Research Article

A Comparative Study on The Effect of Fermentation on Anti-nutrient Composition and Bioactivity of Extracts from Common Tubers- *Amorphophallus paeoniifolius* (Dennst.) Nicolson and *Manihot esculenta L*

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ABSTRACT

The present study was aimed to investigate the effect of fermentation on the antinutrients like tannins, oxalates and phytates present in common tubers like *Amorphophallus paeoniifolius* (EFY) and *Manihot esculenta* (Cassava). Antinutrients decrease the nutritive value and also interfere with the absorption of minerals such as calcium, magnesium, iron, etc. in the intestines. Effect of solid-state fermentation using Baker's Yeast and *Lactobacillus* cultures was found to significantly decrease the amount of the antinutrients in tubers which was estimated quantitatively. The phytates content was seen to decrease maximally by about 98% amongst the antinutrients after fermentation. The bioactivity like antioxidant activity and hepatoprotectant activity of these tubers using their aqueous extracts (10%) was assessed. The antioxidant activity was estimated by DPPH radical scavenging activity and reducing power assay where the Fermented Control samples of both the tubers showed a high scavenging activity with values of 37.6% for EFY and 110.13% for Cassava and a good reducing activity with values of 27.36 µg and 26.16 µg respectively. Hepatoprotective activity was assessed using the biomarkers and calculated as percentage of cytotoxicity. The *Lactobacillus* fermented EFY extract gave a low cytotoxicity for LDH and ALP and Yeast fermented EFY extract gave a low cytotoxicity for SGPT and SGOT. The *Lactobacillus* fermented Cassava extract gave a low cytotoxicity for LDH, SGPT and SGOT and the Yeast fermented extract gave a low cytotoxicity for ALP.

Keywords: Anti-nutrients, fermentation, *Amorphophallus paeoniifolius, Manihot esculenta*, Anti-oxidant, Hepatoprotective activity.

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INTRODUCTION

Anti-nutritional factors are substances that decrease the use of nutrients and/or consumption of plants or plant products as human foods. However, some antinutrients at low concentrations can exert beneficial health effects. For example, it has been shown that phytic acid, tannins, amylase inhibitors and saponins to reduce the blood glucose and insulin responses to starchy foods and/or the plasma cholesterol and triglycerides also they have been related to reduce risk of cancer. The balance between beneficial and harmful effects of plant bioactive and antinutrients depends on their concentration, chemical structure, time of exposure and association with other dietary components. They can therefore be considered as anti-nutritional factors with negative effects, or non-nutritional substances with positive health effects.[1] Tannins exhibit antinutritional properties by impairing the digestion of various nutrients and preventing the body from absorbing beneficial bio available substances. [2] Tannin-protein complexes may cause digestive enzymes inactivation and protein digestibility reduction caused by protein substrate and ionisable iron interaction.[3] Presence of phytic acid is seen in the hulls of nuts, seeds, legumes, tubers and grains, which has a strong binding affinity for calcium, magnesium, iron, copper, and zinc, preventing their absorption. Oxalic acid and oxalates, present in many plants, especially in the members of the spinach family, bind calcium to prevent its absorption.[4]

Tubers are a delicacy and rich in nutrients and the plant's tuberous roots have blood purifying properties and have been used traditionally for the treatment of piles, abdominal disorders, tumours, enlargement of spleen, asthma and rheumatism.[5] The tubers have also been reported to possess tonic, stomachic and appetizer properties.[6]

Amorphophallus paeoniifolius is a perennial, terrestrial underground hemispherical depressed dark brown corm of approximately 20-25 cm in diameter that bears flowers and fruits in the month of April – May.[7]

Manihot esculenta, commonly called cassava is a woody shrub of the spurge family, Euphorbiaceae, originally from South America. While it is a perennial plant, cassava is widely cultivated for its edible starchy tuberous roots, a major source of carbohydrates, as an annual crop in tropical and subtropical regions. Cassava is the third-largest source of food carbohydrates in the tropics, after rice and maize.[8] Cassava is mostly consumed in boiled form, but large quantities are used to extract cassava starch, known as tapioca, which is used for food,

animal feed, and industrial purposes. Tapioca, which is used for food, animal feed, and industrial purposes. It is also used in various industrial applications including biofuels, textiles, paper, adhesives, sweeteners, glues, plywood, and many others.[9]

