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DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF GERMINATED BROWN RICE*oryza Sativa L.* USING ALUMINIUM INDUCED CHICK EGGS

Biochemistry

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ABSTRACT

Brown rice is most versatile grain with plenty of nutrient. Studies proved that germinated brown rice has so many advantages when compared to white rice. Inflammation is a immune response that is triggered by various factors such as pathogens, damaged cells and other toxic compounds. There plenty of commercial drug available for the treatment of inflammation but most of them have side effect. Some times the risk factors leads life threatening. In this study we check the anti-inflammatory activity of GBR rice using Aluminium toxicity induced chick eggs. We focus on the phytochemical analysis, GC-MS analysis, inhibition of albumin denaturation and HET-CAM test.

KEYWORDS

Anti-inflammatory, cytotoxicity, GBR ,Aluminium

1.INTRODUCTION

Rice is the one of the most versatile cereal foods,has been a primary food for many people around the world and it is the most extended cereal crop(1). The scientific name of rice is *oryzasativa* and *oryzaglaberrima* where*oryza sativa* was originated in Asia and *oryzaglaberrima* in is cultivated in Africa.Rice are of different types depending upon their colour, size,shape etc. But two main types are brown rice and white rice.Brown rice is a whole grain in which inedible outer layer (husk) is removed where as in the white rice husk, bran and Germ are removed (2). The brown rice has much nutrient content than white rice. Brown rice consist of more fibers and antioxidants, as well as lots of essential vitamins and minerals where as white rice is mainly the source of empty calories and carbohydrates with little nutrient (2).Thus, the undigested proteins can irritate the intestines, which leads to inflammation and allergic reactionsby neutralizing the phyticacid releasing the proteins, vitamins and enzymes allows the absorption of important nutrient during digestion. white rice has so many disadvantages as it increases the risk of developing type 2 diabetes and also several metabolic syndromes. Brown rice are recommended more because of its plenty of benefits such as it is the good source of most of vitamins and minerals in the grain, it reduces the risk of developing type 2 diabetes as its glycemic index value is low,enhances the heart health and also its better weight controller (3).

1.1. GBR

The unprocessed brown rice is germinated to upgrade the flavour and texture and also to increase the level of nutrient is called germinated brown rice or GBR (4). It's considered as healthier than white rice. It consists of plenty of vitamins, minerals, dietary fibers and essential amino acids. It also contains bioactive components such as ferulic acid and gamma aminobutyric acid. Consumption of GBR reduces the risk 0f disease such as obesity, cardiovascular disease, type 2diabetes and neurodegenerative disease. So many phytochemical compounds such as flavonoids, functional lipids, essential amino acids are present (5).

1.2. Anti-inflammatory

The inflammation is usually process of causing damage to living tissue resulting from the bacterial, viral, fungal infection or by physical agents and defective immune system(6).The primary aim of inflammatory response is to localize and eliminate the harmful agents. Secondary aim is to remove the damaged tissue and healing. The ability of a substance or treatment that can reduce inflammation or swelling is called as Anti-inflammatory (6). The drug or substances that cure the inflammation is called as anti-inflammatory agents. These agent's block's the substances that causes inflammation in the body. Non-steroid anti-inflammatory drugs (NSAIDs) will reduce the pain by balancing the enzyme cyclooxygenase(cox) (7). The cyclooxygenase enzyme synthesizes prostaglandin which creates inflammation in the tissues. The NSAIDs prevents the synthesis of prostaglandin by balancing the activity of COX enzyme and reduces inflammation and pain (7). But most of the commercial drugs have side effect which are some time it become life threatening.

1.3. Phytochemical screening

Plants produces variety of chemical compound called phytochemicals which has a special biological function in those plants(8). It includes both primary and secondary metabolites (9). The plants growth and metabolism are contributed by primary metabolites, where as the secondary metabolites are involved in functions such ascompetition, species interaction and protection from damage and disease. Secondary metabolites are alkaloids, flavonoids, terpenoids, tannins, glycosides, phenols, saponins, phytosterols, proteins and amino acids (9). The plants have been used as the primary source of medicine phytochemicals have various biological functions, including anti-inflammatory, anti-allergic, anticancer, antibacterial, antiviral, and analgesic function. Plants are considered as the excellent source of anti-inflammatory drugs. Phytochemicals are found in all parts of the plants(10).

