

Physicochemical and marker based optimization of fermentation process of *Drakshasava*, an Ayurvedic polyherbal formulation

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Received 11 March 2019; revised 31 October 2019

Drakshasava a classical herbal formulation containing *Vitis vinifera* L. (grapes) as a chief ingredient. It is one of the best *asava* preparation, referred in Ayurvedic Formulary of India (AFI). The present study explores the changes brought by fermentation during the processing of *Drakshasava* with respect to physicochemical parameters using correlation marker based approach. The grapes were extracted to form decoction and further fermented with *Woodfordia* flowers along with *prakshepadravyas* (additional drugs in small quantity) and sugar source, as per AFI, part II. The fermented broth was analyzed daily for 14 days for various physicochemical parameters. Further marker based profiling was done by High Performance Thin Layer Chromatography (HPTLC) with respect to caffeic acid and kaempferol. The fermentation took 3 days lag period for generation of quantifiable alcohol (0.5%) on 4th day to 16.7% on 14th day. The admissible limit of alcohol for *Drakshasava* as per API (5-10%) has been reached on 9th day itself. The pH, specific gravity, solid content has been decreased from day 0 (5.12; 1.32; 28.25) to 14th day (4.35; 1.15; 22.81) respectively whereas phenolic content has been upgraded remarkably (0.040 to 0.165 g/100 mL) during the study. Kaempferol quantity was found to be decreased (0.186% to 0.0810%) after fermentation whereas caffeic acid quantity has been significantly increased in post fermented sample with respect to decoction (0.0168% to 0.0233%). The change brought in quantity of the phytochemicals, indicates the metabolic conversion during fermentation due to glycosylation or deglycosylation. The research limits the fermentation time for preparation of *Drakshasava* from 21 days to 8-9 days, saving time and money. The new optimized method adheres to the Ayurvedic principles and opens the scope for better quality industrial research and large scale production.

Keywords: API, *Drakshasava*, Fermentation, Grapes, HPTLC, *Vitis vinifera*

IPC Code: Int. Cl.²⁰: A61K 36/00, A23F 3/08, A61K 36/87, C07K 1/16, A61K 36/87

Ayurveda is one of the traditional systems of healthcare being practiced in Indian Sub-continent since ages, often known as 'Mother of All Healing'¹. Its origin dates back to 5-10000 BC as per the ancient Indian literature². It is a holistic medicinal system with multidimensional approach to diagnose and treat various ailments including cardiovascular, neurological, inflammatory, cancer and many others. It employs vast use of single, poly or herbomineral drugs and formulations, documented in various Ayurvedic texts namely *Charaka Samhita*, *Shushruta Samhita*, *Ashtanga Hridaya*, *Kashyapa Samhita*, *Chakradatta*, *Gada Nigraha*, *Sharangadhara Samhita*, *Yogaratanakara*, *Bhaisajya Ratnavali*, Ayurvedic Formulary of India (AFI) etc.³⁻⁷ Ayurveda is developed with time from relationship between human with nature universe through ideas, instincts and daily life

experience⁸. All Ayurvedic formulations involve transfer of active constituents of herbs in menstruum by different extraction processes. Among these, '*Sandhana kalpana*' (*Asava and Arista*) is a unique dosage form which may be acidic and involves hydroalcoholic extraction of active compounds⁹⁻¹⁰. *Asavas* (fermented herbal infusion) and *Aristas* (herbal decoction) are the fermented Ayurvedic herbal drug preparations; significantly prescribed in AFI. These preparations are considered superior over tinctures for absorption in gut, former being digested partly only⁵⁻⁷.

Fermentation involves a gradient of rising alcohol which leads to extraction and biochemical transformation of wide range of phyto-compounds through enzymatic activity of added microorganisms¹¹. Self-generated ethyl alcohol is produced along with acetic acid and extraction of active principles of the herbal drugs is done¹²⁻¹³. Thus, the fermented Ayurvedic formulations (*Sandhana Kalpana*) in

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comparison to other Ayurvedic dosage forms have better absorption, longer shelf life and high therapeutic efficacy. The process removes toxicity (bind with heavy metals) and undesirable sugars rendering the product more bio-available¹⁴. Also, *prakshepa dravyas* are added in *Sandhana Kalpana* formulations for improving their assimilation.

