PATENT FORM NO. 4

CRP: 009283

FEDERAL REPUBLIC OF NIGERIA

Certificate of Registration of Patent

(Patents and Designs Act; CAP 344 Laws of the Federation of Nigeria 1990)

RP: **F/PT/NC/2024/12354**
Date of Patent: **14/05/2024**
Date of Sealing: **14/06/2024**

President of the Federal Republic of Nigeria and Commander-in-chief of the Armed Forces
BOLA AHMED TINUBU, *GCFR.*

Whereas a request for the grant of a patent has been made by: **ZHEJIANG UNIVERSITY of NO. 866 YUHANGTANG ROAD, XIHU DISTRICT, HANGZHOU CITY, ZHEJIANG PROVINCE, 310058, CHINA C/O CROWN AND SHIELDS LEGAL OF SUIT 14, FLOOR 1, OCEAN CENTER, PLOT 1018 CADASTRAL ZONE B18, OFF OLADIPO DIYA ROAD, APO GUDU, ABUJA.**

For the sole use and advantage of an invention for: **HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT**

AND WHEREAS the Federal Government being willing to encourage all invention which may be for public good, is pleased to accede to the request:

KNOW YE THEREFORE, that I do by this Instrument give and grant unto the person(s) above named and any successor(s), executor(s), administrator(s) and assign(s) (each and any of whom are hereinafter referred to as the patentee) by special licence, full power, sole privilege and authority, that the patentee or any agent or licensee of the patentee may subject to the conditions and provisions prescribed by any statute or order for the time being in force at all times hereafter during the term of years herein mentioned, make, use, exercise and vend the said invention throughout the Federal Republic of Nigeria, and that the patentee shall have and enjoy the whole profit and advantage from time to time accruing by reason of the said invention during the term of twenty years from the date first above written on this Instrument: AND to the end that the patentee may have and enjoy the sole use and exercise of the full benefit of the said invention, I do by this Instrument strictly command all citizens of the Federal Republic of Nigeria that they do not at any time during the continuance of the said term either directly or indirectly make use of or put in practice the said invention, nor in anywise imitate the same, without the written consent, licence or agreement of the patentee, on pain of incurring such penalties as may be justly inflicted on such offenders, and of being answerable to the patentee according to law for damages thereby occasioned:

PROVIDED ALWAYS that this patent shall be revocable on any of the grounds from time to time by law prescribed as grounds for revoking patents granted by me, and the same may be revoked and made void accordingly:

PROVIDED ALSO that nothing herein contained shall prevent the granting of licenses in such manner and for such considerations as they may by law be granted

MADE this: **14TH DAY OF JUNE, 2024**

JANE IGWE
Registrar

FEDERAL REPUBLIC OF NIGERIA
FEDERAL MINISTRY OF INDUSTRY, TRADE AND INVESTMENT
COMMERCIAL LAW DEPARTMENT
PATENTS AND DESIGNS ACT CAP 344, LFN 1990

PATENT ACKNOWLEDGMENT FORM

RRR	**321046293913**	OAI	**IPONMW638512945053984548**
FILING DATE	**May 14, 2024**	FILE NO	**F/PT/NC/2024/12354**
TRANSACTION AMOUNT	**27900.00**	FILING TIME	

PATENT INFORMATION

APPLICATION TYPE	**Patent**	PATENT TYPE	**NON-CONVENTIONAL**

TITLE OF PATENT

HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT

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S/N	**COUNTRY**	**APPLICATION NO**	**DATE**
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STATE	**FCT-ABUJA**	EMAIL	**info@crownandshieldslegal.com**
--- DOCUMENTS ATTACHED DURING APPLICATION FILING---			
Claims	ATTACHED		
Deed Of Assignment	ATTACHED		
Letter Of Authorization	ATTACHED		
Complete Specification Form	ATTACHED		

YOUR APPLICATION HAS BEEN RECEIVED AND IS RECEIVING DUE ATTENTION
TRADEMARKS, PATENTS AND DESIGNS REGISTRY
COMMERCIAL LAW DEPARTMENT
FEDERAL MINISTRY OF INDUSTRY, TRADE AND INVESTMENT