2. MATERIALS AND METHODS

To evaluate the anti-inflammatory activity of Oryza sativa L. chicken eggs were used 12 healthy chick eggs are used which were kept in incubator at particular temperature for growth. They were divided 3 groups (4 eggs in each). first group was treated as positive control, second group injected with GBR, third group injected with aluminium chloridesolution and fourth group injected with sample and aluminium chloride solution. . The eggs were induced with aluminium chloride. on 7th day of experiment from each group the embryo developed was collected and subjected to albumin-denaturation method for ati-inflammatory.

2.1. Sample collection:

Brown rice sample were collected from the Research seed farm Mannuthy, Thrissur Kerala.

2.2. Preparation of powder:

About 1kg of the raw brown rice was taken washed thoroughly with water and its kept in wet cloth for germination for 48 hours in a dark room. Then after germination its washed and dried in the shaded condition at room temperature. The germinated brown rice(GBR) was crushed to powder using grinding machine. Powder was stored at 4^{o}c in light air container.

2.3. Extraction of plant material:

20gm of germinated brown rice (GBR) powder was taken and mixed with 200ml of solvent (aqueous, ethanol, methanol, chloroform and petroleum ether)separately during 3 hours of soxhlation, till extracts precipitated in the extractor. The obtained liquid extract was subjected to distillation chamber.

2.4. Qualitative phytochemical analysis

Standard procedures were followed for the qualitative screening of phytochemicals such as amino acids, saponins, proteins, phenols, alkaloids, carbohydrates, tannins, flavonoids, phytosterols and glycosides

2.4.1.**Test for Alkaloids:**
Mayer's test: To 1 ml of filtrate, a few drops of Mayer's reagent (Mix 1.358 g of mercuric chloride in 60 ml distilled water and 5 g of potassium iodide in 10 ml distilled water and made up to 100 ml) was added along the sides of the test tube. A white or creamy precipitate indicates the presence of alkaloids in the sample

2.4.2. **Test for Carbohydrates:**
About 400mg of crude extract was dissolved in 20ml of distilled water and filtered.

The was subjected to following test:
Molisch's test: To 2 ml of extract, 2 drops of alcoholic solution of α-naphthol was added and shaken well. 1 ml of concentrated sulfuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates

2.4.3.**Test for Glycosides**
With concentrated hydrochloric acid 50mg extract was hydrolysed for 2 hours on a boiling water bath, filtered and hydrolysate is subjected to the following:

Legal's test: The extract is dissolved in pyridine and then its treated with sodium nitroprusside. Using 10% of NaOH, solution is made alkaline. Formation of pink to blood red colour indicates the presence of glycosides.

2.4.4.**Test for Saponins**
Foam test: The presence of saponin was tested by mixing 2 ml of extract with 6 ml of distilled water. The mixture was shaken vigorously and observed for the formation of persistent foam, which confirmed the presence of saponins.

2.4.5. **Test for Phytosterols**
Libermann-Burchards test: To 0.5 ml of the plant extract, equal volume of chloroform was added followed by the addition of a few drops of concentrated sulfuric acid. The appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicated the presence of Phyto steroids.

2.4.6. **Test for Phenols**
Ferric chloride test: the extract was dissolved in distilled water. To this 0.5ml of 10% Ferric chloride solution was added. Formation of dark green colour indicates the presence of phenols

2.4.7.**Test for Taninns**
Gelatin test: to the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

2.4.8. **Test for Flavanoids**
Lead acetate test: The extract was treated with few drops of lead acetate. Formation of white precipitate indicates the presence of flavonoids

2.4.9. **Test for Proteins and Amino acids**
Xanthoproeictest: The extractswere treated with few drops of concentrated sulphuric acid. The formation of yellow colour indicates the presence of proteins.

Ninhydrin test: to the extract, 0.25% w/v ninhydrin reagent was added and boiled. The appearance of blue colour indicates the presence of amino acids.

