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ANTIOXIDANT AND XANTHINE OXIDASE INHIBITORY ACTIVITY OF *Euphorbia hirta* LEAVES CRUDE EXTRACT

(Antioksidan dan Aktiviti Perencatan Xantina Oksidase Bagi Ekstrak Mentah Daun *Euphorbia hirta*)

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Abstract

Today, exposure to free radicals and gout cases have been a concern, showing several health effects from synthetic medications and clinical drugs for antioxidant effect and xanthine oxidase inhibition effect, respectively; all of which lead to the discovery of natural inhibitors from medicinal plants. In this study, a green solvent known as the deep eutectic solvent is synthesised to replace conventional solvents for the extraction of free radical inhibitors and xanthine oxidase inhibitors from a medicinal plant that has shown many health benefits and is widely used in traditional medicine, the *Euphorbia hirta.* The leaves extract from *Euphorbia hirta* showed the highest inhibition (87.95 ± 0.0061%) on free radical assays. The IC_{50} for free radical inhibition of the leaves extract is 10.56 ± 0.58 µg/mL. The IC_{50} value for xanthine oxidase inhibition of allopurinol was the lowest, which was at 6.94 ± 0.32 µg/mL; while the leaves extract of *Euphorbia hirta* exhibited value at 9.40 ± 0.13 µg/mL. The correlation value between the antioxidant effect and the xanthine oxidase inhibition of the leaves extract was 0.960, which indicated that there is a strong relationship between the two said parameters. From the Lineweaver-Burk plot and solver function analysis, the mode of enzyme inhibition was a non-competitive inhibition.

Keywords: antioxidant, deep eutectic solvent, *Euphorbia hirta*, gout treatment, xanthine inhibitor

Abstrak

Pendedahan kepada radikal bebas dan kes-kes gaut telah menjadi kebimbangan pada hari ini di mana ubat sintetik untuk kesan antioksidan dan kesan perencatan xantina oksidase dari ubat klinikal telah menunjukkan beberapa kesan dalam kesihatan yang membawa kepada penemuan perencatan semulajadi dari tumbuhan perubatan. Dalam kajian ini, pelarut hijau yang dikenali sebagai pelarut eutektik dalam telah disintesis untuk menggantikan pelarut konvensional untuk pengekstrakan perencatan radikal bebas dan perencatan xantina oksidase dari tumbuhan perubatan yang telah menunjukkan banyak manfaat kesihatan dan digunakan secara meluas dalam ubat tradisional, *Euphorbia hirta*. Ekstrak daun dari *Euphorbia hirta* menunjukkan perencatan tertinggi (87.95 ± 0.0061%) pada asai radikal bebas. Nilai IC_{50} untuk perencatan radikal bebas daripada ekstrak daun ialah 10.56 ± 0.58 µg/mL. Nilai IC_{50} untuk perencatan xantina oksidase terhadap alopurinol adalah paling rendah pada 6.94 ± 0.32 µg/mL manakala ekstrak daun

Euphorbia hirta menunjukkan nilai dekat pada 9.40 ± 0.13 μg/mL. Nilai korelasi antara kesan antioksidan dan perencatan xantina oksidase bagi ekstrak daun terbukti pada nilai 0.960 yang menunjukkan hubungan yang kuat. Dari plot Line-weaver Burk dan analisis fungsi penyelesai, mod perencatan enzim adalah perencatan yang tidak kompetitif.

Kata kunci: antioksidan, larutan eutektik dalam, *Euphorbia hirta*, rawatan gaut, perencat xantina

Introduction

Our unhealthy lifestyle and constant exposure to harmful sources, such as pollution, smoking, and radiation, have gradually increased the rate of gout occurrence and free radical exposure. Studies have shown that the occurrence of gout has been rising over the past 50 years worldwide [1]. Gout is a type of disease caused by the accumulation of uric acid crystal deposits in the joints as a result of the high level of uric acid. Meanwhile, aging and other diseases are triggered by high exposure of free radicals that severely damages the cells, DNA, and proteins in the body, resulting in the malfunctioning of cells and molecules in their respective roles [2, 3]. In order to treat gout and other diseases that are resulted by free radical exposure, synthetic antioxidants and synthetic xanthine oxidase inhibitor are usually advocated. In the case of gout, allopurinol is commonly prescribed to suppress gout attacks, but there are several side effects to its long-term intake, such as fever, nausea, rashes, renal impairment, and hepatic dysfunction, among others [4]. Xanthine oxidase is an enzyme in a form of xanthine oxidoreductase, which catalyses the oxidation of hypoxanthine and xanthine to uric acid. Its high expression will cause an increased production of uric acid, thus leading to gout [5]. In the case of free radicals, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-Butylhydroquinone (TBHQ), are commonly recommended although with little effectiveness [6, 7].