Chronologically, the fermentation technique is being used from past *centuries* to present date in different forms of alcoholic drinks (wines and beer) and adapted by numerous civilizations all over the world. Usually these drinks are formulated from variety of cereals, tree barks, fruits like grape and maple. Grapes (*Vitis vinifera* L.) are chief ingredient of the *Drakshasava*, a well-known classical herbal formulation¹⁵. It is out of the 27 *Asava* preparations, mentioned in AFI with 10 additional herbs added apart from grapes providing flavour, enhanced bioavailability and assimilation to the formulation. Its a blood purifier and widely used in treatment of anaemia, piles, digestive disorders with advised remedy in cardiac, respiratory, circulatory and other inflammatory problems due to its powerful antioxidant properties¹⁶⁻¹⁹. *Charaka* has described *Drakshasava* as *Phalasavas* (fruit preparation) mentioned in *Charaka Samhita* (hymn 25:48). *Drakshasava* is used for the promotion of healthy life as a general tonic.

The primary principles behind various fermented products remained similar fundamentally from modern era to those widespread in ancient times. The basic difference was noticed is in type of equipment used, raw drugs, methodology of preparation, sterilization and essentially standardization²⁰. To cope up with growing needs of public health worldwide, along with herbal industries and market all around the world, optimization and standardization of existing Ayurvedic formulations with multi-marker approach is required. In the present research, *Drakshasava* preparation and the process optimization with modified, non-conventional technique has been studied. Further its standardization with non-established biomarker (caffeic acid and kaempferol) has been carried out by High performance Thin Layer Chromatography (HPTLC). Both the selected biomarkers belongs to group of polyphenols, i.e., rich in antioxidants; widely found in *vitis* species thus, *Drakshasava* has been selected as area of research being prepared majorly from grapes and to evaluate the quantitative changes in the selected phenolic markers before and after its preparation.

Material and methods

Standards and chemicals

The organic solvents of analytical grade were procured from Merk (Darmstadt, Germany). All the weighing procedures were performed using electronic balance from Citizen (Mumbai, India). For pH determinations, pre calibrated digital pH meter was used. Spectro-photometric analysis was done using Shimadzu (Kyoto, Japan) double beam Spectrophotometer (UV 1800). HPTLC analysis was performed using CAMAG Linomat V applicator operated using win CATS software (CAMAG, Muttenz, Switzerland). HPTLC Plates were aluminium backed silica gel 60 F₂₅₄ from Merk. Marker compounds (Caffeic Acid and Kaempferol), purity (98%) were procured from Natural Remedies Pvt. Ltd. (Bangalore, India).

Plant materials and formulations

The herbs used in *Drakshasava* preparation were procured from Ayurvedic shop from the local market (Delhi). It was deposited and authenticated (Ref no. PP-V0117) under the supervision of Prof Vidhu Aeri at Pharmacognosy Lab, Jamia Hamdard, New Delhi Materials were stored in air tight containers. The marketed formulation of *Drakshasava* was purchased from the Unjha Ayurvedic Pharmacy, Delhi.

Preparation of *Drakshasava*

The in-house *Drakshasava* (non conventional formulation) was prepared as per AFI (Part-ii) with certain modifications. The composition is given in Table 1. The required quantity of the drug (dried grapes) was overnight soaked in water. The mixture was grinded afterwards and boiled to prepare decoction (1/4) which was further filtered with muslin cloth. It was followed by adding jaggery sugar (*Sarkara*) and honey; stirred properly until homogeneous solution was obtained, following a final filtration. All the other ingredients (*Prakshepa dravyas*) were powdered and passed through sieve no 44. To the filtrate, the *Prakshepa dravyas* were added along with *Woodfordia fruticosa* L. (dhataki flowers). The mixture was kept in sterile, air tight containers at 30±20°C in incubator for fermentation (Fig. 1). The samples were taken out daily for physicochemical studies of 15 days. The samples of decoction, in-house fermented formulation and marketed formulation were weighed each day and their physicochemical studies were done²¹.

