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Original Research Article

In Vivo Assessment of Nanocapsules of Black Rice (*Zizania aquatica*) Yeast Extract in Diabetes Mellitus Type 2-Induced Mice (*Mus musculus*)

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ABSTRACT

Nano-products are commonly used in pharmaceuticals. This study investigated the *in vivo* antiglycemic effect of nanocapsules of black rice (*Zizania aquatica*) yeast aquadest extract in mice (*Mus musculus*) induced with type 2 diabetes mellitus (T2DM). Black rice yeast was prepared and extracted with distilled water. The black rice yeast extract was used to produce nanocapsules using a poloxamer 88 support matrix. The *in vivo* antiglycemic effect of black rice yeast extract (BRY-AE) nanocapsules was investigated using T2DM-induced male Swiss mice. The mice were divided into 4 groups, including the positive control group (DM + commercial feed), the negative control group (no DM + commercial feed), the BRY-AE nanocapsule test group (DM + BRY-AE nanocapsules+feed), and the extract test group (DM + BRY-AE+commercial feed). Changes in glucose levels, profiles of glucose tolerance, profiles of insulin resistance, and histological observations of liver tissue were determined. The results showed that blood glucose levels in all groups reduced after the second week, however, some still did not reach normal levels (no DM). The glucose tolerance test for the nano-treatment of BRY-AE at 800 ppm and BRY-AE at 1000 ppm revealed a good profile, indicating that it was comparable to the negative control and metformin. When compared to the negative control, the insulin sensitivity of the nanocapsules from BRY-AE at 800 ppm was relatively low. The findings of this study suggest that the nanocapsules BRY-AE can be used to lower blood glucose levels in mice with T2DM.

Keywords: Antiglycemic effect,Glucose tolerance, Insulin resistance, Poloxamer 88, Type 2 diabetes mellitus

Introduction

Yeast has been used for nutritional supplements since ancient times since it is high in protein, lipids, RNA, vitamins, and minerals. It also contains B complex vitamins and other minerals that the body needs, including chromium (Cr^{3+}). Baker's yeast contains Cr^{3+} (112.8 x 10^{-4}), Cr^{6+} (53 x 10^{-4}), protein (40.19%), crude fiber (6.00%), and starch (5.424%).[1] Yeast may grow on a variety of carbohydrate-rich media, including black rice, which is known to be high in anthocyanins, which function as antioxidants (IC_{50} of black rice is 102.74 ppm).[2] Yeast grown on black rice (yeast-black) can lower glucose levels by having a high glycemic index, so it must be removed by enzymatic hydrolysis when used as an anti-diabetes mellitus type 2. Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder due to the inability of the pancreas to produce insulin or the body's inability to use the insulin it produces adequately. The cause of T2DM is insulin receptor dysfunction. Insulin is unable to carry out its function without the presence of ionic compounds, namely chromium.[3] A single fraction of yeast contains chromium.

Black rice yeast (BRY) is produced through yeast fermentation in black rice media, so BRY also contains chromium. BRY can lower blood glucose levels (BGL) by 130 mg/dL in mice with type 2 diabetes.

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Because BRY has a high rate of repair of the liver cell damage caused by BGL (41%), it is thought that BRY has the potential to be employed as an anti-T2DM agent and promote liver regeneration following T2DM exposure.[1] Type 2 diabetes is a chronic metabolic disorder characterized by the body's inability to utilize insulin, the hormone that regulates blood glucose levels. The global prevalence of diabetes is relatively high. Indonesia has one of the highest diabetes prevalence rates in the world. Diabetes affected 8.5% of Indonesians aged ≥15 in 2018, with T2DM being the most common type.Type 2 diabetes will triple in Indonesia by 2030. In 90% of cases, the disorder is characterized by insulin resistance and deficiency.[5]

