ORIGINAL ARTICLE

Effect of Fermentation on CHEMICAL changes in *Vitis vinifera* Fruits used in Alcoholic Ayurvedic Formulation

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Abstract

Background: *Arishtas* are weakly alcoholic Ayurvedic formulations prepared by fermenting the decoction of medicinal plants in an airtight sealed vessels. The presence of *Vitis vinifera* is considered essential to initiate the fermentation process. The fruits of *V. vinifera* were reported to have different polar components such as phenolics, phenolic acids, flavonoids, and flavonoid glycosides. Literature has mentioned the impact of fermentation in altering the chemical skeleton of molecules. **Materials and Methods**: Considering this fact, the present study was designed to understand the possible impact of fermentation on the chemical profile of *V. vinifera* by reversed-phase high-performance liquid chromatography (RP-HPLC) studies. Both fermented and non-fermented extract of *V. vinifera* was prepared by the traditional method and analyzed for chemical changes by RP-HPLC-ultraviolet method. **Results and Conclusions**: It was observed that the chromatogram of non-fermented extract of *V. vinifera* showed comparatively very few peaks as compared to the chromatogram of its fermented extract. This is probably due to the fact that the large molecular weight polyphenolics particularly flavonoid glycosides and tannins are hydrolyzed during ferat mention and degraded into small molecules of phenolics or phenolic acids. This change will modify significantly the biological activity of the fermented extracts and the final Ayurvedic formulation, particularly fermented biomedicine like *Arishtas*.

Key words: Arishtas, chemical changes, fermentation, high-performance liquid chromatography, *Vitis vinifera*

INTRODUCTION

Ayurveda is a traditional Indian medicinal system being serving the public health using plants and plant products in various Ayurvedic formulations. *Arishtas* are weakly alcoholic ayurvedic medicinal preparations made traditionally in airtight sealed vessel by anaerobic fermentation of the decoction of plant materials with a solution of jaggery for a specified period of time.[1] The alcohol, so generated also served as a preservative. Examples: *Ashokarishta, Abhayarishta,* and *Dashmularishta.* Fermentation of *Arishtas* is brought about by addition of some source of sugar along with *Vitis vinifera*. The presence of *V. vinifera* is considered essential to initiate the fermentation process, and thus it is also known as fermentation initiator.[2]

V. vinifera, commonly known as draksha, grapes and is considered an important traditional medicinal plant belonging to the family Vitaceae. The plant is abundantly present throughout India and also in the majority of the countries of South East and Far East Asia such as Malaysia, Indonesia, Sri Lanka, China, Japan, and Pakistan as well as Tropical Africa.[3] The fruits of *V. vinifera* were reported to have different polar components including phenolic compounds such as gallic acid, catechin, epicatechin,

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epigallocatechin, epicatechin gallate, epigallocatechin gallate, procyanidins B1, procyanidin B2, and series of flavonol -O- glycosides such as chrysophanol-8-O-D-glucopyranoside, quercetin glycosides [3-rhamnoside], 3-*O*-(6"-galloyl)-β-d-glucopyranoside, naringenin 7-glucoside, and kaempferol 3-*O*-glucoside.[4-7]

Over the past few years, polyphenolic compounds had gained much more attention due to their efficiency in reducing the oxidative stress. As an efficient radical scavenger, natural polyphenolic molecules are considered a powerful tool in the treatment of several cardiovascular, microbial, and neurodegenerative disorders.[8-11] Due to the rich chemical diversity and attractive therapeutic potential, *V. vinifera* fruits are used as an essential ingredient in most of the alcoholic Ayurvedic formulation such as *Arishtas*. Ayurvedic Formulary of India, 2003 (AFI) reported 19 *Arishtas* in which *V. vinifera* along with jaggery or honey is used as a sugar medium and fermentation initiator.[12] Fermentation facilitates the dissolution and degradation of several plant constituents of plant materials which may lead to the possibility of having some changes in chemical diversity as well as the therapeutic potential of the final formulation. Although *V. vinifera* fruits are used either as flavoring agent or fermentation initiator, its rich chemical and biological properties motivated us to determine the changes in its chemical profile after fermentation. It was already reported in literature that several factors such as generation of acid and alcohol during fermentation of decoction stimulate the hydrolysis of the process, thus causing biotransformation of chemical substances.[13,14] Various reports have been published which explained the degradation pattern of polyphenolics substances during fermentation.[15] Publication of these data promoted us to explore the effect of fermentation on chemical changes in *V. vinifera* fruits.

