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Original Article

Antioxidant and Antidiabetic Activities of Mempening (*Lithocarpus bancanus*) Leaves

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ABSTRACT

Background: *Lithocarpus bancanus* or commonly called as mempening in Talang Mamak Tribe, Indonesia is a plant that is used as a traditional medicine. **Objective:** This study aim to evaluated antioxidant and antidiabetic activities of *L. bancanus* leaves extract. **Material and Methods:** The methanol extract was obtained by maceration of the leaves. The *n*-hexane, dichloromethane and ethyl acetate fractions were prepared by successive partition process of the methanol extract. Antioxidant activities were evaluated by various antioxidant assays, including DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (ferric reducing antioxidant power), CUPRAC (*cupric reducing antioxidant capacity)*, and ABTS (*2,2'-azonobis 3-ethylbenzothiazoline-6-sulfonic acid*) method. Total phenolics were estimated based on the Folin–Ciocalteu method, while, aluminum chloride methods were employed to estimate total flavonoids. Antidiabetic activies was determined by inhibiting the activity of α-glucosidase method. **Results:** antioxidant activity assay against DPPH radical as well as the total phenolic and flavonoid content of *L. bancanus* leaves showed that the methanol extract possessed IC_{50} value of 39.469 ± 0.273 µg/mL with total phenol and flavonoid were 11.426 ± 0.432 mg GAE/g dry weight sample and 15.423 ± 0.213 mg QE/g respectively. The FRAP, CUPRAC and ABTS values of methanol extract were 3494.302 ±0.456 26665.501 ± 5.940 and 2857.977 ± 0.715 µM TE/g dry weight sample respectively. Antidiabetic activity of methanol extract with IC_{50} value of 30.565 ± 0.331 µg/mL. **Conclusion:** It could be concluded that leaves of *L. bancanus* have antioxidant and antidiabetic properties.

Key words: Antioxidant; Antidiabetic; *Lithocarpus bancanus*; Talang Mamak Tribe.

INTRODUCTION

Diabetes mellitus is the common serious metabolic disorder due to disturbance of carbohydrate, lipid and protein metabolism. It is characterized by hyperglycaemia resulting from insulin resistance or decreased production of insulin by the β-cells of the pancreas. Approximately 90% of all cases of diabetes in developed and developing countries are type-2 diabetes.[1,2] Hyperglycemia is found to increase the production of free radicals that are associated with long-term damage, dysfunction, and failure of various organs, especially eyes, kidneys, nerves, hearts, and blood vessels. Several other factors such as hyperlipidaemia and enhanced oxidative stress play a major role in diabetes. The development of diabetes and progression of complications are usually associated with oxidative stress which is as a result of overexpression of reactive oxygen species (ROS) or free radicals. Free radicals are generated during autoxidation of glucose in diabetes mellitus resulting in oxidative stress. ROS is involved in the process of signal transduction in the pancreatic β-cells and has the potential to regulate glucose-stimulated insulin secretion. However, insulin secretion can reduce when excessive ROS synthesis is produced by elevated glucose or fatty acid oxidation.[3,4]

Traditional medicine is gaining so much interest recently due to their multiple modes of actions with minimal adverse effects in humans. Medicinal plant are rich source of secondary metabolites used in various therapies, including diabetes mellitus. Thus, considering the high Indonesia biodiversity, it is essential to explore potential plant species, including *L. bancanus* (mempening). This species belongs to Fagaceae family found in the Talang Mamak tribe in Kelayang District, Indragiri Hulu Regency, Riau Province. It is usually used by the peoples as a medicine to treat pain and inflammation. Some species of this genus have been previously reported to contain various secondary metabolites, including terpenoids, steroids and flavonoids as the major components. Likewise, bioactivities from the genus have been evaluated including antioxidants, antidiabetic, anticancer, antimicrobial and other activities.[5-8] In regard to explore antidiabetic agent from Talang Mamak medicinal plants[9], we reported the antioxidant and antidiabetic activity of *L. bancanus* leaves extract and fractions.