Black rice used as a yeast fermentation medium is known to contain anthocyanins, a red flavonoid that acts as an antioxidant.[6]Also, it is one of the bioactive components that can be used as a strong nutraceutical,[7] traditional medicine, including a phytopharmaceutical, an anti-diabetic,[8-12,] and anti-obesity.[13-15] Regeneration of cells damaged by exposure to DM also requires protein or amino acids as raw materials. If yeast is to be employed as an anti- or type-2 DM medication, the presence of chromium, anthocyanins, proteins, and other components must be maintained. Many new drugs have been developed, however, some of them have side effects that prevent the advantages from always outweighing the risks. Several medications are quite successful *in vitro* but cannot inhibit endogenous enzymes, rendering them ineffective *in vivo*.[16,17]

Nanotechnology with nanoencapsulation has improved diagnosis and therapy, including formulations with different structures designed for

biomedical uses.[18] Several anti-DM medications have been produced using diverse technologies. Nanotechnology is currently being developed, which is a technology used to modify matter at the atomic, molecular, and supramolecular scales. This technique, also known as molecular nanotechnology, intends to precisely manipulate atoms and molecules for the manufacture of macro-scale products.[19]Public health research institutions, including the National Institute for Occupational Safety and Health, are actively investigating the health impacts of nanoparticle exposure. Nanotechnology comes in many forms, one of which is nanoencapsulation. Encapsulation is a process by which one or more materials are coated by another material. Both the substrate to be coated and coated materials are mainly liquids, but some gas particles can also be present.[20] Microencapsulation is a particle with a diameter between 3 and 800 μm, while nanoparticles are particles with a diameter ranging from 10 to 1,000 nm. Nano-sized particles allow for better distribution of the product and can expand the contact surface of the particles with the material. Furthermore, nanoencapsulation permits active substances to be released regularly through the encapsulant layer, boosting the efficiency with which active compounds are used.[21] The potential of nanotechnology as an anti-DM can be tested for its activity in several ways, including glucose tolerance test,[22] insulin resistance test,[23] and hypoglycemic activity.[24] Therefore, research combining chromium-rich yeast with nanotechnology (encapsulation approach) is necessary to generate an effective anti-T2DM product.

The present study was aimed at conducting an *in vivo* assessment of nanocapsules of black rice yeast extract in mice (*Mus musculus*) induced with T2DM.

Materials and Methods

Sources of materials used

Black rice(*Zizania aquatica*), organic black rice of Mlatiharjo brand, a product of KSU Citra Kinarya, Java, Indonesia were obtained. The identification of the black ricewas carried out by the Laboratory of Biology, Department of Biology, Faculty of Science, Universitas Negeri Surabaya, Indonesia. Other materials used in this study include α-amylase and glucoamylase (liquid, commercial), dry bakery yeast (Maury-pan brand), distilled water, poloxamer 88 powder (New Green Health Industry Co., Ltd), liquid fat (commercial), liquid fructose (Rose brand), glucose (p.a, Merck), metformin tablet, hematoxylin (Merck), and eosin (Path chem). Also included are paraffin (Sakura brand), Bouin's solution (picric acid/Merck, formaldehyde/ Merck, acetic acid/ Merck, aquadest), and physiological salt (Merck).

Preparation of black rice yeast

Black rice was used as a yeast growth medium, and its preparation included washing, grinding, and sifting through a 100-mesh screen to make black rice flour. The flour was then cooked until it formed a gel (gelatinization). The hydrolysate produced by the enzymatic hydrolysis of black rice flour gel with α--amylase and glucoamylase was used as a yeast growth medium. The yeast was then fermented. To make black rice yeast, commercial yeast (bakery yeast) was added to black rice hydrolysate media and fermented for 20 days.[2]

Extraction of black rice yeast

Black rice yeast was initially macerated in distilled water for 24 hours. The macerated filtrate was then filtered through a vacuum pipe and evaporated through a freeze drier.[2] The yeast extract obtained (BRY-AE) was used as a core material in nanoencapsulation. The water content of the resultant yeast extract was evaluated using the gravimetric technique. The next step was to prepare BRY-AE solutions in various concentrations of 100, 200, 400, 600, 800, 1000, and 2000 ppm.

