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POLYPHENOL ANALYSIS OF ASAVAS AND ARISHTAS – AN IMPORTANT MARKER FOR STANDARDIZATION

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ABSTRACT:
Ayurveda has gained worldwide attention due to its efficacy. Ayurvedic medicines are formulated from medicinal plants using modern scientific techniques. Standardization of these prepared medicines plays a very crucial role for their authentication. As the need for safer drugs is increasing, an awareness has been drawn to the quality and standards of the ayurvedic formulations. As these formulations are based on plant materials, it is rich in phytochemicals. The main aim of the present work was to quantify different polyphenols present in the samples of *Lohasava, Punnarnavasa, Amritarishta* and *Kirayatikadha*. The polyphenols identified by RP-HPLC could be used as phytochemical analytical markers for these formulations. These findings can certainly help in the quality assurance during the manufacturing of the given formulations.

Keywords: *Polyphenols, asavas, arishtas, fermentation, biotransformation.*

INTRODUCTION:

Ayurvedic system of medicine is accepted as the oldest written medical system that came into existence in about 900 B.C.; which is more effective in some cases than in modern therapies (Jerald EE, 2007). A variety of ayurvedic medicines are available in order to meet the diverse requirement in the treatment of human illness (Sekar & Mariappan, 2008). Asavas are medical preparations made by soaking the drugs in coarse powder form, in a solution of jaggery or sugar as cited in he literature, for a specified period of time during which it undergoes a process of fermentation thereby leading to production of alcohol; which facilitates extraction of active principles contained in the drugs. The alcohol, thus generated also acts as a preservative (The Ayurvedic Pharmacopoeia of India). Arishtas, are however fermented decoctions. According to a report of WHO nearly 80% of the earth's population rely on traditional medicines for their primary health care needs. The therapyfor treatment involves using the plant extracts or their active components (Kalaiselvan et al, 2010). These active components are the phytochemicals. One amongst them is polyphenols which may or may not be affected during the course of fermentation.

Polyphenols are the natural compounds found mainly in fruits, vegetables, cereals and beverages (Spencer et al, 2008). Polyphenols are the source of antioxidants in our diet. Since the average daily intake is about 1g which is almost 10 fold intake of vitamin C; 100 fold intake of vitamin E and 500 fold intake of carotenoids (Scalbert et al, 2000; 2005). Polyphenols are secondary metabolites of plants and protects the plants by aiding in defence against UV radiation or aggression by pathogens (Beckman, 2000). Apart from their antioxidant properties, polyphenols show some other biological activities including antimutagenic, anti-oestrogenic, anticarcinogenic and anti-inflammatory effects that might potentially be beneficial in human health such as in treatment and prevention of cancer, cardiovascular diseases and other pathologies (Bravo, 1998). There is a clear evidence that they have the potential to act in three general areas specified i.e. transition metal ion complexation, as antioxidants in cellular pro - oxidant states, and by

association with proteins and peptides (Haslam, 1996). Almost all polyphenols possess antioxidant property and hence are responsible for their purported beneficial health effects associated with conditions such as cancer, cardiovascular diseases, neurodegenerative diseases and aging (Manach et al, 2004). Whereas several issues related to disease protective activities of polyphenols are still unsolved. One of the burning issues is bioavailability of these polyphenols, since it plays important role in evaluating the efficiency as well as efficacy of these compounds (Kanti Bhooshan Pandey, 2009). As these polyphenols are responsible for the therapeutic action of specific formulation, they can be used as an analytical marker for the standardization of the formulation.

An attempt was thus made in the present study to analyze the different polyphenols present initially and after the process of fermentation in the final product. For this, two asavas namely Punarnavasava and Lohasava were chosen. On the other hand, two arishtas chosen were Amritarishtha and Kirayatikadha.

METHOD AND MATERIAL:

Chemicals and Reagents - Methanol (HPLC grade), Acetone (GR), Sodium dihydrogen phosphate dihydrate ($Na_2H_2Po_4.2H_2O$), Sodium Carbonate (Na_2Co_3), Folin – Ciocalteu (F-C), Dimethyl sulfoxide (DMSO) Reagent were highest analytical grade and purchased from Merck. Reference compounds for HPLC were from Extrasynthase and Sigma Aldrich.

Samples - The samples were collected from the manufacturing unit directly after regular time intervals 0day, after 10 days, 20 days and after 30 days i.e. completion of fermentation. The samples were stored in the refrigerator till the processing was done. The samples were shaken properly and used. Fixed amount of sample was taken and were extracted in extraction solution 25ml two times. The extracted sample was vortexed and centrifuged at 7000rpm for 10 minutes, the supernatant was collected and this is known as the raw extract. The raw extracts were evaporated using rotary evaporator (B'U'CHI, R-215, made in Switzerland) and the samples were reconstituted with 5ml and 10ml of DMSO. The extracts were centrifuged at 4200rpm for 10 minutes and filtered through a Whatman 0.2µm syringe filter (PTFE) before analysis.