With the above perspective of evaluating, the effect of fermentation, reversed-phase high-performance liquid chromatograms (RP-HPLC) of pre- and post-fermented extract of *V. vinifera* fruits was prepared and compared with each other to know the possible changes in chemical profile by observing the retention time and ultraviolet (UV) spectrum of separated peaks.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade and were purchased from CDH, S.D. fine chemicals. The triple distilled water passed through 0.45 µm filter (Sartorius, Germany) was used in the mobile phase. Methanol and acetic acid were of HPLC grade and purchased from JT Baker and S. D. Fine Chemicals (India), respectively.

Plant material

Dried fruits of *V. vinifera* were purchased from the local market of Chandigarh and were identified by Dr. A. S. Sandhu (Garden Supervisor), Department of Natural Products, NIPER, Mohali.

Extraction procedures

Approximately 100 g of plant material was weighed and dipped insufficient amount of water. The decoction was made by continuous boiling till volume is reduced to 1/4th. It was kept for cooling and then filtered. The filtrate was divided into two portions. One portion of the filtrate was extracted with ethyl acetate by liquid-liquid extraction method. Second portion of the filtrate was kept for anaerobic fermentation (in a closed earthen vessel for 45 days) and was filtered again after a specified period. The filtrate was further extracted with ethyl acetate by liquid-liquid extraction method. Both the pre-fermented and post-fermented extracts were concentrated and evaluated for RP-HPLC studies.

Analytical HPLC

Completely dried both crude extracts were dissolved in HPLC grade methanol and allowed to sonicate for 20 min. Samples were then filtered through 0.5 µm MTFE filter before injection of 10 µl into HPLC system. In this work, HPLC analysis was performed using a Shimadzu HPLC system LC-10AVP with PDA and LC solution for data processing. The separation was performed on a reversed phase C-18 Column, Luna® (250 mm × 4.6 mm i.d., 5 µ) Phenomenex, USA. The mobile phase consists of 1% acetic acid in water (solvent A) and 80% methanol in solvent A (solvent B). The binary gradient elution program was as follows: 0–10 min, 5–10% B; 10–15 min, 10–17% B; 15–20 min, 17–25% B; 20–40 min, 25–40% B; 40–50 min, 60–70% B; 50–60 min, and 70–10% B with a flow rate of 1.0 mL/min. The peaks in a chromatogram were identified by comparing the HPLC retention time and UV spectra against the data reported in literature.

RESULTS AND DISCUSSION

Both the pre- and post-fermented extracts of *V. vinifera* were successfully prepared, and their samples for the RP-HPLC analysis were prepared in similar manner so that a comparison between the two can be made. Polyphenols were extracted from the plant material and identified by RP-HPLC coupled with UV DAD detector. The chromatograms of both the fermented and non-fermented extracts with the UV spectrum of their selected peaks are shown in Figure 1. All the major components were separated within a time period of 55 min. Qualitative evaluation of UV absorption pattern of different peaks under UV spectra revealed that most of the