Antioxidants ensure organism's defence against the attack of free radicals which are very unstable and reactive, they capture electrons from other molecules to acquire a stable electronic configuration causing oxidative damage in the human body when high concentrations of these species and other pro radical's reactive species such as hydrogen peroxide or nitrogen monoxide are present. A lack of equilibrium between free radicals and antioxidants contributes to a condition called 'oxidative stress'[10] which Sies has described as a disruption in the prooxidant antioxidant balance in favour of the prooxidants, leading to potential damage. [11]

Liver is the first recipient of intestinal toxins, and is thus involved in the degradation and removal of toxins such as drugs and other foreign chemical substances.[12] It also detoxifies drugs, alcohol, chemicals, heavy metals, infectious organisms, as well as toxin by products from the blood.[13] Liver diseases are considered one of the leading global health issues prevalent in developing countries. These diseases are classified into different categories, namely hepatosis (non-inflammatory), acute or chronic hepatitis (inflammatory), and cirrhosis or fibrosis (degenerative) caused commonly due to heavy metals, toxins, malnutrition, and over the counter drug use without doctors' prescription.[14] Protecting a healthy liver is therefore vital for good health and wellbeing. Plant based products being less toxic have been used as hepatoprotective agents in recent times. Continuous exploration of plant diversity for novel hepatoprotective potential has thus been an important area of active research. Thus, researching readily available and cheap medicinal plants that do not require strenuous pharmaceutical production processes appears to have gained worldwide attention as potential therapeutic agents for the diseases.[15]

In the present study, antinutrient content in tuber extract was quantified. The effect of fermentation on antinutrient content and biological activity of tubers was compared with unfermented tuber extract.

MATERIALS AND METHODS

Sample collection

The samples *A. paeoniifolius* and *M. esculenta* were collected from the Local Vegetable Market, Bengaluru. The samples were labelled as EFY (Elephant Foot Yam) for *A. paeoniifolius* and Cassava for *M. esculenta*.

Preparation of sample extracts

10% aqueous extract of tubers was prepared by homogenizing 10g of sample using pestle and mortar with 100ml distilled water. The homogenised sample was centrifuged at 3,000 rpm for 10 min. at room temperature. The supernatant obtained was labelled and used as sample extract for further analysis.

Fermentation

Sample preparation: 25 g each of samples of EFY and Cassava tubers were distributed into 3 beakers each respectively. The samples were washed, peeled and cut into small cubes. Surface sterilization was done by repeated washing of the samples with Tween 20 and distilled water followed by Ethanol and again distilled water. The samples were then taken in beakers and were treated with 0.2mg of sodium metabisulphite in 100 ml of distilled water and kept aside for 1 hour for surface sterilization. After 1 hour the samples were again washed with distilled water. Fermentation medium: The medium used for fermentation process was sterile 20 ml sugarcane juice dispensed into 6 different conical flasks respectively for each of the samples. To one flask each of EFY and Cassava, 20 ml of 24hr yeast culture and *Lactobacillus* in sugarcane juice was added and the flasks are incubated at 25˚C for 5 days under sterile conditions with intermittent shaking of the flask. One control conical flask each was maintained for both the samples wherein only 20 ml of sugarcane juice was added. The normal microflora present in the samples was allowed to ferment the samples for 5 days respectively. After 5 days of fermentation, the flasks were taken and the samples were homogenised and centrifuged at 10,000 rpm for 10 min at room temperature to obtain clear supernatant. The obtained supernatant was used for further analysis. The extracts were labelled as Control EFY, Yeast fermented EFY and *Lactobacillus* fermented EFY and Control cassava, Yeast fermented Cassava and *Lactobacillus* fermented Cassava.

