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Identification of Gamma-Aminobutyricacid (GABA) of Tempeh Made from Koro Kratok (*Phaseolus lunatus*) Bean and Beluntas (*Plunchea indica*) Leaves as Glucose Stabilizer

Istiqomah[1,*], Dina Mustika Rini[2], Larissa Tania[1], Sholihatil Hidayati[3], Mohammad Rofik Usman[3]
[1]Program of Pharmacy, College of Health Sciences, Harapan Bangsa School of Health, Indonesia.
[2]Graduate School of Integrated Sciences for Life, Hiroshima University, Japan.
[3]Departement of Pharmacy, Faculty of Health, Universitas dr. Soebandi, Indonesia.

Corresponding author*
istihariyanto@gmail.com

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Abstract

The tempeh available on the market is predominantly made from imported soybeans, but alternative formulations using locally sourced legumes, such as *koro kratok* (*Phaseolus lunatus*), have been explored. This study investigates the potential of *koro kratok* tempeh enriched with *Plunchea indica* (beluntas) leaves to improve its functionality. The study will evaluate gamma-aminobutyric acid (GABA) levels and physicochemical changes during fermentation. Tempeh was produced using different *koro kratok*-to-*P. indica* leaf ratios (100:0, 75:25, and 50:50). Key parameters observed included mycelium development and the content of fat, moisture, ash, protein, carbohydrates, and pH. Adding *P. indica* increased the fat content by 0.66–2.28%, the moisture content by 68.81–78.42%, and the ash content by 0.36–1.09%. Meanwhile, the protein content ranged from 6.62–8.87%, the carbohydrate content from 12.07–21.31%, and the pH between 4.21–4.25. The inclusion of *P. indica* also altered the profile of dominant volatile compounds. GABA was detected in all samples, and in vivo studies showed improved blood glucose levels and glucose tolerance in prediabetic rats. These results suggest that *koro kratok* tempeh enriched with *P. indica* leaves could be a promising functional food for regulating blood glucose.

Keywords: *P. lunatus bean*; *P. indica leaves*; GABA, Blood Glucose.

INTRODUCTION

Tempeh, a widely consumed plant-based protein in Indonesia, can be made from soybeans or other local beans, such as *koro kratok* (*Phaseolus lunatus* L). *Koro kratok*, also known as lima beans in some countries, thrives in tropical climates and is an excellent source of protein and carbohydrates, with low fat content. According to (Diniyah et al., 2013), *Koro kratok* contains 19.93% protein, 1.21% fat, and 61.42% carbohydrates. The high protein content of *koro kratok*, along with its local accessibility (in contrast to imported soybeans), forms the basis for its application as the primary component in soybean alternatives.

Tempeh contains higher levels of gamma-amino butyric acid (GABA) when the *R. oligosporus* microspores strain is used in an aerobic process followed by anaerobic fermentation. GABA is the main neurotransmitter in the central nervous system that acts by inhibiting certain brain signals and neuroactive molecular compounds produced by members of the human gut microbiota that regulate the neurological system in humans. In addition, this neurotransmitter may have a therapeutic effect by improving pancreatic β-cells function in patients with hyperglycemia. Activation of GABAA and GABAB receptors expressed on β-cells has been shown to stimulate insulin secretion and induce proliferation of β-cells. In addition, GABAA receptors are widely expressed on CD4 T cells and several studies have demonstrated a role for this neurotransmitter as an immunomodulator, suggesting an alternative mechanism that may reduce the risk of systemic inflammation (Patterson et al., 2019).

Meanwhile, *beluntas* leaf shoots (*Pluchea indica* less) were added to tempeh *koro kratok* to enhance its functional content. A previous study indicated that *beluntas* leaves possess active caffeoylquinic acid compounds that function as antihyperglycemia agents by inhibiting α-glucosidase enzyme activity, which plays a role in the conversion of carbohydrates to glucose (Nopparat et al., 2020). Another study found that *beluntas* leaves and roots contain caffeoylquinic acid and terpene glycosides as bioactive compounds. In addition, beluntas leaves exhibit stronger antioxidant activity than the rhizomes of *Curcumin longa* (turmeric) and *Camellia sinensis* (tea leaves). Moreover, both the leaves and roots of beluntas exhibit antimicrobial and anti-inflammatory

properties. As an antimicrobial agent, it can inhibit Gram-positive and Gram-negative bacteria. Therefore, the leaves and roots of the beluntas plant present considerable potential for product development, encompassing a range of items such as antibiotics, deodorants, foot sprays, creams, and gels, among others (Chan et al., 2022).

A previous study extracted *Beluntas* leaves at different stages of maturity using ethanol. DPPH, ABTS, and FRAP analyses indicated that young *Beluntas* leaf shoots had stronger antioxidant activity than mature leaves at the pre-flowering and flowering stages. The high antioxidant activity observed in the young *beluntas* leaf shoots, especially in the buds, was attributable to the increased presence of bioactive and phenolic compounds (Suriyah et al., 2019). This study focuses on developing indigenous food constituents derived from probiotic-based *koro kratok*, which are combined synergistically with herbs like *beluntas* leaves, recognized for their potential functional effects on metabolic syndrome. Tempeh, a traditional Indonesian probiotic-rich food, is a staple in the daily diet of Indonesian communities. Thus, this study aims to objectively evaluate the physicochemical characteristics of fermented *koro kratok* with the inclusion of beluntas leaves and determine the ideal composition for producing probiotic foods as a glucose stabilizer.