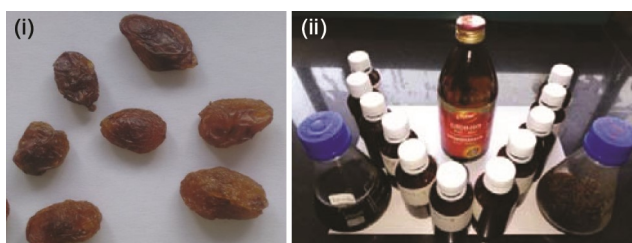
Physicochemical characterization

Preliminary evaluation

Determination of organoleptic characteristics, viz., odour, taste and colour of the prepared *Asava* was carried out.

Table 1 — Composition of *Drakshasava* formulation

S. no	Name of plant	Botanical name	Part used	Quantity prescribed (as per AFI)	Quantity used
1	Draksha API	<i>Vitis vinifera L.</i>	Dried fruit	4.8 kg	2.4
2	Jala for decoction	Water	-	49.152L to 12.288L	24.57 L to 6.14 L
3	Sarkara API	-	-	4.8 kg	2.4
4	Madhu API	honey	-	4.8 kg	2.4
Prashpekadravya					
5	Dhataki API	<i>Woodfordia fruticosa</i>	Flower	336	168
6	Jati API	<i>Jasminum officinale</i>	Flower	24	12
7	Lavaga API	<i>Syzygium aromaticum</i>	Flower bud	24	12
8	Kakkola (Kankola) API	<i>Piper cubeba</i>	Fruit	24	12
9	Lavaliphala API	<i>Ciccaacida</i>	Fruit	24	12
10	Candana (sáveta Candana) API	<i>Santalum album</i>	Heart wood	24	12
11	krsna API	<i>Piper longum</i>	Fruit	24	12
12	Tvak API	<i>Cinnamomum zeylanicum</i>	Stem bark	24	12
13	Ela API	<i>Elettaria cardamomum</i>	Seed	24	12
14	Patra (Tejapatra) API	<i>Cinnamomum tamala</i>	Leaf	24	12

Fig. 1 — (i) *Draksha* fruit; and (ii) sterile containers for *Drakshasava* preparation**Total solids**

Accurately weighed 50 mL of clear *Asava* was extracted with four equal quantities (10 mL) of ethanol, and evaporated to a thick extract. Then, dried diatomite (1 g) was added. The mixture was dried at 105°C till constant weight. The weight of the solid content was calculated with respect to the volume taken by deducting diatomite weight²¹.

pH

The pH of the formulation was determined by using Digital pH meter.

Specific gravity

The weight difference method was used for specific gravity determination. A 25 mL of water (at 25°C) was taken into a clean, dry pycnometer and weighed. It was emptied, dried and further; 25 mL of each sample was filled in the same pycnometer and weighed at same constant temperature. Specific gravity of the sample was determined by dividing the weight of the sample by the equal weight of water (at 25°C).

Alcohol content (Distillation method)

The 25 mL of each prepared sample was diluted with 150 mL of water and transferred to the

distillation flask. It was further distilled (not less than 90 mL of the distillate) and collected into a 100 mL volumetric flask. The temperature was adjusted to 24.9°C to 25.1°C, and diluted to make up the volume with distilled water. Specific gravity was determined. Through relative density table, alcohol content was analyzed as per USP/NF²¹

Total phenol content (TPC)

The samples were prepared and equally diluted with water. Then 0.25 mL Folin Ciocalteu reagent (1N) and 1.25 mL sodium carbonate solution (20%) was mixed into it and vortexed. The mixture was kept in incubator at 45°C for 45 min and further analysed at 725 nm spectro-photometrically. The samples were prepared in triplicate for analysis and the mean value of absorbance was obtained. The standard solution of gallic acid was prepared similarly and the calibration curve was constructed. The total phenolic content of extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract)²².

Total Flavonoid Content (TFC)

Prepared samples with equal quantity of water were diluted. To the sample equal quantity of 2% AlCl₃ solution in methanol was added. The samples were incubated for 1 h at room temperature. The absorbance was determined at 415 nm using UV spectrophotometer. The triplicate samples were prepared and the mean absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration curve was constructed. Based on the measured absorbance, the total content flavonoid content in the sample

was expressed in terms of quercetin equivalent (mg of QU/g of extract)²³.