Phytochemical Analysis

Estimation of tannins

0.2 to 1.0 ml aliquots of standard tannic acid (50 µg/ml) was taken in different test tubes and the volume was made up to 1.0 ml using distilled water. 1.0 ml each of the different sample extracts was taken in tubes and labeled. 5.0 ml of Folin Denis reagent and 1.0 ml of 10 % sodium carbonate solution was added to the test tubes and the absorbance was measured at 700 nm against a suitable blank. The extracts were performed in triplicates and the amount of tannin was expressed in terms of mg of tannin equivalent/ ml of plant sample extract.[16]

Estimation of oxalates

5.0 g each of fresh samples were weighed and crushed into fine pulp using mortar and pestle. The crushed pulp was transferred into a beaker and was boiled with 50 ml of dilute H_2SO_4 for 10 minutes. The contents were then cooled and filtered into a 100 ml measuring flask and the volume in the flask was made up to 100 ml using distilled water. 10 ml of the flask solution was taken along with 10 ml of dilute H_2SO_4 in a 100 ml flask and heated to about 60˚C. The mixture was then titrated against 0.1 N $KMnO_4$ solution in the burette till the end point of pale pink colour

appeared. The process was repeated for concordant values.[17]

Estimation of phytates

0.5 to 2.5 ml aliquots of standard ferric nitrate were utilized. 5.0 ml of sample solution was used for estimation. The volume in each of the tubes was made up to 10 ml with 1 N hydrochloric acid. A blank test tube was prepared with 10 ml of 1 N hydrochloric acid. 10 ml of 1.5N potassium thiocyanate was added to each of the test tubes. The contents of the tubes were mixed well and the absorbance was read at 450 nm against a suitable blank. The readings were noted and a standard curve was plotted to determine the phytic acid content in the samples. This was repeated twice for each of the samples for concordant values.[18]

Antioxidant Activity

DPPH radical scavenging activity

A slightly modified procedure of Braca *et al.,* 2002[19] was used for determination of DPPH scavenging capacity of the plant extracts. Standard solution of ascorbic was prepared (100 µg/ml) so 0.2 ml to 1.0 ml aliquots was taken in different test tubes and the final volume was made up to 1.0 ml using distilled water. 3.0 ml of DPPH (20 µg/ml) was added and incubated for 10 minutes at room temperature. The contents of each tube were mixed well and the absorbance was measured at 517 nm against ethanol/ distilled water as blank. The percentage inhibition of DPPH by the samples was calculated as follows:

$$\text{Scavenging effect (\%)} = \frac{(\text{OD of Control} - \text{OD of Test sample})}{(\text{OD of Control})} \text{ X } 100$$

Determination of Hepatoprotective Activity [20]

Liver tissue source

To determine the *in vitro* effect of aqueous extract of EFY and Cassava, goat liver was used as the mammalian tissue. The fresh liver was collected from the slaughter house located in Frazer town, Bengaluru, Karnataka. The liver slices were immediately transferred to sterilized Krebs Ringer HEPES [-4-(2-hydroxyethyl) -1-piperazineethanesulphonic acid] buffer medium (KRH 2.5mM HEPES, pH 7.4, 118mM NaCl, 2.85mM KCl, 2.5mM $CaCl_2$, 1.15mM KH_2PO_4, 1.18mM $MgSO_4$ and 4.0mM glucose). The thinly cut slices, weighing approx, 100 mg were used for the study. These slices were washed with 10 ml KRH medium every 10 minutes over a period of 1 hour and then pre-incubated for 1 hour with 10 ml of KRH medium on a shaker incubator at 37^{o}C.

Preparation of lead acetate solutions:

30 mg of lead acetate was dissolved in 100 ml of distilled water to prepare the stock solution of 100 ppm (3 ppm/ml). A definite volume of the stock solution was used in the reaction mixture to induce heavy metal toxicity and to study its effect on liver tissue.

Liver Enzyme Assays

LDH Activity Assay

1.0 ml of the buffered substrate, 0.2 ml of NAD solution and 0.1ml of the respective supernatants were taken in respective tubes. The reaction mixture was incubated at 37ºC for 15 minutes. Activity of the enzyme in each tube was arrested by adding 1.0 ml of 2,4-DNPH reagent and the tubes were again incubated at 37ºC for 15 minutes. At the end of incubation, 7.0 ml of 0.4 N NaOH was added in all the tubes. A blank containing 1.0 ml of buffered substrate, 0.1 ml of NAD solution, 1.0 ml of 2,4-DNPH reagent and 7.0 ml of 0.4 N NaOH was used. Similarly, the test was performed with standard sodium pyruvate solution (80 µg/ml) by pipetting 0.05 ml to 0.25 ml of the standard in different test tubes, the final volume in each tube was made up to 1.3 ml using distilled water. 1.0 ml of 2,4-DNPH reagent was added to the tubes and incubated at 37ºC for 15 minutes followed by addition of 7.0 ml of 0.4 N NaOH after the incubation period. The absorbance was read at 540nm and a standard graph is plotted.