MATERIALS AND METHODS

Study area

This research material was obtained from a local supermarket in Jember, Indonesia. *Beluntas* leaves were collected from Jember, Indonesia. Raprima (PT Aneka Fermentasi Industri, Bandung, Indonesia) and Palape (Demi Bumi, Indonesia) yeast were purchased from a local supermarket.

Preparation of Tempeh

Koro kratok was first sorted and washed prior to use. It was then soaked in distilled water for 20-24 hours to soften the *koro kratok*. Subsequently, the outer skin of the *koro kratok* was peeled away, allowing it to become partially soft. The *koro kratok* was then boiled for 2-3 hours and the epidermis was removed until it was completely clean, followed by the drying and cooling process. Meanwhile, the beluntas shoot leaves were sorted, washed, and air-dried until half withered. The dried leaves were then ground and sieved into a coarse powder ready to be used in tempeh formulations.

Koro kratok and beluntas powder were used as the main tempeh ingredients. Three different ratios of *koro kratok* and beluntas leaf were used in this study: 100:0, 75:25, and 50:50, which were then named KK100, KKB75, and KKB50, respectively. To initiate the fermentation process, *koro kratok* and beluntas were mixed with Raprima and Palape yeast. The mixture was then wrapped in banana leaves, and the fermentation takes 24 hours. The fermentation proceeded for 20 hours under aerobic conditions, with a subsequent 4-hour period under anaerobic conditions at a room temperature. For analysis, tempeh was grounded and milled to a finely powdered state using a food processor. The powdered samples were then stored at 4°C to avoid potential damage. 200 grams of the material were used for each analysis.

Mycelium Observation

The physical properties were analyzed by examining the growth of mycelium. The appearance of tempeh *koro kratok* with and without the addition of *beluntas* leaves was subjectively observed, with assessments made on mycelium growth and tempeh compactness as a whole (Radiati, 2016). Tempeh samples were documented using a Xiaomi Redmi Note 8 camera at 40x magnification, positioned at an equidistant point via a tripod. Consistency of the base substrate was maintained, and subjective measurements of mycelial growth, hyphae, texture, and other physical appearances were conducted.

Procedures

Fat Content

The boiling flask was dried in an oven, then cooled in a desiccator and weighed. Approximately 1-2 grams of the sample was placed in a filter paper thimble lined with cotton. The stoppered thimble containing the sample was dried in an oven at a temperature not exceeding 80°C for approximately 1 hour and then transferred to a Soxhlet extraction flask. A condenser apparatus was placed on top of the flask, with the boiling flask underneath. Sufficient hexane solvent was added to the boiling flask, followed by extraction for 6 hours until the solvent returning to the flask was clear. The solvent in the boiling flask was distilled and the solvent collected. The boiling flask containing the extracted fat was heated in an oven at 105°C until a constant weight was reached, then cooled in a desiccator and weighed (AOAC, 2005).

Carbohydrate Content

The carbohydrate content was evaluated by the by-difference method. The carbohydrate content was calculated as the residue after determining the moisture, ash, protein and fat content of the sample. The total carbohydrate content of the sample can be calculated using the following formula: Carbohydrate content = 100 – (moisture content + ash content + protein content + fat content).

Protein Content

Protein content was determined as previously described (Utami et al., 2016). 0.51 gram sample was placed in a 100 mL Kjeldahl flask, followed by adding 2 grams of a selenium mixture and 25 mL of concentrated H_2SO_4. The

Kjeldahl flask containing the sample and reagents was heated to boiling on a hot plate until the solution turned a clear greenish color for approximately 2 hours. After cooling, the solution was diluted and transferred to a 100 mL graduated flask. Then 5 mL of the solution was pipetted into a distillation apparatus, followed by adding 5 mL of 30% NaOH and a few drops of PP indicator. Distillation was performed for 10 minutes, with 10 mL of a 2% borate solution mixed with an indicator as the receiver. Titration was performed with a 0.01 N HCl solution. The protein content of the sample can be calculated using the following formula:

$$\%N = \left(\frac{(Vol\ sample\ -\ Vol\ blanko) * N * 14{,}008 * 100}{Sample\ weight\ (mg)}\right) * 6{,}25$$

$$\%Crude\ Protein\ =\ \%N\ x\ Conversion\ factor$$

Water Content

The moisture content was determined by the oven method at a temperature of 105°C. Samples (1-2 grams) were weighed and placed in a pre-dried evaporating dish that had been previously weighed. The sample and the evaporating dish were then dried in an oven at 105°C for 3 hours, cooled in a desiccator and weighed until a constant weight was obtained (AOAC, 2005).