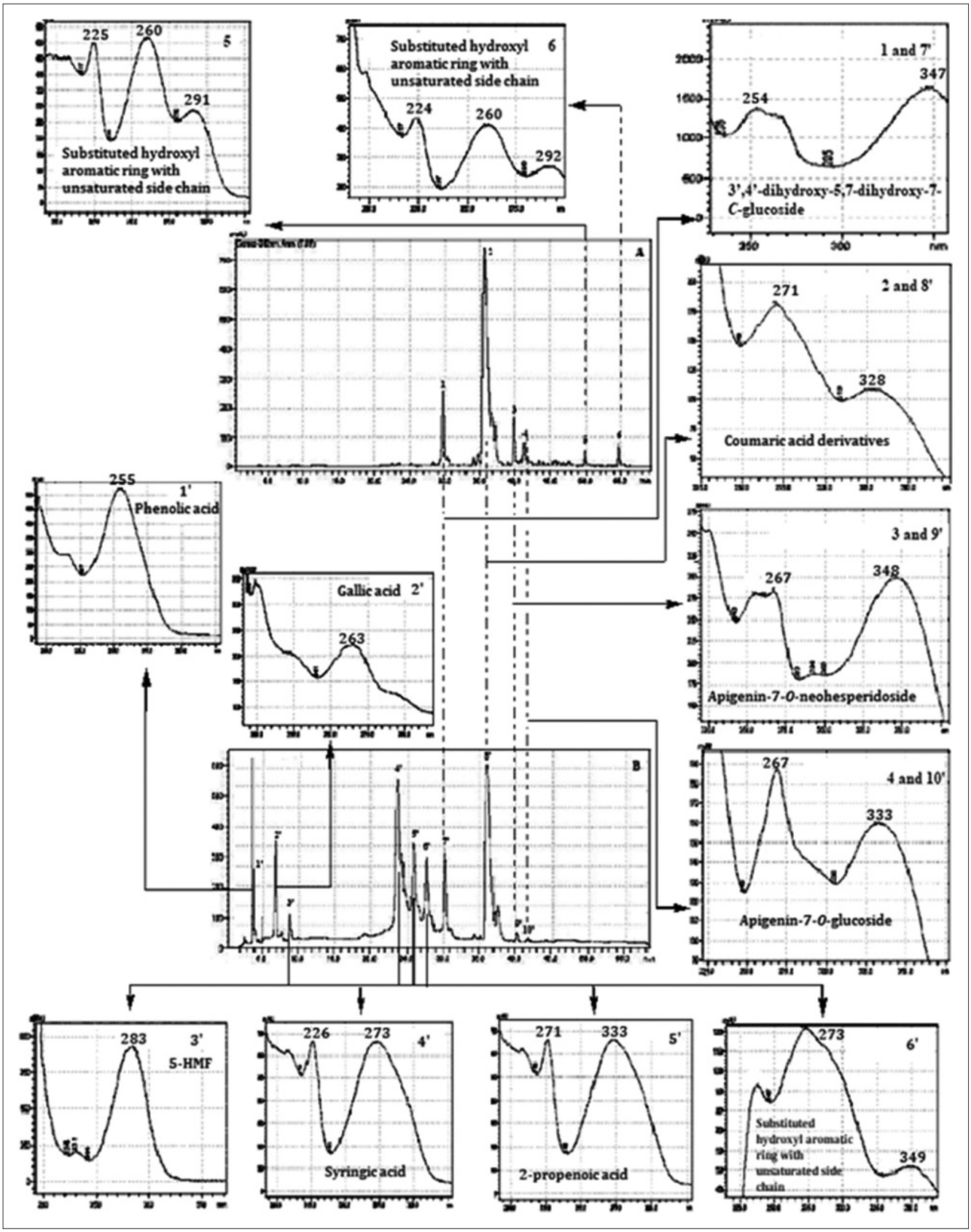

Figure 1: Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatogram of *Vitis vinifera* fruits and ultraviolet spectra of all the separated peaks (a) HPLC chromatogram of non-fermented *V. vinifera* fruits (b) HPLC chromatogram of fermented *V. vinifera* fruits

components in both the fermented and non-fermented extracts are polyphenolic in nature. Most of the components exhibit their UV spectrum with two major absorption peaks (Band I 300–380 nm and Band II 240–280 nm) in the region of 220–350 nm. These two peaks are considered a characteristic feature of flavonoids.[16] Since most of the peaks in Band I region are in the range of 328–350 nm, thus it was concluded that flavonoids in both the fermented and non-fermented extracts are of specifically hydroxyl substituted flavones.[17] These two major absorption peaks are either corresponds to two aromatic rings (A and B) or one aromatic moiety with an unsaturated side chain. Band I is more pronounced if molecule contains hydroxyl substitution in both aromatic rings A and B or having unsaturated side chain attached with aromatic ring A whereas, Band II is more pronounced when molecule is having only one aromatic ring (A ring) substituted with hydroxyl group without any unsaturated side chain shown in Figure 2.[17] It was observed from the UV spectra, that most of the components in both the extracts showed pronounced Band II comparatively which confirms the presence of a ring substituted hydroxyl polyphenolic compounds.

Further, it was also observed from the RP-HPLC chromatogram of both the extracts of *V. vinifera* that the density of peaks in its fermented extract was higher than the non-fermented extract which indicates that during fermentation some of the high molecular weight substances especially flavonoids get hydrolyzed into small molecules such as phenolic acids. Comparative analysis of HPLC chromatogram revealed that both the fermented and non-fermented extracts exhibited four common peaks separated at same retention time with same UV absorption pattern. Peak 1, 2, 3, and 4 of non-fermented extract correspond to peak 7', 8', 9', and 10' of fermented extract at R_t 29.5, 36.0, 40.0, and 41.5 min, respectively. It indicated that the phenolic substances corresponding to these peaks were not qualitatively affected by fermentation. The UV spectra of peak 1 and 7' displayed pronounced Band I with λ_{max} 347 which resembles with the UV spectra of 3',4'-dihydroxy-5,7-dihydroxy-7-C-glucoside. [18] This molecule is a type of flavonoid -C- glycosides, and it was assumed that C- glycosides are more stable than O-glycosides; thus, it is not affected by fermentation. However, peak 2, 8'; 3, 9'; and 4, 10' displaying pronounced Band II indicated that such compounds may have either substituted aromatic ring a or an aromatic ring with an unsaturated side chain. The comparison of UV spectra of 3, 9' and 4, 10' peaks with literature identifies such compounds as apigenin-7-O-neohesperidoside and apigenin-7-O-glucoside whereas peak 2, 8' corresponds to coumaric acid derivatives.[16] It was clearly shown in the chromatogram of fermented extract that the area of peak 9' and 10' is relatively less compared to area occupied by same molecule corresponding to peak 3 and 4 of non-fermented extract, respectively, which confirms that O- glycosides get easily hydrolyzed during fermentation in comparison to C- glycosides. The appearance of various peaks in fermented extract such as 1' – 6' under R_t 4–28 min depicted that these were originated as hydrolyzed product of peak 3, 4, 5, and 6 of non-fermented extract. All the hydrolyzed products were identified by comparing their UV absorbance pattern with the literature, and these peaks exhibited λ_{max} corresponding to phenolic acids.[16] The proposed type of phenolic compounds corresponding to the separated peaks was shown in Table 1.