SGPT Activity Assay

The enzyme assay was carried out by adding 1.0 ml of buffered substrate into different test tubes which were pre-incubated at 37ºC for 15 minutes. 0.2 ml of respective supernatant was added to the respective tube and incubated at 37ºC for 15 minutes. Addition of 1.0 ml of 2,4-DNPH reagent after the incubation period arrests the enzyme activity. The tubes are allowed to stand at 37ºC for 15 minutes. After cooling the tubes 5.0 ml of 0.4 N NaOH was added.

Similarly, the test was performed with standard sodium pyruvate solution (200 µg/ml) by pipetting 0.1 ml to 0.5 ml of the standard in different test tubes, the final volume in each tube was made up to 1.2 ml using phosphate buffer. 1.0 ml of 2,4-DNPH reagent was added to the tubes and incubated at 37ºC for 15 minutes followed by addition of 5 ml of 0.4 N NaOH after the incubation period. The absorbance was measured at 540 nm and plot a standard calibration curve.

SGOT Activity Assay

Enzyme assay was carried out by adding 1.0 ml of buffered substrate into different test tubes which were pre-incubated at 37ºC for 15 minutes. 0.2 ml of respective supernatant was added to the respective tube and incubated at 37ºC for 15 minutes. Addition of 1.0 ml of 2,4-DNPH reagent after the incubation period arrests the enzyme activity. The tubes are allowed to stand at 37ºC for 15 minutes. After cooling the tubes 5 ml of 0.4 N NaOH was added.

Similarly, the test was performed with standard sodium pyruvate solution (200 µg/ml) by adding 0.1 ml to 0.5 ml aliquots of the standard in different test tubes, the final volume in each tube was made up to 1.2 ml using phosphate buffer. 1.0 ml of 2,4-DNPH reagent was added

to the tubes and incubated at 37°C for 15 minutes followed by addition of 5.0 ml of 0.4 N NaOH after the incubation period. The absorbance was measured at 540 nm and plot a standard calibration curve.

Alkaline Phosphatase Activity Assay

Enzyme assay was carried out by adding 3 ml of substrate (1 mM) which is the standard p-nitro phenyl phosphate solution, 3.0 ml of sodium carbonate buffer (pH 10) and 0.5 ml of respective supernatant into respective test tube. The reaction mixture is incubated at 37ºC for 15 minutes. After the incubation period, 3.0 ml of 0.1 N NaOH solution was added to all the tubes.

Similarly, the test was performed with standard p-nitro phenol solution (30 µg/ml) by adding 0.2 ml to 1.0 ml aliquots of the standard in different test tubes, the final volume in each tube was made up to 3.0 ml with distilled water. 3.0 ml of 0.1 N NaOH was added and the absorbance was read at 540 nm to plot the standard curve.

RESULTS AND DISCUSSION

Phytochemical Analysis

Estimation of Tannins

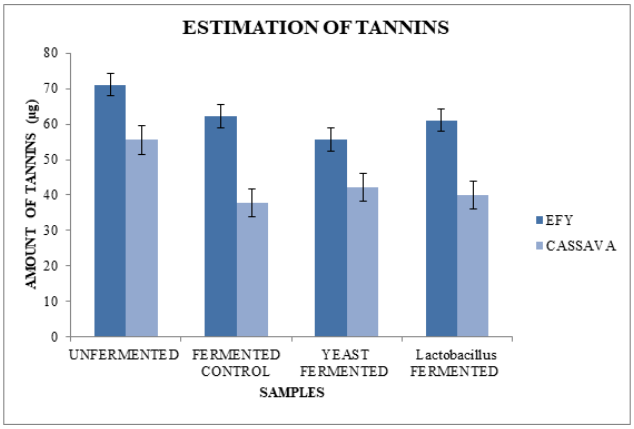

Figure 1: Graphical representation of amount of Tannins in EFY and Cassava samples