Ash Content

An empty porcelain crucible was dried in an oven at 105°C for 15 minutes, then cooled in a desiccator and weighed. A sample weighing 2-3 grams was placed in the crucible and weighed. The crucible containing the sample was ignited until complete volatilization, then placed in an electric furnace at a temperature of 550°C until complete ashing. The crucible containing the sample was removed from the furnace, cooled in a desiccator, and weighed to a constant weight (AOAC, 2005).

pH

The pH of the samples was measured using a pH meter, which was previously calibrated using pH 4, 7, and 9 buffer solutions. Powdered samples were then dissolved in distilled water prior to pH measurement.

GC-MS Analysis

GC-MS analysis was used to identify the volatile compound of the sample. The samples were filtered using a syringe filter membrane. The samples were then injected into the GC-MS instrument and the qualitative volatile compounds were determined. The GC-MS instrument was a GCMS-QP2010 Plus Shimadzu equipped with a splitless injector set at a temperature of 80°C. The temperature of the MS detector was set at 200°C. An Rtx-50 column (with an internal diameter of 0.25 mm, length of 30 m, and thickness of 0.25 µm) was used. The detector temperature was initially programmed at 80°C for 10 minutes and then increased to 230°C for 15 minutes at a rate of 5°C/min. Helium was used as the carrier gas at a 19 mL/minute flow rate. A 1 µL sample was injected using the splitless method.

Thin Layer Chromatography Analysis of GABA

GABA production was determined by Thin Layer Chromatography (TLC) using a 10% GABA solution as a standard reference. The samples were filtered with a syringe filter membrane. The samples were previously prepared as supernatant. The resulting supernatant from the sample was transferred to a fresh tube. Sample concentrations were prepared by diluting each sample to 0.01 g in 10 mL of 96% ethanol to give a 10% concentration. Subsequently, 1 mL of the sample concentration was applied to the TLC plate using a capillary pipette. A 10% pregabalin-GABA solution was used as a positive control, followed by sequential application of the supernatants of the isolates from the 2% samples to the TLC plates. TLC analysis was performed for 30 minutes using a developing solution (eluent) of n-butanol, acetic acid, and distilled water in a 5:3:2 ratio (Qiu et al., 2010). After analysis, the TLC plate was treated with a 0.5% (w/v) ninhydrin solution and then oven-dried at 60°C for 60 minutes. The GABA compound produced within the Tempe formulation was identified based on the retention factor (Rf), similar to the Rf value obtained from the pregabalin solution used as the GABA standard reference. The Rf value was calculated as previously described (Yogeswara et al., 2018):

$$Rf\ =\ \frac{distance\ traveled\ by\ the\ compound}{\text{distance traveled by the solvent front}}$$

Animals Preparation

Male Wistar Balb/c mice, aged 2–3 months and weighing approximately 200 grams on average, were used in this study. A total of 25 rats were selected, individually weighed, and marked before grouping. The animals were randomly divided into five groups, each consisting of five rats. Prior to treatment, the rats were fasted for 16 hours and subsequently induced with Streptozotocin (Sigma-Aldrich). All experimental procedures were approved by the Ethical Committee of Medical Research, Faculty of Dentistry, University of Jember, under registration number 2836/UN.25.8/KEPK/DL/2024.

Anti-Hyperglycemic Test

The antidiabetic testing began with weighing the rats and grouping them based on similar body weights. The animals were then fasted for 16 hours. On the first day, baseline blood samples were collected prior to treatment, and initial blood glucose levels were measured. Subsequently, a Streptozotocin (STZ) solution was administered intraperitoneally at 0.5 mg per rat. Following a 3-day stabilization period post-induction, rats with confirmed diabetes mellitus (blood glucose

levels >200 mg/dL) were selected and regrouped. Each group then received different treatments:

- Normal group
- The negative control group received STZ and 0.5% CMC and NaCl,
- The positive control group received STZ and metformin suspension at 0.013 mg/20 g body weight,
- Treatment groups received STZ solution one of the following (all dosed at 100 mg/20 g body weight):
  - *Koro kratok* tempeh extract,
  - *Koro kratok* tempeh extract and *beluntas* leaf extract (75:25 ratio),
  - *Koro kratok* tempeh extract and *beluntas* leaf extract (50:50 ratio).

All treatments were administered orally once daily in the morning for 14 consecutive days. Blood samples and body weight measurements were recorded on days 0, 3, 7, and 14 after treatment initiation. Blood samples were collected from the tail vein by puncturing with a sterile needle. A drop of blood was then applied to a glucometer strip and analyzed using a validated and calibrated glucometer to determine blood glucose levels.

Data analysis

Data were analyzed using one-way ANOVA statistical analysis followed by the Tukey test. Statistical analysis was performed using Sigma Stat 4.1.

