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Study on the Free Radicals and Oxidative Stress in Biotechnology

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ABSTRACT

Resveratrol, a stilbene phytoalexin, has been gaining importance as an antioxidant with anti-carcinogenic and cardio protective properties. The polyphenol, mainly found in grapes and red wines, has also been characterized from berry fruits and peanuts. However, literature concerning its occurrence in underutilized fruits of India is sparse. With the objective to add value to underutilized fruits with low economic value, resveratrol was quantitated. The study envisages expansion of fruit juice industry in terms of functional fruit juices with several health benefits. In this regard, mulberry (Morus rubra), Indian blackberry or jamun or jambul fruit (Syzygium cumini) and jackfruit (Artocarpus heterophyllus) of Indian origin were explored for processing in terms of resveratrol. Since fermentation brings about quality changes in fruit beverages, studies were focussed to analyze changes in polyphenols, anthocyanins, antioxidant capacity and individual phenolic acids and resveratrol content in fruit wines prepared by fermentation. Wine polyphenols are known to provide protection against atherosclerosis and resveratrol is the main polyphenol responsible for French paradox phenomenon. Nowadays polyphenols are gaining importance in treating metabolic disorders like Type 2 diabetes. This study describes effects of feeding polyphenol rich mulberry and jamun wines to streptozotocin-induced diabetic rats.

Keywords: *Antioxidan, Jackfruit, Fruits, Polyphenols.*

INTRODUCTION

Epidemiological studies have shown that consumption of fruits and vegetables more than 5 servings a day has inverse association with cardiovascular and other degenerative diseases. Polyphenols which forms the major antioxidants in diet are associated with the lower incidence of chronic diseases and their intake is 10 times greater than that of vitamin C and 20 times than that of vitamin E or the carotenoids. Some foods such as tea, wine and cocoa are extremely rich in polyphenols, and the polyphenols contained in these foods are highly effective as antioxidant defences. The antioxidant properties of polyphenols may explain some of their beneficial effects (Quiñones, Miguel, & Aleixandre, 2017). Resveratrol is one such stilbene polyphenol mainly found in grapes and wines and has gained importance in nutritional field due to its involvement

in French paradox phenomenon and is an antioxidant and an inhibitor of platelet aggregation (Kopp, 2018). The ability of resveratrol to modulate the activity of various enzymes and thus to interfere in signaling mechanisms in various cellular processes and participate in different metabolic cellular oxidation– reduction reactions has ascribed it to be a potent anticancer, antiatherogenic and antidiabetic compound (Bhat, Kosmeder, & Pezzuto, 2017).

Present work concentrates on analysis of resveratrol in underutilized fruits and their fermented beverages, belonging to different plant families which are under explored due to regionalization of consumption of indigenous fruits and non commercialization of exotic wild fruits though rich in bioactive components. Also there is emphasizing on invivo antidiabetic effect of fermented beverages from the selected underutilized fruits.

OBJECTIVE OF THE STUDY

1. To study the resveratrol from underutilized fruits and effect of fermentation for functional properties.

2. To study the free radicals and oxidative stress in biotechnology.

MATERIALS AND METHODS

Fruit samples

Wine varietal Red (Vitis vinifera cv.Shiraz) and white grapes (Vitis vinifera cv. Chardonnay) were procured from Zampa Vineyards (Valle de Vin), Nasik Valley, Maharashtra, India. Jamun (Syzygium cumini) fruits were purchased from local farmers, Srirangapatna, Mysore. Jackfruit (Arterocarpus heterophyllus) was procured from local market, Mysore. Mulberry (Morus rubra), fruits were kindly provided by Central Sericulture Research & Training Institute (CSRTI), Central Silk Board, Mysore. The grapes used in our experiments were cultivated in the same vineyard and were harvested during same seasons. Likewise jamun and mulberry fruits were harvested from the same garden and season every time, so the significance of factors, cultivation methods, soil and climatic conditions are not considered to interfere the experimental results. All fruits were stored at -20oC and protected from light until use.

