NANOEMULSIFYING OF ETHANOLIC PAITAN LEAF EXTRACT (*Tithonia Diversifolia* (Hemsley) A. Gray) TO ENHANCED ANTIOXIDANT AND ANTIDIABETIC PROPERTIES

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ABSTRACT

Paitan (*Tithonia diversifolia* (Hemsley) A. Gray) extract is formulated to nanoemulsion to reduce the dose *In-vivo* diabetes mellitus treatment by increasing distribution and absorption. It is necessary due to concentrated extract is reported to be inefficient and harm the kidneys. Nanoemulsion was carried out by the ionic gelation method of chitosan crosslinking: Na-TPP: extract (5:1:1). It was successfully formed with a size of 346.0 nm, a polydispersity index of 0.406, and the zeta potential of +14.1 mV confirmed by particle size analyzer with dynamic light scattering method. Antioxidant evaluation with DPPH shows a value of IC₅₀ 16.801 ppm and is classified as an excellent antioxidant activity. The antidiabetic activity was evaluated *In-vivo* in Wistar strain rats and the results were analyzed using a One-Way ANOVA test of 95% confidence level. This nanoemulsion can reduce blood glucose levels up to 24% for 13 days at 150 mg/kg BW. This is more effective than glibenclamide (18%) ($p > 0.05$) as a positive control. Based on phytochemical screening, the extract contains alkaloids, tannins, terpenoids, and saponins.

Keywords: *Tithonia diversifolia*, Nanoformulation, *In-vivo*, Diabetic Treatment.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease caused by abnormally insulin condition (no insulin produce, or the body not effectively use insulin¹). DM is a silent killer because people are unaware, and only known when complications occur. Unhealthy lifestyles and consumption of high sugar foods and beverages cause DM sufferers to get higher every year. DM is noticed by a high blood glucose level (≥ 200 mg/dL or fasting blood glucose levels (FGBL) ≥ 126 mg/dL). DM Type 1 DM occurs due to damage to pancreatic cells caused by inflammation that causes β -cells unable to secrete insulin or only secrete in small amounts. However, in type 2, β -cells remain healthy. Therefore, the amount of insulin is normal. On the other hand, the insulin receptors are measly found on the cell surface. Consequently, the glucose intake of the cell is less than normal. This causes an increase in blood glucose levels.¹

Various types of modern and traditional medicines have been consumed and developed in the community. Unfortunately, the use of synthetic drugs has side effects such as digestive disorders, liver disorders, dizziness, nausea, and vomiting.² Based on these, a safer alternative medicine is needed. Bioactive components from natural ingredients have relatively fewer side effects than synthetic medicine.³ Paitan (*Tithonia diversifolia* (Hemsley) A. Gray) contains chemicals that are valuable in various fields of health, including as an antidiabetic.⁴ Phytochemical content in paitan plants is reported to have the ability to reduce blood glucose.⁵⁻⁷ The infusions and the extract of this plant are usually used as antidiabetic. However, the application was very concentrated (up to 2000 mg/kg) and resulted in a relatively long decrease (28 days) in glucose levels.^{7,8} Concentrated extracts in herbs are not effective for consumption and can harm the kidneys.^{4,9} It is also heavier to remove the remaining unabsorbed active ingredients. This extract is also hardly dissolved in water, inhibiting the distribution of drugs in the blood to the target. Nanoemulsion is one way to increase effectiveness and reduce dosage by increasing the distribution and absorption¹⁰ of the drug. Nanoemulsion as a carrier for drug delivery to optimize dispersion and permeation, prolong drug release and distribution in the circulatory system towards treatment targets.¹¹

Nanoemulsion has been applied to several extracts (Curcumin¹², Cinnamon, Ginger leaf, *Alpinia galanga* L), essential oils (Lemongrass, clove, tea tree, thyme, geranium, marjoram, palmarosa, rosewood, sage, mint¹³ as well as drugs and vitamins^{10,14} and is proven to increase bioavailability.^{15,16} So far, there have been no studies that explain the effects of *Tithonia diversifolia* (Hemsley) A. Gray Extract-Nanoemulsion (TDE-NE) as antidiabetic therapy. On this basis, it is expected that the preparation of paitan leaf nanoemulsion becomes an innovation that can increase the activity of DM pharmacological therapy. Therefore, in this study TDE-NE was prepared and evaluated for its antioxidant level, and its effect on reducing blood sugar was tested in mice with diabetes.

