Rancidity	GG	Negative	Negative	Negative	Negative
		A3GG	Negative	Negative	Negative	Negative
		A7GG	Negative	Negative	Negative	Negative
2	Specific gravity (gm/dl)	GG	0.9135	0.9082	0.9114	0.911
		A3GG	0.9475	0.9067	0.9129	0.9224
		A7GG	0.9098	0.9096	0.9094	0.9096
3	Refractive index (rf)	GG	1.4608	1.4604	1.4606	1.4606
		A3GG	1.4616	1.461	1.4606	1.4611
		A7GG	1.4601	1.4602	1.4603	1.4602
4	Acid value (mgKOH/gm)	GG	0.35	0.45	0.54	0.45
		A3GG	1.15	1.09	1.08	1.11
		A7GG	1.57	1.54	1.56	1.56
5	Saponification value (mgKOH/gm)	GG	221.17	227.34	218.43	222.31
		A3GG	225.04	232.34	227.87	228.42
		A7GG	229.87	235.69	232.06	232.54
6	Viscosity (cP)	GG	10.5	9	8.93	9.48
		A3GG	13.5	9.13	9.07	1057
		A7GG	13.92	11.36	9.42	11.6

Observations during the Experimental study

Triple antigen injection in the hind paw caused the occurrence of paw oedema in rats. The volume of immunological oedema thus produced was measured using vernier callipers[17] and the mean of paw oedema on 7th day immediate after injection, after 24 hour and 48 hours has been compared in ***table 4.***

Table 4: Mean Paw oedema on 7th day before injection, immediately after injection, after 24 h and48 h.

Group	7th day before injection	7th day immediately after injection	After 24 hrs	After 48 hrs
Disease control	3.74	5.42	8.41	8.37
Test Drug 1 (GG)	3.80	6.16	7.56	7.49
Test Drug 2 (A3GG)	3.75	5.98	6.70	6.59
Test Drug 3 (A7GG)	3.80	6.19	7.01	6.85

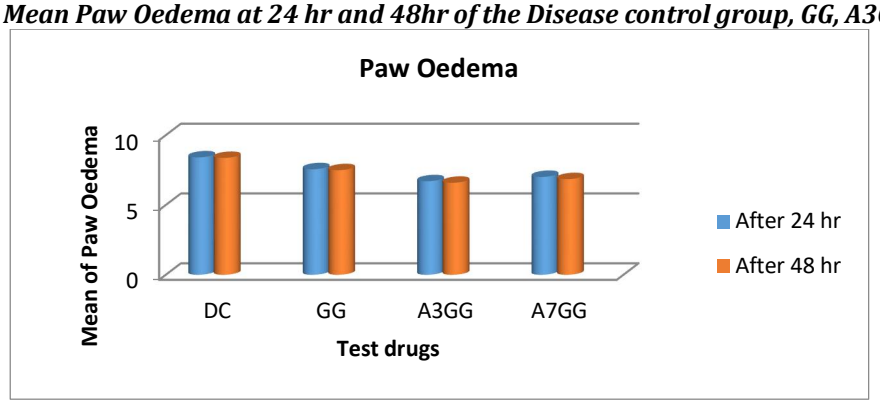
Percentage of Inhibition of paw oedema can be calculated with the following formula,

[(A-B)/A] X 100, where, A is the paw oedema of Control group, B is the paw oedema of test drug treated group.

When compared to the disease control, the formation of paw oedema after 24 h and 48 h was slow in test drug treated groups. The test drug 2 (A3GG) showed a significant inhibition (P<0.001) of occurrence of paw oedema when compared to the GG and A7GG.