FEDERAL REPUBLIC OF NIGERIA
FEDERAL MINISTRY OF INDUSTRY, TRADE AND INVESTMENT
COMMERCIAL LAW DEPARTMENT
PATENTS AND DESIGNS ACT CAP 344, LFN 1990

PATENT ACCEPTANCE LETTER

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NATIONALITY	**China**		
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YOUR APPLICATION HAS BEEN ACCEPTED
TO VERIFY THIS DOCUMENT, SCAN THE BARCODE BELOW

Patents From No. 1(b)
N4 for each application for
protection in a convention country

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PATENTS AND DESIGNS DECREE 1970
(1970 No. 60)

APPLICANTS OR AGENTS REF:
PT_CP_NG00002151
HKJP20240402668

CONVENTION APPLICATION FOR A PATENT
(To be accompanied by two copies of Patents Form No. 3)

1. We, (a) **Zhejiang University** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China
   hereby declare that applications for protection for an invention or for inventions have been made in the following country or countries and on the following official dates, namely -

   in
   on
   by

   and that each of the said applications was the first application in a Convention country in respect of the relevant invention by us or by any person from whom we derive title.

2. I am (e) the assignee Zhejiang University of the said patent entitled (d) HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT by virtue of (f) assignment.
   ~~or (e) the personal representative of the said (d) ..... .~~

3. We declare that to the best of our knowledge and belief there is no lawful ground of objection to the grant of a patent to us on this application, and pursuant to subsection 2 of section 27 of the Act, we pray that a patent may be granted to us with priority founded on the above-mentioned application(s) in a Convention country or countries as provided by subsection (4) of section 3 for the invention described in the accompanying complete specification under the title HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT.

4. ~~And I/we request that the patent may be granted as a patent of addition to (e). Patent No .... the patent to be granted on application No . .....~~

5. And we request that all notices, requisitions, and communications relating to this application may be sent to CROWN AND SHIELDS LEGAL at Suit 14, Floor 1, Ocean Center, Plot 1018 Cadastral Zone B18, Off Oladipo Diya Road, Apo Gudu, Abuja who are hereby appointed to act for us.

KIMI ISAAC ONANA
FOR: CROWN & SHIELDS LEGAL

To The Registrar of Patents and Designs,
Patents Branch.
Federal Ministry of Trade,
Federal Capital Territory, Abuja, Nigeria.

FORM 2

PATENT AND DESIGNS ACT
LFN 2004 CAP 344
AUTHORISATION OF AGENT

We, **Zhejiang University** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China

have appointed

Crown and Shields Legal of Suit 14, Floor 1, Ocean Center, Plot 1018 Cadastral Zone B18, Off Oladipo Diya Road, Apo Gudu, Abuja, Nigeria

to act as our agent for the registration of patents and designs in our name.

And request that all notices, requisitions and communications relating thereto be sent to such agent at the above address.

We hereby revoke all previous authorizations, if any, in respect of the same matter.

Dated this 9th of May 2024

WANG, Yue

For: Zhejiang University
Signatory: WANG, Yue
Capacity: Deputy Director

To: Registrar of Patents Patents Office, Patents Branch Federal Ministry of Commerce Abuja, Nigeria

NIGERIA

DEED OF ASSIGNMENT

Agent reference: PT_CP_NG00002151
Client reference: HKJP20240402668

BY AND BETWEEN:

(1) **SUN, Chongde** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China
(2) **WANG, Yue** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China
(3) **CAO, Jinping** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China
(hereinafter referred to as the "Assignors")

AND

Zhejiang University of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China
(hereinafter referred to as the "Assignee")

WHEREAS the Assignors are the inventors of the invention disclosed in the patent application:

HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT

AND the Assignee has for good and sufficient consideration acquired the said invention from the Assignors.

NOW THEREFORE the Assignors hereby confirm having assigned the said invention to the Assignee as far as **NIGERIA** is concerned with the right to apply for a Nigerian patent in their name and the Assignee hereby accepts the Assignment.