MATERIAL AND METHOD

Chemical reagents

DPPH (1,1-diphenyl-2- picryl hydrazyl), gallic acid, quercetin, ascorbic acid, Trolox®, TPTZ (2, 4, 6-tripydyl-s-triazine), neocuproine (Nc), α-glucosidase enzyme and *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) substrate from Sigma-aldrich Chemical Co (Singapore). Folin-Ciocalteu,

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Na_2CO_3, $NaNO_2$, $AlCl_3$, NaOH, $CuCl_2$. $2H_2O$ 10 mM, neocuproine (Nc), $K_2S_2O_8$, organic solvents p.a (*n*-hexane, dichloomethane, ethyl acetate and methanol from Merck (Germany).

Collection of plant material

Samples was collected from Kelayang (Bukit Tiga Puluh National Park (TNBT) of Indragiri Hulu Regency, Riau Province and identification of sample plants was carried out in the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Riau. Samples were dried and finely ground and stored at 4^0C until analysis.

Extraction

Dried *L. bancanus* leaves (100 g) were ground into powder and then macerated for 48 hour followed by ultrasound for 1 hour and the macerates were collected and concentrated with a rotary evaporator at 50^0C. Methanol extract were fractionated with *n*-hexane, dichloromethane and ethyl acetate respectively. Each fraction were evaporated to get extracts.

DPPH radical scavenging activity assay

Antioxidant activity assay was carried out by using DPPH method (1,1-diphenyl-2- picryl hydrazyl) by the standard method, with a slight modification.[10-12] Samples with a finally concentration of 1000 µg/mL were diluted by two fold dilution method (1000 - 31.25 µg/mL) in 96 well clear polystyrene microplate. A total of 50 µL of sample was added with 80 µL of DPPH 100 µg /mL then incubated for 30 minutes in a dark place. Absorbance were measured by microplate reader (Berthold, Germany) at 520 nm. The same method were conducted for ascorbic acid and quercetin as positive control.

The % Inhibition value is calculated by the following formula:

% Inhibition = $((A_0-A_s))/A_0 \times 100$

Where A_0 represents the absorbance of the DPPH radical solution without sample while A_s represents the absorbance of the sample with DPPH radical solution. A graph of inhibition percentages (I%) versus concentrations of the sample was plotted to provide value of IC_{50}.

Determination of total phenolic content (TPC)

Determination of the total phenolic sample was carried out by using the Folin-Ciocalteu method.[13-16] Gallic acid was used as a standard. A total of 100 µL of sample, gallic acid and blank were each mixed with 50 µL of the Folin-Ciocalteu reagent 0.25 N in 96-well microplate. After 5 minutes, 100 µL Na_2CO_3 7.5% (w v) was added. The mixture was incubated for 30 minutes in a dark place at room temperature before absorbance was measured at a wavelength of 765 nm by microplate reader. The total phenolic content is expressed as milligrams of equivalent gallic acid per gram dry matter of sample (mgGAE/g) throught the calibration curve gallic acid. Linearity range of calibration curve was 10 -50 µg/mL (y = 0.016x + 0.0081, r = 0.992).

Determination of total flavonoids content (TFC)

Determination of the total flavonoid content of extracts was carried out by using the colorimetric method of aluminum chloride with quercetin as a standard.[13] A total of 50 µL samples, quercetin and blanks were each mixed with 10 µL $NaNO_2$ 5% (w/v), 10 µL $AlCl_3$ 10% (w/v) in 96 well micoplates. After 5 minutes 100 mL of 1 M NaOH was added. The mixture was added with 30 mL of distilled water and the mixture was incubated in a dark place at room temperature for 30 minutes. Absorbance of the mixture was measured at a wavelength of 510 nm by microplate reader. The total content of flavonoids is expressed as milligrams of equivalent quarsetin per gram dry matter of sample (mgQE/g). Linearity range of calibration curve was 10 -50 µg/mL (y = 0.0162x + 0.0755, r = 0.999).

Ferric reducing antioxidant power assay (FRAP)

Antioxidant activity was measured with FRAP according to the method with Trolox as standard.[17] FRAP reagents was made from 0.2 M acetate buffer solution (pH 3.6), TPTZ solution (2, 4, 6-tripydyl-s-triazine) 10 mM in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution were prepared and then the solution was mixed with ratio 10: 1: 1. Some 100 µL of sample was added to *96*-well clear polystyrene microplates which contained 100 µL of FRAP reagent. The mixture was incubated for 30 minutes in a dark place at room temperature. The absorbance of sample was measured at wavelength of 595 nm by microplate reader and calculated as micromolar of Trolox equivalent per gram of dry weight (mg TE/g dry weight) and using the Trolox as standard curve. Linearity range of calibration curve was 2 - 10 µM/mL (y = 0.0641x + 0.0644, r = 0.991).