Preparation of BRY-AE nanocapsules

A poloxamer 88 supporting matrix was used in the production of BRY-AE nanocapsules. The nanocapsules were produced with the help of a sonicator. The preparation of yeast nanoencapsulation started by dissolving black rice yeast extract (BRY-AE) and matrix (poloxamer) into 10 mL of distilled water. Nanoencapsulation of yeast was made in a ratio of 1:2 and dissolved in distilled water until homogeneous. Furthermore, the sonication process was carried out for 5 minutes at a frequency of 12.5 Hz and then allowed to stand for 24 hours so that a relatively perfect encapsulation occurred. The resultant BRY-AE nanocapsules were stored in a closed container for use in anti-DM *in vivo* tests. The BRY-AE nanocapsules were dissolved in distilled water with various concentrations of 100, 200, 400, 600, 800, 1000, and 2000 ppm.

In vivo assessment of BRY-AE nanocapsules as anti-diabetes mellitus

In vivo testing of BRY-AE, which has been processed with nanoencapsulation technology (BRY-AE nanocapsules) was carried out using Swiss strain mice to investigate its ability to lower blood glucose. BRY-AE was also evaluated as a comparator. The initial stage of the *in vivo* test was carried out with the adaptation of male Swiss mice. Mice weighing 25-30 g were acclimatized by being given water and food in general, typically commercial feed, for one week. Type 2 diabetes was induced by a diet high in fat and fructose.[1]Type 2 DM was confirmed in the mice with blood glucose levels ≥150 mg/dL. Mice with T2DM were placed in cages and administered BRY-AE nanocapsules before their blood glucose levels were measured. A positive control (DM + commercial feed), a negative control (no treatment + commercial feed), and a test group (administered BRY-AE in various concentrations and BRY-AE nanocapsules in various concentrations) + commercial feed were employed in the study. For three weeks, each mouse was housed in a separate cage.

Determination of glucose tolerance profile

The determination of glucose tolerance was carried out by a tolerance test according to the Fitriani method.[23] The mice were fed for 7 days and fasted for 18 hours before their blood glucose levels were measured at time intervals of 0, 60, 90, 120, and 150 minutes. The initial blood glucose levels of mice were measured by taking a sample from the tail (TO) and then administering the test substance orally. After 30 minutes, all mice were given a 3 g/bb glucose solution orally. Blood glucose levels were checked again after 60, 90, 120, and 150 minutes.

Determination of insulin resistance profile

The insulin resistance test was used to determine the insulin resistance profile to determine antidiabetic activity in enhancing insulin sensitivity.[23] The mice were divided into five groups: a negative control group (normal mice), a positive control group (DM), the BRY-AE extract test group, the BRY-AE nanocapsule test group, and the comparator group with metformin 65 mg/kg body weight. Except for the negative control group, all groups were given a high-fat and glucose diet for 14 days to establish insulin resistance. Insulin sensitivity testing was performed by injecting 0.1 U/kg of insulin intraperitoneally and then measuring blood glucose levels every 15 minutes for one hour. Blood glucose levels were measured using an Easy Touch@7 device.

Histological observation of liver

Histological examinations of mouse livers were performed to determine the extent of cell damage. Mice were dissected after 10 weeks of being fed a high-fat and fructose diet, and their liver organs were washed with physiological solution, placed in Bouin's solution, and histological preparations were made. The paraffin-embedded specimens were stained with hematoxylin and eosin. The prepared organ slides were examined under a light microscope from five different angles at a magnification of 400x. The total number of cells and cells that were damaged (apoptosis and necrosis) were counted for

each sub-view, then the percentage of damage that occurred was calculated.[24]

Results and Discussion

Table 1 presents the results of glucose levels in mice after treatment. The results showed that blood glucose levels in mice after the induction of diabetes ranged from 212 to 500 mg/dL. The nanocapsules BRY-AE (2000 ppm) and BRY-AE (2000 ppm) showed the greatest reduction. In the second week, the initially high glucose level (diabetes) returned to normal. Nanocapsules (100 ppm) and yeast extract (100 ppm) still had high glucose levels, indicating that the treatment had not restored a normal condition, and the decrease was the smallest of the treatments tested. The negative control revealed a 12-point rise in treatment for two weeks, but the glucose level remained normal. After two weeks of treatment, yeast extract and metformin were able to reduce elevated glucose levels and return them to normal. One of the factors that affects the decrease in glucose level is the chromium content of BRY-AE and BRY-AE nanocapsules. BRY-AE contains chromium (Cr^{3+}). Three-valent chromium is known to play a role in the mechanism of glucose transport across the cell membrane by insulin.[25]The results of the glucose tolerance test for positive and negative controls are shown in Figure 1. The glucose level in the negative control group was steady, while in the positive control group, the results of the glucose tolerance test highlighted a high glucose level at 150 minutes.