The figure 1 illustrates the tannin content in EFY and Cassava unfermented and fermented samples. The amount of tannins is seen to decrease after fermentation. In EFY, the unfermented sample showed a value of 71.11µg as the amount of tannins which was higher than the values after fermentation. Amongst the fermented samples, the Yeast fermented sample showed a value of 55.55 µg which was comparatively lesser than the other two fermented samples. Similarly, in Cassava the amount of tannins is seen to decrease significantly after fermentation. The unfermented sample showed a value of 55.55 µg as the amount of tannins which was higher than the values after fermentation. Amongst the fermented samples, the fermented Cassava Control showed a value of 37.77 µg which was comparatively lesser than the other two fermented samples. According to the study, Cassava is seen to have less tannins as compared to EFY naturally. Tannins reduced by approximately 21.8% in EFY and by 32% in Cassava after fermentation.

Estimation of Oxalates

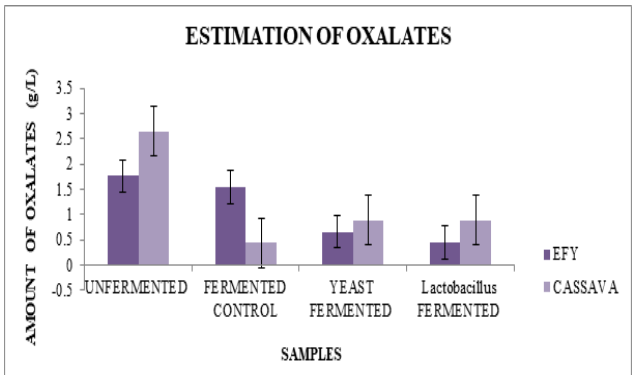

Figure 2: Graphical representation of Estimation of Oxalates of EFY and Cassava samples

In EFY, the content of oxalates seemed to have decreased to quite low levels after fermentation. The unfermented EFY sample showed an amount of 1.76 g/L. After fermentation, the *Lactobacillus* fermented EFY showed a value of 0.44 g/L which was lower than the other two fermented samples (figure 2). In Cassava samples, the content of oxalates seemed to have decreased to quite low levels after fermentation. The unfermented Cassava sample showed an amount of 2.64 g/L. After fermentation the fermented Cassava Control showed a value of 0.44 g/L which was lower than the other two fermented samples.

The presence of oxalates was seen to be higher in Cassava as compared to EFY but due to fermentation, a decrease in the content of oxalates was seen to prove the positive effect of fermentation. Oxalates were seen to reduce by 75% in EFY and by 83.3% in Cassava after fermentation.

Estimation of Phytates

The process of fermentation has seen to significantly reduce the amount of phytates in the samples.

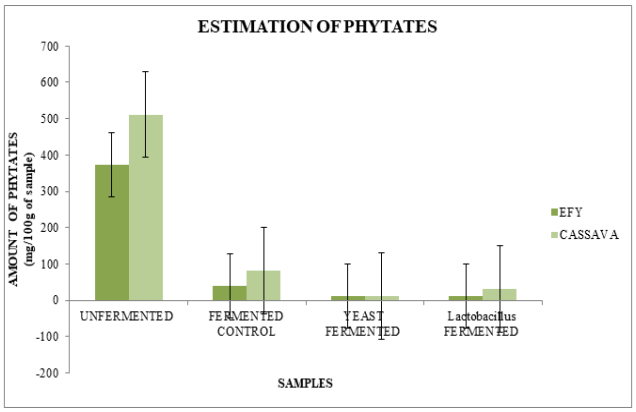

Figure 3: Graphical representation of Estimation of Phytates of EFY and Cassava samples

Figure 3 represents the amount of phytates expressed as mg/ 100 g of sample for unfermented and fermented EFY and Cassava samples. The unfermented EFY sample showed a value of 372 mg/ 100 g of sample. After

fermentation, both the Yeast fermented and *Lactobacillus* fermented samples showed a value of 11.16 mg/ 100 g of sample which indicated quite a good reduction in the amount of phytates. The unfermented Cassava sample showed a value of 511.5 mg/ 100 g of sample. After fermentation, both the Yeast fermented sample showed a value of 11.16 mg/ 100 g of sample which indicated quite a good reduction in the amount of phytates.

