Comparative Analysis of Secondary Metabolites in Selected Rice Varieties.

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Comparative analysis of secondary metabolites in selected rice varieties" is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences And Biotechnology, Goa University under the Supervision of Dr. Siddhi K. Jalmi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Comparative analysis of secondary metabolites in selected rice varieties" is a bonafide work carried out by Ms Mahima Dayanand Naik under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline Botany at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The study deals with the profiling of secondary metabolites in selected rice varieties grown in Goa.The varieties selected were indigenous rice varieties like*Korgut*and *Assgo*,and hybrid high yielding varieties like IR,*Jaya*,*Karjat* and *Jyoti*. Qualitative and quantitative analysis were done using rice seed samples and husk samples of all the selected rice varieties. The secondary metabolites were first detected for their presence in qualitative analysis with the help of histochemical studies.The quantification of secondary metabolites was done using biochemical analysis. Also,GC MS analysis gave the list of overall groups of metabolites present in seeds and husk of different rice varieties. Finally,comparative analysis was done by comparing the data obtained from all the tests.

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TABLES

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ABBREVIATIONS USED

Entity	Abbreviation
Korgut	Ko
Jaya	Ja
Assgo	As
Karjat	Ka
Jyoti	Jy

ABSTRACT

Rice is a staple food for wide population of Asia which reasons for the wide range of research done on the nutrition quality of rice.Rice, being the staple food of Goa,is grown in the state since times immemorial with many different types of varieties grown in different soil types.Keeping in mind the grain quality and properties of different rice varieties,six selected rice varieties were analysed for their secondary metabolite content qualitatively and quantitatively.

The amountof secondary metabolites were found to be higher in seeds and husk of pigmented rice varieties than in seeds and husk non-pigmented rice varieties.Among the pigmented ricevarieties, seeds and husk of indigenous rice varieties*Korgut*and *Assgo* showed higher secondary metabolite content followed by high yielding variety *Jyoti*. The amount of secondary metabolites in grains of different rice varieties and their comparison brings their nutritional quality in light as secondary metabolites have got wide range of health benefits to human health.Bringing the results of such comparative nutritional quality analysis in light of society will help in increased demand,cultivation and preservation of varieties possessing higher amount of these nutritional aspects.

1.INTRODUCTION

1.1 Background

1.1.1Rice as a primary food source

Rice (*Oryza sativa* L.),which belongs to the Gramineae family,has been consumed by humans for almost 5000 years. Rice is a widely diffuse staple food, providing energy and nutrients for more than half of the world population, especially in Asia. The most common rice consumed by humans is white rice (about 85%)due to the taste and texture of the cooked rice, and the rest is pigmented rice(Wang, et al. 2018).However, high starch content of the white rice may cause chronic diseases such as type 2 diabetes. Unlike white rice, the consumption of pigmented rice and unpolished rice is known to prevent the risk of degenerative diseases such as type 2 diabetes(Nurnaistia, et al. 2018).

1.1.2 Secondary metabolites

Secondary metabolites: Plants are capable of producing and synthesizing diverse groups of organic compounds that are divided into two major groups: primary and secondary metabolites. Secondary metabolites are metabolic intermediates or products which are not essential to growth and life of the producing plants but rather required for interaction of plants with their environment and produced in response to stress. Their antibiotic, antifungal and antiviral properties protect the plant from pathogens.Secondary metabolites are studied under three main classes: Phenolic acids which include flavonoids and tannins,Terpenoids and Alkaloids.Terpenes constitute a large class of natural products which are composed of isoprene units. Terpenes are only hydrocarbonsandterpenoids are oxygenated hydrocarbons. The general molecular formula of terpenes are multiples of $(C_5H_8)_n$,where 'n' is number of linked isoprene units. Hence, terpenes are also termed as isoprenoid compounds.

Classification is based on the number of isoprene units present in their structure.Phenolics are a chemical compound characterized by the presence of aromatic ring structure bearing one or more hydroxyl groups. Phenolics are the most abundant secondary metabolites of plants ranging from simple molecules such as phenolic acid to highly polymerized substances such as tannins. Classes of phenolics have been characterized on the basis of their basic skeleton.Alkaloids are a diverse group of nitrogen-containing basic compounds. They are typically derived from plant sources and contain one or more nitrogen atoms. Chemically they are very heterogeneous.

Plants produce a wide range of secondary metabolites that play vital roles for their primary functions such as growth, defence, adaptations or reproduction.Some of the plant secondary metabolites are beneficial to mankind as nutraceuticals and pharmaceuticals. These metabolites are a means of communicating with nature, for their survival and an adaptation to the local environment (Erb and Kliebenstein, 2020).Because of their usefulness and productivity,plant-originated secondary metabolites have been part of human welfare. These metabolites are exploited for their nutritive values, utilized as a source of medicinal components, and agrochemicals.