EXPERIMENTAL

Material and Instrumentations

Materials use in this experiments are ethanol, methanol, alloxan monohydrate, chitosan, glibenclamide, Na-CMC, anhydrate acetic acid, HCl, Dragendroff Reagent, Mg powder, Liebermann-Burchard Reagent, Folin Ciocalteous Reagent, ascorbic acid, tannic acid (all from Merck), DPPH (*Sigma Aldrich*), STPP 94% food-grade, male Wistar rats' strain, Paitan leaves (*Tithonia diversifolia* (Hemsley) A. Gray). The equipment used in this research is a particle size analyzer (Horiba-scientific: sz-100), UV-Vis spectrometer (Hitachi UH-5300), Glucometer (EasyTouch®GCU).

General Procedure

Extraction and Phytochemical Screening

Paitan leaves were identified by taxonomists as *Tithonia diversifolia* (Hemsley) A. Gray from the Compositae family. Paitan leaves are washed, air-dried, and coarse to powder. Subsequently, 30 g of powder was macerated with 96% ethanol in 24 hours, filtered, evaporated under reduced pressure in a rotary evaporator, weighed and the yield obtained is calculated. Phytochemical screening evaluation includes the flavonoid test with a Wiltater reagent, alkaloid test with Dragendorft reagent, terpenoid test with Liebermann-Burchard reagent, tannin test using the FeCl₃ method, and saponin test by hydrolysis method with water.^{3,17-22} These screening results are provided in Table-1.

Antioxidant evaluation

Antioxidant evaluation is done by the DPPH method⁷. Ascorbic acid was used as a comparison with the same treatment as the test sample. Antioxidant activity can be seen from the IC₅₀ value (Table-2.) of the concentration of the sample solution which gives 50% inhibition of DPPH radicals.

Preparation and Characterization of TDE-NE

Chitosan 0.1% (w/v) is prepared in acetic acid 0.1% (v/v), while STPP 0.1% (v/v) and TDE (150 mg/kg BW) are dissolved in distilled water. Nanoparticles were made with the ratio 5:1:1 of chitosan: STPP: TDE. The prepared TDE is dropped into the STPP solution while stirring for 15 minutes. The homogeneous solution was then poured into chitosan solution while stirring for 1 hour followed by ultrasonication (Bio-Logics, 12 minutes, 30Hz, and 35-watt power). The droplet size, polydispersity index, and zeta potential of nanoemulsion are characterized by PSA with the dynamic light scattering method.

In-vivo Anti-diabetic Study of TDE-NE

Ethical Clearance

This study was approved by the Medical and Health Research Ethics Committee of the Medical Faculty of the Indonesian Islamic University Number 23/Ka.Kom.Wt/70/KE/II/2020.

Adaptation and Alloxan Induction in Rats

An amount of 15 male Wistar strain rats aged 2-3 months, 110-200 g, has been declared healthy and has never received any treatment, were adapted to the research environment for 7 days with the same conditions and treatments. On day 0, all rats were weighed and fasted for 8-12 hours before their blood sugar was measured and induced alloxan (325 mg/kg BW) intraperitoneally except in control mice.

Inclusion Criteria

Group I: male Wistar strain rats with FBGL <126 mg/dL. Groups II, III, and IV: Wistar strain male rats with FBGL >126 mg/dL.

Exclusion Criteria

Mice get sick/die during treatment.

FBGL Measurement

FBGL is measured 6 times, i.e. 1 day before and 3 days after alloxan induction, and 4 times during 13 days after with a time interval of 3 days. Measurements were made using a glucometer by taking blood through the tail without anesthesia. Based on the inclusion criteria, the rats were divided into 4 groups, i.e. group I (basal control) Na-CMC 0.2%, group II (negative control) Na-CMC 0.2%, group III (positive control) glibenclamide 0.45 mg/kg BW, group IV TDE-NE treatment (150 mg/kg BW). This treatment is given for 15 days orally.