RESULTS AND DISCUSSION

Mycelium Observation

Based on Figure 1, observations of the physical appearance of KK100 tempeh without the addition of beluntas leaves show evenly distributed mycelium growth. The developed hyphae also show an even distribution. This contributes to the compactness of the tempeh, which is dense and tends to be dry. Conversely, KKB50 tempeh, consisting of a 50% composition of koro kratok and 50% addition of beluntas leaves, had a low level of mycelial growth and the developing hyphae were barely visible. This significantly affected the fragility of the tempeh, making it prone to breakage and less dense in texture.

The fermentation of KK75 showed a denser result with more mycelium growth than KKB50; although solid in shape, it tended to be brittle. The grown hyphae were unevenly distributed on the surface of the tempeh, with a greater concentration on one side. This appearance suggests that KK100 fermented tempeh has a texture and density similar to soy-based tempeh, while the addition of 25% beluntas leaves in the KK75 treatment shows relatively favorable visual results.

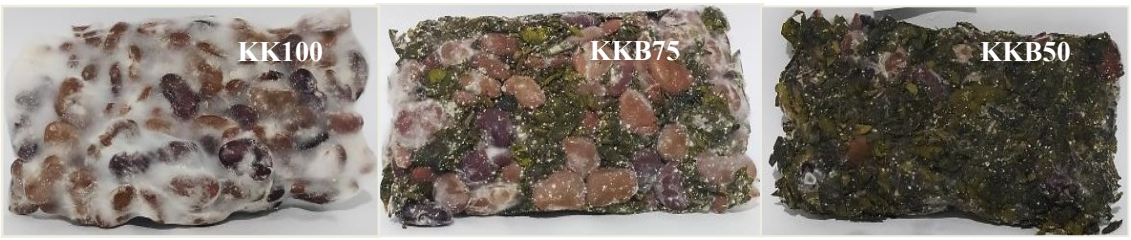

Figure 1. Physical appearance of tempeh koro kratok with beluntas leaves addition.

Fat Content

The fat content of KK100 was significantly lower than that of KKB50, with values of 0.67% and 2.28%, respectively, indicating an increase in fat content with the addition of *Beluntas* leaves (Table 1). These results suggest that the fermentation process can alter the formation of fat content with the addition of *Beluntas* leaves. A previous study showed that after 24 hours of fermentation, the levels of short-chain fatty acids were found in samples fermented with Bifidobacterium animalis subsp. The microbial strain from the fermentation pathway possesses a metabolic pathway capable of optimizing the production of short-chain fatty acids as the final fermentation product. The function of the fatty acids is as an energy source with the highest caloric content and as a source and solvent for vitamins A, D, E, and K (Annunziata et al., 2020).

Protein Content

The protein content was significantly different among the groups, with the highest protein content recorded in KK100 at 8.88% (Table 1). It was consistent with the composition of KK100 without adding *Beluntas* leaves, which had a higher protein content. Meanwhile, adding *Beluntas* leaves does not contribute to the increase of protein content during *koro kratok* fermentation. *Beluntas* leaves play a more significant role in the high fiber content than the formation in protein content during *koro kratok* fermentation. The protein content of tempeh increased as the fermentation time increased. This increase in protein content could be attributed to R. oligosporus engaging in metabolism during its growth phase and producing protease enzymes capable of breaking down proteins into free amino acids containing N groups, thus increasing protein content (Dewi et al.,

2014). Dietary protein has been reported to affect intestinal health by modulating barrier function (Rini et al., 2020).

Water Content

The water content of KK100 was significantly different from that of the KKB50 groups (Table 1). This observation was consistent with the composition of KKB50, which contained the highest concentration of *beluntas* leaves, resulting in a higher water content during the fermentation process than KK100, which lacks *beluntas* leaves. The water content of the fermentation product also affects the quality of the fermentation process using the *R. oligosporus starter*. The increase in water content during fermentation is due to microbial digestion of the substrate, which produces water, carbon dioxide, and a certain amount of energy. Fermentation time is a critical factor influencing the increase in water content; therefore, as the fermentation time increases, the moisture content also increases (Qomariyah & Utomo, 2016).

Adding materials such as *beluntas* leaves also contributes to increased water content. A characteristic of *beluntas* leaves is their tendency to produce more water compared to fermented tempeh, resulting in a higher overall water content. The accelerated mycelial growth in tempeh without *beluntas* leaves allows *R. oligosporus* hyphae to grow more compactly, resulting in a lower water content than that observed in fermented tempeh containing *beluntas* leaves.

Ash Content

The ash content showed a significant difference between the KK100 and KKB50, with values of 0.36% and 1.10%, respectively (Table 1). These results indicated that the fermentation of *koro kratok* and *beluntas* leaves provides a safe level for consumption as a food ingredient. Total ash content is used to assess the nutritional value of a food by indicating the total amount of minerals present, some of which may be toxic (Mardiah et al., 2020). Ash content analysis is also often used as an indicator to determine the quality of other food materials. This study showed that KK100, KKB75 and KKB50 groups has a good level of quality, making them suitable for use as food materials.

