Kaulath, a new fungal fermented food from horse gram

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Abstract Horse gram (*Macrotyloma uniflorum*) is used in the traditional method for treatment of several health complications. It is also known that fermentation of such substrates yields a number of compounds that enhance the overall activities against several disease states. Solid state fermentation of horse gram using *Penicillium camemberti* showed an inhibition of pancreatic lipase and alpha glucosidase activities. The fermented material, termed *Kaulath*, showed 60 % increase in fat content. A reduction in sodium and increased levels of potassium and calcium was observed in *Kaulath*. In addition, a higher free radical scavenging activity was noted in this product compared to unfermented horse gram. Antinutritional factors, such as phytic acid and trypsin inhibitors showed a reduction in *Kaulath*. Furthermore, *Kaulath*, upto 1 g per kg body weight, did not exhibit any mortality or toxic effects in experimental rats after 14 days of administration. The hematological and clinical parameters were within safe limits between the groups, supported by the histopathology of liver and kidney. These results indicate potential food use of *Kaulath* in diets and as functional ingredients in formulated foods.

Keywords Acute toxicity · Enzyme inhibition · Horse gram · *Kaulath* · *Penicillium camemberti* · Solid state fermentation

Introduction

Legumes are good source of proteins in developing countries (Doval 2013) and accord major health benefits, such as reduction of cholesterol and in managing the complications of diabetes. Horse gram (*Macrotyloma uniflorum*), often termed as poor man's pulse, is utilized more as cattle feed than for human consumption. It is used in the traditional method for treatment of kidney stones, diabetes, obesity, hypertension etc. (Bhuvaneshwari et al. 2014). The reported antioxidant activities, angiotensin converting enzyme and trypsin inhibitory potentials in horse gram put a spotlight on this legume (Siddhuraju and Manian 2007; Sreerama et al. 2012). In addition, an α -amylase inhibitor from horse gram seed has been reported to display anti diabetic property in streptozotocin-nicotinamide-induced diabetic mice (Laxmi et al. 2011). Recent reports point towards this bean as a good source for many healths beneficial uses, such as in reduction of cholesterol, glucose level, and also in insulin resistance by inhibiting protein tyrosine phosphatase with a significant antioxidant activity (Tiware et al. 2013).

Normally, food fermentations utilize bacterial cultures and the use of fungi in fermented foods are relatively limited (Nout and Aidoo 2002). In East- and South East Asian countries, filamentous molds are predominant organisms in the fermentation process, whereas in Europe, Australia and America, fermented foods are produced by bacterial or bacteria-yeast combinations (Tamang 2010). Although lactic acid bacteria is mostly used for bacterial fermentation of cereals and grains for the production of fermented products, there are few examples of fungal fermented foods in the Eastern countries, such as

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miso, tempe and shoyu (Shurtleff and Akiko 2011; Sasaki and Mori 1991). An example of fungal food application in Europe is the use of *Penicillium camemberti*, a surface mold, grown to aid the ripening process of soft cheeses like Camembert or Brie (Bizet et al. 1997). Generally, functional properties of fungi in fermented foods are mainly in the production of enzymes, such as amylase, lipases, etc., and degradation of anti-nutritive factors to improve the bioavailability of minerals (Nout and Aidoo 2002). One of the benefits of fermented foods is the ability of the fermentation process to transform compounds to promote health giving abilities. Therefore, the aim of this work is to use *Penicillium camemberti* culture that is typical of the Western cuisine to ferment horse gram, an underutilized legume in India with perceived health benefits to create new fermented health food. This manuscript deals with various aspects of a new fermented product, termed as *Kaulath* (derived from Sanskrit, meaning “made from horse gram”).

Materials and methods

Materials

Horse gram was purchased from a local market in Mysore, India, and brought to the laboratory in air tight polyethylene bags, cleaned and kept in a cool and dry place prior to use. Care was taken to purchase all the grains from a single batch. All reagents were of analytical grade. *Penicillium camemberti* MTCC-418 was procured from the Microbial Type Culture Collection (Chandigarh, India) and maintained on potato dextrose agar (PDA) slants.

Fermentation

A loopful of spores from a 5 day old PDA slant were transferred to 10 ml sterile distilled water with 0.1 % (v/w) Tween 20. One ml of this spore suspension was inoculated in 30 g of coarse horse gram grits, moistened with 15 ml distilled water (previously autoclaved at 121 °C for 60 min) in a 500 ml Erlenmeyer flask. The flasks were incubated in a slanting position for 5 days at 25 °C.