Bodyweight Measurement

The rat's body weight was measured to get a comparison value before and after treatment. It is also re-measured together with blood glucose measurements (days 0, 3, 6, 9, 12, 15).

RESULTS AND DISCUSSION**Plant Extraction and Phytochemical screening**

Phytochemical screening showed that this TDE contains alkaloids, terpenoids, tannins, saponins, and does not contain flavonoids (Table-1). Alkaloids and tannins are the secondary metabolites that are reported to be the most abundant in the leaves and roots of plants in this family^{20,23-24}. In addition to self-defense from pathogens and herbivores, this compound is widely used as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities.²⁴

Table-1: Phytochemical Constituents

No	Constituents	Method	Visualization	Results
1	Flavonoids	Wilstater	No color changed (still green solution)	-
2	Alkaloids	Dragendorff	The green solution changed to orange precipitated	+
3	Terpenoids	Liebermann-Burchard	The green solution changed to purplish black	+
4	Tannins	FeCl ₃	The green solution changed to dark green	+
5	Saponins	Water hydrolysis	1 cm stable persistent froth solution	+

Antioxidant Evaluation

Based on the phytochemical screening, paitan leaves contain tannin which acts as antioxidants to deactivated free radicals. In β -pancreatic cells, alloxan undergoes a redox reaction process to become dialuric acid which can be re-oxidized to alloxan. The redox reaction that occurs will result in the formation of Reactive Oxygen Species (ROS) and superoxide radicals. ROS free radicals will undergo dismutation into hydrogen peroxide and increase the concentration of cytosolic calcium, causing rapid destruction of β -pancreatic cells²⁵. ROS will cause DNA fragmentation and resulting in β -pancreatic cells damaged. This damage will reduce insulin production and cause hyperglycemia²⁶. Antioxidants in DM sufferers can inhibit free radicals through several mechanisms including working as an enzyme that destroys free radicals, the ability to bind with metals which stimulate free radical production and thus inhibit the formation of free radicals, and act as free radical scavengers²⁷. Antioxidant evaluation of this extract (Table-2) shows a value of IC₅₀ 16.801 ppm. This result is classified as an excellent antioxidant activity (<50 ppm)^{28,23}. This result is stronger than TDE from a previous report (41 ppm²⁹ and 21 ppm³⁰). The difference in the place of growth, geographical location, age, and part of the plant used will produce different activity values.

TDE-NE Characterization

Chitosan is a type of polysaccharide with varying molecular weights. This research used chitosan with 40-50 kDa. Chitosan molecular weight is very influential on the size of nanoparticles. The greater the

molecular weight of chitosan used, the larger the particle size produced³¹. The particle size and distribution of TDE-NE are presented in Table-3.

Table-2: Antioxidant Measurement with UV-Vis

Concentration (ppm)	Abs Sample	% Inhibition	IC ₅₀ (ppm)
10	0.149	9.690	16.801
20	0.124	24.840	
30	0.109	37.350	
50	0.013	91.390	

Table-3: Particle Size and Zeta Potential

Chitosan (%)	TPP (%)	TDE (mg/kg BB)	Volume Ratio Chi:TPP:TDE	Distribution	Z-Ave (nm)	PI	ZP (mV)
0.1	0.1	150	5 : 1 : 1	Monodisperse	346.0	0.406	14.1

Note Z-Ave (Average size); PI (Polydispersity Index); ZP (Zeta Potential).

The polydispersity index indicates the uniformity of the size of the preparation particles. The lower the polydispersity index value, the higher the uniformity of the preparation of particle sizes.³² If the polydispersity index value is between 0.08-0.7 this indicates that the particles are relatively uniform, whereas values greater than 0.5 indicate high heterogeneity. This research produces particles with a polydispersity index that is 0.406, this shows that TDE-NE particle size is relatively identical. The measurement of zeta potential is based on the electrophoretic movement of the drug in the medium. Suspensions that have a high potential zeta value will prevent particles from flocculating and aggregating. A stable potential zeta value of more than +30 mV or less than -30.³³ The main characteristic of the chitosan-STPP nanoparticle is its zeta potential. It is positively charged due to the presence of amine groups in chitosan.³⁴ Chitosan is positively charged in the digestive tract because the acid environment can therefore interact with the active compound which is coated more optimally by the electrostatic force.³⁵ The making of nanoemulsion by ionic gelation method produces greater mucoadhesion ability compared to other methods and prolongs retention time and increases the time of drug delivery.³⁶ The bioadhesive ability of chitosan can increase the absorption of active ingredients.³⁷ The increase in chitosan molecular weight can improve the bioadhesive ability of chitosan.