1.1.3 Secondary metabolites in human health

Secondary metabolites (SM) are compounds which are not only very important for plant survival but also play an essential role in their mechanism of action and have a number of medicinal properties to improve human health. These compounds have antioxidant activity that alleviates many ailments and promotes good health. These SM have the potential to improve human health by mitigating the negative effects of certain chronic diseases and aging. Certain components found in horticultural crops are considered to reduce the cancer of risk,protect the genome, develop immune system, and eliminate toxins.Among the various plant metabolites,polyphenols act primarily as free scavengers, reduce oxidative stress, are anti-mutagenic, and play a role in cancer and heart disease prevention and the development of atherosclerosis(Lal, et al. 2023)

1.1.4 Secondary metabolites in rice

There are diverse secondary metabolites produced in rice. These metabolites are organ- and tissue-specific. For example, diterpenoid phytoalexins are mainly present in the leaves, whereas phenolic acids, flavonoids, sterols, and triterpenoids are mainly present in the bran.Rice secondary metabolites play roles either as defence agents, by providing disease resistance and exerting anti-nematode, anti-insect, and allelopathic activities against biotic and abiotic stresses, or as plant growth regulators. They also show various kinds of biological activities, such as antimicrobial, antioxidant, cytotoxic, and anti-inflammatory properties, which are implicated in various health-promoting and disease-preventive effects. Rice metabolites mainly include phenolic acids, flavonoids, terpenoids, steroids, alkaloids. Some metabolites such as phenolic acids and flavonoids are also distributed in other plant species(Wang, et al. 2018).

It has been known that secondary metabolites play important roles in disease resistance, antioxidant, antibiotic in germination stage or production of rice.Phenolic and flavonoid compounds are the most widely distributed secondary metabolites in plants. They play as growth inhibitors against weeds, pests as increased the resistance of plants against pathogenic stresses.Phenolics are also important in growth and reproduction of plants and provide protection against predators.Besides, many phenolics are reported to correlate with food quality such as appearance, flavour and health-promoting properties(Xuan, et al. 2018).

1.1.5 Traditionally cultivated rice varieties of Goa

Rice is the predominant staple food crop of Goa occupying more than 39 per cent (52,442 ha)of the total cultivated area in the State. It is cultivated under three distinct ecologies during kharif season. The crop is cultivated in Morod lands (lateritic uplands covering 16.4 % of area accounting to 8,600 ha), midlands or kher lands totalling to 32 per cent of area (16,900 ha) and the Khazan lands 32 per cent (17,200 ha) and the rest covered under rabi season (Manjunath, et al. 2009).

Goan farmers have been traditionally cultivating wide varieties of rice grown in different soil types and different seasons. Among many varieties of rice grown in the state,local names of some are noted here,including those which are used in this study:*Annapurna*,*Assgo*,*Atthavis*,*BarikKadi*,*Bello*,*Chudi*,*Damgo*, *Dhave*, *Dodga*, *KalaNovan*,*Karjat*,*Karz*, *Kenal*,*Kendal*,*Khochro*,*Korgut*, *Mangala*,*Ner*,*Novan*, *Revati*,*Salsi*, *TamdeJyoti*,*VadloKenal*, IR8,*Jyoti*,*Jaya*,*Babri*,*Khochri*,*Patni*, *Dhavi-Patni*,*Belo*, *Tamdi* and *Nermar*(Manjunath, et al. 2009).

1.1.6 The rice varieties selected for the study

Variety *Korgut*is the only variety grown in Khazan lands of Goa and is endogenous to Goa. Out of the six varieties, *Korgut*,*Assgo* and *Jyoti* have brown to dark brown bran colour while others are white.Grains of *Korgut* are broader as compared to all other varieties and those of IR are thinnest and shortest.

The study involved first qualitative analysis for the presence of four types of secondary metabolites: Phenolic acids, Flavonoids, Terpenoids and Alkaloids with different chemical tests. Moving further,using the data from qualitative analysis, quantitative studies were done on all six varieties for three types of secondary metabolites: Phenolics, Flavonoids and Terpenoids using UV-Visible spectroscopy. Finally, localisation of the secondary metabolites was done with different stains.

1.1.7 Studies done on rice varieties in Goa

A study on grain quality analysis of traditionally cultivated rice varieties was done earlier which included testing the grains for the grain appearance, nutritional value, cooking and eating quality, Head rice recovery (HR), Gel consistency (GC), Chalkiness of endosperm, Aroma, Amylose content (AC), Volume expansion ratio and elongation ratio and Water uptake(Bhosle, et al. 2010).

Though there has been a study on grain quality of various rice varieties cultivated in Goa, secondary metabolite composition needed some light to understand the presence and quantity of them in these rice varieties. In the study total six rice varieties were selected for analysis,local names of which are *Korgut*, IR, *Jaya*, *Assgo*,*Karjat* and *Jyoti*.

1.1.8 A brief summary of the work

The study involves first qualitative analysis to detect for the presence of four types of secondary metabolites: Phenolic acids, Flavonoids, Terpenoids and Alkaloids in selected rice varieties and their respective husk with different chemical tests. Further,using the data from qualitative analysis, quantitative studies were done on all six varieties for three types of secondary metabolites: Phenolics, Flavonoids and Terpenoids using UV-Visible spectroscopy.Finally, localisation of the secondary metabolites was done with different stains.

1.2 Aim and Objectives

1.2.1. Aim: To perform comparative profilingof secondary metabolites in grains of selected rice varieties.

1.2.2 Objectives:

a. Qualitative and quantitative analysis of secondary metabolites in grains of selected rice varieties.

b. Localisation of secondary metabolites in grains of selected rice varieties.

c. Comparing secondary metabolitecontent in grains of selected rice varieties.