Dated this 9th of May 2024

Assignors

SUN. Chongde
SUN, Chongde

WANG, Yue
WANG, Yue

CAO, Jinping
CAO, Jinping

Assignee

WANG, Yue
Zhejiang University

Patents From No. 3
Fee:

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APPLICANTS OR AGENTS REF: PT_CP_NG00002151 HKJP20240402668	**PATENTS DECREE NO 60 OF 1970 COMPLETE SPECIFICATION (To be furnished in duplicate - one without fee)**	**Where Foreign Priority is desired in respect of one or more specification. Quote Nos and date or dates PRIORITY DATA:**

(a) Insert titles of Invention.	(a) HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT
(b) State (in full) name, address and nationlity of applicant or applicants as stated in the application form.	(b) We, **Zhejiang University** of No. 866 Yuhangtang Road, Xihu District, Hangzhou City, Zhejiang Province, 310058, China do hereby declare the invention, for which I pray that a patent may be granted to me, and the method by which it is to be performed, to be particularly described in and by the following statement:
(c) (a) Here begin full description of invention. The continuation of the specification should be upon paper of the same size as this form, on one side only with the lines well spaced and a margin of one inch and a half on the of the paper. The completion of the description should be followed by the words “what I (or we) claim is” After which should be written the claim/claims numbered consecutively (see note below). The specification and duplicate thereof must be signed at the end.	(c) SEE ATTACHED

NOTE.- The claims must relate to a single invention, must be clear and succinct and must be fairly based on the matter disclosed in the specification. They should define the scope of the invention claimed. Applicants should be careful that their claims include neither more nor less than they desire to protect by their patent. Any unnecessary multiplicity or claims or prolixity of language should be avoided. Claims should not be made for the efficiency or advantages of the invention.

HYPOGLYCEMIC USAGE OF MANDARIN ORANGE FRUIT EXTRACT

TECHNICAL FIELD

[01] The present invention belongs to the field of medicine, and relates to application of natural mandarin orange fruit extract in preparing hypoglycemic functional food, a health product or preventive drug, in particular to application of mandarin orange fruit extract in reducing blood glucose in diabetic KK-A^y mice.

BACKGROUND ART

[02] Diabetes is one of the most common and serious metabolic diseases in the world, affecting nearly 500 million people. According to different pathogenesis, diabetes can be divided into three main forms, namely type 1, type 2 and gestational diabetes mellitus. Type 1 diabetes is mainly an autoimmune disease, and the function of islet cells declines or even loses in the early stage. Most diabetes patients in the world are type 2 diabetes, which is closely related to lifestyle. The main pathogenesis is insulin resistance, with or without different degrees of islet dysfunction, and often accompanied by other metabolic disorders. Type 2 diabetes previously only appeared in adults, but now it is more and more common in children and adolescents. Diabetes seriously affects the social economy, and the annual cost of dealing with diabetes worldwide exceeds 827 billion dollars. By 2030, it is estimated that the global economy loses as much as 1.7 trillion dollars due to diabetes, with developed countries and developing countries each accounting for about half. Therefore, it is urgent to prevent and control diabetes and its complications.

[03] *Citrus reticulata cv. Suavissima* is a cultivated variety of the genus Citrus in Rutaceae, and it is also a traditional specialty in Wenzhou and Lishui of Zhejiang Province. It has been cultivated for more than two thousand years. Mandarin orange has special food and medicine functions in folk, such as removing heat, promoting the secretion of saliva or body fluid, preventing phlegm from forming and stopping coughing, resolving toxin with clearing coolness. However, there is no report about whether the mandarin orange fruit extract has hypoglycemic activity in or in vivo.

Polymethoxylated flavonoids (PMFs) refer to flavonoids with four or more methoxy substituents, which mainly come from citrus plants in Rutaceae, especially in the peel of orange fruits. Polymethoxylated flavonoids in orange fruits have good activities in anti-tumor, anti-inflammatory, antimutagenesis and cardiovascular protection, and the research on the biological activity mechanism of polymethoxylated flavonoids has gradually deepened.

SUMMARY

[04] An objective of the present invention is to provide application of mandarin orange fruit extract in preparing a hypoglycemic agent. The mandarin orange fruit extract of the present invention includes solid-phase extracted (SPE) powder of mandarin orange peel (oil gland layer and albedo).

[05] Another objective of the present invention is to provide application of mandarin orange fruit extract in preparing hypoglycemic functional food and a health product. The mandarin orange fruit extract of the present invention includes the solid-phase extracted (SPE) powder of mandarin orange peel (oil gland layer and albedo).