2.4.10.**Test for Diterpenes**
Copper acetate test: Extract was dissolved in water and treated with few drops of copper acetate solution. Formation of emerald green indicated the presence of diterpenes.

2.5. Gas chromatography-mass spectrometry (GC-MS)
Gas chromatography-mass spectrometry is an analytical technique that combine the features of gas chromatography and mass spectrometry to identify different substances within a test sample. It is a separation technique of choice for smaller and volatile molecules such as benzenes, alcohols and aromatics, and simple molecules such as steroids, fatty acids and hormones. It is widely used for chemical analysis, and especially for drug and environmental contamination testing.

2.5.1. **Instrumentation**
The two major building blocks up on which GC-MS is made up of are the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions such as length, diameter, film thickness as well as the phase properties. The variation in the chemical properties between different molecules of a mixture and also their relative affinity for the stationary phase of the column will assist separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute from the column at different times (retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect and detect the ionized molecules individually. The mass spectrometer does this by breaking each molecule in to ionized fragments and detecting them using their mass-to-charge ratio.

2.5.2. **Ionisation**
After the molecules travel the length of the column, pass through the transfer line and enter in to the mass spectrometer where they are ionized by several normally methods with typically only one method being used at any given time. After sample fragmentation then it shoul be detected, propby an electron multiplier, which essentially turns the ionized mass fragments in to an electrical signal that is then detected.

2.5.3**Analysis**
The primary goal of instrument analysis is to quantify an amount of a substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library. This is best performed by a computer. Computers can also simultaneously correlate more data such as retention time identified by GC to more accurately related data in the library. Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. A full spectrum analysis considers all the peaks within a spectrum.

2.5.4. **GC-MS sample analysis method**
The chemical composition of the *oryza sativa* samples extracted in aqu was analysed using GC-MS. The samples were filtered through 0.22 µm syringe filter prior to analysis. 1 µl each of filtered sample was analysed using GC-MS (QP-2010-Shimadzu) equipped with a Rxi-5Sil MS column of 30m in length, 0.25mm in diameter, and 0.25µm thickness. The GC-MS was employed with helium as carrier gas at a constant flow of 1ml/minute. The oven temperature was started at 80℃ and remained at this temperature for 4 minutes and, increasing to 280℃ at 5℃/min ramp rate. Injection port was adjusted at 260℃ and split less injection mode was used. EI mode was at 70 eV, while mass spectra were recorded in 50-500 amu range and ion source temperature was maintained at 200℃. The components of *Oryza sativa*samples were identified by comparing the retention time of chromatographic peaks using quadrupole detector with NIST library.

2.6. Anti-inflammatory activity
Inhibition of Albumin Denaturation
Protocol
The anti-inflammatory activity of Sprouted Brown Rice extract was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al and Sakat et al followed with minor modifications. The reaction mixture consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 º C for 20 min, after cooling the samples the turbidity was measured at 660nm,(UV-Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition= (Abs Control – Abs Sample) X 100/ Abs control

2.7. HET- CAM Test
Hen's egg- chorioallotonicmembrane testis used to detect the irritation potential of substances.The incubated hens eggs on the 9th day is opened carefully and CAM membrane is exposed. Four eggs are taken in to which 4 solutions are added. A positive control -sterilized water, a negative control - NaOH solution, commercial drug and sample-

brown rice. These four solution are placed directly on the exposed CAM membrane using a micropipette. Then the membrane is observed carefully through aMicroscope to check whether there is any haemorrhage has taken place