Cupric reducing antioxidant capacity assay (CUPRAC)

Antioxidant activity was measured using the CUPRAC analysis.[18] Some 50 µL of $CuCl_2$. $2H_2O$ 10 mM, 50 µL Neocuproine (Nc) 7.5 mM and 50 µL ammonium acetate buffer were added to *96* well- clear polystyrene microplates which contained 100 µL of samples, standard and blank. Then, distilled water was added up to 300 µL. The mixture was incubated for 30 minutes in a dark place at room temperature and the absorbance was read at 450 nm by microplate reader and calculated as micromolar of Trolox equivalent per gram of dry weight (mg TE / g dry weight) and using Trolox as standard curve. Linearity range of calibration curve was 20 - 100 µM/mL (y = 0.0073x + 0.1274 r = 0.999).

2,2′-azonobis 3-ethylbenzothiazoline-6-sulfonic acid assay (ABTS)

Antioxidant activity was carried out with the ABTS method.[19] ABTS reagents was prepared by dissolving 0.077 g ABTS powder in 10 ml of distilled water. 10 ml of ABTS solution was reacted with 10 ml of $K_2S_2O_8$ (5 mM) and was saved in a dark place at room temperature for 16 hours to produce ABTS radical cation. The solution was diluted with distilled water to obtain an absorbance of 1.00 at a wavelength of 734 nm. Some 100 µL of $ABTS^{\bullet +}$ solution was added to 96 well clear polystyrene microplates which contained 200 µL of sample, standard, and blank. The mixture was incubated for 30 minutes in a dark place at room temperature. The absorbance of the sample was measured at a wavelength 734 nm by microplate reader (Berthold, Germany). and the results were calculated as micromolar of Trolox equivalent per gram of dry weight (mg TE/g dry weight) using the Trolox as standard curve. Linearity range of calibration curve was 2 - 10 µM/mL (y = 0.0738x + 0.0473, r = 0.999).

Antidiabetic activity assay

The antidiabetic test used a method of inhibiting the activity of α-glucosidase enzyme with *p*-NPG as a substrate by the standard method, with a slight modification.[20-22] The sample was diluted by the two fold dilution method of concentration 1000 - 31.25 µg / mL. A total of 10 µL of DMSO (B_0) and 10 µL of sample (S_0) were added with 50 µL of pH 7 phosphate buffer, 25 µL of p-NPG 20 mM and 10 µL of DMSO (B_1) and 10 µL of sample (S_1) with 50 µL of phosphate buffer pH 7.25 µL p-NPG 20 mM and 25 µL α-glucosidase 0.2 U/mL were mixed in 96-well microplate and incubated for 30 minutes at 37^0C. The reaction was stopped by adding 100 µL of 0.1 M Na_2CO_3 then absorbance was measured by microplate reader at a wavelength of 405 nm.

The % Inhibition value is calculated by the following formula:

% Inhibition = $\frac{(B_1 - B_0) - (S_1 - S_0)}{(B_1 - B_0)}$ x 100

Where B_0 represents the absorbance without sample and enzyme, B_1 represents the absorbance without sample and contain enzyme, S_0 represents the absorbance contain sample and without enzyme while S_0 represents the absorbance contain sample and enzyme. A graph of inhibition percentages (I%) against concentrations of the sample was plotted to provide value of IC_{50}.

Statistical analysis

All assays were carried out in triplicate and their results were expressed as mean ± standard deviation. Data analysed by one - way ANOVA by using IBM SPSS statistics 20 (Version 20.0, IBM. Corp., U.S.A). The significance of difference was calculated by using Duncan's multiple range test, while Pearson correlation test was conducted to determine the correlation among variable. A $P < 0.05$ were considered statistically significant levels. All measurements were carried triplicate.