[06] The mandarin orange fruit extract of the present invention is prepared by the following method:

[07] (1) sample extraction: extracting a certain mass of freeze-dried mandarin orange peel powder twice with ethanol having a concentration of 95% (a solid-liquid ratio of 1:20) by ultrasound at extraction temperature of 25℃ for 30 min, after vacuum filtration, combining supernatant, evaporating the combined supernatant to an ethanol-free phase on a rotary evaporator, and dissolving the ethanol-free phase in deionized water; and

[08] (2) performing sugar removal and segmentation on Sep-pak® C18 solid-phase extraction column: loading an aqueous solution obtained in step (1) on the Sep-pak® C18 solid-phase extraction column, and after loading, eluting 25 times of column bed volumes (BV) of the solid-phase extraction column with deionized water to remove highly polar impurities, and then performing elution with 12 BV of methanol having a concentration of 35% to remove impurities, then performing elution with 3 BV of methanol having a concentration of 100% at an elution flow rate of 1 mL/min,

collecting eluent, and evaporating the eluent at 36°C on a rotary evaporator to dryness to obtain mandarin orange fruit extract powder rich in polymethoxylated flavone components.

[09] According to the present invention, it is found that in KK-A^y mice with spontaneous diabetes (in vivo), the mandarin orange fruit extract may significantly reduce fasting blood glucose of the diabetic KK-A^y mice, increase glucose tolerance and reduce liver injury.

[10] The present invention emphasizes latest discovery of mandarin orange fruit extract which is the natural product in prevention and treatment of diabetes.

[11] The present invention provides application of mandarin orange fruit extract in preparing hypoglycemic functional food, a health product or hypoglycemic agent. In the diabetic KK-A^y mice (in vivo), a compound rich in polymethoxylated flavones separated and purified from mandarin orange fruits may significantly reduce the fasting blood glucose of the diabetic KK-A^y mice, increase the glucose tolerance and reduce the liver injury of the mice. The extract may be used as the functional food, the health product or preventive medicine for preventing and treating diseases related to abnormal glucose metabolism.

BRIEF DESCRIPTION OF THE DRAWINGS

[12] FIG. 1 shows an effect of mandarin orange fruit extract powder rich in polymethoxylated flavone components on fasting blood glucose in diabetic KK-A^y mice as a fed object. Administration is performed for 7 weeks, and fasting blood glucose is measured once a week. C57BL/6 and water is used as a background group; KK-A^y and water is used as a control group; KK-A^y and extract is used as a treatment group, and dose is 15 mg/kg bw d, n = 8-10. A significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with *$p<0.05$, and **$p<0.01$, compared with the control group.

[13] FIG. 2 shows an effect of the mandarin orange fruit extract powder rich in polymethoxylated flavone components on an oral glucose tolerance test (OGTT) in the diabetic KK-A^y mice as the fed object. Administration is performed for 7 weeks, and

oral glucose tolerance of the mice is measured at the seventh week. C57BL/6 and water is used as a background group; KK-A^y and water is used as a control group; KK-A^y and extract is used as a treatment group, and dose is 15 mg/kg bw d, n = 8-10. A significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with *$p<0.05$, and **$p<0.01$, compared with the control group.

[14] FIG. 3 shows an effect of the mandarin orange fruit extract powder rich in polymethoxylated flavone components on serum biochemical indexes AST/ALT in the diabetic KK-A^y mice as the fed object. Administration is performed for 7 weeks, after a test, eyeballs of the mice are removed, blood is taken, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are measured, and an AST/ALT value is calculated. C57BL/6 and water is used as a background group; KK-A^y and water is used as a control group; KK-A^y and extract is used as a treatment group, and dose is 15 mg/kg bw d, n = 8-10. A significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with *$p<0.05$, and **$p<0.01$, compared with the control group.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[15] The present invention will be further described with reference to the accompanying drawings and examples.

[16] Example 1: Preparation of mandarin orange fruit extract, etc.

[17] 10 g freeze-dried mandarin orange fruit peel powder was extracted third times (25°C) with ethanol having a concentration of 95% (solid-liquid ratio of 1:20) by ultrasound for 30 min each time, after vacuum filtration, supernatant was combined, the combined supernatant was evaporated to an ethanol-free phase on a rotary evaporator, and the ethanol-free phase was dissolved in deionized water to obtain a crude extract aqueous solution.