Figure 2 depicts the glucose tolerance test results for each treatment using nanocapsules and yeast extract at varying concentrations, along with positive and negative control comparisons. The glucose tolerance of mice with the nanocapsules at100 ppm treatment was poor, with a high glucose level of 319 mg/dL at the end of the measurement (150^{th} minute), as shown in Figure 2. Treatment with nanocapsules (800-1000 ppm) resulted in an excellent glucose profile, comparable to a negative control profile (no DM, commercial feed). Except for the 100 ppm nanocapsule treatment, the trend of the glucose level at 60 minutes increased for all treatments and then gradually decreased towards normal at 150 minutes. The glucose tolerance profile of 1000 ppm yeast extract treatment, nanocapsules at 800 ppm, metformin, and negative (normal) controls is shown in Figure 3. The glucose tolerance test results for the nano-treatment and the extract were normal. This indicates that they were close to the negative control, and metformin levels were similar (800 ppm for nanocapsules and 1000 ppm for BRY-AE yeast extract), which was not nanoencapsulated. It is suggested that nanocapsules at 800 ppm concentration and 1000 ppm yeast extract be taken as anti-diabetic agents.

Table 1: Effects of treatments on blood glucose levels in mice.

Treatment	Blood glucose levels/week			Delta (week 0 & 2)	Description
	0	1	2		
Positive control (DM+commercial feed),	431	485	384	47	Week 2, glucose level is down but still very high
Negative control (no DM + commercial feed),	90	120	102	-12	2nd week glucose level is normal
Metformin	325	300	108	217	glucose level in the 2nd week, decreased, normal category
The BRY-AE nanocapsule test group (DM+ BRY-AE nanocapsules +feed					
100 ppm	500	421	407	93	Glucose level in the 2nd week fell, still high
200 ppm	250	332	137	113	glucose level on the 2nd week, still moderately low
400 ppm	287	165	146	141	glucose level in the 2nd week, decreased, still in the moderate category
600 ppm	318	222	139	179	glucose level in the 2nd week, decreased, still in the moderate category
800 ppm	300	137	135	165	glucose level in the 2nd week, decreased, still in the moderate category
1000 ppm	288	189	92	196	glucose level in the 2nd week, decreased, normal category
2000 ppm	456	197	111	345	glucose level in the 2nd week, decreased, normal category
The BRY-AE extract test group (DM+BRY-AE +feed)					
100 ppm	319	298	313	6	glucose level in the 2nd week, decreased, high category
200 ppm	289	354	168	121	glucose level in the 2nd week,
400 ppm	238	159	128	110	glucose level in the 2nd week,

					decreased, normal category
600 ppm	296	205	127	170	glucose level in the 2nd week, decreased, normal category
800 ppm	212	157	123	90	glucose level in the 2nd week, decreased, normal category
1000 ppm	333	152	121	212	glucose level in the 2nd week, decreased, normal category
2000 ppm	349	341	107	242	glucose level in the 2nd week, decreased, normal category

One of the methods of testing the pharmacological efficacy of drugs *in vivo* is to use experimental animals, including for testing anti-diabetic activity. Various animal-based research approaches for both T1DM and T2DM have been established, using various diabetogenic drugs (alloxan and streptozotocin) that cause damage to pancreatic β-cells, reducing the pancreas' ability to secrete insulin. Furthermore, insulin is a peptide hormone secreted by the β cells of the islets of Langerhans.[26,27] This hormone has a role in maintaining normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid, and protein metabolism, and promoting cell division and growth through its mitogenic effect. Figure 4 shows the positive and negative control glucose levelsin the insulin resistance test. Figure 5 presents the glucose level given the nanocapsule treatment in the insulin resistance test.