1.3 Hypothesis /Research questions:

Traditionally cultivated rice varieties,having coloured bran and aroma,are expected to contain higher amount of secondary metabolites than hybrid rice varieties with colourless bran.Comparative analysis reveals which varieties contain high secondary metabolites,thus supporting their nutritional value.

1.4 Scope:

Bringing in light the scientific data on comparative analysis of secondary metabolite content in different rice varieties will promote people to opt for varieties promising a high amount of secondary metabolites and also lead to increased production and preservation of these varieties.Due to revelation of the nutritional values of these varieties,more research can be done on genes involved in the pathways for secondary metabolites in these varieties and use of this knowledge in making hybrid or recombinant varieties.

Introduction

2. REVIEW OF LITERATURE

2.1 Studies of rice secondary metabolites

Rice can accumulate a large number of secondary metabolites, such as phenolic acids,flavonoids,terpenoids, steroids, and alkaloids. These molecules play various physiological and ecological roles(i.e., antimicrobial, insecticidal, growth regulatory, and allelopathic activities).They also exhibitfeatures beneficial to humans, including cytotoxic, anti-tumor, anti-inflammatory, antioxidant, and neuroprotective properties. For example, many phenolic acids, flavonoids, tocopherols, tocotrienols, γ-oryzanol, and phytic acid from rice exhibit antioxidant activities.

Studies on secondary metabolites in rice have been conducted to understand their composition in response to pathogen attack, stress and the signaling that is transduced in response to them.Varieties of secondary metabolites also has been done in rice in different varieties throughout the world. Characterization of secondary metabolites in rice varieties also has been done using techniques like GC - MS and HPLC. Review articles providing crisp information of the plethora of secondary metabolites and their content, structure is also been published. A study on grain and cooking qualities of traditionally cultivated rice varieties of Goa had been conducted to understand the texture, chalkiness,cooking characteristics,etc of traditionally cultivated rice.

2.2 Phenolic acids in Rice

As the research and review articles suggest, the pigmented rice contains phenolic acids with larger structural diversity and in higher content than the non-pigmented rice. About 32 phenolic acid analogues have been identified in rice(Wang, et al. 2018). Rice phenolic acids can be classified as soluble-free, soluble-conjugated, and insoluble-boundforms. The rice insoluble-bound phenolic acids covalently bind to structural components of cells like cellulose, hemicellulose, lignin, pectin, rod-shaped structural proteins, etc. The distribution of rice phenolic acids exhibit varietal differences, and rice bran has the highest total phenolic acid content among four different fractions of whole rice grain.Overall, p-hydroxybenzoic acid, caffeic acid, protocatechuic acid, ferulic acid,sinapic acid, syringic acid, and vanillic acid are present in the whole rice grain, and ferulic acid is the most abundant phenolic acid in the insoluble-bound fraction(Wang, et al.2018).

The main rice phenolic acids are protocatechuic acid, p-coumaric acid, ferulic acid,sinapic acid, and vanillic acid. A total of 12 phenolic compounds were identified in all rice varieties in studies conducted by Ding et al. Protocatechuic acid, ferulic acid, gallic acid, and syringicacid were the dominant phenolic compounds in rice bran, while p-hydroxybenzaldehyde was the main phenolic acid in rice husk. Bran and husk fractions provide more than 90% of phenolic acids and antioxidant activity of the whole rice plant. In addition, the rice subspecies japonica has significant higher phenolic acids content and antioxidant activity than the indica subspecies. Ferulic acid has also been found as the major phenolic compound in black rice bran, indicating the potential use of black rice bran as a natural source of antioxidants. Phenolic acids are considered to be natural antioxidants, being able to scavenge free radicals that may increase oxidative stress and potentially damage large biological molecules such as lipids, proteins, and nucleic acids. Therefore, the phenolic acid content is positively correlated with rice antioxidant capacity.The development and utilization of phenolic acid analogues from rice husk and bran are important for improving the functionality of rice by-products. Some phenolic acids are released from rice roots as allelochemicals (Wang, et al. 2018).

2.3 Flavonoids in Rice

According to the structural features, rice flavonoids can be classified as flavones, flavanols, flavanones, flavanonols, flavanols, and anthocyanins,along with their glycosides.Rice flavonoids mainly have antioxidant properties, though some of them have not been evaluated for their antioxidant activities. Among them, anthocyanins are mainly distributed in pigmented rice plants.The flavonoids in rice include aglycones(i.e., quercetin, kaempferol and tricin) and their glycosides(Wang, et al. 2018).

2.4 Terpenoids in Rice

Rice terpenoids include monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids. Some monoterpenoids and sesquiterpenoids are volatile components and are often distributed in rice leaves. Rice diterpenoids play roles as phytohormones and phytoalexins. The triterpenoids are usually distributed in rice bran. The monoterpenoids, sesquiterpenoids, and triterpenoids usually play functions as allelochemicals. Monoterpenoids are mainly volatile compounds which confer rice its good aroma character. At least 18 monoterpenoids have been identified in rice. Sesquiterpenoids are also volatile components which contribute to the aroma quality of rice. They can be analyzed and identified by GC and GC-MS. The relative content of sesquiterpenoids was much lower, on average, than that of monoterpenoids in rice. Sesquiterpenoids are usually produced and released from wounds or microbe-infected sites.They act as signaling molecules that induce defense against tissue damage caused by herbivores or plant pathogens.Almost all rice diterpenoids are members of the labdane-related superfamily, which includes not only phytohormone gibberellins (GAs) but also phytoalexins (i.e., phytocassanes, oryzalides, and oryzalexins), participate in the defense against pathogens, and are allelochemicals (i.e., momilactone B) inhibiting the growth of other plant species.Triterpenoids are usually distributed in rice bran. Eight hydroxylated triterpene alcohol ferulatewere isolated from rice bran. They showed moderate cytotoxic activity. The seed coats (or bran) usually contain large amounts of bioactive metabolites (Wang, et al. 2018).