Total Reducing Sugar

A 20 mL of prepared *Asava* was neutralized with sodium hydroxide. It was then evaporated to half volume on water bath to remove alcohol. After cooling 10 mL of 21.9 g zinc acetate, 3 mL glacial acetic acid followed by 10.6 g potassium ferrocyanide was added and volume was made up to 100 mL with distilled water. To the Fehling Solution (10 mL), burette solution was added drop wise. It was boiled till blue colour. Further two drops of methylene blue were added and titration was carried out till the appearance of brick red colour²¹.

Determination of non-reducing sugars

A 20 mL of sample was mixed with equal amount of distilled water and further boiled for 30 min. The solution was cooled and adjusted to pH 7. The volume was further made up to 100 mL by addition of distilled water. To the Fehling Solution (10 mL), solution from burette was added drop wise. It was heated to boiling till blue colour appeared, over the hot plate. Then two drops of methylene blue were added and the titration was carried out till the appearance of brick red colour²¹.

HPTLC Analysis

Preparation of test sample

The three samples (Decoction, in-house fermented and one marketed formulation) of 50 mL each were dried on a water bath until the alcohol was completely removed. Then, 50 mL water was added to the residue to make up the volume. It was subjected to successive solvent extraction, with n-hexane (50*3 mL) followed by chloroform (50*3 mL) and ethyl acetate (50*3 mL). The, ethyl acetate fractions were evaporated to dryness and reconstituted with methanol as per AFI, Part-I. Each sample was prepared in concentration of 10 mg/mL of which 2 µL of each was applied on TLC plates for HPTLC analysis.

Preparation of stock solution (reference) and Calibration curves

Separate stock solutions (1 mg/mL) of caffeic acid and kaempferol were prepared respectively by dilution method with methanol. To determine the linearity, calibration curves were plotted. A 2 µL of each concentration range was applied on TLC plates to get final concentration 20–200 ng/spot for caffeic acid and 100–200 ng/spot for kaempferol. The densitometry scanning was performed for both

standard and their presence in samples were quantified by means of calibration plot.

HPTLC instrumentation

TLC plates with a dimension of 10 cm × 10 cm pre coated with 0.20 mm layers of silica gel 60 F254 (Merck, Darmstadt, Germany) were used for chromatography. 2 µL of samples were applied as 8 mm wide bands and 10 mm was the distance kept between the two bands by use of sample applicator CAMAG HPTLC System (Muttens, Switzerland) with Linomat V sample applicator equipped with a syringe of 100 µL capacity. The plates were developed using Toluene: Ethyl Acetate: Formic acid: Methanol with dilution ratio (5.5:3:1:0.5) for caffeic acid and (3:3:0.8:0.2) for kaempferol respectively in CAMAG Twin trough HPTLC Chamber pre saturated with mobile phase. The developed plates were dried and scanned at various lamda max from 254 to 366 nm using a spectro-densitometer (Scanner 3., CAMAG) equipped with win CATS planar chromatography manager (Version 1.30, CAMAG) software.

Results

The present research developed a modified method of *Drakshasava* preparation and optimized it as per parameters prescribed in AFI. The developed formulation was further quantified by HPTLC with respect to marker compounds (kaempferol and caffeic acid) and evaluated for the changes brought by fermentation during its preparation.

Physicochemical characterization

The prepared *Asava* was similar in colour to decoction however, taste and odour changed to sweetish sour and acid-alcoholic respectively (Table 2). The pH of the decoction initially 5.1 which gradually decreased to 4.4 on 9th day and reached 4.3 by 14th day. The specific gravity and total solid content of the decoction decreased from 1st to 14th day due to the increased growth of microorganism (fungus) with time and conversion of sugars present in decoction into alcohol to form *Asava*. The alcohol content was increased upto 16.7% on 14th day whereas permissible

Table 2 — Preliminary analysis of *Draksha* pre and post fermentation

S. no	Parameters	Decoction	<i>Drakshasava</i>
1	Colour	Dark brown	Brown
2	Odour	Slight fruity	Alcoholic -aromatic (wine like)
3	Taste	Sweet astringent	Sour sweet

limit as per API (5-10%) has been reached on 9th day itself. Since fermentation takes lag time to enter log phase, hence the alcohol content of the decoction remained nil, initial for 3-4 days. With the formation of *Asava*, there was prominent hike in total phenolic and flavonoid content with respect to the decoction whereas the content of reducing and non-reducing sugar has been gradually decreased with time (Table 3).