At 60 minutes, the mice given the nanocapsule treatment had a glucose profile that was nearly identical to the negative control, but the positive control had a high glucose level. The glucose level profile in the insulin resistance test is depicted in Figure 6. At 60 minutes, the glucose level profile of mice treated with yeast extract was nearly identical to the negative control, whereas the positive control and treatment with 100 ppm yeast extract revealed high glucose levels, poor insulin resistance, and low insulin sensitivity. Mice treated with 800 ppm nanocapsule yeast had a comparatively low sensitivity index, indicating a negative control comparison. When compared to metformin treatment, the sensitivity index of mice treated with 800 ppm was still lower.

Histological observation of the liver

Medications can damage the liver, the body's metabolic center, because these drugs are removed through the liver's metabolism.[4] Hepatocytes account for 80% of liver mass. They are in charge of metabolic and detoxifying processes.[28]

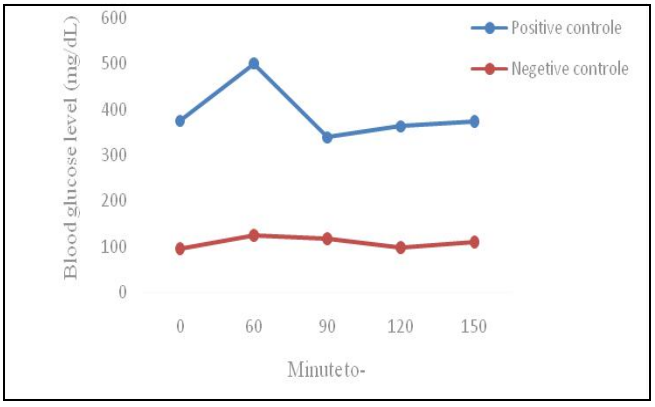

Figure 1: The results of the mice glucose tolerance test.

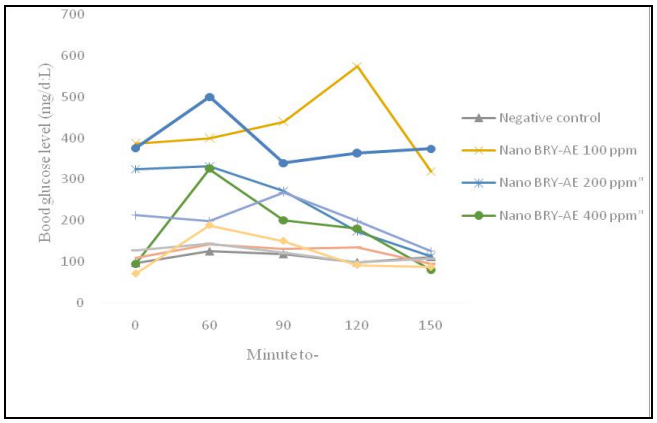

Figure 2: The effects of nanocapsules on glucose tolerance.

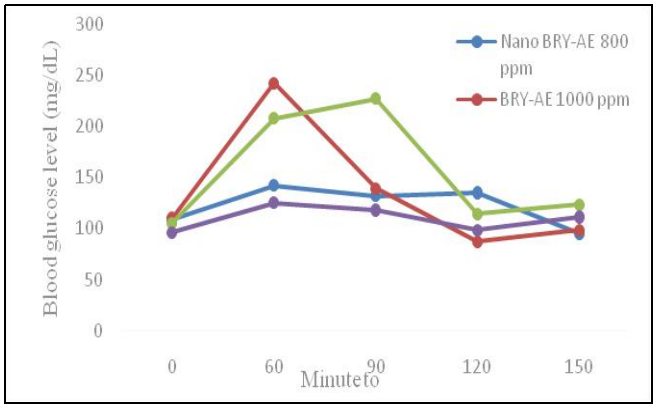

Figure 3: Profile of glucose tolerance in various treatments.