Table 1 Chemical characteristics of tempeh made from *koro kratok* and *beluntas* leaf

Group	Fat (%)	Protein (%)	Water (%)	Ash (%)
KK100	0,67^{a}	8,88^{a}	68,81^{b}	0,36^{a}
KKB75	1,03^{ab}	7,34^{b}	73,27^{ab}	0,61^{ab}
KKB50	2,28^{b}	6,61^{c}	78,42^{a}	1,10^{b}

*Values followed by the same letter indicate no significant difference (P>0.01)

Carbohydrate Content

The carbohydrate content showed a significant difference between KK100 sample and KKB50, with values of 21.31% and 12.07%, respectively (Table 2). The highest carbohydrate content was observed in the fermentation of 100% *koro kratok* compared to the addition of *beluntas* leaves. This result was attributed to the role of hyphal growth forming mycelium during fermentation by *R. oligoporus*, which can break down polysaccharide components and release amylase enzymes, resulting in the generation of new carbohydrate compounds (Indriyani et al., 2010). It was also supported by another study which suggests that *beluntas* leaves (*Pluchea indica* less) contain active caffeoylquinic acid compounds capable of inhibiting the action of α-glucosidase enzymes responsible for the conversion of carbohydrates into glucose as an anti-hyperglycemic agent (Nopparat et al., 2020). Therefore, the higher the concentration of *beluntas* leaves, the higher the carbohydrate content. In addition, KK100 has a compact tempeh texture due to uniform hyphal growth, resulting in higher fiber formation compared to KKB50 and KKB75, which have lower carbohydrate content. High fiber has been reported to help maintain intestinal health (Rini et al., 2023), as well as its metabolites such as short-chain fatty acids (Isayama et al., 2023; Xu et al., 2023).

Table 2. Carbohydrate and pH of tempeh *koro kratok* with *beluntas* leaves addition

Group	Carbohydrate (%)	pH
KK100	21,31 ± 0,05^{a}	4,23
KKB75	17,90 ± 0,42^{ab}	4,25
KKB50	12,07 ± 0,07^{b}	4,21

*Values followed by the same letter indicate no significant difference (P>0.01)

pH level

The pH values were not significantly different among the three samples and ranged from 4.21 to 4.25, indicating an acidic pH of tempeh (Table 2). These pH measurements indicated that the tempeh had undergone a successful fermentation process with increased lactic acid content as a source of probiotics. A previous study showed that the strain type of Lactobacillus sp influenced the decrease in pH during the fermentation of red beans with *Lactocbacillus plantarum* WCFS1. The pH decreased from 6.6 to 3.76 after 48 hours of fermentation at room temperature **(Gan et al., 2017)**. The acidifying ability of *Lactobacillus* is also influenced by the role of carbohydrates present in the composition of legumes during the fermentation process. Oligosaccharide components present in legumes are used by certain *Lactobacillus* species to initiate microbial enzymatic activities, resulting in varying pH reductions (Mardiah et al., 2020).

Volatile Compounds

The shift in the volatile composition of tempeh made from *koro kratok* and *beluntas* leaves was shown in

Figure 2. The dominant volatile compounds in the KK100 group were carbamic acid, acetic acid and 2,3-butanediol. Meanwhile, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and L(+) Milchsaeur were more dominant in the KKB75 group. Other volatile compounds such as 1-(4-methoxyphenethyl)-1-methylbiguanidine, benzenethanamine, mevalonic lactone and coumarin were found in similar amounts with carbamic acid, acetic acid and 2,3-butanediol in KKB75 group. On the other hand, the KKB50 group was dominated by similar compounds to those found in the KKB75 group, with the addition of volatile compounds such as ethanol, propinoic acid, glycerol, isopentane, tetramethylene sulfone, 1-methyl-4-aminocyclohexane, cis-6-methyltetrahydro-pyran-2-yl)-acetic acid, 2,3-dihydro-benzofuran, benzenepropanoic acid, and melitol. These results suggest that the addition of *beluntas* leaves changes the composition of volatile compounds in tempeh. The dominant volatiles in each group may contribute to the aroma of tempeh. However, less dominant volatile compounds may also be involved in forming subtle flavors. This finding is consistent with the previous study that showed that the production of volatile compounds depends on the substrate (Mei Feng et al., 2007).