RESULT AND DISCUSSION

DPPH radical scavenging activity

DPPH radical scavenging activity from L. bancanus leaves exibited various activity (**Table 1**). Methanol extract and ethyl acetate fraction showed high antioxidant activity with IC_{50} value of 39.469 ± 0.273 µg/mL and 52.546 ± 0.557 respectively with no significantly different ($P<0.05$) with quercetin. The *n*-hexane fraction showed no activity with IC_{50} values greater than 1000 µg/mL while the dichloromethane fraction exhibited moderate antioxidant activity with IC_{50} values of 334.464 ± 0.361 µg/mL. In this result showed that the solvent with high polarity exhibited high activity, and this might be due to the presence of flavonoids and phenolics.

Total phenolic (TPC) and flavonoid content (TFC)

The determination of TPC is based on the reduction of the phosphomolybdate-tungstate complex with its active center is Mo (VI) by phenolic compounds forming a blue product.[18] Totcal phenolic content of extract and fraction of *L. bancanus* leave were differed significantly ($P<0.05$) (**Table 2**). Methanol extract and ethyl acetate fraction showed high TPC compared to *n*-hexane and dichloromethane fractions with value of 11.426 ± 0.106 and 6.525 ± 0.188 mg GAE/g dry weight sample, respectively. In order to determined TFC, the samples were reacted with $AlCl_3$ to form a complex in the ortho hydroxy ketone group which gives a batochromic effect from flavonoid.[23] The results showed that methanol extract and ethyl acetate fraction exhibited high TFC with value of 15.422 ± 0.306 and 9.144 ± 0.138 mg QE/dry weight sample, respectively (p<0.05)

Flavonoids are diphenyl propanoids consisting of two of rings connected by chains with three of carbon atoms. The plants extracts contained phenolic and flavonoid compounds which showed effective antioxidant properties and could lower cellular oxidative stress.[24] In this study, we reported correlation between the phenolic and flavonoid content with DPPH radical scavanging with coeficient correlation (r) = 0.996 and 0.994, respectively (**Table 3)** and it is in an agreement with Jacobo-Velazquez and coworkers.[25]

Ferric reducing antioxidant power (FRAP)

The FRAP method was used to measure the ability of antioxidants by reducing ferric in acidic conditions. Complex ferric-tripyridyltriazine (Fe^{+3}-TPTZ) is reduced to form Fe^{+2} (Fe^{+2}-TPTZ) with maximum absorbance at 595 nm.[26] The results showed significanly different among the tested sampel ($P<0.05$), however, the methanol extract and quercetin exhibited no sifnificantly different (**Table 3**).

Cupric reducing antioxidant capacity (CUPRAC)

In this assay, Cu (II) was reduced to Cu (I) by antioxidants. Neocuproin (Nc) chromophore reagent reacts with $CuCl_2$ to form complex Cu (I) –Nc at pH 7 at a wavelength of 450 nm, from bright blue to yellow-orange.[27] The results showed that extract and fractions differed significantly ($P<0.05$) (**Table 3**). The methanol extract and ethyl acetate fraction possessed activity with valueo of 26665.501 ± 5.940 and 15146.556 ± 3.107 µM TE/dry weight, respectively, and these results exibited high activity compared to *n*-hexane and dichloromethane fractions.

2,2'-azonobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The ABTS (*2,2'-azonobis 3-ethylbenzothiazoline-6-sulfonic acid)* assay is based on the ability of antioxidants to capture the cation radical of ABTS. In this assay, the radical cation of ABTS is produced from ABTS oxidation by potassium persulfate ($K_2S_2O_8$) which produces a greenish blue color. Color loss will occur when antioxidant compounds donate H atoms to the ABTS cation radical.[27] The antioxidant activity of a sample in reducing ABTS cation radical compared to Trolox, and

Table 1. Antioxidant activity of *L. bancanus* leaves against DPPH radical.

Sample	IC_{50} (µg/mL)
n-Hexane fraction	1151.808 ± 17.458[a]
Dichloromethane fraction	334.464 ± 0,361[b]
Ethyl acetate fraction	52.546 ± 0.557[c]
Methanol extract	39.469 ± 0.273[c]
Quercetin	40.063 ± 1.604[c]
Ascorbic Acid	11.043 ± 0.154[d]

Note: Data expressed as mean ± standard deviation (n = 3). Same letters in each column mean no significant difference ($P < 0.05$).

Table 2. Total phenolic and flavonoid content of *L. bancanus* leaves.