[18] 5 mL of crude extract aqueous solution was loaded on a Sep-pak® C18 solid-phase extraction column, and after loading, 25 times of column bed volumes (BV) of the solid-phase extraction column was eluted with deionized water to remove highly polar impurities, and then elution was performed with 12 BV of methanol having a

concentration of 35% to remove impurities, then elution was performed with 3 BV of methanol having a concentration of 100% at an elution flow rate of 1 mL/min, about 12 mL of eluent was collected, and the eluent was evaporated at 36℃ on a rotary evaporator to dryness to obtain preliminarily impurity-removed powder rich in polymethoxylated flavone components. After purification in the step, 201.2 mg of preliminarily purified and impurity-removed powder was obtained, which is mandarin orange fruit extract powder rich in polymethoxylated flavone components.

[19] **Example 2**

[20] Diabetic KK-A^y mouse test with mandarin orange fruit extract

[21] Diabetic KK-A^y mice and feed thereof were purchased from Beijing Huafukang Biotechnology Co., Ltd., and C57BL/6 mice and feed thereof were purchased from Shanghai Slac Animal Experimental Center. 6-week-old male diabetic KK-A^y mice were put into an animal room and fed with special feed for 2 weeks to make the mice adapt to an experimental environment. Fasting blood glucose was measured once a week, 20 mice with a fasting blood glucose level greater than or equal to 8 mmol/L for two weeks were randomly divided into two groups, and the mice in a control group were fed with the special feed and gavaged with water. The mice in a mandarin orange fruit extract group were fed with the special feed and gavaged with and gavaged with, with dose of 15 mg/kg bw d. 10 C57BL/6 mice were used as normal mouse control.

[22] The test lasted for 7 weeks, and successive administration was performed for 6 days in a week and stopped for 1 day. Fasting is performed for 4 hours on the fifth day of each week, caudal veins of the mice were cut to take blood, a fasting blood glucose level was measured by Johnson One Touch Ultra ZSJ 843ETT blood glucose meter, and a curve of fasting blood glucose levels of the mice for 7 weeks was drawn. Data are expressed by an average value ± a standard error, a significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with $*p<0.05$, and $**p<0.01$, compared with the control group. FIG. 1 shows specific fasting blood glucose measurement values of the diabetic KK-A^y mice for 7 weeks.

[23] An OGTT was measured in the seventh week of the test. Before the OGTT was measured, the mice were fasted for 12 h and given with medicine, and the OGTT was

measured 1 h later. The blood was taken from the caudal veins as a blood glucose level of 0 min, then glucose having a concentration of 20% (2 g/kg BW, volume of medicine of 10 mL/kg BW) was given to stomachs, blood was taken from the caudal veins at 30, 60 and 120 min separately, the blood glucose level was measured by Johnson One Touch Ultra ZSJ 843ETT blood glucose meter, and an oral glucose tolerance curve was drawn. Data are expressed by an average value ± a standard error, a significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with *$p<0.05$, and **$p<0.01$, compared with the control group. FIG. 2 shows the oral glucose tolerance curve of the diabetic KK-A^y mice.

[24] 7 weeks after the test, the mice were killed, and serum was collected. Indexes of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by Roche Cobas 8000 biochemical analyzer, AST/ALT values were calculated, and an AST/ALT histogram was drawn. Data are expressed by an average value ± a standard error, a significant difference is analyzed by SPSS software, and LSD multiple comparisons are made, with *$p<0.05$, and **$p<0.01$, compared with the control group. FIG. 3 shows specific measurement results of the AST/ALT indexes.

WHAT IS CLAIMED IS:

1. Application of mandarin orange fruit extract in preparing a hypoglycemic agent, wherein the mandarin orange fruit extract is powder rich in polymethoxylated flavone components separated and purified from peel.

2. Application of mandarin orange fruit extract in preparing hypoglycemic functional food and a health product, wherein the mandarin orange fruit extract is powder rich in polymethoxylated flavone components separated and purified from the peel.