HPTLC Analysis

The *Drakshasava* was evaluated for the presence of kaempferol and caffeic acid for the first time. Both the marker compounds were quantified by HPTLC, before (decoction) and after fermentation (*asava*) in comparison with marketed formulation. The calibration curve plotted for caffeic acid and kaempferol had $y=0.843x-0.114$ and $y=1.509x-22.434$; with R^2 values 0.9965 and 0.9971 respectively. Kaempferol quantity was found to be decreased (0.186% to 0.0815%) after fermentation with marketed formulation quantified to 0.0813% whereas caffeic acid quantity has been increased significantly in post fermented sample compared to decoction (0.0168% to 0.0233%) with marketed formulation 0.0216% (Fig. 2). The increase or decrease in quantity of phytochemicals may be, due to metabolic changes taken place during fermentation of grapes and bioconversion of its active constituents in less toxic novel compounds.

Discussion

Traditional systems of medicine have been in use for centuries, concurrently with modern allopathic system in different parts of the world. But with time, modern

medicine has come up with toxic side effects and lack of proper medicine for various diseases which has enforced reemergence of the traditional system as better alternative. These days many multifactorial disease like diabetes, cancer, heart and brain disorders has become evident, which necessitate therapeutic intervention at multiple level. Since Ayurveda deals with one or more herb in its formulation having complex phytochemical biomolecules with advantage over single molecules in treating such ailments. Although Ayurvedic formulations are multi targeted and therapeutically sound, they have constraints of time consumption, validation and proper standardized method. A systematic approach is required to develop a well-designed methodology for standardization of herbal drugs and formulations. Emphasizes on certain types of Ayurvedic formulations which are particularly relevant to the pharmaceutical industry is majorly necessary. The characterization of a few constituents (bio markers) or group of constituents to mark acceptable ranges leads to standardized manufacturing process. It promotes quality control and batch-to-batch consistency.

The *Asava (Drakshasava)* development describes the course of fermentation and changes brought within the period without any analytical processing. In case of *Arista* and *Asava* (fermented Ayurvedic formulation), physicochemical screening is foremost step of standardisation while marker based fingerprinting is the next. The Fig. 3 depicts the changes brought by invertase enzyme present in *woodfordia* flowers to initiate fermentation which leads to conversion of sugar

Table 3 — Physico-chemical studies of in-house *Drakshasava* formulation

DAYS	pH	Specific Gravity	T. Solid content	Ethanol content	TPC	TFC	Reducing Sugar	Non-Reducing Sugar
DECOCTION	5.12	1.32	28.25	0	0.0401	0.00645	38.87	13.31
0								
1	5.10	1.30	28.03	0	0.055	0.00677	37.62	10.53
2	5.08	1.28	27.69	0	0.0483	0.00692	35.05	7.43
3	5.01	1.28	27.63	0	0.0502	0.00714	32.12	6.18
4	4.95	1.26	26.52	0.55	0.0645	0.0077	25.60	5.55
5	4.87	1.25	25.48	2.34	0.0764	0.0089	20.71	3.792
6	4.73	1.25	25.45	4.15	0.0802	0.0096	18.98	2.061
7	4.62	1.24	25.33	5.80	0.0815	0.0103	18.10	1.56
8	4.53	1.21	25.21	7.80	0.0992	0.0154	17.44	0.78
9	4.48	1.20	25.09	9.85	0.1035	0.0181	16.83	0.85
10	4.46	1.21	25.05	11.60	0.1282	0.0192	15.35	0.76
11	4.44	1.19	24.78	12.82	0.1331	0.0186	12.33	0.71
12	4.35	1.18	23.67	13.94	0.1460	0.0168	9.57	0.64
13	4.39	1.15	22.84	14.53	0.1565	0.0153	8.96	0.62
14	4.35	1.15	22.81	16.75	0.1658	0.0158	8.57	0.61