Sample	Total phenolic (mg GAE/g dry sample)	Total flavonoid (mg QE/g dry sample)
n-Hexane fraction	0.472 ± 0.241[d]	0.463 ± 0.110[d]
Dichloromethane fraction	0.788 ± 0.125[c]	0.925 ± 0.216[c]
Ethyl acetate fraction	6.525 ± 0.188[b]	9.144 ± 0.138[b]
Methanol extract	11.426 ±0.106[a]	15.422 ± 0.306[a]

Note: Data expressed as mean ± standard deviation (n = 3). Same letters in each column mean no significant difference (P< 0.05). GAE: Gallat acid equivalents, QE: Quercetin equivalents.

they showed significantly different ($P<0.05$) (**Table 3**). The value of ABTS from methanol extract and ethyl acetate fraction were 2857.977 ± 0.715 and 1402.082 ± 0.371 μM TE/dry weight sample, respectively, and they exibited highest ABTS values compared to *n*-hexane and dichloromethane.

Correlation analyses between phenolic and flavonoid contents with antioxidant and Inhibitor α-glucosidase activities

Correlation analyses (**Table 4**) between phenolic and flavonoid content with antioxidant (DPPH, FRAP, CUPRAC, ABTS) and antidibetic (inhibitor *α*-glucosidase) activities were performed. Extract and fraction of *L. bancanus* leaves exhibited significant ($P<0.01$) linear corelations between TPC and TFC, TPC and IC_{50} DPPH, TPC and FRAP, TPC and CUPRAC, TPC and ABTS, and TPC and IC_{50} *α*-glucosidase inhibitor. By comparing the correlation coeficient (*r*) between TPC and CUPRAC exibited highest *r* value ($r = 1$), followed by TPC and TFC ($r = 0.999$), TPC and ABTS (r = 0.997), TPC and FRAP (r = 0.996), TPC and IC_{50} *α*-glucosidase inhibitor ($r = 0.944$) and TPC and IC_{50} DPPH ($r = 0.944$). There were linear correlation ($P < 0.01$) between TFC and CUPRAC, TFC and ABTS, TFC and FRAP, TFC and IC_{50} *α*-glucosidase and TFC and IC_{50} DPPH with $r = 0.999$, 0.994, 0.993, 0.955 and 0.739, respectively.

Through these correlation analysis, the phenolic and flavonoid contents diplayed association with antioxidant activities (DPPH radical, FRAP, CUPRAC and ABTS) and antidiabetic activities. The results are consistent with those found by Sahreen who reported that there was existence of a strong relationship between phenolic and flavonoid contents and DPPH and FRAP, CUPRAC and ABTS radical scavenging.[28]

Antidiabetic activity assay

The *α*-glucosidase enzyme is the enzyme which responsible for breaking down disaccharides and complex carbohydrates into glucose. Inhibition of this enzyme can delay the absorption of glucose in the digestive tract, and to prevent an increasing in blood glucose concentration after eating.[29] α-glucosidase inhibition activity is one of method to determined antidiabetic acivity. The mechanism of this assay was observed by interfering with the carbohydrate hydrolysis process, inhibits the absorption of glucose and other monosaccharides. Inhibition of this enzyme can effectively to reduce the digestion of complex carbohydrates and their absorption, so as to reduce the increase in postprandilla glucose levels in diabetics.[30] The antidiabetic activity results showed significantly different ($P<0.05$), where methanol extract showed high activity followed by ethyl acetate fraction with IC_{50} 30.565 ± 0.331 μg/mL, 44.901 ± 0.128 μg/mL, respectively. (**Table 5**). There are significant corrrelation between total phenolics and flavanoids and the activity ($P<0.01$) with coeficient correlation (r) = 0.944 and 0.955 (**Table 4**).

Table 3. Antioxidant activities (FRAP, CUPRAC and ABTS) of *L. bancanus* leave.