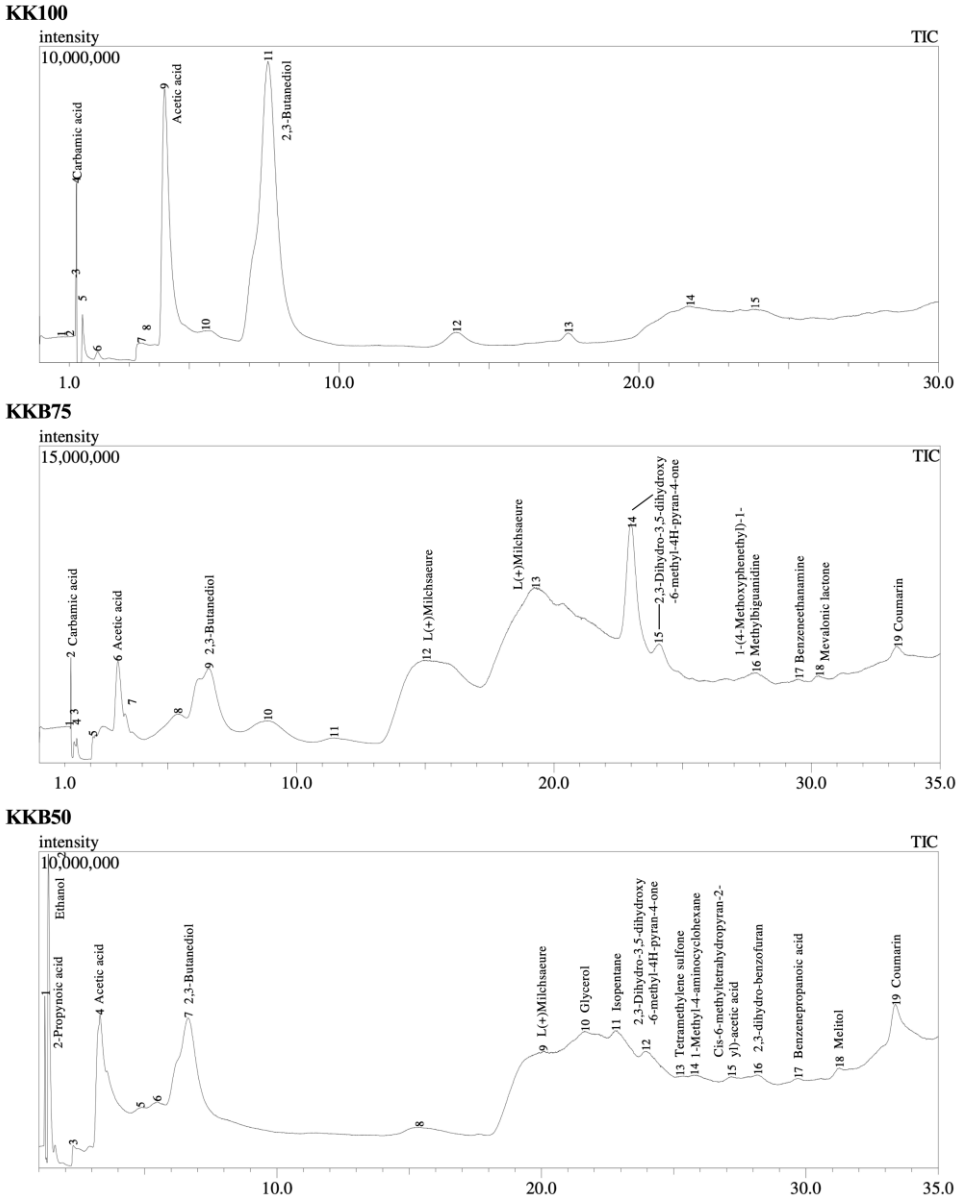

Figure 2 Profile of volatile compounds on tempeh *koro kratok* with *beluntas* leaves addition.

Identification of GABA

Figure 3 illustrates the identification profile of GABA in tempeh *koro kratok* with *beluntas* leaves. Purple spots indicate the presence of chemical compounds that are accelerated by the mobile phase of the eluent and stained by the dye solution. The first lanes show the GABA

profile of pregabalin as a standard at a concentration of 10 mg. The next lane shows the profile of KK100, KKB75, and KKB50 isolates, respectively. The presence of GABA can be readily confirmed by the appearance of purple spots upon applying ninhydrin (Yogeswara et al., 2018). Table 3 shows Rf values for pregabalin were determined to be 0.62, while the Rf values of KK100, KKB75, and KKB50 were 0.60, 0.53, and 0.51, respectively. The equivalence between the Rf values of the isolates and the Rf value of pregabalin serves as evidence affirming the capability of all isolates to generate GABA. KK100 was demonstrated to produce GABA in a manner comparable to the standard used, in contrast to the other groups. Certain strains or species within the lactic acid bacteria (LAB) have been documented to possess the ability to produce GABA. The majority of GABA-producing bacterial strains or species are typically derived from traditional fermented food sources. Furthermore, scientific reports have indicated that only four isolates belonging to the LAB, namely *L. paracasei* PF6, *L. bulgaricus* PR1, *L. lactis* PU1 and *L. brevis*, have been extracted from different types of cheeses, exhibiting the most substantial GABA production and the highest GABA levels (Siragusa et al., 2007). In addition, microorganisms synthesize GABA during the fermentation process. *R. oligosporus*, in conjunction with *L. plantarum* 202, has been observed to produce GABA during the fermentation of soybean residues in a time-dependent manner. A comparative analysis of the respective fermentation times of 24 and 48 hours revealed that co-inoculation with GABA-producing *L. plantarum* 202 did not result in an additive effect on GABA content, as observed in *R. oligosporus* fermented soybean residue (Hariyanto et al., 2022). This improvement in insulin function is suspected to be partly due to the presence of GABA (gamma-aminobutyric acid), which may support pancreatic recovery by helping stabilize insulin and glucagon hormones under homeostatic conditions. However, it is also possible that other bioactive compounds present in the 100% tempeh contribute to the observed improvement in fasting glucose tolerance.