2.5 Alkaloids in rice

Almost all research papers suggest absence of secondary metabolites. However,some review articles provide a mention for some alkaloids in rice. The main alkaloids in rice are phenylamides containing an indole ring. Rice plants accumulate phenylamides in response to a pathogen attack (Wang, et al. 2018).

2.6Effect of germination on secondary metabolite profile of rice

Germination can change the secondary metabolite profile and antioxidant activity of rice. The antioxidant activity,which plays important roles in both plant and human health, can decrease or increase upon germination depending of rice species. The varying antioxidant activity of extract depends on the content of the phenolic compounds of which in the pigmented rice are anthocyanin, a group of phenolic compounds that is water soluble. In addition,phenolic compounds in rice are found in the soluble outer layer so that the soaking can decrease the content of the compound. Germination of pigmented rice without removing the hull of rice can preserve the antioxidant compound and its antioxidant activity(Nurnaistia, et al. 2018).

2.7 Role of secondary metabolites in biotic and abiotic stress to rice

Much studies have been done on role of secondary metabolites in rice - pathogen interaction or secondary metabolite composition in rice in stress conditions.Terpenoids are known to be involved in animal attraction.Saponosids (heterosides) are compounds that have varied biological and pharmacological principles. They are known for their antimicrobial and insecticidal activity.Triterpenoids include groups of biologically important substances that comprise sterol and steroids hormones. The basic structure is the common sterane molecule which has insecticide and repulsive actions.The total phenols are very essential to protect crops against pests.They act on the behavior, physiology and metabolism of pest insects and hence reduce the pest population on plants.Tannins are a complex group of polyphenolic compounds found in a wide range of plant species commonly consumed by ruminants.Tannins may sometimes act as toxins for herbivorous insects but as facilitators of digestion.Flavonoids are a complex group of polyphenolic compounds found in a wide range of plant species. They are known to have very complex roles in plant insect interactions and may have a positive or negative role in the life of the herbivorous larvae depending on the species of pests and the type of chemical composition(Xuan, et al. 2018).

2.8Studies done on rice varieties in Goa

Screening of rice varieties for tolerance to salinity: Soil salinity is a serious problem in the coastal saline soils ofGoa. These lands locally known as Khazan lands are subjected to periodical inundation of sea or creek water during high tides. As a result, these lands are progressively rendered saline. Even ifthese lands are protected from the ingression of sea or creek water by constructing embankments, salts from the shallow water table rises to the surface through capillaries making the surface soils, saline in Goa. The problematic area is estimated to be around 18,000 ha, out of which about 12,000 hectares are utilized for cultivation of rice in kharif. To overcome the problem and decide the strategy for increasing production and productivity of rice in the problematic saline soil, efforts have been made to identify the high yielding salt tolerant rice varieties suitable for saline (Khazan) lands as local rice varieties like *Korgut* and *Assgo* which are tall and lodging type are low yielders (Manjunath, et al. 2009).

Introduction and evaluation of Red kernelled Rice: There has been a preference for red kernelled rice in Goa and adjoining coastal region. Par boiled red rice being the most preferred along with fish curry, nine high yielding red kernelled rice genotypes were introduced from RARS, Moncompu, Kerala and evaluated for their local adaptability and suitability during the period from 2003 to 2005 (Manjunath, et al. 2009).

Introduction and selection of high yielding medium duration rice varieties: To improve the productivity levels of paddy, improved varieties of rice were introduced from similar agro-climatic conditions in the country and were evaluated for the local situations. During the year 1977, five rice varieties namely Co-40, Co-39, Bhavani, Intan and Phalguna were evaluated along with the local check (IR-8), Recommended manuring and cultural practices were followed for all the varieties and growth observations were recorded periodically. Observations recorded five weeks after transplanting indicated very good growth and stand ofCo-40 and Bhavani (Manjunath, et al. 2009).

Introduction and feasibility studies on Scented Rice: Northern parts ofIndia has been a domain of Basmati and known for its quality all over the world. The research on this high value rice was specifically attempted to provide a cushion against the spiralling cost in traditional paddy cultivation in order to make it remunerative with a better benefit-cost ratio. In an attempt to introduce Basmati / fine grain rice, six varieties including Pusa Basmati-l as control were included for evaluation during the period 1995 to 1998. Among these varieties, variety Indrayani out yielded rest of the varieties with yield of 6.36 t/ha as compared to Pusa Basmati-l (3.89 t/ha). Further, the work done in this area suggested that Basmati can be successfully grown in the region if planted early in kharif season. The results showed accumulation of adequate amounts of 2 Acetyl-l pyrroline content in the grain. The quantification of the active ingredient was to an extent of 0.030 ppm under Goa conditions as against 0.061 ppm recorded in marketed brand of Basmati rice (Manjunath, et al. 2009).