Nutrient composition and metal analysis

The moisture content of unfermented horse gram and *Kaulath* was determined by drying the samples at 105 °C to constant weight. Analyses for protein, fat and ash contents were carried out according to the standard methods of the AOAC (1990). The carbohydrate content was determined as the weight difference using protein, fat and ash content data. Metal analysis was done as described by AOAC method (2012) using a Thermo Fisher Scientific, (Waltham, MA, USA, Model: icE 3000)

atomic absorption spectrophotometer. The values were evaluated from the mean of three determinations for each sample.

Biological activities

At the end of the fermentation, 100 ml of ethyl acetate was added to each flask and placed on a rotary shaker operating at 150 rpm at room temperature for an hour. The contents were filtered through Whatman No. 1 filter paper followed by addition of 1 % (w/v) anhydrous sodium sulphate. The extract was concentrated to dryness under reduced pressure using a rotary flash evaporator. Stocks of the extract at 2 mg/ml were prepared in dimethyl sulphoxide (DMSO) to carry out the biological activities. The enzyme inhibition assays were carried out for lipase (Shirai and Jackson 1982), alpha glucosidase (Pistia-Brueggeman and Hollingsworth 2001) and lipoxygenase (Axelrod et al. 1981). Free radical scavenging activity of extracts was determined by the DPPH method (Abe et al. 2000) and inhibition of topoisomerase was carried out by agar disc diffusion method using Dh5 α and wild type *E. coli* strain (Pererva et al. 2007).

Determination of anti-nutritional factors

Tannic acid content was determined by the method described by Saxena et al. (2013). Phytic acid and trypsin inhibitors was determined according to the methods described by Vaintraub and Lapteva (1988) and Hamerstand et al. (1981) respectively, while the alkaloid content was determined gravimetrically (Harborne 1973).

Acute toxicity studies

Eight week old adult male wistar rats weighing 180–200 g ($n = 3$) were obtained from Institute animal house and kept in individual stainless steel cages. These were maintained at 25 ± 2 °C with a relative humidity 60–70 % and exposed to a light and dark cycle of 12 h duration. These were fed with commercial rat diet purchased from Sai Durga Feeds and Foods, Bangalore and had free access to water. All animal experiments were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee (IAEC, Registration number: 49/1999/CPCSEA) regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi, India. Handling and care of animals was carried out according to the standard guidelines laid by the IAEC.

A single dose of horse gram and *Kaulath* was orally intubated at a dose of 1 g/Kg body weight and the animals were observed every 30 min up to 4 h on first day and thereafter, once in 24 h for 14 days. Individual records were maintained on physical or behavioral changes and mortality. At the

Table 1 Nutrient composition of unfermented and fermented (*Kaulath*) horse gram

Constituents (% dry matter)	Inhibition (%)	
	Unfermented Horse gram	<i>Kaulath</i>
Moisture	32.89 ± 1.8 ^b	36.05 ± 0.9 ^a
Protein	21.8 ± 1.0 ^b	19.6 ± 0.6 ^a
Fat	1.8 ± 0.3 ^a	2.9 ± 0.5 ^b
Ash	2.4 ± 0.4 ^a	4.1 ± 0.5 ^b
Total carbohydrate ^a	74.0 ± 1.9 ^a	73.4 ± 1.6 ^a

Means with the same letter (a, b) within the same row do not differ significantly ($p < 0.05$)

^a By difference as 100 - (protein + fat + ash). Values are mean ± standard deviation of three independent determinations

end of the experiment, all animals were sacrificed under light ether anesthesia after 14 days and kidney, liver and lungs were dissected and processed for histological studies. The determination of activities of various enzymes and markers, such as lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) creatinine, urea and cholesterol, was carried out using commercial biochemical kits (Aggappe Diagnostics Ltd., Kerala, India). The kits were calibrated as per manufacturer's instructions.