A locally available herb, *Euphorbia hirta* (locally known as *ara tanah* or *pokok susu nabi*) was introduced as a new and potential natural source of gout medication, in which it is deemed a promising substitute for synthetic xanthine oxidase inhibitors as well as for antioxidant effect. This herb has been used traditionally for the treatment of gout and other diseases. However, there has yet to be a scientific report on the application of this plant extract as a xanthine oxidase inhibitor.

Conventional methods of extracting bioactive compounds using organic solvents, such as methanol and ethanol, have been indicative of several adverse health and environmental effects [8]. As such, a green solvent known as the deep eutectic solvent (DES) has been chosen to be used in the study of natural inhibitor, *Euphorbia hirta via* green extraction method. Deep eutectic solvent is produced from the mixing of two or more components, where one acts as a hydrogen bond donor and the other a hydrogen bond acceptor. This solvent has several advantages, such as low in cost and toxicity, is biodegradable, and requires simple preparation [9].

Materials and Methods

Preparation of choline chloride-glucose (ChCl-Glu)-based deep eutectic solvent (DES)

A choline chloride with D-glucose (ChCl-Glu) solvent was prepared with a molar ratio of 2:1. The choline chloride and D-glucose used in the DES were dried in a vacuum oven at 80 °C overnight to eliminate moisture from the powder [8]. Then, the mixture was heated and stirred at 80 °C in a conical flask wrapped with aluminium foil until a homogenous, colourless liquid was formed. Next, the DES solution was stored in a vacuum desiccator containing silica gels to prevent humidity [10,11].

Extraction of *Euphorbia hirta* using ChCl-Glu-based deep eutectic solvent

An amount of 0.5 g of the plant leaves crude extract was weighed and subsequently mixed with 10 mL of DES. The mixture was stirred using a magnetic stirrer for 2 hours at 60 °C, and then centrifuged at the speed of 3000 rpm for 30 minutes. Centrifugation was repeated twice to ensure the layers were evenly separated. Then, the supernatant and pellet were collected [11, 12].

1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging assay

Methanol with a concentration of 0.2 mM of DPPH was prepared by adding 2 mL of the solution into 200 µL of extracts and subsequently mixed with 800 µL of methanol. Next, the mixture was left at room temperature for 30 minutes. A corresponding blank sample was prepared using only methanol. Then, a control solution was prepared by mixing 2 mL of methanolic solution of DPPH radical scavenging assay and 800 µL of methanol. In this case, gallic acid was used as reference. The whole process was carried out in a dark environment, and an absorbance was measured at 517 nm after 30 minutes [13, 14].

Inhibitory activity assay of xanthine oxidase (XO)

An amount of one millilitre (100–500 µg/mL) of sample solution from *Euphorbia hirta* pellet extract of leaves was mixed with 2.9 mL of 0.05 M of sodium phosphate buffer (with a pH of 7.5), where the solution was further added with 0.1 mL of XO. The assay had then undergone preincubation at 25 °C for 15 minutes, where 2 mL of 150 mM of xanthine solution was then added into the assay mixture and further incubated for 30 minutes at 25 °C. Using the water bath, the solution was heated to 90 °C for 5 minutes, and the enzymatic reaction halted. The absorbance was measured at 295 nm by using UV-Vis spectrophotometer. Allopurinol was used as reference and positive control by through similar preparation method and concentration. Dimethyl sulfoxide (DMSO) was used to replace the test sample in order to obtain the maximum formation of uric acid. The activity of XO inhibitory was calculated as equation 1:

$$\% \text{ XO Inhibition} = (1-\beta/\alpha) \times 100\% \quad (1)$$

where α is the activity of XO without any sample extract, and β is the activity of XO with a sample extract [11, 15, 16].

Inhibitory concentration of 50% (IC_{50}) of XO analysis

The IC_{50} for the inhibition of XO can be calculated by transforming it to probit analysis using Microsoft Excel and probit table. Equation from the graph obtained via the regression method in probit analysis was used to determine the IC_{50} values for allopurinol and *Euphorbia hirta* [17].

Correlation study of xanthine oxidase inhibition (XOI) and free radical inhibition

A correlation study between the antioxidant and XOI activities was done by plotting a graph of correlation for a range of concentration: 100, 200, 300, 400, and 500 µg/mL of sample extracts to assess the antioxidant and XOI activities. A correlation between the two independent variables was obtained using correlation and regression analysis in Microsoft Excel to relate antioxidant activity with xanthine oxidase inhibitory activity of the leaves pellet of *Euphorbia hirta* extract.

Kinetic study of enzyme

The Lineweaver-Burk plot analysis was adopted to perform a kinetic study of the enzyme. This assay was carried out with the presence or absence of the sample extract from *Euphorbia hirta*, with varying substrate concentrations of xanthine at 10, 20, 40, 60, 80, 100, and 120 µM; while the tested sample was set at 500 µg/mL by using the xanthine oxidase assay methodology [11, 12].