***In-vivo* anti-diabetic study of TDE-NE**

Measurement of normal blood glucose levels is carried out before alloxan induction to determine differences in normal glucose levels and after alloxan induction. Based on the comparison of glucose levels test animals have increased after alloxan induction. On the 0th day, the average FBGL of all groups was 103.35 mg/dL and rose to 260.83 mg/dL on the 3rd day. An increase in blood glucose levels was tested by the T-test. The results showed that there was a significant difference between fasting blood glucose levels before induction and after alloxan induction ($p < 0.05$).

Alloxan and glucose compete to enter the β -pancreas cells. The presence of glucose can inhibit alloxan from entering β -pancreatic cells.^{3,4,7} Therefore, to minimize the amount of blood glucose, the control test rats must be fasting. Alloxan has a similar shape to a glucose molecule so that the glucose transporter GLUT2 in the plasma membrane of the pancreatic β cell accepts it as a glucose analog and carries it into the cytosol. Alloxan is accumulated and has a toxic effect on pancreatic beta cells.

The FBGL curve (Fig.-4.) in the basal group did not change significantly and was stable with normal FBGL (<126 mg/dL) during the observed period. In other words, the 0.2% Na-CMC treatment did not cause a hyperglycemic effect on normal rats, while in the control negative group on days 3, 6, 9, 12, and 15 showing diabetes FBGL. A fluctuating data (high rise in blood glucose on the 9th day and then decreases on the 12th day and again rises on the 15th day) occur because alloxan has damaged the β -pancreas cells.

Conversely, the FBGL curve in the positive control group on days 3, 6, 9, 12, 15 shows diabetes FBGL tends to decrease because glibenclamide treatments improve β -pancreatic cells even though they have not reached normal values/control. Similarly, the FBGL curve of the TDE-NE treatment group on the 3, 6, 9,

12, and 15 days showed a decrease in diabetes sugar levels. From this data, the decrease in sugar levels in the treatment group is better than the glibenclamide group even though it has not reached normal control. The results of this study indicate that nanoemulsion increases antidiabetic activity at a dose of 150 mg/kg BW and can reduce blood glucose levels by 23.98% for 13 days. The reduction of blood glucose by this TDE-NE is higher than paitan leaf extract (15% at 200 mg/kg BW for 4 weeks)³⁸. Conversely, it is lower compared with the infusion at a dose of 800 mg/kg BW (96.42% for 14 days)³⁹ and 500 mg/kg BW (98%)⁴⁰. However, judging from the concentration, this result is 4x more effective than using infusion³⁹. TDE-NE dosage variations can be further investigated to get the efficient concentration in the nanoemulsion formulation.

Table-4: Average FBGL in Various Conditions

Group Treatments	FBGL Changes Day 3-15 (%)
Normal Control	-35.58
Negative Control	+55.07
Positive Control (glibenclamide)	-18.00
TDE-NE	-23.98

Fig.-4: Average FBGL Curve

CONCLUSION

Based on phytochemical screening, TDE contains alkaloids, tannins, terpenoids, and saponins which may work synergistically to reduce blood glucose in TDE-NE. The antioxidant evaluation shows an excellent value of IC_{50} 16.801 ppm. The ionic gelation method succeeded in producing crosslinking nanoemulsion between chitosan-STPP and paitan leaf extract (5:1:1) with a size of 346 nm, PI 0.407, and Potential Zeta of 14.1 mV. TDE-NE formulation at 150 mg/kg BW was able to reduce blood glucose in male Wistar-induced alloxan strains for 13 days (23.98%) and better than glibenclamide (18.00%).

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