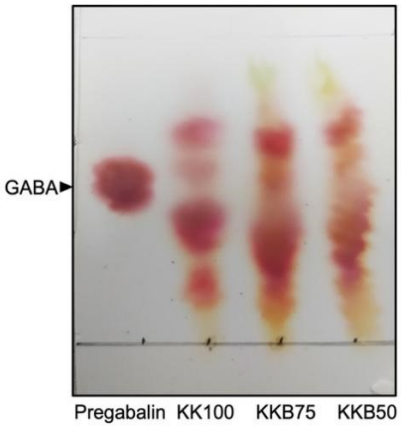

Figure 3 Identification profile of GABA with Thin Layer Chromatography on tempeh *koro kratok* with *beluntas* leaves addition.

Table 3. Retention factor (Rf) GABA of tempeh *koro kratok* with *beluntas* leaves addition.

Group	GABA	KK100	KKB75	KKB50
Rf Value	0,62	0,60	0,53	0,51

Body Weight and Blood Glucose Level

The results of the body weight measurements of rats in Figure 4, which had been induced with STZ, showed no significant weight gain in either the control or treatment groups. However, the KK100 and KKB75 treatment groups exhibited increased body weight from day 0 to day 14. In contrast, the KKB50 group did not show significant weight gain and experienced a weight loss by day 14. This indicates that the KKB50 group had a lower protein content than KK100 and KKB75. Changes in the rats' body weight suggest that plant-based proteins derived from legumes may reduce plasma cholesterol, triacylglycerols, and blood glucose levels, acting as potential antioxidants and improving coronary endothelial function. The findings support that replacing animal-based protein with legume protein can improve lipid profiles, prevent atherosclerosis, and enhance overall metabolism, thereby contributing to body weight stabilization (Sun T. et al., 2022).

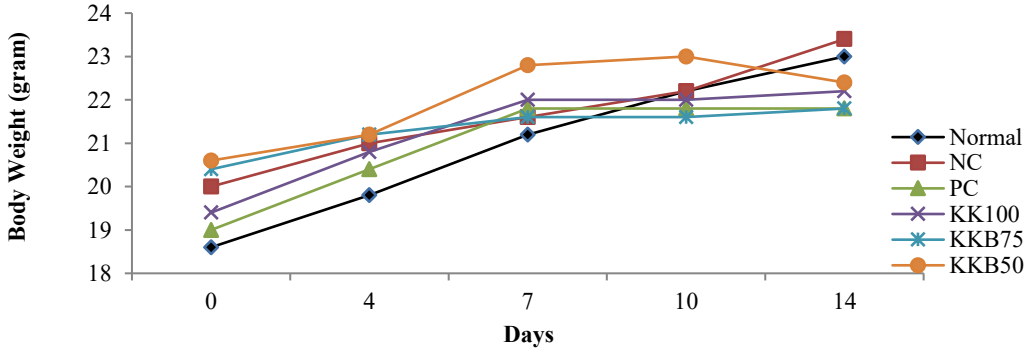

Figure 4. Body weight levels in mice induced with STZ under different treatments: Normal, NC (Negative Control), PC (Positive Control), KK100 (100% *koro kratok* extract), KKB75 (*koro kratok* 75% and *Beluntas* 25%), and KKB50 (*koro kratok* 50% and *Beluntas* 50%).

Based on the blood glucose levels shown in Figure 5, measured over a 0–12 day period, the KK100 treatment group demonstrated a reduction in blood glucose levels compared to the Positive Control (PC) group, which showed improved insulin levels on days 6, 9, and 12. Meanwhile, the KKB75 treatment group exhibited improved on day 12, as indicated by a decrease in blood glucose levels accompanied by increased insulin levels. In contrast, the KKB50 group showed a continuous increase in glucose levels with no significant improvement, similar to the Negative Control (NC) group. This suggests possible mortality among the rats during the testing period due to persistently elevated glucose levels observed on day 12. This indicates that adding *beluntas* leaves during the fermentation process to make tempeh did not significantly affect blood glucose reduction. This finding indicates that adding *beluntas* (*Pluchea indica*) leaves during the fermentation process of *koro kratok* tempeh does not have a significantly effect on lowering blood glucose levels. It is hypothesized that incorporating *beluntas* leaves during fermentation may inhibit the reduction of phytic acid in *koro kratok*, thereby increasing its antinutrient capacity compared to fermentation without *beluntas* leaves (Hariyanto, et al., 2022). Moreover, the protein content in the KKB75 and KKB50 samples was lower than in the KK100 group, suggesting that the glucose-lowering effect was more pronounced in the KK100 group, where no *beluntas* leaves were added. This finding underscores the potential role of protein in reducing fasting blood glucose levels. Nevertheless, further research is required to identify the specific bioactive compounds involved in this mechanism.