HPLC analysis for characterization of the phenolics in samples was detected by using the following individual standards namely Gallic acid, Gallocatechin, Protocatechuic acid, 3,4-Dihydroxy benzoic acid, Epigallocatechin, Chlorogenic acid, Catechin, Syringic acid, Vanillic Acid, 3-OH Benzoic acid, Epicatechin, Caffeic acid, Sinapic acid, (-)Catechin gallate, Procyanidin B2, Epigallocatechin gallate, Ferulic acid, Isoferulic aicd,4-Coumaric aicd, Hesperdien, Gallocatechin gallate, Epicatechin galate, 2-Coumaric acid, Rosmaric acid, Isovitexin,Luteolin-7-O-Glucoside, Hespertin, Quercetin -3-Beta-Galactoside,Hyperoside,Ellagic acid, Rutin, Physcion, Daidzein, Galangin, Salicyclic acid, Rhoifolin, Naringenin, Flavonone(internal standard), Quercetin, Luteolin, Chrysophanol, Chalcone, Rhein, Isorhamnetin, Myricetin, Emodin, Kaempferol, Cinnamic aicd and Curcumin. Calibration curves were made by diluting stock solutions with DMSO to give concentration of the standard in the range 1mg/1ml for standards. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard. Linear calibration curves were obtained with all phenolic standards within the concentration range studied (r = 0.9992–0.9999). The limits of detections (LOD) were calculated from the parameters obtained from calibration curves, using the formula LOD = 3.3 Sa/b, where Sa is the standard deviation of the y-intercept of the regression line and b is the slope of the calibration curve (Ribani et al, 2007).

The HPLC system employed was a Dionex HPLC series Ultimate 3000 (Germany) equipped with Dionex model Chromeleon software, autosampler U-3000, and 3000 RS diode array detection system to monitor at all wavelengths from 200 to 600nm. For the column, Dionox PA_2 RSLC 120 A, Column C_{18} Acclaim RSLC, (100mm x 2.1dm) i.e., 2.1µ Thermo Scientific Ltd was used at 35 ºC. Gradient elution was performed with solution A, composed of 50mM Sodium phosphate (pH 3.3) and 10% methanol, and solution B, comprising of 70% methanol, delivered at a flow rate of 0.47 ml/min as follows: initially 100% of Solution A; for the next 0.03 min, 70% A; for another 2.65 min, 65 % A; for another 7.9 min, 60% A; for another 11.5 min 50% A and finally 0% A for 13.1 min , again 17 min 100 % A and 20.25 min 100% A. The injection volume of the extract was 5µl (Sakakibara et al, 2003).

RESULTS AND DISCUSSION

The asavas and arishtas were prepared by the process of fermentation as stated in the literature and RP-HPLC analysis was carried out of the non fermented sample as also of the samples withdrawn after specific time intervals till the completion of the fermentation process. The elution of standard polyphenols on HPLC showed the above chromatogram. The results obtained were tabulated as given in the table 1 and table 2.

It was determined that during the process of fermentation significant chemical change was observed in the quantity of polyphenols. Thus the fermentation resulted in considerable changes in the quality and quantity of both high and low molecular weight phenolic compounds which modify the biological activity of the fermented extracts, particularly fermented biomedicines like asavas and arishtas (Gill et al, 2018). This modified activity could be responsible for the therapeutic action of these medicines.

The polyphenol concentration decreased in the biomedicines after fermentation with few exceptions. Similar observations were made for bean polyphenols by Cuellar Alvarez and Zapata et al (2013). In Amritarishta, the concentration of polyphenols decreased with the exception of chlorogenic acid whose concentration increased significantly from 1.765mg/100g to 6.436mg/100g, lutelion-7-O-glucose which was initially not detected increased to a concentration of 0.044mg/100g. This polyphenol has been used as a marker polyphenol in Amritarishta by Wadkar et al. In Lohasava, the concentration increased from 0.358mg to 0.693mg for vanillic acid, from 0.350mg to 0.724mg for OH-benzaldehyde and from 6.738mg to 0.794mg for chlorogenic acid. For Punnarnavasava, the polyphenols like kaemperferol, lutelion-7-O-glucose, vanillic acid, chlorogenic acid, caffeic acid were initially not in the detectable range. However, their concentration reached to 0.076mg, 0.062mg, 2.326mg, 5.781mg and 0.437mg respectively. Ferulic acid which initially had a concentration of 0.089mg reached a value of 1.755mg/100g. Exceptionally for Kirayatikadha, all the polyphenols which were present initially showed an increase in concentration.