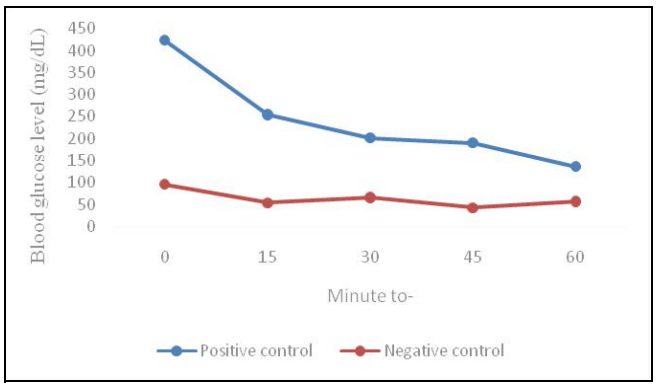

Figure 4: Comparison of insulin resistance for negative and positive controls.

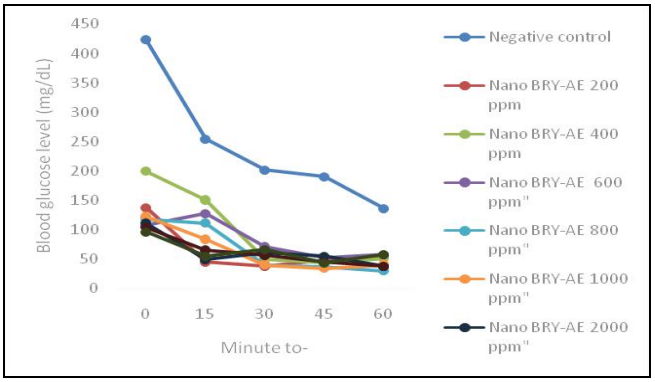

Figure 5: Insulin resistance for nanocapsule treatment.

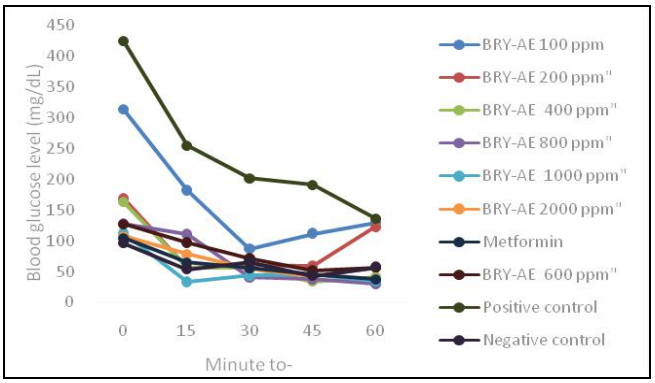

Figure 6: Profile of insulin resistance for BRY-AE treatment.

Liver cells can be destroyed, but they can also regenerate. Hepatocytes, although highly differentiated cells, have a limitless proliferation capacity. In a normal liver, hepatocytes are dormant, have a very long life span of about 5 months, and experience very slow turnover (1-2 times per year). With a long-life span, hepatocytes are capable of at least 68 cell divisions.[29] The liver is the most commonly injured organ as a result of the metabolism of various chemicals or medicines. Consumption of certain drugs or foods can induce liver damage in a matter of days, weeks, or even months. Liver tissue damage might manifest as hepatocyte necrosis, cholestasis, or the gradual emergence of liver disease. The results of the observations made on microscopic images of livers that were given various treatments after 2 weeks revealed normal hepatocytes and hepatocytes that changed the form of parenchymatous degeneration, and hydropic degeneration (apoptosis and necrosis).

Figures 7 to 11 depict the histology of mouse hepatocytes after various treatments. The number of liver cells (healthy hematocytes, apoptotic cells, and empty/no cell nuclei) is shown in Table 2. Apoptosis causes cell shrinkage and pyknosis on a morphological level. Cytoplasmic compaction occurs as a result of DNA fragmentation and chromatin condensation. The condition is accompanied by swelling of the plasma, and eventually, nuclear rupture (karyorrhexis). Following that, cells separate from the surrounding tissue, a condition leading to apoptosis.[30] The total number of liver cells counted in the following order: BRY-AE 2000 ppm > normal > metformin >nano BRY-AE 2000 ppm > positive control (normal/no treatment) as shown in Table 3. As observed in Table 4, the highest number of liver cells that were not damaged were mice exposed to T2DM and treated with BRY-AE 2000 ppm. Table 5 shows the number of cells undergoing apoptosis in the order of DM < BRY-AE 2000 ppm Metformin < nano = positive control (normal/no treatment). The number of empty cells (Table 5) follows the order: nano BRY-AE 2000 ppm < positive control (normal/without treatment) < DM < BRY-AE at 2000 ppm < Metformin. Mice with T2DM that were not treated showed a substantial number of empty liver cells (32%), as did mice treated with metformin, which showed a 50% empty number of cells.