Sample	FRAP (μM TE/g dry sample)	CUPRAC (μM TE/g dry sample)	ABTS (μM TE/g dry sample)
n-Hexane fraction	126.508 ± 0.996^{e}	380.082 ± 0.544^{f}	77.517 ± 0.776^{f}
Dichloromethane fraction	190.050± 0.581^{d}	536.926 ± 0.579^{e}	131.634 ± 0.741^{e}
Ethyl acetate fraction	1695.239 ± 0.372^{c}	15146.556 ± 3.107^{d}	1402.082 ± 0.371^{d}
Methanol extract	3494.302 ±0.456^{b}	26665.501 ± 5.940^{b}	2857.977 ± 0.715^{b}
Quercetin	3492.846 ± 2.930^{b}	25848.774 ± 5.940^{c}	2742.498 ± 0.589^{c}
Ascorbic Acid	35220.782 ±0.674^{a}	28571.197 ±5.941^{a}	2911.909 ±0.889^{a}

Note: Data expressed as mean ± standard deviation ($n = 3$). Same letters in each column mean no significant difference ($P < 0.05$), TE: Trolox equivalents.

Table 4. Correlation between phenolic and flavonoid contents with antioxidant and antidiabetic (inhibitor *α*-glucosidase) activities of *L. bancanus* leaves.

	TPC	TFC	IC_{50} DPPH	FRAP	CUPRAC	ABTS	IC_{50} α-glucosidase
TPC	-	0.999**	0.730**	0,996**	1,000**	0.997**	0.944**
TFC		-	0.739**	0.993**	0.999**	0.994**	0.955**
IC_{50} DPPH			-	-0.697*	0.722**	0.701*	0.844**
FRAP				-	0.995**	1,000**	0.916**
CUPRAC					-	0.996**	0.947**
ABTS						-	0.920**
IC_{50} *α*-glucosidase							-

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

Table 5. Antidiabetic (Inhibitor *α*-glucosidase) activity of *L. bancanus* leaves.

Sample	IC_{50} (μg/mL)
n-Hexane fraction	116.607 ± 1.379^{a}
Dichloromethane fraction	102.189 ± 1.631^{b}
Ethyl acetate fraction	44.901 ± 0.128^{c}
Methanol extract	30.565 ± 0.331^{d}
Acarbose	18.173 ± 0.122^{e}

Note: Data expressed as mean ± standard deviation ($n = 3$). Same letters in each column mean no significant difference ($P < 0.05$).

CONCLUSION

The leaves extract and its *n*-hexane, dichloromethane and ethyl acetate fractions of *L. bancanus* showed high antioxidant and antidiabetic activities, especially ethyl acetate fraction and methanol extracts. It could be concluded that leaves of *L. bancanus* has antioxidant and antidiabetic properties.

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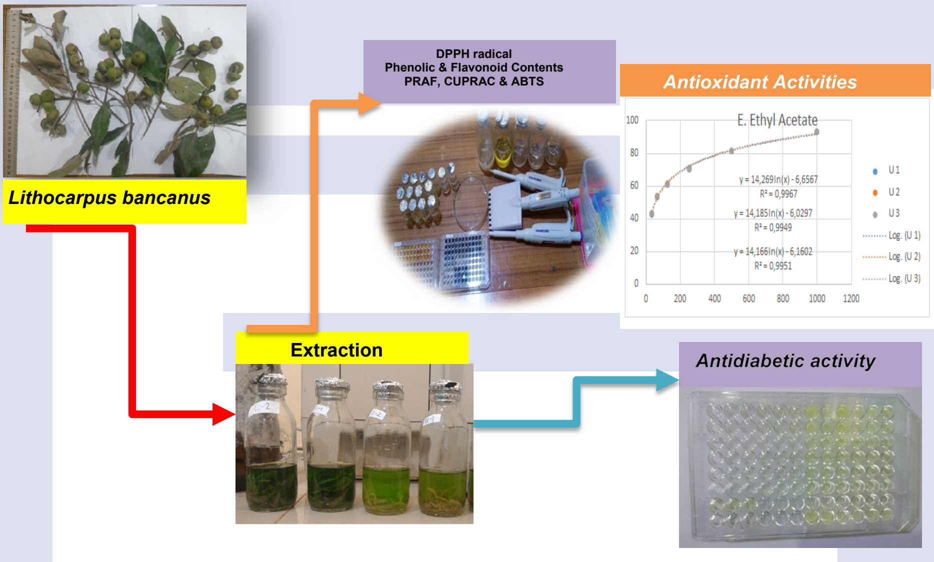
GRAPHICAL ABSTRACT



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