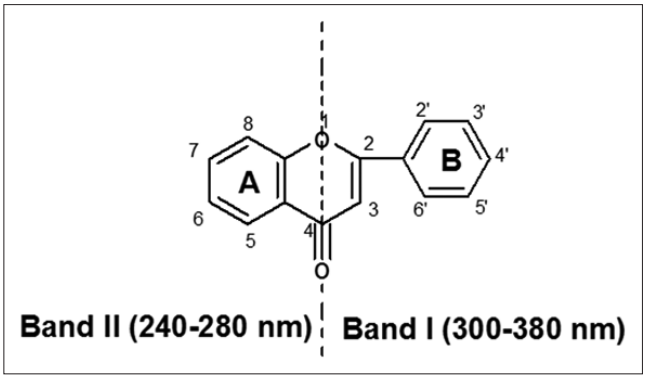

Figure 2: Flavone skeleton associated with ultraviolet absorbtion in Band I and Band II

It was also determined in the RP-HPLC-UV analysis that significant chemical change observed in the quality of polyphenols during fermentation. After fermentation, 5 hydroxymethylfurfural (5-HMF) was observed at R_t 9 min with its characteristic λ_{max} 283 nm, which could be considered as a marker in determining the effect of fermentation.[13] It was considered that fructose from *V. vinifera* fruits is transformed to 5-HMF under acidic conditions raised during fermentation. However, quantitative analysis of separated compounds was also required to determine the effect of fermentation on polyphenolic content in plant-drug as well as finished Ayurvedic formulation.

CONCLUSIONS

V. vinifera reports on different clinical implication declared it an important alternative as protective applications against the decline of physiological disorder and the usefulness of this plant is supported by its rigid phytochemical framework. Its use in Ayurveda as an important ingredient in fermented biomedicine in the form of fermentation initiators will never be neglected. The fermentation process resulted in considerable changes in the quality and quantity of both high and low molecular weight phenolic compounds which modifying significantly the biological activity of the fermented extracts and thus the final Ayurvedic formulation, particularly fermented biomedicine like *Arishtas*. By controlling each step of the fermentation process, particularly the incubation time, it should be possible not only to predict the final content and composition of formulation but also to prevent the degradation of pharmacologically important phytoconstituents, thus maintaining the therapeutic potential of the final product.

Table 1: Retention time and UV spectrum of separated peaks of fermented and non-fermented *V. vinifera* fruits

Sample	Peak no.	Retention time	UV spectrum		Proposed type of phenolic compound
			Band I	Band II	
Non-fermented *V. venifera* extract	1	29.5	347	254	3',4'-dihydroxy-5,7-dihydroxy-7-*C*-glucoside
	2	36	328	271	Coumaric acid derivatives
	3	40	348	267	Apigenin-7-*O*-neohesperidoside
	4	41.5	333	267	Apigenin-7-*O*-glucoside
	5	50		225, 260, 291	Substituted hydroxyl aromatic ring with unsaturated side chain
	6	55		224, 260, 292	Substituted hydroxyl aromatic ring with unsaturated side chain
Fermented *V. venifera* extract	1'	4		255	Phenolic acid
	2'	7		263	Gallic acid
	3'	9		283	5-hydroxymethylfurfural (5HMF)
	4'	23.8		226, 273	Syringic acid
	5'	26	333	271	2-propenoic acid
	6'	28	349	273	Substituted hydroxyl aromatic ring with unsaturated side chain
	7'	29.5	347	254	3',4'-dihydroxy-5,7-dihydroxy-7-C-glucoside
	8'	36	328	271	Coumaric acid derivatives
	9'	40	348	267	Apigenin-7-O-neohesperidoside
	10'	41.5	333	267	Apigenin-7-O-glucoside

UV: Ultravoilet, *V. vinifera*: *Vitis vinifera*

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