Statistical analysis

All measurements were carried out in triplicate. The analytical values were evaluated from the mean (±standard deviation) and the results for animal experiments were represented as

Table 2 Mineral content of unfermented horse gram and *kaulath*

Mineral (mg/100 g)	Inhibition (%)	
	Unfermented	<i>Kaulath</i>
Calcium	106.5 ± 0.04 ^a	107.2 ± 0.04 ^a
Potassium	861 ± 0.41 ^a	884 ± 0.32 ^a
Sodium	304.4 ± 0.06 ^a	238.5 ± 3.2 ^a
Magnesium	136.4 ± 0.36 ^a	131.8 ± 0.33 ^a
Iron	9.74 ± 0.06 ^a	6.11 ± 0.04 ^b
Manganese	1.67 ± 0.05 ^a	1.85 ± 0.01 ^a
Copper	0.79 ± 0.003 ^a	0.77 ± 0.003 ^a
Phosphorus	251.3 ± 0.69 ^a	261.0 ± 0.57 ^a
Zinc	2.45 ± 0.05 ^a	2.43 ± 0.02 ^a
Arsenic (mg/kg)	BDL of 0.02	BDL of 0.02
Mercury (mg/kg)	BDL of 0.02	BDL of 0.02

Values are mean ± standard error of three individual experiments

Means with the same letter (a, b) within the same row do not differ significantly ($p < 0.05$)

BDL below detection limits

Table 3 Biological activities of unfermented horse gram and *Kaulath*

Biological activity	Inhibition (%)	
	Unfermented	<i>Kaulath</i>
Alpha glucosidase	9.3	10.8
Lipase	10	38.7
2, 2'-diphenyl picryl – 1- hydrazyl (DPPH) radical scavenging activity	19.3	26.7

Topoisomerase, Tyrosinase, Lipoxygenase were not detected in above samples

mean ± standard error for each experimental group of animals. Data obtained were analyzed using one-way analysis of variance; p values ≤ 0.05 were considered as statistically significant. All statistical analysis was performed using SPSS statistical software package version 17.0.

Results and discussion

Camembert cheese is a surface ripened cheese with *Penicillium camemberti* and its typical flavor reveals a number of volatile compounds mainly methyl ketones and their corresponding secondary alcohols, fatty acids, and the alcohols 3-methylbutanol, 2-methylpropanol, 3-octanol, and 1-octen-3-ol (Jollivet and Belin 1993). Hence, in order to obtain newer metabolites with potent biological activities and composition when the mold *P. camemberti* is grown on a substrate, such as horse gram, we have attempted to create a new fermented food designated as *Kaulath*.

Nutrient composition and mineral analysis

The nutrient composition of flours produced from fermented and unfermented horse gram seeds is presented in (Table 1). The moisture content of fermented grain flours was higher than that of unfermented grain flours. However, fermentation brought about a slightly decrease in protein content

Table 4 Determination of anti nutritional factors in unfermented horse gram and *Kaulath*

Anti nutritional factors	Inhibition (%)	
	Unfermented horse gram	<i>Kaulath</i>
Phytic acid (mg/100 g)	10.4 ± 0.28 ^b	9.13 ± 0.03 ^a
Tanin (mg/100 g)	2.6 ± 0.03 ^a	2.6 ± 0.01 ^a
Trypsin inhibitor activity (mg/100 g)	2.4 ± 0.11 ^a	0.5 ± 0.08 ^b
Alkaloid (%)	1.61 ± 0.02 ^a	1.95 ± 0.07 ^b

Values are mean ± standard error mean of three independent determinations ($P > 0.05$)

Means with the same letter (a, b) within the same column do not differ significantly ($p < 0.05$)

Table 5 Effect of unfermented horse gram and *Kaulath* on relative organ weights in rats

Organ weight g/100 g body weight	Liver	Lungs	Kidney	Heart	Adrenal	Spleen	Gonads
Control	2.97 ± 0.22 ^a	0.61 ± 0.10 ^a	0.61 ± 0.03 ^a	0.30 ± 0.01 ^a	0.27 ± 0.05 ^a	0.36 ± 0.02 ^a	1.31 ± 0.08 ^a
Horse gram	3.07 ± 0.43 ^a	0.49 ± 0.03 ^b	0.59 ± 0.07 ^a	0.25 ± 0.04 ^a	0.05 ± 0.03 ^a	0.90 ± 0.34 ^b	1.29 ± 0.04 ^a
<i>Kaulath</i>	2.95 ± 0.21 ^a	0.56 ± 0.13 ^b	0.53 ± 0.04 ^a	0.11 ± 0.07 ^b	0.11 ± 0.07 ^a	0.33 ± 0.06 ^a	1.17 ± 0.09 ^a