Basically fermentation is a microbiological process which occurs spontaneously and results into biochemical changes. The micro organisms are activated by the temperature changes during the fermentation process (Yader et al, 2014). The concentration of most of the polyphenols is lower in fermented sample as compared to the non fermented sample. This is due to the liquid reduction caused by the fermentation process (Wollgast & Anklam, 2000). Another mechanism of polyphenol loss is the action of polyphenol oxidase enzyme E.C.1.14.18.1, which is responsible for catalyzing the polyphenol oxidation to high

molecular weight condensed polyphenol (Weisberger, 2001).

The polyphenols are present in foods in the form of esters, glycosides or polymers that cannot be absorbed in the native form. Therefore these substances must be hydrolyzed by enzymes present in the gut such as β-glucosidases and lactase-phlorizin hydrolase or by the colonic microflora, before they can be absorbed (Nemeth, 2003). A reduction in the concentration of specific polyphenol indicates their hydrolysis or oxidation and conversion into simpler forms which could be better absorbed. Similar observations were made in rats by Carbonaro et al. According to Skrabanja V, polyphenol may be bound in protein polyphenol complexes are modified by enzymes i.e. some biotransformations are taking place by the microbes present during fermentation.

According to literature, before carrying out the process of fermentation soaking of some components is to be done. There might be loss of some tannins during the soaking of components. The reason for their loss can be attributed to their dominant presence in the seed coat and also as they are water soluble, readily leach out into the liquid medium (Reddy & Pierson, 1994; Kumar et al, 1979). A decrease in the concentration of polyphenols may be due to improper storage conditions. Storage is a factor that affects the content of polyphenols that are easily oxidized. Oxidation results in the formation of more or less polymerized substances which leads to changes in quality of foods particularly in colour and organoleptic characteristics (Sosulski, 1982).

CONCLUSION

Based on surveys, the use of traditional medicines from medicinal plants and plant products is apparently increasing throughout the world in the treatment of diseases and also for healthy life. Use of traditional medicines is widespread in many countries. In India, about 40% of the total medicinal consumption is attributed to traditional tribal medicines.

Most of the research studies have confirmed that the natural polyphenols are responsible for the beneficial effects of herbal medicines. A protective role of polyphenols against degenerative diseases is supported today by many studies carried out on humans and different mechanisms of action have been proposed to explain such protective efforts. However, study on polyphenols is quite complex because of heterogeneity of different molecular structures and scarcity of data on bioavailability and biotransformation. An attempt was made in the present study to find out the active polyphenols in some asavas and arishtas and their fate during the course of fermentation. Some of these polyphenols could be used as analytical markers for these medicines. However, there is need for more research and development so as to find out the biotransformation of the polyphenols to exactly determine the role of these bioactive compounds with respect to human health.

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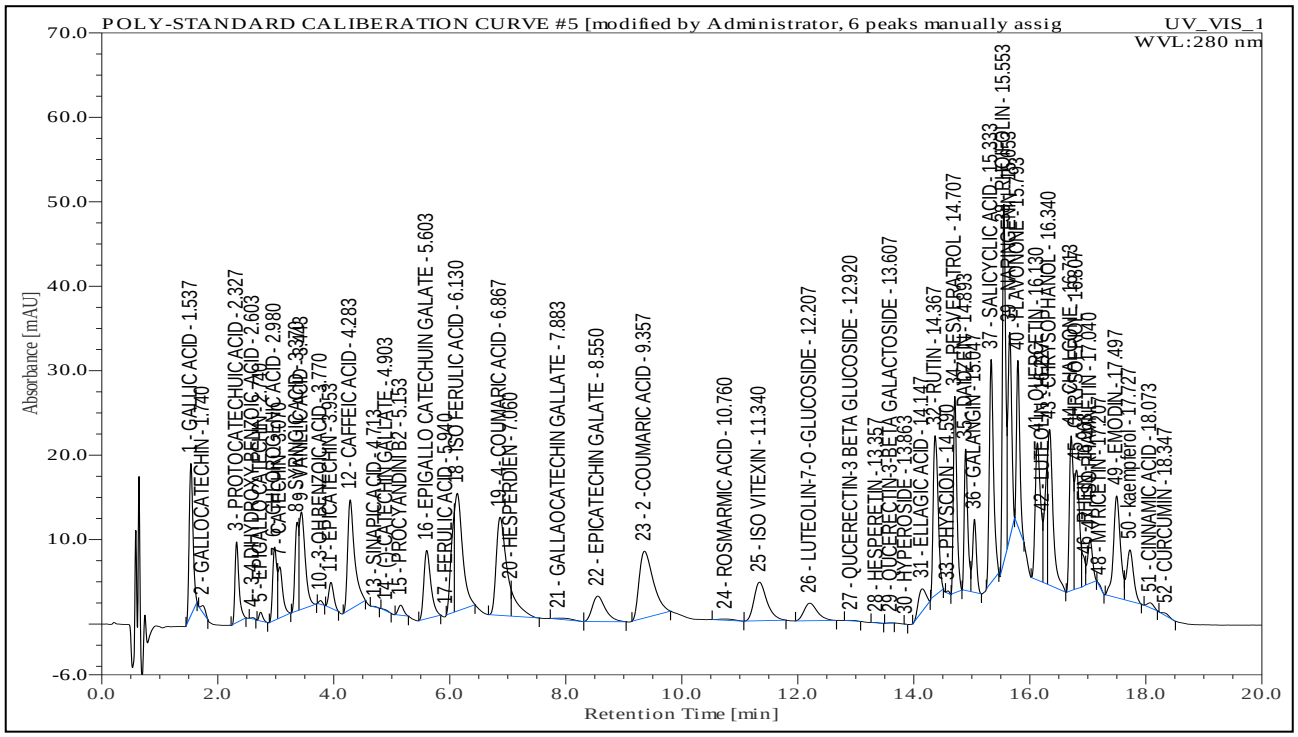