Rice Hybrids in breaking the yield barriers: In order to break the yield barriers in the traditional inbred varieties popularly cultivated, the research efforts were further intensified for introduction and evaluation of rice hybrids. During the period 1998-99 to 2001, the Institute under it's rice research programmes has introduced and evaluated 27 rice hybrids from the ICAR network as well as private companies. Under the Cess fund project (1998-2000), eleven hybrids were evaluated along with the best inbred varieties viz *Jaya*, *Jyoti* and Triguna as checks. Among the eleven hybrids tested, the evaluation of the proven public bred rice hybrids viz, Sahyadri, KRH-2 and DRRH1, was continued. The studies resulted in recommendation of hybrids like Sahyadri (7.53t/ha) and KRH-2 (7.04 t/ha) which were found to be highly significant over the three check varieties *Jaya* (6.1 tlha), *Jyoti* (4.84 tlha) and Triguna (5.88 t/ ha) for the region. The yield advantage of Sahyadri over the check varieties *Jaya*, *Jyoti* and Triguna was 23.44 per cent, 55.57 per cent and 28.06 per cent, and that ofKRH-2 was found to be15.40 per cent, 45.45 per cent and 19.72 per cent, respectively. This corresponded to a yield advantage of 1,430 kg, 2,690 kg and 1,650 kg/ ha in case of Sahyadri and 940 kg, 2,200 kg and 1,160 kglha in case ofKRH-2, respectively (Manjunath, et al. 2009).

Standardisation of Seed production practices in Hybrid rice: Availability of quality hybrid seeds in bulk at an affordable cost and at an appropriate time is very essential to bring more area under the rice hybrids. Thus, seed production is the most crucial link between development of hybrids and their cultivation by the farmers. Hybrid rice seed production involves several intricacies which have to be managed efficiently to obtain a higher seed yield. Further,development of an economic and efficient seed production package is a prerequisite for commercial viability of the hybrid rice technology. Achieving proper synchronization between parental lines, determining the optimum row ratio, identifying appropriate dosage and stage for application of GA3 finding out precise and appropriate timing for supplementary pollination etc., are some of the aspects which need to be standardized for different locations. Keeping these points in view, attempts were made to standardize the seed production practices for agro-climatic conditions ofGoa (Manjunath, et al. 2009).

A study on grain quality analysis of traditionally cultivated rice varieties was done earlier which included testing the grains for the grain appearance, nutritional value, cooking and eating quality, Head rice recovery (HR), Gel consistency (GC),Chalkiness of endosperm, Aroma, Amylose content (AC), Volume expansion ratio and elongation ratio and Water uptake (Bhosle, et al. 2010).

3. METHODOLOGY

3.1 Rice varieties,their collection and storage

Grains from a total of six rice varieties were obtained directly from farmers involved in the cultivation of these varieties. The names of varieties and their respective place of collection is as given in Table No 3.1. Pictures of the selected rice varieties with husk and without husk is presented in Fig Nos. from 3.1 to 3.12.

The collected rice varieties were stored in labelled polythene bags at room temperature and used as and when needed.

3.2 Dehusking and converting the rice and its husk into fine powder

All six rice varieties were dehusked for obtaining their husk.The dehusked rice and the husk of all six varieties was ground separately to convert it into fine powder. Finally, the powdered rice and husk was stored in closed glass containers to avoid contamination by moisture and pathogens.Total of twelve powdered samples (six of dehusked rice powder and six of husk powder) were stored.

3.3Preparation of extract and its storage

Extraction of secondary metabolites from the above mentioned twelve powdered samples was tried for their results using four different solvents: Methanol,Ethyl acetate,N – hexane and distilled water.The extraction was done using maceration method wherein 5g of respective powdered samples were mixed with 50mL of solvent in respective labelled beaker and kept in water bath for 30min at temperature of 60 degrees and shaken repeatedly.The mixtures in the beakers were then filtered using grade 1 filter paper and the filtrate was used as extract.The mentioned extraction procedure was performed using all four solvents. After repeating the extraction of twelve powdered samples with all four solvents, methanol was selected as the solventgiving best extraction results due prominent pinkish colored extract obtained unlike a colorless extract produced from other solvents.

At the end of extraction,six (1 from each variety) extracts were obtained of dehusked rice and six(1 of each variety) extracts were obtained of husk.The twelve extracts thus obtained from methanol were stored in glass vials and stored at 4 degrees in cold room throughout their use in further work.Extracts prepared from rice powder and husk powder are presented in Fig Nos. 3.13 and 3.14,respectively.

3.4 Qualitative analysis (Preliminary testing)

For testing the presence or absence of secondary metabolites in the samples of rice and husk,different tests were employed for different classes of secondary metabolites.

3.4.1 Preliminary tests for Phenolic acids:

a. Ferric chloride test: To 1mL extract add few drops of 5% $FeCl_3$ solution. Green color indicates the presence of phenolic acids.
b. Gelatin test: To 1 mL of extract add 2mL of 1% gelatin solution containing 10% sodium chloride solution.A white ppt. indicates the presence of phenolic acids.
c. Lead acetate test: To 1mL extract add 3mL 10% lead acetate solution.Awhite bulky ppt. indicates the presence of phenolic acids.

3.4.2 Preliminary test for flavonoids:

a. Lead acetate test: To 1mL of extract add few drops of lead acetate and shake. A yellow – white ppt. indicates the presence of flavonoids.