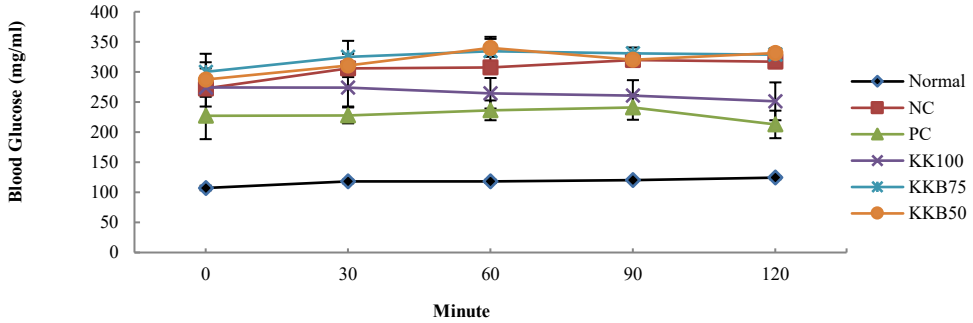

Figure 5. Blood glucose levels in mice induced with STZ under different treatments: Normal, NC (Negative Control), PC (Positive Control), KK100 (100% *koro kratok* extract), KKB75 (*koro kratok* 75% and *beluntas* 25%), and KKB50 (*koro kratok* 50% and *beluntas* 50%).

Glucose Tolerance Results

The results of the glucose tolerance test, which measured the test animals blood glucose sensitivity every 30 minutes (as shown in Figure 6), illustrate the animals' tolerance to increased glucose levels. Fasting glucose tolerance is commonly used to assess prediabetic conditions, with blood glucose levels categorized as follows: <140 mg/dL (normal), 140–199 mg/dL (prediabetes), and ≥300 mg/dL (diabetes) [11]. Based on the test results, the KK100 treatment group showed an improvement in glucose tolerance compared to the baseline (0 minutes). In contrast, the KKB75 and KKB50 treatment groups did not improve fasting glucose tolerance compared to the Positive Control (PC) group. These findings suggest that tempeh made from 100% *koro kratok* may enhance insulin activity, thereby lowering blood glucose levels in prediabetic conditions. KK100, which was fermented without the addition of *beluntas* leaves, had a higher total protein content, which may have contributed to the reduction in glucose tolerance levels in diabetic rats. Although the addition of *beluntas* leaves in the fermentation of *koro kratok* tempeh led to an increase in beneficial fatty acids, the results indicated that it did not have a significant effect on fasting blood glucose or glucose tolerance in diabetic rats with insulin resistance. Further studies are needed to understand the specific mechanism.

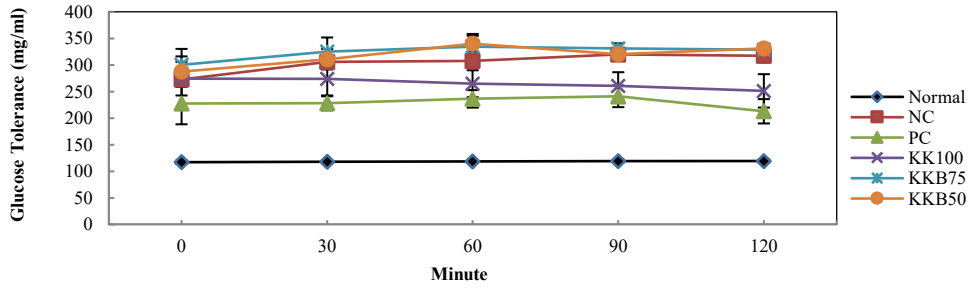

Figure 6. Glucose tolerance over a 0–120 minute period under different treatments: Normal, NC (Negative Control), PC (Positive Control), KK100 (100% *koro kratok* extract), KKB75 (*koro kratok* 75% and *Beluntas* 25%), and KKB50 (*koro kratok* 50% and *Beluntas* 50%).

CONCLUSIONS

Our study demonstrated that *koro kratok* could serve as a viable alternative to soybeans in tempeh production. The results showed that the fat content ranged from 0.66% to 2.28%, protein content from 6.62% to 8.87%, moisture content from 68.81% to 78.42%, ash content from 0.36% to 1.09%, carbohydrate content from 12.07% to 21.31%, and pH ranged from 4.21 to 4.25. The addition of *beluntas* leaves during fermentation significantly affected both the physical and chemical characteristics of the tempeh. Specifically, it increased the fat, ash, and moisture content, while decreasing the protein and carbohydrate levels. The pH value, however, remained unchanged. Tempeh containing *beluntas* leaves exhibited a more brittle texture and less evenly spread mycelium growth than the control groups. Additionally, including *beluntas* leaves enhanced the volatile compounds in tempeh, potentially contributing to its distinctive flavor. Importantly, our analysis confirmed the presence of (GABA) in *koro kratok* tempeh, both with and without the addition of *beluntas* leaves, suggesting its potential as a functional food. However, an *in vivo* study revealed that only koro kratok tempe without beluntas leaves can significantly reduce fasting blood glucose. Thus, further research is needed to evaluate the bioactive components responsible for these effects and to assess consumer acceptance of the product.

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