3. The application of mandarin orange fruit extract according to claim 1 or 2, wherein the mandarin orange fruit extract is prepared by the following method:

(1) sample extraction: extracting freeze-dried mandarin orange peel powder twice with ethanol having a concentration of 95% by ultrasound at extraction temperature of 25℃ for 30 min, with a solid-liquid ratio of 1:20, after vacuum filtration, combining supernatant, evaporating the combined supernatant to an ethanol-free phase on a rotary evaporator, and dissolving the ethanol-free phase in deionized water;

(2) performing sugar removal and segment purification on Sep-pak® C18 solid-phase extraction column: loading an aqueous solution obtained in step (1) on the Sep-pak C18 solid-phase extraction column, and after loading, eluting 25 column bed volumes (BV) of the solid-phase extraction column with deionized water to remove highly polar impurities, and then performing gradient elution with a methanol solution having a concentration of 35% and a methanol solution having a concentration of 100% separately, with an elution sequence of 12 column bed volumes with methanol having a concentration of 35% and 3 column bed volumes with methanol having a concentration of 100%; and collecting 100% eluted components, and evaporating the eluted components to dryness at 36℃ on the rotary evaporator, and the obtained powder is powder rich in polymethoxylated flavone components separated and purified from mandarin orange peel.

ABSTRACT OF THE DISCLOSURE

The present invention provides application of mandarin orange fruit extract in preparing hypoglycemic functional food, a health product or hypoglycemic agent. In diabetic KK-A^y mice (in vivo), the extract rich in polymethoxylated flavone components separated and purified from mandarin orange fruits can significantly reduce fasting blood glucose, increase glucose tolerance and reduce liver injury of the mice. The extract can be used as the functional food, the health product or preventive medicine for preventing and treating diseases related to abnormal glucose metabolism.