Chemicals

Polyphenols used in the study were trans-resveratrol, pterostilbene, piceatannol, gallic acid, cinnamic acid, caffeic acid, p-coumaric acid, ferulic acid, chlorogenic acid, (+)-catechin, (–)-epicatechinquercetin , and indole harmones melatonin and serotonin. All were purchased from Sigma-Aldrich (Steinheim, Germany). Piceid was obtained from Apin chemicals Ltd. Oxfordshire, UK.

Chemicals needed for wine elaboration and further analysis were as follows: Sucrose was from Rankem (RFCL, New Delhi), Trizyme P 5000 which contained cellulose and pectinase was obtained from Triton Chemicals, Food industry complex (Metagalli, Mysore). Sodium metabisulphite, Bentonite, sodium carbonate anhydrous, hydrochloric acid, aluminium chloride were obtained from Merck (Worli, Mumbai); HPLC grade methanol, Folin – Ciocalteu's phenol

reagent, sodium nitrite, were procured from SRL, Mumbai. Bile salts, salivary α-amylase, pancreatin and mucin were from Sigma-Aldrich (Steinheim, Germany).

Microorganisms

Saccharomyces cerevisiae 20A yeast strain was used in the alcoholic fermentation of fruits. The culture was maintained in CSIR-CFTRI yeast culture collection. Yeast was maintained on Yeast Extract Peptone Dextrose (YEPD) agar slants at 4oC. The slants were sub-cultured every 2 months and 4 days old slants grown at 30oC were used for experiments.

All media preparation and culturing of organisms were carried out as routine microbiological methods. Culture media, glass and plastic wares are sterilized at 1210C for 20 min (15 lbs pressure). Inoculation of cultures was performed in Laminar Air Flow (LAF) hood and all other aseptic methods described for microbiological work were generally followed. All media preparations were carried out using single glass distilled water. For biochemical experiments such as buffer preparation, enzyme assay, antioxidant assays, triple glass distilled water was used.

FREE RADICALS AND OXIDATIVE STRESS

Global population is suffering from chronic diseases such as cancer, type 2 diabetes, atherosclerosis, osteoporosis, depression, dementia, alzheimer's and obesity. Unhealthy diet and lifestyle has led to tremendous increase in these diseases causing damage to human resource and economy globally. Reactive oxygen species (ROS) and oxidative stress has been implicated in a number of human diseases as well as in the ageing process (Harman, 2016). Mitochondrial electron transport chain reacts with molecular oxygen generating many free radicals mainly superoxide anion radical (O2 •−) which further starts a chain reaction of radical species. Dismutation of O2 •− forms hydrogen peroxide (H2O2) which can further react to form the hydroxyl radical (•OH) (Figure 1). Overproduction of ROS results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA (Cadenas & Davies, 2017).

Reactive oxidants are counter balanced by complex cellular antioxidant defense systems in body regulated by a web of pathways to ensure that the response to oxidants is adequate for the body's needs. When this balance goes wrong and there are alterations in signaling pathways oxidative stress emerges. Hence, oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage.

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing (Bar-Or, Bar-Or, Rael, & Brody, 2015). These diseases could be grouped into first group involving diseases characterised by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance, the so called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus) and the second group involves disease characterised by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidaseinduced formation of ROS

(implicated in ischemia and reperfusion injury). The process of ageing is to a large extent due to the damaging consequence of free radical action (lipid peroxidation, DNA damage, protein oxidation) (Valko et al., 2017).