3.4.3 Preliminary test for terpenoids:

a. To 2mL of extract ad 2mL chloroform. To this add 1mL of conc. sulfuric acid. A reddish - brown coloration indicates the presence of terpenoids.

3.4.4 Preliminary tests for Alkaloids

a. Mayer's test: To 1mL of extract add a drop of Mayer's reagent by side of the test tube.A white ppt. indicates the presence of Alkaloids.
b. Wagner's test: To 1mL of extract add few drops of Wagner's reagent by side of the test tube.A reddish-brown ppt. indicates the presence of Alkaloids.
c. Hager's test: To 1mL of extract add 2mL of Hager's reagent.A prominent yellow ppt. indicates the presence of Alkaloids.

3.4 Quantitative analysis

Quantification of the secondary metabolites detected in the qualitative tests was done using UV – Visible spectroscopy. For each extract,sample preparation was done in triplicates prior to recording its absorbance in spectrophotometer.The sample preparation,standard solution and wavelength at which the absorbance is recorded differs for each class of secondary metabolite and is given below in detail.

3.4.1 Sample preparation for phenolic acids:

- Take 0.2mL of extract in a test tube and dilute it to 3mL with distilled water.
- Add 0.5mL Folin -Ciocalteu reagent.
- After 3minutes,add 2mL of 20% Sodium carbonate and mix the contents thoroughly.
- Finally,test tube is placed in boiling water bath for exactly 1minute.
- Cool and record absorbance at 650nm.
- Standard used was Catechol with stock solution of 1mg/mL.

3.4.2 Sample preparation for flavonoids

- Take 1mL extract in atest tube and dilute it by adding 4mL distilled water.
- Add 0.3% Sodium nitrite and wait for 5min.
- Add 0.3% Aluminium chloride and mix.Wait for 5min.
- Add 2mL of 1M NaOH and dilute the mixture to 10 mL with distilled water.
- The absorbance is determined against reagent blank at 510nm.
- Standard used was quercetin with stock solution of 1mg/mL.

3.4.3 Sample preparation for terpenoids

- Take 1mL of extract in a test tube and to it add 3mL chloroform.
- Vortex thoroughly and close using ice packs.
- Add 400microliter of conc.H_2SO_4.
- Incubate in dark for four hours at 25degrees.
- Reddish brown ppt. formed at bottom.
- Colored part made to final volume of 4mL using 95% Methanol.
- Absorbance is recorded at 538nm with 95% Methanol as blank.
- Standard used was Linalool with stock solution of 1mg/mL.

3.4 Localisationof secondary metabolites in rice grains

Varieties of secondary metabolites was done for all six selected rice varieties. The dehusked rice grains were kept for soaking overnight and the following day their thin sections were taken and stained with stains for different classes of secondary metabolites.The stains and procedure for staining was as follows:

3.4.1 Localization of Phenolic acids: $FeCl_3$ was used for staining the sections of rice grains for localizing phenolic acids. Greenish areas visible when the section is viewed under compound microscope localize the areas of phenolic acids.

3.4.2 Localization of Flavonoids: NH_4OH was used for staining the sections of rice grains for localizing flavonoids. Yellow-green areas visible when the section is viewed under compound microscope localize the areas of phenolic acids.

3.4.3 Localization of terpenoids: Cupric acetatesolution was used for staining the sections of rice grains for localizing terpenoids.Bluish - green areas visible when the section is viewed under compound microscope localize the areas of terpenoids.

3.5 Comparative analysis:

Comparative analysis on the secondary metabolite content in different rice varieties was done and is presented in this report in the form of tables and charts.

4 RESULTS AND DISCUSSION

4.1 Results of qualitative analysis

Overview:

i. Phenolic acids were found to be present in rice of *Korgut*,*Assgo* and *Jyoti* and husk of all six varieties.
ii. Flavonoids were found to be present in rice and husk of all six selected varieties.
iii. Terpenoids were found present in rice of *Korgut*,*Assgo* and *Jyoti* and husk of all six varieties.
iv. Alkaloids were found absent in all the samples.

(Here,presence imply that the particular secondary metabolite was **detected** in the sample extract indicated by the test result).

Results of qualitative tests are presented in tabular form in Table No.4.1.

Results of qualitative tests in pictorial form are presented in figures from Fig No.4.1 to Fig No. 4.16.

4.2 Results of Quantitative analysis

4.2.1 Sample color, optical density,standard graph and concentrationof phenolic acids:

Sample color for phenolics was dark blue in color, with that for husk sample being darker (Fig No. 4.17 and Fig No.4.18). Phenolic acids content was found higher in *Korgut*, Assgo and *Jyoti* than other three varieties which reasons their detection of phenolics in these varieties in qualitative tests.Among these,*Assgo* showed the highest content of phenolics (Table No.4.2) followed by *Korgut* and *Jyoti*. Content of phenolics in other three varieties was almost similar. When the content in rice is compared to that in husk,husk has higher phenolics than rice (Table No.4.2). The trend of phenolics being higher in *Korgut*, *Assgo* and *Jyoti* is followed even in husk. In concentration graph,concentration of phenolics in different varieties is presented (FigNo.4.24).