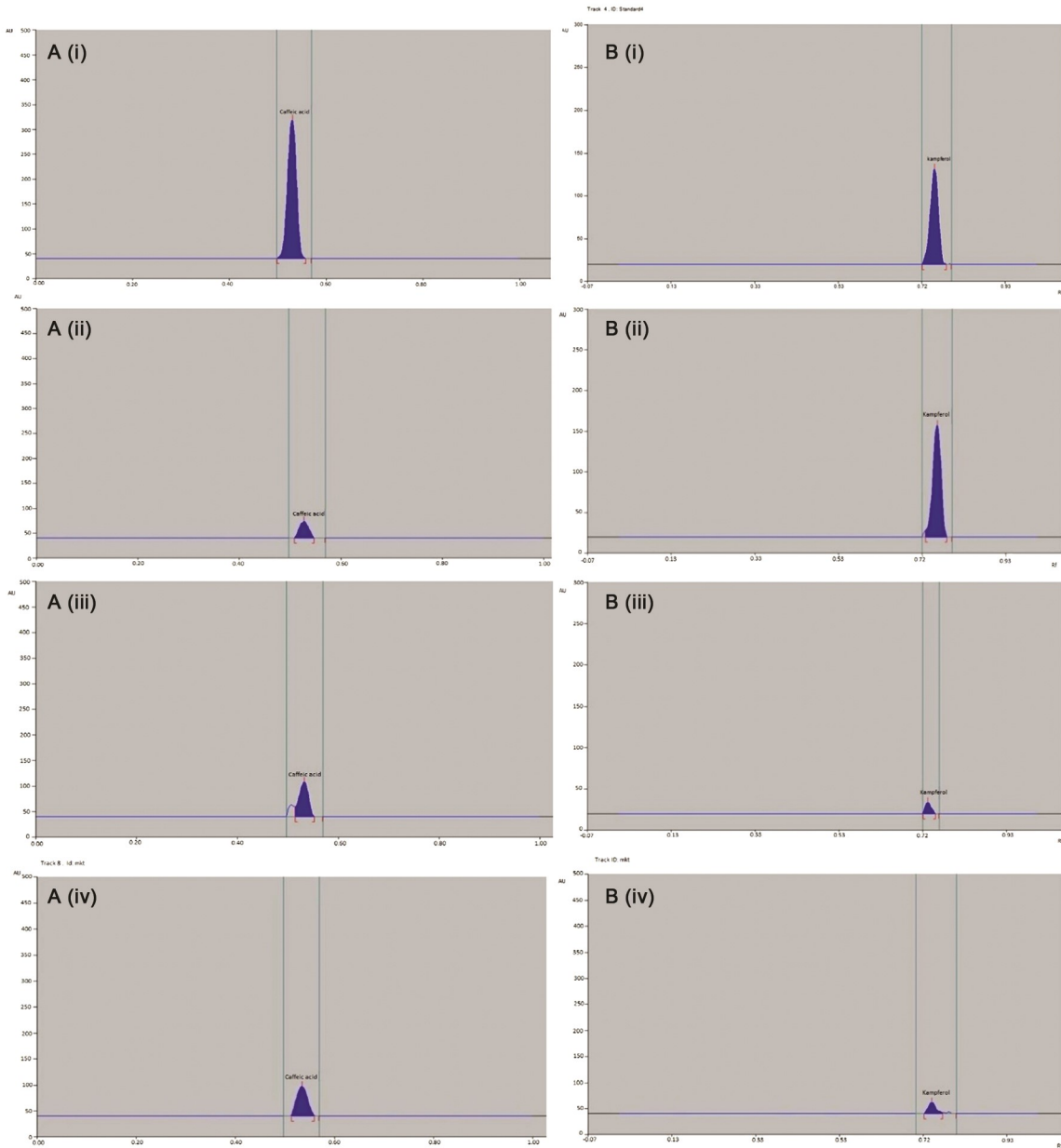


Fig. 2 — HPTLC chromatogram of (A). Caffeic acid; (B). Kaempferol (i) Standard; (ii) Decoction; (iii) Fermented; (iv) Marketed formulation

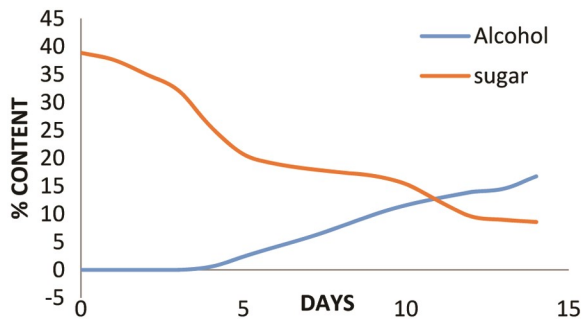


Fig. 3 — Changes in % content of sugar and alcohol with days

to alcohol with time and bioconversion of polyphenols simultaneously²⁴. The Fig. 4 graphically represents formation of alcohol (Alc percentage) by conventional method versus non-conventional (new modified method) with respect to days. The non-conventional method reports formation of 5-10% alcohol on 9th day of fermentation as compared to conventional process of 21 days mentioned in AFI.