Values are mean ± SEM of three animals. No significant difference between control, native and *Kaulath* groups ($p < 0.05$)

Means with the same letter (a, b) within the same column do not differ significantly ($p < 0.05$)

of horse gram flour. Although this observation is in accordance with that of Khetarpaul and Chauhan (1989), who had shown that *Saccharomyces* and lactic fermentations either decreased the protein content or remained unchanged in pearl millet flour, it differs from those reported for naturally fermented African locust-bean seeds where an increase of crude and true protein content was observed (Ibrahim and Antai 1986). The decrease in protein content in *Kaulath* indicates that *P. camemberti* utilizes the available nitrogen for its vegetative growth initially. On the other hand, fat content of *Kaulath* increased by 61 % as compared to the unfermented grains, which could be attributed to the production of fatty acids during fermentation (Higashiyama et al. 2002; Akubor and Chukwu 1999). Significant increase ($p < 0.05$) was also observed in the ash content upon fermentation, whereas the change in total carbohydrate was insignificant.

Kaulath was analysed for the presence of various minerals and metal ions and the observations are presented in Table 2. Both control and *Kaulath* showed low levels of toxic metals (all of them were present below detection limits of 0.2 mg/kg). *Kaulath* and unfermented horse gram have high levels of

mineral content, with potassium, sodium and phosphorus at higher values than other metals. Upon fermentation, in comparison to control, *Kaulath* exhibited a substantial increase of potassium, phosphorus and manganese levels (about 10%), while copper and sodium were reduced by 12 and 24 %, respectively. Iron content was reduced drastically (38 %) upon fermentation while no changes were observed for zinc, calcium, magnesium and heavy metals.

Biological activity

Ethyl acetate extracts of *Kaulath* and unfermented horse gram were screened for their biological activity against lipase and α -glucosidase which have important implications in a number of diseases. *Kaulath* exhibited 38.7 and 10.8 % inhibition of lipase and α -glucosidase, respectively compared to 10 and 9.3 % inhibition observed in unfermented horse grains (Table 3). Furthermore, *Kaulath* extract showed strong free radical scavenging activity than control. The *Kaulath* extract showed 26 % DPPH activity as compared to 19.3 % observed in unfermented horse gram, perhaps due to the production of new or transformation of existing grain phenolic and flavonoids during the course of fermentation.

Anti nutritional factors

Anti nutritional factors in cereals and grains reduce the nutrient utilization or food intake in human subjects (Soetan and Oyewole 2009). Table 4 shows that there was a reduction of 12.2 % in phytic acid content and 79.16 % of trypsin inhibitors in *Kaulath* as compared to unfermented horse gram, whereas no changes were observed in tannin and alkaloids content.

Single dose acute toxicity tests in experimental animals

Three groups of rats were intubated with unfermented grains, *Kaulath* and normal diet, at a single dose of 1 g/kg bodyweight. This dosage is the maximum allowed for acute toxicity studies in evaluating new foods under OECD guidelines (2010). The animals did not show any visible signs of toxicity immediately or during the course of experiment. Some of the signs that were monitored were salivation, convulsions, and diarrhea, lethargy, sleep, coma and respiration

Table 6 Hematological profile of rats fed with unfermented horse gram and *Kaulath*

Parameters	Control	Unfermented	<i>Kaulath</i>
Hb (g/dl)	13.43 ± 0.4 ^a	13.23 ± 0.28 ^a	14.47 ± 0.72 ^a
RBC ($\times 10^6/\mu\text{l}$)	7.09 ± 0.6 ^a	7.09 ± 0.38 ^a	7.54 ± 0.24 ^a
WBC ($\times 10^3/\mu\text{l}$)	11.56 ± 1.2 ^a	12.57 ± 1.47 ^a	13.10 ± 3.90 ^a
HCT (%)	38.96 ± 1.45 ^a	37.60 ± 3.93 ^a	41.73 ± 1.79 ^a
MCH (pg)	18.96 ± 0.8 ^a	18.70 ± 0.10 ^a	19.17 ± 0.37 ^a
MCHC (g/dl)	34.53 ± 0.45 ^a	34.30 ± 0.40 ^a	34.63 ± 0.25 ^a
MCV (fl)	55.03 ± 1.64 ^a	54.63 ± 0.40 ^a	55.33 ± 0.58 ^a
LYM (%)	71.96 ± 8.24 ^a	91.50 ± 3.18 ^b	72.30 ± 1.45 ^a
PLT ($\times 10^3/\mu\text{l}$)	632.0 ± 46.86 ^b	595.67 ± 92.5 ^{ab}	735.33 ± 120.8 ^b