4.2.2 Sample color,optical density,standard graph and concentration of flavonoids:

Sample color for flavonoids was orange in color, with color for husk being darker (Fig No.4.19 and 4.20). Flavonoid content was found higher in*Korgut*,*Assgo* and *Jyoti* than other three varieties in case of both rice and husk(Table No.4.3).Among these three prominentvarieties,*Korgut* showed the highest content of flavonoids followed by *Assgo* and *Jyoti* in rice.Content of phenolics in other three varieties was almost similar. When the content in rice is compared to that in husk,husk has higher phenolics than rice. The trend of phenolics being higher in *Korgut*, *Assgo* and *Jyoti* is followed even in husk.But here in husk flavonoid content in the three prominentvarieties is higher in *Assgo*followed by *Jyoti* and *Korgut*. In concentration graph,concentration of flavonoids in different varieties is presented (Fig No.4.26).

4.2.3 Sample color, optical density,standard graph and concentration of terpenoids:

Sample color for terpenoids was light brown in color, with color for husk being darker than that in seeds (Fig No.4.21 and 4.22). Terpenoid content was found higher in *Korgut*,*Assgo* and *Jyoti* than other three varieties in case of both rice and husk (Table No.4.4).Among these three prominentvarieties,*Assgo* showed the highest content of flavonoids followed by *Korgut* and *Jyoti* in both rice and husk.Content of terpenoids in other three varieties was almost similar. When the content in rice is compared to that in husk,husk has higher terpenoids than rice. In concentration graph,concentration of terpenoids in different varieties is presented (Fig No.4.28).

4.3 Results of Histochemical study:

4.3.1: Localization of phenolics

Phenolic acids appear green when stained with $FeCl_3$ solution.In *Korgut*,Phenolic acids can be seen near the embryo region and along the bran layer (Fig No.4.30).The phenolics decrease as we go towards endosperm.In IR too,phenolic acids are found towards embryo and in bran layer; they decrease towards endosperm(Fig No.4.31). However,the amount of phenolics is less in IR as compared to *Korgut* as seen by the high intensity of stain in*Korgut* as compared to IR.In *Jaya* the phenolics are seen in the endosperm region and in the bran layer (Fig No.4.32).In *Assgo*,Phenolics are more concentrated in embryo region and highly concentrated in bran layer but they are almost absent in endosperm (Fig No.4.33).Hence distribution of phenolics in *Korgut* and *Assgo* is more towards embryo side. In *Karjat*,like in *Jaya*, phenolics are seen in the endosperm region and in the bran layer (FigNo.4.34).In *Jyoti*,phenolics are more concentrated in the embryo region and in bran layer and they decrease in the endosperm region(Fig No.4.35).

Hence,it can be concluded that,in varieties having higher phenolic acid content (*Korgut*,*Assgo* and *Jyoti*),the phenolics are more towards embryo and in bran layer and they are almost absent in endosperm region.Invarieties having low phenolic acids content like *Karjat* and *Jaya*(but not IR),phenolics are present in embryo region.

4.3.2: Localization of flavonoids

Flavonoids appear yellow when stained with NH_4OH.In *Korgut*,as yellow coloration can be seen only in the bran layer, it can be said that flavonoids are in bran layer(Fig No.4.36).In IR,Flavonoids are present in embryo region (In IR, phenolics were also localized in the embryo region)(Fig No.4.37).In *Jaya* and *Karjat*,clear visualization of flavonoids couldn’t be made as there was no yellow coloration (Fig No.4.38 and 4.40).Hence it proves the less content of flavonoids in *Jaya*and*Karjat*.In *Assgo* and *Jyoti*,slight presence of flavonoids can be seen at the tip of embryo region (Fig No.4.39 and 4.41).

Hence, it can be said that in varieties having high flavonoid content (*Korgut*,*Assgo* and *Jyoti*),some presence of flavonoids can be seen near the embryo region. In varieties having low flavonoid content like *Karjat* and *Jaya*(but not IR),flavonoids are not localized to a distinguishable extent.IR,though showing low presence of flavonoids in quantitative tests,has flavonoids distributed in embryo region.

4.3.2: Localization of terpenoids

Terpenoids appear bluish-green when stained with cupric acetate.In *Korgut*,as clearly visible,Terpenoids are more concentrated towards embryo along the line that travels in the middle of endosperm (Fig No.4.42 and 4.43).In IR,there's a slight appearance of terpenoids at the junction of embryo region and the start of endosperm(Fig No.4.44).In *Jaya*,the section did not take anystain but instead the stain crystallized to form small blue crystals of cupric acetate which proves the absence of terpenoids in the section(Fig No.4.45).In *Assgo*,though not as clearly distinguishable as in *Korgut*,the terpenoids are slightly localized in the embryo region and their content is almost absent in the endosperm region (Fig No.4.46).In *Karjat*,the section did not take stain but the stain instead got crystallized,proving the absence of terpenoids in *Karjat* grain (Fig No.4.47).In *Jyoti*, terpenoids are localized in the embryo region,especially at the junction of embryo and endosperm (Fig No.4.48 and 4.49).But terpenoids are almost absent in endosperm region.

Hence it can be concluded that *Korgut* shows a very clear visualization of terpenoids in the embryo region.In other two high terpenoid containing varieties(*Assgo* and *Jyoti*)terpenoids are present near the embryo region.In low terpenoid containing varieties,terpenoids were not localized indicating their negligible presence.