DNA DAMAGE

Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. To date, more than 100 products have been identified from the oxidation of DNA. ROS-induced DNA damage involves single or doublestranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis. Human cell is exposed to approximately 1.5×105 oxidative hits a day from hydroxyl radicals and other such reactive species. The hydroxyl radical •OH can further oxidizes the nucleotides in DNA and RNA and is known to react with all components of the DNA molecule damaging both the purine and pyrimidine bases and also the deoxyribose backbone. 8-hydroxydeoxyguanosine (8-OH-dG) in DNA and 8-hydroxyguanosine in RNA are the major oxidized products from damaged nucleic acids. In DNA, this base can be mutagenic and, thus, carcinogenic in principle (Kasai, 2017). Though the exact effect of 8-OH-dG is not known in carcinogenesis it's hypothized that the DNA damage is predominantly linked with the initiation process of cancer (Valko, Izakovic, Mazur, Rhodes, & Telser, 2018). Although less studied, the RNA base can lead to accelerated degradation and a reduced capacity for protein translation. Varieties of DNA repair enzymes exist to remove oxidized deoxyguanidine from DNA and thus eliminate its potential mutagenicity. Perhaps more intriguing from the point of view of acute illnesses is the fate of oxidized guanidine in RNA. RNA miscoding or mRNA degradation are rapid events, rendering them more significant to acute disease development than the effects of DNA mutation. 8-Oxoguanine, an oxidized form of guanine, has the potential to pair with both cytosine and adenine, and thus, the persistence of this base in messanger RNA is of great danger. RNA molecules carrying 8-OH-dG are prevented by cellular defensive enzymes from entering into the translational machinery so as to prevent m-RNA from causing further translational errors (Hayakawa, Kuwano, & Sekiguchi, 2017)

CARDIOVASCULAR DISEASES (CVDS)

Cardiovascular diseases (CVD's) are the leading cause of death globally. Worldwide rise in CVD's has become a major concern among various health care organisations like AHA (American Heart Association) and NIH (National Institute of Health), USA. A report from AHA estimated that there will more than 23.6 million deaths due to CVD's by 2030 (Mozaffarian et al., 2015). Cardiovascular diseases are a group of disorders which includes metabolic abnormalities of heart or blood vessels. ROS mediated oxidative stress affects the cardiovascular system and results in cardiac diseases like atherosclerosis, ischemic heart disease, cardiomyopathy and complications of vascular diseases of the brain and kidney resulting in Alzheimer's disease (AD) and peripheral arterial systems (Heitzer, Schlinzig, Krohn, Meinertz, & Münzel, 2018). Hypertension, hyperglycemia, mental stress, physical inactivity and elevated blood cholesterol form the risk factors of CVD's (Kannel, 2016). These risk factors are modifiable with the changes in the lifestyles and dietary intervention of healthy foods.

Epidemiologic studies have consistently shown that diet plays a crucial role in the prevention of chronic diseases. A change in dietary behavior such as increasing consumption of fruit, vegetables, and grains is a practical strategy for significantly reducing the incidence of many degenerative diseases. Functional foods that contain significant amounts of bioactive components may provide desirable health benefits beyond basic nutrition and play important roles in the prevention of CVD's.

ANTIOXIDANTS AND PHYTOCHEMICALS

Endogenous antioxidant defence system exists in living organisms to act against free radical damage. Antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). The enzymes act against reactive oxidant species and have a role in preventing several pathologies involved in human diseases like cancer, ischemia, and endocrine dysfunctions. SOD, GPX, and CAT within cells remove superoxide and peroxides before they react with metal catalysis to form more reactive species. Finally, peroxidative chain reactions initiated by reactive species that escaped enzymatic degradation are terminated by chainbreaking antioxidants, including among others water-soluble ascorbate, lipid-soluble vitamin E, and ubiquinone. To optimize performance, oxidative stress must be controlled by supplying known group of nutrient antioxidants which is mainly acquired through diet.

SCREENING OF UNDERUTILIZED FRUITS FOR RESVERATROL AND OTHER PHENOLICS AND THEIR ANTIOXIDANT PROPERTIES

Extraction of fruits

The experimental procedure was performed as described in the reference with slight modification (Soong & Barlow, 2016). Frozen fruit samples were thawed and different parts of fruits- skin, pulp and seeds were separated and lyophilized. However mulberry was used as a whole as it was tough to separate the parts. 1-2 g of lyohilized fruit samples were homogenized in mortar and pestle with 25 ml of ethanol/water (80:20 v/v) and kept in shaking water bath at 60oC for 30 min. The samples were filtered through Whatman No.1 filter paper by applying vacuum. The crude extracts were then concentrated by flash evaporation to 10 ml and used for antioxidant assays.