Values are mean ± SEM of three animals. No significant difference between control, native and *Kaulath* groups ($p < 0.05$)

Values are ± standard error mean of three animals. Means with the same letter (a, b) within the same row do not differ significantly ($p < 0.05$)

Hb haemoglobin, RBC red blood cells, WBC white blood cells, HCT Haematocrit, MCH mean corpuscular volume, MCHC mean corpuscular haemoglobin concentration, MCV mean corpuscular volume, PLT platelet, LYM lymphocytes

Table 7 Effect of unfermented horse gram and *Kaulath* on plasma enzymes and molecules

Parameters	Control	Unfermented	<i>Kaulath</i>
SGOT (u/l)	133.24 ± 2.65 ^a	130.12 ± 2.74 ^a	127.50 ± 0.09 ^a
SGPT (u/l)	31.62 ± 0.5 ^b	30.69 ± 1.92 ^a	31.33 ± 1.32 ^a
ALP (u/l)	183.95 ± 3.43 ^a	182.39 ± 1.64 ^a	187.31 ± 3.42 ^a
LDH (u/l)	1126.0 ± 11.52 ^a	1160.6 ± 9.89 ^a	1137.2 ± 5.40 ^a
Creatinine (mg/dl)	0.59 ± 0.03 ^a	0.51 ± 0.17 ^a	0.63 ± 0.17 ^a
Urea (mg/dl)	57.15 ± 9.27 ^a	48.92 ± 7.98 ^a	52.82 ± 3.51 ^a
*Triglycerides (mg/dl)	108.0 ± 31.91 ^a	84.85 ± 10.81 ^b	89.18 ± 9.74 ^b
*Cholesterol (mg/dl)	84.4 ± 2.43 ^a	70.4 ± 6.42 ^b	69.2 ± 7.25 ^b

Values are mean ± SEM of three animals. No significant difference between control, native and *Kaulath*, * except in cholesterol and triglycerides

Values are ± standard error mean of three animals. Means with the same letter (a, b) within the same row do not differ significantly ($p < 0.05$)

SGOT serum glutamate oxaloacetate transaminase, SGPT serum glutamate pyruvate transaminase, ALP alkaline phosphatase, LDH lactate dehydrogenase

patterns. At the end of the experiment, the animals were dissected and different organs were weighed. No abnormality was observed among the control, unfermented horse gram and *Kaulath* in gross organ weights and histological slices of kidney, liver and lungs (Table 5). Further, there was no adverse effect on cellular structure of these organs in both groups as compared to the control.

Haematological and clinical profile

The data on various haematological parameters are presented in Table 6. No significant changes were observed in any haematological parameters between control and *Kaulath*, respectively. Similarly, no significant changes in any of the marker enzymes for liver toxicity such as lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP), were observed in *Kaulath* compared to unfermented horse gram and control (Table 7). Similar results have been reported in fermented rice grains using *Monascus sp.* (Mohan kumari et al. 2009), who indicated no adverse effect on liver and kidney functioning. However, significant decrease in total cholesterol and triglycerides contents (16 and 22 %, respectively) were observed in rats fed with both unfermented horse gram and *Kaulath*. These results, probably, are the reflection of the increase in lipase inhibition in the crude extracts (Table 3).

Conclusion

In an effort to obtain new metabolites with possible therapeutic value and composition, a new fermented food, *Kaulath*, has been developed on a substrate that is distinctly of Indian origin by growing a generally recognized as safe generally

recognize as safe (GRAS) organism, which is typically consumed in the Western cuisine. This new fermented horse gram-based product showed a reduction in sodium content with an increase in potassium and reduction in alpha glucosidase activity aid to the health benefits of the product, particularly for those with cardiovascular and diabetic complications. The reduction of anti-nutritional factors like phytic acid and trypsin inhibitors and increase in antioxidant activities further aid value to the product. Preliminary safety studies on this unique food in experimental rats did not cause any toxic effects. As this fermented material holds promise for alleviating the symptoms of lifestyle diseases, elaborate toxicological, safety and sensory studies need to be carried out.

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