4.3 Results of comparative analysis:

After analyzing the data from all the tests,it can be concluded that grains of pigmented varieties contain higher amount of secondary metabolites than grains of non-pigmented rice varieties.Among pigmented rice varieties,*Korgut*and *Assgo*contain higher,sometimes equal,amount of secondary metabolites than *Jyoti*.

Qualitative analysis revealed the presence of secondary metabolites like phenolics, flavonoids and terpenoids in *Korgut*, *Assgo* and *Jyoti*. Alkaloids were found absent in all the varieties. Quantitative analysis proved that the concentration of phenolics is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by Jyoti than in seeds of non-pigmented varieties. The concentration of phenolics in husk was always higher than in dehusked seeds. Similarly, the concentration of flavonoids is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by *Jyoti* than in seeds of non-pigmented varieties. The concentration of flavonoids in husk was always higher than in dehusked seeds. The concentration of terpenoids is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by *Jyoti* than in seeds of non-pigmented varieties. The concentration of terpenoids in husk was always higher than in dehusked seeds.

When rice and husk data are compared,husk contains higher amount of secondary metabolites than rice but the trend of higher amountof secondary metabolites in pigmented rice varieties than non-pigmented ones remain the same in husk too.

The results of comparative analysis are presented in the form of a table(Table No.4.5) and a graph(FigNo. 4.29).

4.3 Analysis of characterization data obtained from GC-MS for secondary metabolites in selected rice varieties:

Data on characterized secondary metabolites in four rice varieties(seeds and husk): *Korgut*,*Assgo*,IR and *Jaya*,obtained from GC-MS was analyzed to find which compounds of secondary metabolites are unique to particular category and which are common to all four varieties.The results were drawn in the form of Venn diagrams(Fig No.4.50 and 4.51) using the software Biotool.Using the same software, compounds those are unique to a particular variety and those which are common to all were identified.Also, segregation of compounds into categories like amino acids, fatty acids,etc. was made.

In seeds data,compounds with normalized area of more than 0.05 were analyzedfurther. Venn diagram for seeds data is presented in Fig No.4.50. In seeds, *Korgut*showed the presence of three unique varieties namely, D-Glucitol, 6TMS derivative, 1,5-Anhydroglucitol, 4TMS derivative, Dulcitol, 6TMS derivative. In *Assgo* seeds there were only one compound, D-Psicose, pentakis(trimethylsilyl) ether, methyloxime (syn), was unique. Three compounds were unique to IR seeds namely, N-Methyltrifluoroacetamide, TMS derivative, Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]propyl ester, 1,4-Butanediol, 2TMS derivative. Total six compounds were unique to *Jaya* seeds and one of the compounds, Maltose, octakis(trimethylsilyl) ether, methyloxime (isomer 1), was found common in both *Jaya* seeds and *Jaya* husk.Four compounds were common to seeds of all the 4 varieties (Table No.4.6).

In husk data,compounds with normalized area of more than 0.05 were analyzed further.Venn diagram for seeds data is presented in Fig No.4.51.In husk, *Korgut* and *Assgo*had seven compounds common to each of them.IRhad six compounds unique to itself and *Jaya* had nine compounds unique to itself.There were five compounds common to husks of all the five varieties (Table No.4.7).

Further, categorization of compounds was made into categories like amino acids, fatty acids, sugars and alcohols. Categorization of compounds into different classes is presented in Table No.4.19.

5. CONCLUSION

Qualitative analysis revealed the presence of secondary metabolites like phenolics, flavonoids and terpenoids in *Korgut*, *Assgo* and *Jyoti*. Alkaloids were found absent in all the varieties.Quantitative analysis proved that the concentration of phenolics is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by Jyoti than in seeds of non-pigmented varieties. The concentration of phenolics in husk was always higher than in dehusked seeds. Similarly,the concentration of flavonoids is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by *Jyoti* than in seeds of non-pigmented varieties. The concentration of flavonoids in husk was always higher than in dehusked seeds. The concentration of terpenoids is higher in seeds of pigmented rice varieties like *Korgut* and *Assgo* followed by *Jyoti* than in seeds of non-pigmented varieties. The concentration of terpenoids in husk was always higher than in dehusked seeds.After analyzing the data from all the tests, it can be concluded that grains of pigmented varieties contain higher amount of secondary metabolites than grains of non-pigmented rice varieties. Among pigmented rice varieties, *Korgut*and *Assgo*containhigher, sometimes equal, amount of secondary metabolites than *Jyoti*. Similar contents in *Korgut* and *Assgo* can be evidence of them being a same variety and justification for the confusion existing among farmers on the names of these varieties. Secondary metabolite content being higher in grains of *Korgut* variety can be attributed to it being a salt-tolerant variety grown in Khazan lands of Goa. It can be said that this variety produces higher amount of secondary metabolites as a response to salt stress which provides it resistance against this stress.

Histochemical analysis proved that phenolics and flavonoids are distributed in the embryo region and terpenoids in seeds of pigmented rice varieties and in the endosperm region in seeds of non-pigmented rice varieties. Terpenoids were distributed in the embryo region in *Korgut*. In other two high terpenoid containing varieties (*Assgo* and *Jyoti*) terpenoids were present near the embryo region. Comparative analysis proved that the amount of secondary metabolites is highest in endogenous rice varieties*Korgut* and *Assgo* followed by high - yielding variety *Jyoti*.

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