The mean paw oedema at two different time points after triple antigen injection of disease control and test drug treated groups has been plotted in **Graph 1.**

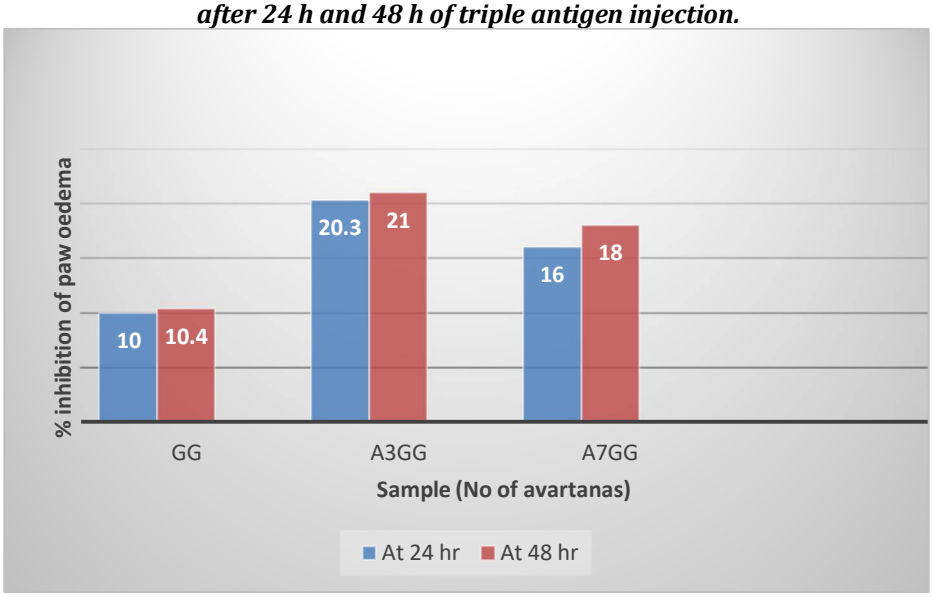
Graph 1: Mean Paw Oedema at 24 hr and 48hr of the Disease control group, GG, A3GG, A7GG



DC: Disease Control Group, GG: Guduchi Ghrita, A3GG: Three times Avartita Guduchi Ghrita, A7GG: Seven times Avartita Guduchi Ghrita.

The percentage inhibition of paw oedema with different test drug treated groups has been plotted in **Graph 2.**

Graph-2: Percentage inhibition of Paw oedema with the test drugs, GG, A3GG and A7GG after 24 h and 48 h of triple antigen injection.



DISCUSSION

Siddha ghrita are the formulations needed to be taken in a higher dose which is quite inconvenient for the patient. Hence an attempt has been made to study the role of the process of *avartana*in potentiation of formulation thereby increasing the therapeutic efficacy. Repeated processing cycles of lipids with similar ingredients may cause certain thermogenic alterations which may simplify the compounds of the medicament and may provide maximum surface area for absorption, enhancing its bioavailability and efficacy.

Cow ghee prepared by traditional method has been used as base material for the process. Docosahexaenoic acid (DHA) is more in the ghee prepared by traditional method using curd starter cultures compared to the ghee prepared with the direct cream method [18]. DHA along with other Conjugated linolenic acids (CLA) are responsible for the health benefits exhibited by the ghee [18].

In the present study murchana process is not carried out. Murchana is a concept in *snehakalpana* which came into light after 19th century. It is not mentioned in *Brihatrayee*. Though GhritaMurchana is spotted for the first time in Chakradatta Ratnaprabha by Nishchalkara commentary, it is explained in detail in *Bhaishajyaratnavalijwara chikitsa* 5/128, where it is mentioned to improve the colour, odour, and shelf life of the preparation by controlling the rancidity.[19] The process of *murchana*, adds on active principles of herbal ingredients used in the process which may have a role in efficacy of the final product. Primary aim of present study was to assess the effect of *avartana* process; hence it was important to use single ingredient *guduchi* for clarity of analysis and interpretation.