Determination of total polyphenols of fruits

Total phenolic content of each crude extract was estimated by FolinCiocalteau method (V. Singleton & Rossi, 1965). To 6.0ml triple distilled water 0.1ml sample and 0.5ml Folin-Ciocalteau reagent was mixed followed by the addition of 1.5ml Na2CO3 (20g/100ml water) and the volume was made up to 10.0 ml with distilled water. The reaction mixture was kept in dark for 30 min at 25oC, the absorbance was measured at 760 nm and the phenolic contents were expressed as gallic acid equivalents (mg GAE/g dry weight).

Determination of total flavonoids of fruits

Flavonoid contents in the crude extracts were determined by a colorimetric method (Zhishen, Mengcheng, & Jianming, 2019). 50µl of each extract were taken, made up to 5 ml with milli Q water and 0.3ml of 5% NaNO2 solution was added. After 5 min, 0.5 ml 10% AlCl3.H2O

solution was added and mixed thoroughly. After 6 min of incubation 2ml 1M NaOH was added and final volume made up to 10ml with distilled water. The mixture was vortexed and the absorbance was measured against a suitable blank at 510 nm by spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan). (+)-Catechin was used to prepare the standard curve (20-200 µg) and the results were expressed as mg of (+)-catechin equivalents (CEs) g-1 of extract.

CHANGES IN POLYPHENOLS AND ANTIOXIDANT PROPERTIES DURING DIFFERENT STAGES OF FERMENTATION

Preparation of Starter Culture

The yeast starter culture method was followed according to (SEMON, EDWARDS, FORSYTH, & Dinn, 2017) with slight modifications. Briefly yeast was inoculated into 18X150mm test tubes containing YEPD broth (5 ml) and incubated for three days at 30oC. Upon reaching the late exponential growth phase, yeast cells were harvested by centrifugation (2,000 X g for 25 min) and washed twice with 4 ml phosphate buffer. 500 ml of diluted fruit juice medium (diluted to Brix 10.2, pH adjusted to 4.5 and 0.1% yeast extract added) was prepared from fruits Mulberry, Jamun fruit, Shiraz and Chardonnay. Fruit juices in 2L conical flasks were pasteurized at 80oC for 15-20 minutes. Cooled flasks were inoculated with 1.0% (v/v) washed yeast cells and incubated for three days at 25oC. After 3 days fruit juice inoculum was used to inoculate respective musts.

Elaboration of wine from mulberry jamun and wine grapes

Vinification protocol was followed as described by MA Amerine (Amerine, 2016). Microvinification of fruits (3L) was carried out in the laboratory. Frozen fruits were thawed, grapes destemmed and seeds were manually removed only from jamun fruits. All the fruits were crushed and the resultant juice was called must. Total soluble solids of musts was determined by an ATAGO RX – 5000 digital refractometer (Tokyo, Japan) and corrected to 24 oBrix using sucrose. The pH of musts was checked and adjusted to 3.6 with freshly prepared saturated tartaric acid solution. Musts were sulfited in the form of K2S2O5 to get the sulphur dioxide concentration of about 30 mg/L of free SO2 to inhibit bacterial growth. Pectinase enzyme was added at a concentration of 2% (2g/100ml). After four hours of sulfiting,musts were inoculated with juice inoculums. Fermentation was carried out in 5L conical flasks incubated in a temperature controlled incubator (Kuhner Lab-Therm LT-X, Switzerland) at 25oC. The incubator was covered with black cloth to avoid light in order to prevent the degradation of polyphenols. Throughout active fermentation, the cap formed in the flasks was punched down daily two times in order to immerse the skins. After 7 days of fermentation, 500ml of wine was sampled for analysis and fermentation was continued for another one week. Following, after 14 days wines were pressed and filtered to clean brown bottles (2.5 L capacity) and 500 ml wine sample was kept aside for analysis.