The hydrolysis of disaccharides (sugar) to monosacchrides and finally alcohol can be depicted through non reducing sugar analysis and percentage

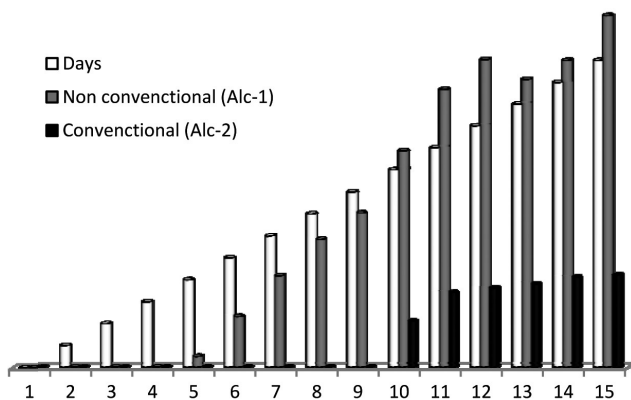


Fig. 4 — Changes in alcohol (Alc) percentage by conventional (marketed sample) versus non-conventional method with time (1-15 days)

alcohol content. Both the factors depict inverse relationship. The increase in acidity (pH) of the post fermented preparation may be due to de-conjugation occurring within the compounds during fermentation. However, other tests including specific gravity, total solid content provides information related to physical properties of the formulation.

Vitis vinifera L. is a powerhouse of polyphenols hence, variety of antioxidants²⁵⁻²⁷. Apart being a powerful antioxidant drug, *Drakshasava* (*Asava* of *vitis*) is reported to be diuretic, cardioprotective and antimicrobial in action²⁸⁻³⁰. A large variety of grapes all over the world are used to make hundreds of fermented products including beverages and fermented drinks (*Aristha* and *Asava*). It has been reported that use of bacterial or fungal fermentation methods, not only amplify the bound phenolics release from the cell walls of plants, but also convert them into different metabolites, through phase I/II i.e., bedeglucosides³¹⁻³³, sulfoconjugates^{34,35} and glucuronides³⁶, which may further exert other bioactivities.

Nowdays, marker profiling for the identification of active compounds present in various multi-herbal formulation having complex natural products has become essential tool³⁷⁻³⁹. Hence the role of fermentation in bioconversion of polyphenols has been significantly described through HPTLC fingerprint profiling of kampferol and caffeic acid in *Drakshasava*. It is the first report, evaluating quantitative changes with respect to the marker compounds in *Drakshasava*. Earlier reported work on *Drakshasava* determine the quantitative estimation of gallic acid, rutin, quercetin and catechin respectively⁴⁰⁻⁴¹ through validated HPTLC method; standardization of marketed formulation as per the

established monograph in AFI, (Part ii)⁴²; resveratrol and pterostilbene occrence in marketed *Drakshasava* by HPLC⁴³.

Conclusion

The present research work was based on modification of already established method of *Drakshasava* preparation mentioned in AFI; its standardization, optimization and further chemoprofiling with respect to two non-reported markers (kaempferol and caffeic acid). The modified method for *Drakshasava* preparation provides reproducible product saving time, money and providing better quality for large scale production. The new validated method adheres to the Ayurvedic principle and satisfies need of industries. It provides scope for further validation of established monograph of *Drakshasava* and finding novel pharmacophores. It enlightens its application in routine qualitative and quantitative analysis with respect to biomarkers; hence directs researchers to study combinatorial effects of multiple markers, which may lead to development of new phytopharmaceutical drug.

Conflict of Interest

We declare no Conflict of Interest.

Acknowledgement

The authors are thankful to ICMR, New Delhi for providing the necessary scholarship grant (SRF) for the research work.

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