Fresh *guduchi* stem was used for the study as per the textual reference of *Sharangdhara*. For the preparation of *guduchikwatha*, stem was cut into pieces of equal size of 1-2 cms and soaked overnight to facilitate maximum extraction of active principles. No water was added while preparing *kalka* as fresh drug was used. During the preparation of *guduchighrita,* after frying *kalka* in ghee for some time, *guduchikwatha* was added initially. Second *dravadravya,* cow milk was added after maintaining the temperature of the mixture and milk at around 70^0c to avoid the spoiling of milk. The reason behind the maintenance of temperature below boiling point is explained as follows. Temperature above boiling point facilitates the clustering of milk protein casein and fats, and this is further catalysed by the presence of acidic *guduchikwatha* in the mixture [20]. Every time after the completion of third *avartana,* there was increase in the yield which may be due to addition of milk fats.

SOP for the process of *avartana* was developed taking *guduchighrita* as the experimental drug. The organoleptic characteristics of *guduchighrita* prepared in 3 batches showed a similar pattern showing a batch-to-batch consistency. The color and taste were getting more and more intense with progression of number of *snehapaka,* which may be due to the increased extraction of active principles of *guduchi* into the ghee, however its qualitative or quantitative analysis was out of preview of the study. The thicker consistency at the third *avartana* may be due to the addition of milk fats into the ghee and further not much change was observed after 3rd*avartana*till 7th which may be suggestive of limit of potentiation.

Specific gravity is a relative ratio which indicates the densities of substances in relation to water. It alters with change in temperature and pressure. In the present study it was found to be increasing till the third *avartana*which may be due to the accumulation of the milk fat and the other chemical constituents of the *guduchi*.

Refractive index (RI) is the ratio of velocity of light in vacuum to the velocity of light in a specific medium. At the third *avartana*, value of RI was found to be slightly greater when compared to the plain cow ghee and *guduchiGhrita*. This may be due to the increase in density of the lipid media due to the accumulation of milk fats as well the poly herbal aqueous extracts of *guduchi*. Subsequent *avartana* again showed a decrease and the values complied with that of cow ghee, which may be due to the dissociation of clustered fats.

Acid value was found to be increasing with each *avartana* as every time the *paka* was done by the addition of fresh liquid media and *guduchikalka*. Acid value indicates the Free Fatty Acids (FFA) present in the *ghrita*. Formation of FFA is an important measure of Rancidity. They are formed due to hydrolysis of triglycerides and may be promoted by the reaction of *ghrita* with moisture. The fatty acid profile affects the shelf life, flavour, and stability of *ghrita*. Hence it can be said that shelf life of *guduchighrita* and *avartitaguduchighrita* is less than the plain cow ghee.

Saponification value gives the average molecular weight and indicates the length of carbon chains of the acid present in that fat or oils. Ghee is rich in short chain fatty acids and a further increase in the saponification value indicates a lower molecular weight and higher percentage of short chain fatty acids. Increase in saponification value indicates that during *ghritapaka* there may be conversion or dissociation of long chain fatty acids into short chain fatty acids.

In the experimental study, appearance of paw oedema immediately after the injection of triple antigen is suggestive of cell mediated immunological response. Immunomodulatory activity of the three trial drug samples i.e., GG, A3GG, A7GG was compared with each other as well as with the disease control. Among the test samples A3GG showed significant decrease of paw oedema at 24 h and 48 h interval than GG as well as A7GG at the same dose. Also, the percent inhibition of paw oedema was also significant with A3GG as compared to GG. This indicates that the cell mediated immunity suppression could contribute to the therapeutic efficacy of the drug. This experiment led us to confirm the *avartana* process endpoint at 3 repetitions. It may make the formulation time-cost effective with maximum potency.

CONCLUSION

In this study, SOP for the process of *avartana* was developed with *Guduchighrita* as the study drug. *Guduchighrita* and *avartitaghrita* have shown batch to batch consistency in pharmaceutical procedures as

well as on the analytical parameters. Experimental evaluation of the test drugs indicated that three times *avartitaghrita* has significant immunomodulatory activity when compared to the *guduchighrita* and seven times *avartitaghrita,* which may be suggestive of increased therapeutic effect of *avartana* as well as limit of potentiation. This may further open a scope to reduce the dose of formulation.

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