Table 2: Effect of treatments on the number of liver cells in mice.

Treatment	Field of view 1-5				Total liver cells
	Hepatocytes	Apoptosis	Empty	Total per field	
Negative control (no DM + commercial feed),	35.2	16	8	59	531
Metformin	15.8	10	26	52	468
Positive control (DM+commercial feed),	18.8	5	11	34	310
Nano BRY-AE 2000 ppm	29.4	16	1	46	412
BRY-AE 2000 ppm	53.4	9	15	77	697

Table 3: The liver cells that were not damaged.

Treatment	Number of hepatocytes per field	Total hepatocyte	Counted total liver cells that were not damaged	% total that is not damaged
Negative control (no DM + commercial feed),	35.2	317	531	60
Metformin	15.8	142	468	30
Positive control (DM+commercial feed),	18.8	169	310	55
Nano BRY-AE 2000 ppm	29.4	265	412	64
BRY-AE 2000 ppm	53.4	481	697	69

Table 4: Number of cells undergoing apoptosis.

Treatment	Total of apoptosis	Total of liver cells counted	% total of apoptosis liver cell
Negative control (no DM + commercial feed),	142	531	27

Metformin	92	468	20
Positive control (DM+commercial feed),	41	310	13
Nano BRY-AE 2000 ppm	142	412	34
BRY-AE 2000 ppm	83	697	12

Table 5: Number of empty liver cells.

Name of treatment	Total ofempty liver cells	Total of liver cells counted	% total of empty liver cells
Negative control (no DM + commercial feed),	72	531	14
Metformin	234	468	50
Positive control (DM+commercial feed),	99	310	32
Nano BRY-AE 2000 ppm	9	412	2
BRY-AE 2000 ppm	133	697	19

Based on the observations in Tables 3, it can be interpreted that the BRY-AE and nano BRY-AE 2000 ppm treatments outperformed the negative control (normal/no treatment) and the treatment with metformin. This is explained by the fact that BRY-AE and nano BRY-AE 2000 ppm can regenerate liver cells in mice with T2DM. The results revealed that BRY-AE is high in phenolic compounds and flavonoids,[2] which are known to play a role in the regeneration of cells, including hepatocytes. These two compounds are also known to act as antioxidants, following the findings of a previous study.[31]

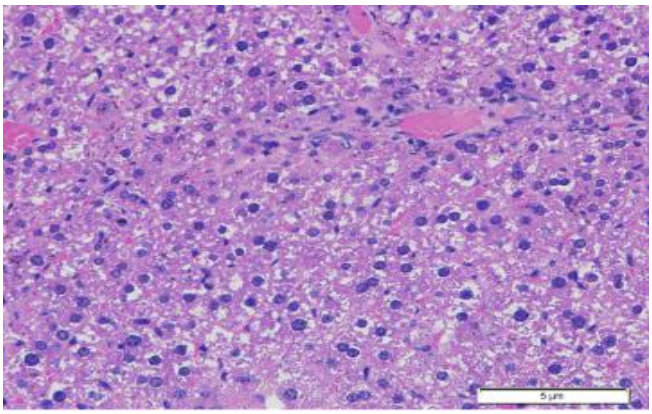

Figure 7: Hepatocyte histology of negative control mice (normal/no treatment).