Fig.. HPLC ANALYSIS CHROMATOGRAM: ELUTION PROFILE OF A STANDARD MIXTURE OF POLYPHENOLS

Table 1 – Polyphenol concentration (mg/100g sample) in different samples

SAMPLE	GALLIC ACID	PROTOCATECHUIC ACID	VANILLIC ACID	OH-BENZALDEHYDE	CHLOROGENIC ACID	CAFFEIC ACID	FERULIC ACID
A1	ND	1.601 ± 0.003	2.217 ± 0.008	0.36 ± 0.007	1.765 ± 0.006	1.813 ± 0.006	1.966 ± 0.006
A2	ND	0.613 ± 0.004	1.514 ± 0.007	0.348 ± 0.007	3.701 ± 0.008	0.795 ± 0.005	0.034 ± 0.005
A3	ND	0.387 ± 0.004	0.314 ± 0.003	0.195 ± 0.005	5.312 ± 0.005	0.422 ± 0.007	ND
A4	ND	0.218 ± 0.005	ND	ND	6.435 ± 0.005	0.033 ± 0.006	ND
L1	2.01 ± 0.003	1.294 ± 0.005	0.357 ± 0.006	0.349 ± 0.007	6.737 ± 0.006	2.741 ± 0.004	0.352 ± 0.007
L2	0.463 ± 0.008	ND	1.606 ± 0.006	0.388 ± 0.006	8.874 ± 0.005	0.723 ± 0.006	0.332 ± 0.005
L3	0.374 ± 0.003	ND	1.867 ± 0.006	0.409 ± 0.007	7.382 ± 0.005	0.357 ± 0.006	0.035 ± 0.007
L4	0.33 ± 0.006	ND	0.691 ± 0.009	0.724 ± 0.005	7.974 ± 0.005	0.343 ± 0.006	ND
P1	0.917 ± 0.003	ND	ND	ND	ND	ND	0.088 ± 0.006
P2	0.525 ± 0.007	ND	0.676 ± 0.006	ND	4.549 ± 0.004	0.074 ± 0.007	0.327 ± 0.006
P3	0.682 ± 0.004	ND	3.06 ± 0.008	0.274 ± 0.008	7.404 ± 0.006	1.354 ± 0.006	0.767 ± 0.006
P4	0.717 ± 0.007	ND	2.326 ± 0.006	ND	5.783 ± 0.006	0.437 ± 0.006	1.755 ± 0.006
K1	0.113 ± 0.004	4.285 ± 0.005	0.238 ± 0.007	ND	13.042 ± 0.006	1.05 ± 0.008	ND
K2	0.114 ± 0.004	4.743 ± 0.005	0.452 ± 0.007	ND	13.54 ± 0.005	1.089 ± 0.006	ND
K3	0.131 ± 0.004	5.857 ± 0.006	0.839 ± 0.007	ND	17.482 ± 0.006	1.513 ± 0.006	ND
K4	0.183 ± 0.004	5.525 ± 0.005	1.432 ± 0.007	ND	16.952 ± 0.007	1.332 ± 0.006	ND

Values are mean of three ± S.D.