The tubers contain high amounts of phytates with Cassava having a slightly higher amount but fermentation successfully reduced the amount of phytates. Phytates also showed a drastic reduction by 98% in EFY and Cassava after fermentation.

Antioxidant Activity

Scavenging activity on DPPH radicals

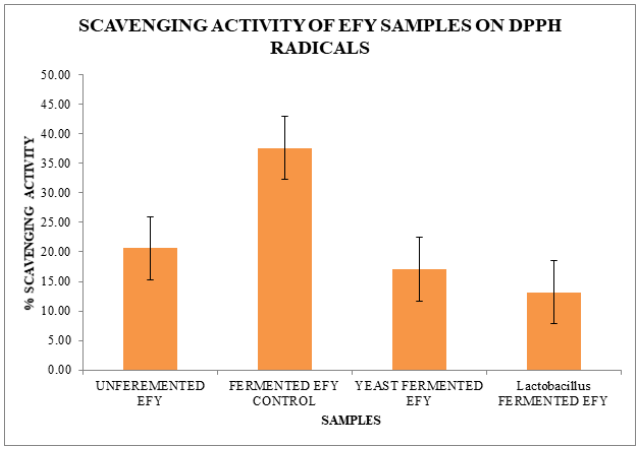

Figure 4: Graphical representation of Scavenging Activity on DPPH Radicals of EFY samples

The free radical scavenging activity of the unfermented and fermented extracts of EFY was evaluated and shown in the figure 4. The antioxidant activity of the aqueous extracts was compared with ascorbic acid where the percentage of antioxidant activity of the three fermented samples was seen to be higher than the unfermented sample. The fermented EFY Control extract showed a scavenging activity of 37.60% which was the highest amongst the respective EFY samples.

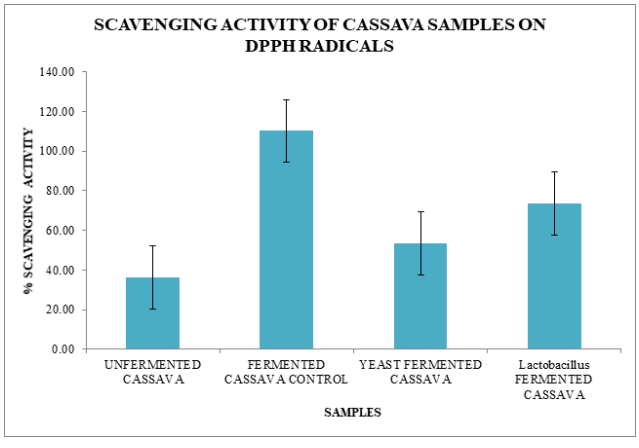

Figure 5: Graphical representation of Scavenging Activity on DPPH Radicals of Cassava samples

The free radical scavenging activity of the unfermented and fermented extracts of Cassava was evaluated and shown in the figure 5. The antioxidant activity of the aqueous extracts was compared with ascorbic acid where the percentage of antioxidant activity of the three fermented samples was seen to be higher than the unfermented sample. The fermented Cassava Control extract showed a scavenging activity of 110.13% which was the highest amongst the respective Cassava samples. A high antioxidant activity indicates that the extract has the ability to decrease the ability to decrease DPPH concentration.

Liver Enzyme Assay

Percentage of cytotoxicity in EFY samples

The amount of enzyme LDH, SGPT, SGOT and ALP released in liver slices treated with lead acetate for 2 hours was found to be 61.50%, 93.75%, 90.50% and 83.40% (figure 6). The amount of each of the enzymes in the untreated experimental set (negative control) was seen to be 50%. The liver samples that were co-exposed with lead acetate and the aqueous extracts of the unfermented and fermented EFY extracts showed a significant decrease and showed appropriate percentages. There was a marked difference between the values of unfermented and fermented samples. The fermented samples showed even lesser values than the unfermented sample proving that fermentation improves the bioactivity. The values expressed as percentage of cytotoxicity in *Lactobacillus* fermented EFY sample were 44.4% for LDH and 50.0% for ALP. For SGPT and SGOT, the Yeast fermented Cassava sample showed a value of 81.82% and 74.43% respectively. These values were found to be lesser in comparison to the other samples.