3.RESULT AND DISCUSSION

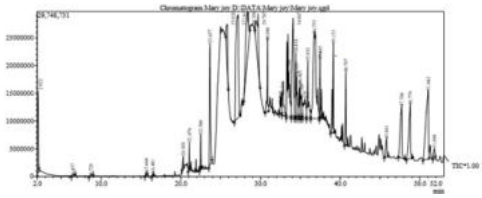
3.1. PHYTOCHEMICAL SCREENING

SL. NO	COMPOUND	AQUEOUS	ETHANOL	METHANOL	CHLOROFORM	PETROLEUM ETHER
1.	Alkaloids	-	+	-	+	-
2.	Flavanoids	+	-	+	-	+
3.	Tannins	-	+	-	-	+
4.	Steroids	-	-	+	-	-
5.	Saponins	-	+	-	-	-
6.	Glycosides	+	+	-	-	-
7.	Phenols	+	+	++	-	-
8.	Carbohydrates	++	+	++	-	-
9.	Proteins and aminoacids	++	++	++	+	+
10.	Diterpenes	-	+	-	-	+

+ = Presence of Phytochemical, - = Absence of Phytochemical

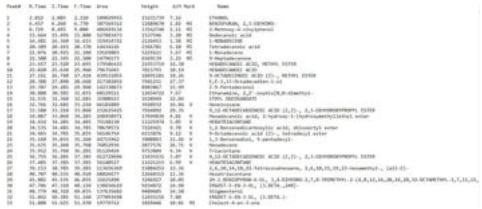
Germinated brown rice represent a source of phytochemicals, such as amino acids, phenolics, dietary fibre and flavonoids. These are rich in almost all solvent extracts above especially in Ethanol, Methanol, and aqueous. The presence of alkaloids and diterpenes are minimum.

3.2. Gc-ms Analysis



Mass Spectrometry Of Gcms Analysis

Gc-ms Chromatogram Of The Germinated Brown Rice – Ethanol Extract



Bioactive Compounds in ethanolic extractofGerminated Brown Rice.

3.3.Anti-inflammatory

Table-2: Effect of Sprouted Brown Riceextract on Inhibition of Albumin

Concentration (µg)	% Inhibition of Albumin Denaturation
Control	-
100	25.21
200	33.98
300	48.11
400	52.01

3.4. Cytotoxicity

Fig 1.CAM exposed with sample

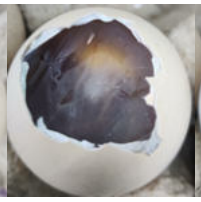


Fig 2. CAM exposed with Drug

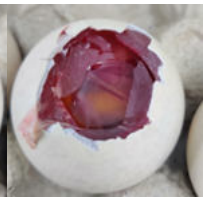


Fig 3. CAM exposed with NaOH

WhenHen's egg- chorioallotonic membrane was exposed to water, sample, sodium hydroxide solution and commercial drug, the lysis occurred only to the egg exposed by NaOH. Others no lysis was observed.

CONCLUSION

The study is conducted on ***Determination Of Anti-inflammatory Activity Of GBR Using Aluminium Induced Chick Eggs***. It is an important grain in the whole word with plenty of nutrients. Its beneficial properties depend up on the knowledge of their chemical constituents. The GBR were extracted in aqueous, ethanol,methanol, chloroform and petroleum ether are subjected to preliminary screening and identified the presence of proteins, amino acids, carbohydrates and phenols are found in huge amount. Other phytochemicals are present in small amount. Mostly in ethyl extract positive results are obtained except forflavonoid and steroids. So, ethyl extract was taken for further studies.

The GC-MS analysis was done using the ethanolic extract.The GC-MS was done to identify and characterize the chemical compounds present in the crude extract. The result revealed the presence of 33 bioactive compound in the ethanolic extract of *Oryza sativa L.* The ethanolic extract of GBR proved to be reservoir of bioactive compounds. Which are effective in treatment of inflammation.

The anti-inflammatory activity of the sample was determined by using albumin-denaturation method. In which the ability of the sample extract to inhibit the protein denaturation was studied. Form the analysis maximum inhibition was at 52.01% with corresponding concentration of 400µg. effective anti-inflammatory activity was found for sample.

HET-CAM is used to check the irritation potential of a substance to the chorioallotonic membrane of chick egg. The eggs cam exposed to NaOH solution was undergone lysis others were remained the same. The membrane has higher irritation potential to the chemical sodim hydroxide when compared to water, commercial drug and sample. No lysis was observed in the egg in which sample was injected. So the sprouted brown rice has no cytotoxic effect on chick embryo.

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