Drawings

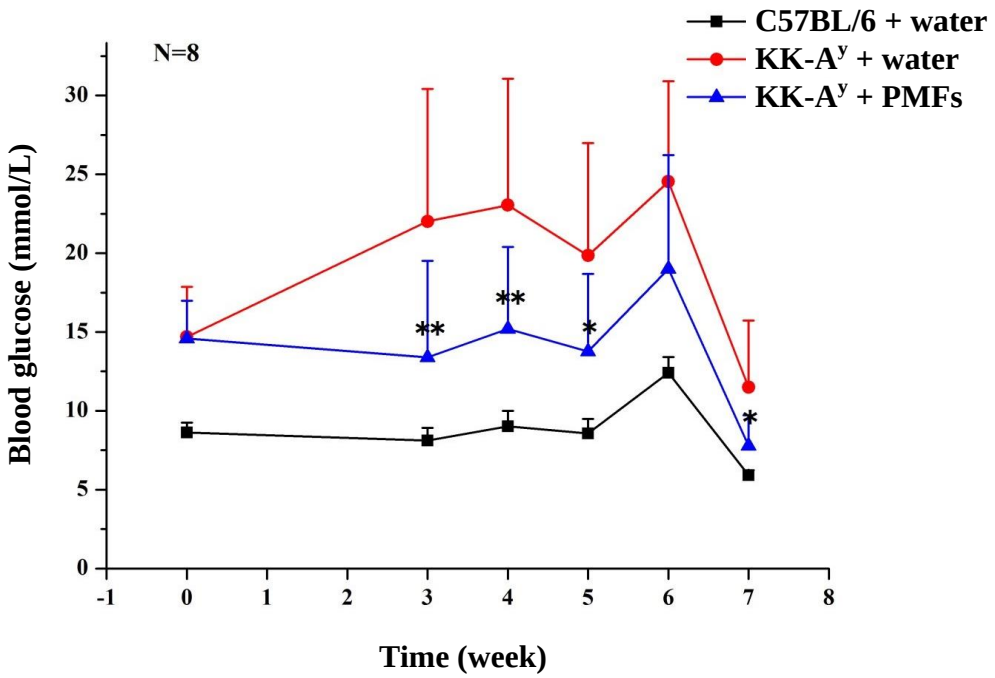

FIG. 1

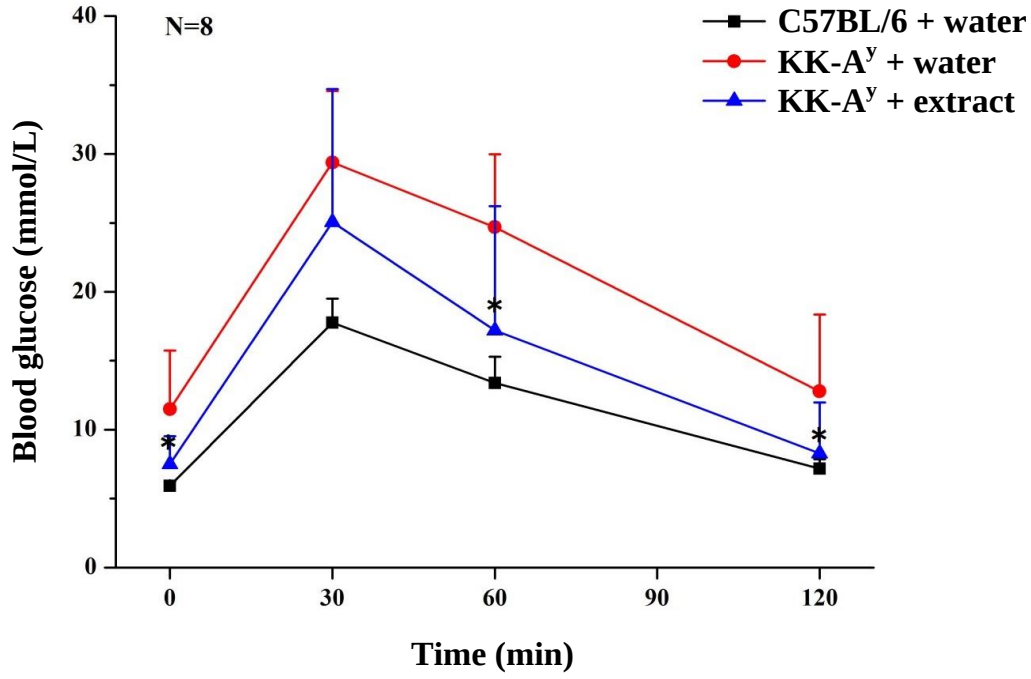

FIG. 2

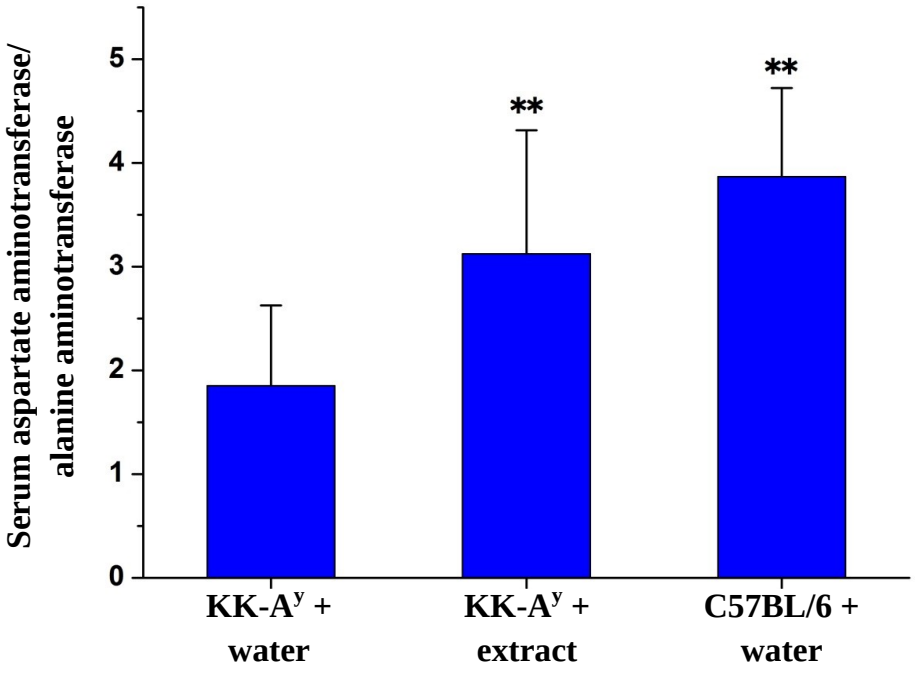
Serum aspartate aminotransferase/
alanine aminotransferase
0
1
2
3
4
5
**
**
KK-A^y +
water
KK-A^y +
extract
C57BL/6 +
water

FIG. 3