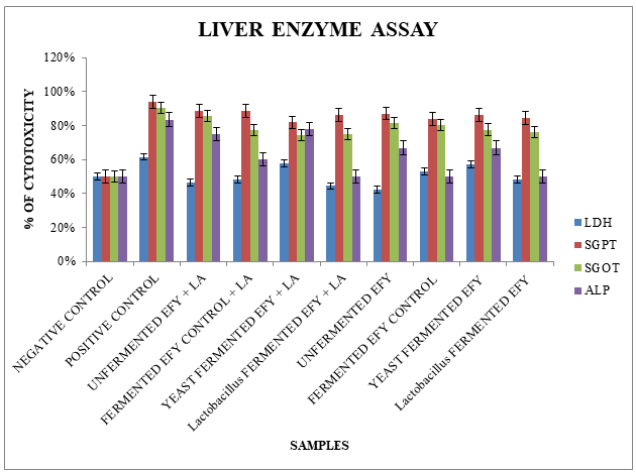

Figure 6: Graphical representation of Liver Enzyme Assay of EFY samples

Percentage of cytotoxicity in cassava samples

The amount of enzyme LDH, SGPT, SGOT and ALP released in liver slices treated with lead acetate for 2 hours was found to be 61.50%, 93.75%, 90.50% and 83.40% (figure 7). The amount of each of the enzymes in the untreated experimental set (negative control) was seen to be 50%.

The liver samples that were co-exposed with lead acetate and the aqueous extracts of the unfermented and fermented Cassava extracts showed a significant decrease and showed appropriate percentages. There was a marked difference between the values of unfermented and fermented samples. The fermented samples showed lesser values than unfermented samples in all cases proving that fermentation improves the bioactivity. The values expressed as percentage of cytotoxicity in *Lactobacillus* fermented Cassava sample were 40.0% for LDH, 86.21% for SGPT, 85.47% for SGOT. For ALP, the Yeast fermented Cassava sample showed a value of 66.74%. These values were found to be lesser in comparison to the other samples.

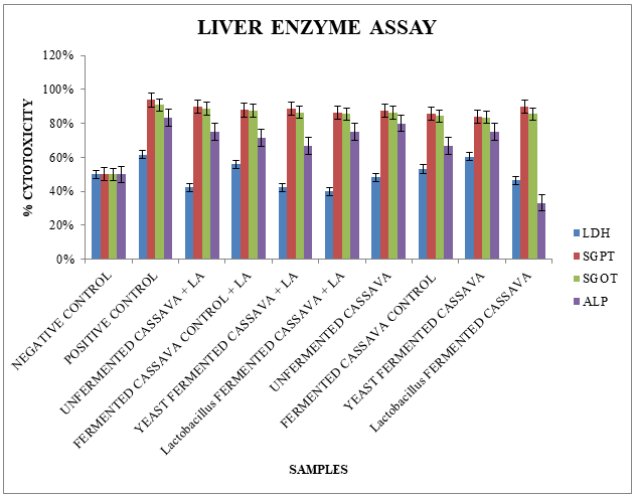

Figure 7: Graphical representation of Liver Enzyme Assay of Cassava samples

CONCLUSION

From the present study we can conclude that, fermentation process reduced the antinutrients in both EFY and Cassava samples. The antioxidant activity exhibited that the fermented samples showed better activity than the unfermented samples. The hepatoprotective activity of the tuber extracts tested against the heavy metal lead induced toxicity in mammalian liver tissue and the *in-vitro* studies on the liver tissue along with the samples assessing the biomarkers or the enzymes- LDH, SGPT, SGOT and ALP showed that the extracts seemed to have lowered the cytotoxicity, proving that it has the potency to protect liver tissues in case of damage.

Further it can be concluded that, fermentation and other methods like cooking, boiling etc. reduces the antinutrient content and enable the tubers to be more biologically potent and improve its nutritional value also bringing more economic value. They can also be cut into desired shape and used as a health food by oven-drying and flavoring the tubers. The tubers when fermented with *Lactobacillus spp.* can also be used as a probiotic by converting them into flour form or any desired form which makes the fermented samples more palatable.

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