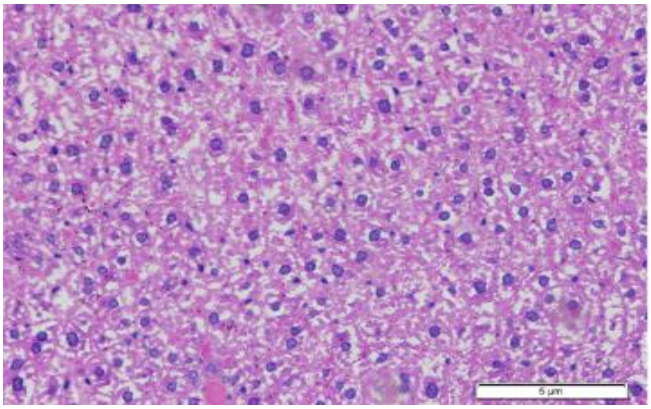

Figure 8: Histology of mice treated with metformin.

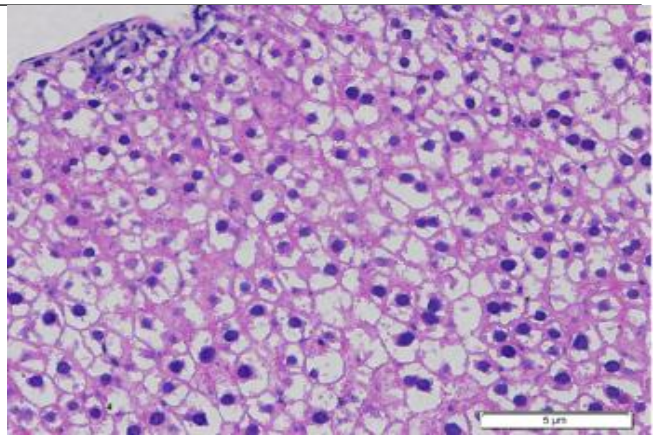

Figure 9: Histology of hepatocytes in type 2 diabetic mice.

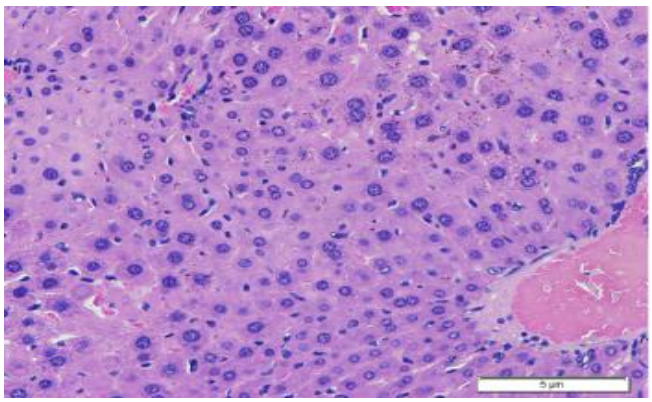

Figure 10: Histology of mice treated with nanocapsules BRY-AE at 2000 ppm.

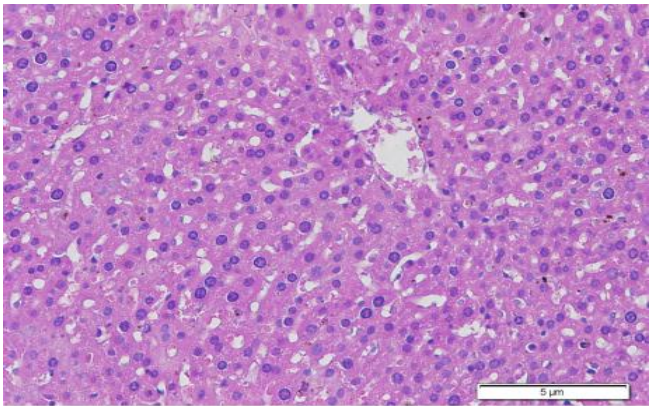

Figure 11: Histology of mice treated with BRY-AE at 2000 ppm.

Conclusion

The findings of this study reveal that after the second week, BGL dropped in all groups. The glucose tolerance test for the BRY-AE and BRY-AE nanocapsule treatments showed a good profile, which was close to a negative control profile. Histological examination of liver tissue revealed that AE-BRY and nanocapsules AE-BRY at 2000 ppm outperformed the negative control (no treatment) and metformin. As a result, the nanocapsules BRY-AE can be used to lower blood glucose levels in mice with T2DM.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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