TOTAL POLYPHENOL CONTENT OF FRUIT EXTRACTS

The total polyphenol content of aqueous ethanol extracts of underutilized fruits are presented in Table 3. The total polyphenols varied significantly (P equivalents (GAE)/g dry weight. Jamun seed was found to have significantly higher polyphenols (55.54 mg GAE/g) compared to other

fruit extracts. On the other hand, jackfruit seed (1.00 mg GAE/g), jackfruit pulp (1.27 mg GAE/g) and grape pulp (1.04 mg GAE/g) extracts contain significantly least amount of polyphenols when compared to others. Similar to jamun, seed extract of grapes (26.88 mg GAE/g) was having significantly higher polyphenols followed by skin (10.76 mg GAE/g) and pulp (1.04 mg GAE/g) extracts. In contrast to jamun and grapes, skin extracts of jackfruit was found to have significantly more polyphenols (13.38 mg GAE/g) compared to pulp (1.27 mg GAE/g) and seed (1.00 mg GAE/g) extracts. Mulberry extract was reported to contain 14.35 mg GAE/g which was significantly lower than jamun skin, jamun seed and grape seed extracts.

Total antioxidant capacity

Total antioxidant capacity of fruit extracts were expressed as Gallic acid equivalents (Table 4). Among all extracts, jamun skin extract had significantly more total antioxidant capacity of 4.21 GAE mM/g when compared to the others. Jackfruit seed extract was found to have significantly lesser antioxidant capacity (0.19 GAE mM/g) than the others. Interestingly, pulp extracts of jackfruit and grapes were found to contain more total antioxidant capacity of 3.25 and 3.66 GAE mM/g respectively followed by skin and seed extracts.

Phenolics of underutilized fruits

Analysis of polyphenols by Folin-Ciocalteu method and flavonoids by aluminium chloride method showed that jamun fruit showed highest polyphenol content both in its skin and seeds and also in its pulp as comparable with jackfruit and grape skin seed and pulp extracts respectively whereas grape seed extract contained highest flavonoids (Experiment section 1). Skin and seeds of analyzed underutilized fruits showed more polyphenol content compared to their respective pulps. Our results are in agreement with the studies conducted by Solomon et al., 2016) where the results showed that skins of different commercial varieties of fig fruits had higher polyphenols than pulps. And they found darker fruit varieties had higher polyphenols owing to anthocyanins present in skins which explains our studies also where mulberry fruit, jamun skin and grape skin has higher polyphenols than their colorless pulps (Solomon et al., 2016).

CONCLUSION

In our laboratory indigenous underutilized fruits of India mulberry, jamun (Indian blackberry) and jackfruit were analyzed for their polyphenol content and antioxidant capacities. Resveratrol, the stilbene polyphenol and our compound of interest was detected in all the fruit extracts. The detailed characterization of polyphenols by HPLC showed highest concentration of resveratrol in mulberry and jamun fruits as compared to grapes. These fruits rich in phytochemicals showed potent antioxidant ability in the form of radical scavenging activity. Further, fruit wines were elaborated and assessed for their main quality parameters during different stages (alcoholic, malolactic, aging stages) of wine processing. In mulberry and chardonnay wines polyphenols and flavonoids increased whereas in jamun and red grape shiraz wines there was decrease in polyphenols. Wines at all stages showed high radical scavenging activity. Anthocyanins were decreased due to various chemical reactions like copigmentation and polymerization. It was found that in mulberry wines resveratrol concentration were highest and increased after fermentation and found to be at its highest during aging of mulberry wines. In jamun wines also

resveratrol concentration increased in malolactic and aged wine samples. There were varied significant differences in individual polyphenol compounds at all stages of wines. The results of simulated gastro-intestinal digestion showed that the bioaccessibility of polyphenols of mulberry, jamun, Shiraz and chardonnay wines was greatly decreased after the in vitro intestinal digestion. The DPPH radical scavenging activity assay showed that the wine digests had very low antioxidant capacity.

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