Results and Discussion

Antioxidant test on different parts of *Euphorbia hirta* extracts using deep eutectic solvent (DES)

Figure 1 shows the comparison made between the percentage of inhibition for each sample and the positive control, which was gallic acid, demonstrated that the flower pellet of *Euphorbia hirta* extract recorded the highest percentage inhibition at 88.61%, followed closely by the leaves pellet extract at 87.95%. In view of this result, the leaves pellet had been chosen throughout the conduction of this work, as it had the second nearest percentage inhibition to flower pellet; this is because the flowers of *Euphorbia hirta* are difficult to harvest due to its scarcity. The high antioxidant activity of the plant pellet might be due to the existence of flavonoids, where the phytochemical analysis of the plant had revealed the presence of two types of flavonoids: quercetin and myricitrin. They are deemed to have a reliable role of having strong anti-oxidative activity with either their reducing capabilities or their influence on intracellular redox status [18].

Xanthine oxidase inhibitory assay

Both allopurinol and leaves pellet extracts showed a gradual increase in their percentage inhibition when the concentration of the sample was increased from 100 to 500 µg/mL (Figure 2). The higher the concentration of the extract, the higher the inhibition activity of XO. This could be due to the increasing presence of flavonoids in the extracts. A strong inhibition activity of XO exhibited by allopurinol proved its effective usage on the clinical treatment of gout [15].

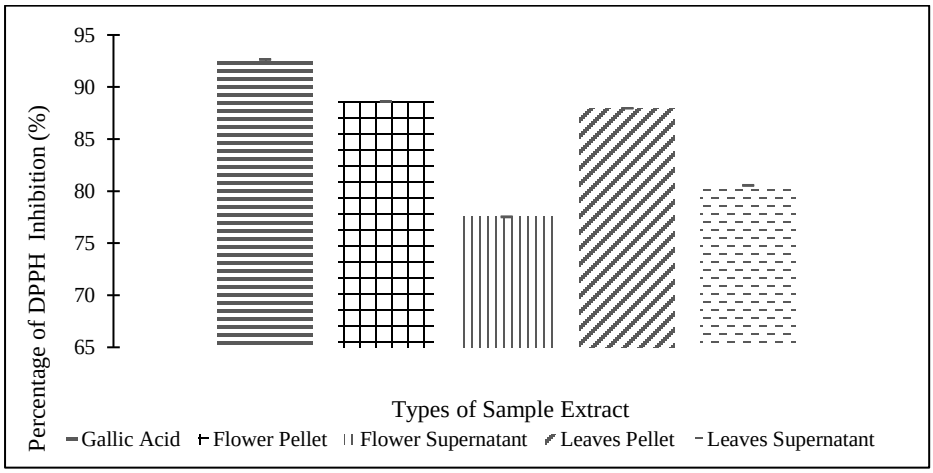

Figure 1. The percentage inhibition of DPPH radical scavenging activity by leaves and flowers crude extract of *Euphorbia hirta*

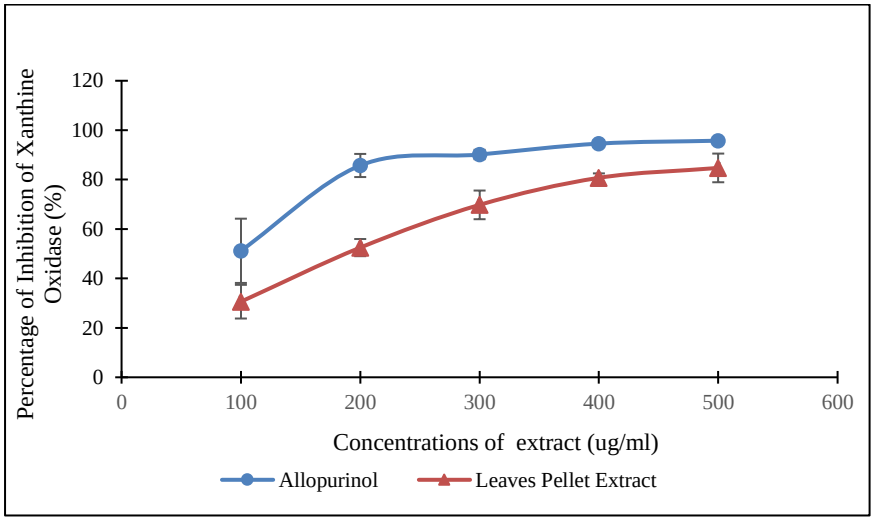

Figure 2. Xanthine oxidase inhibition by *Euphorbia hirta* extract

The IC_{50} of xanthine oxidase inhibition (XOI) and DPPH free radical inhibition

For the IC_{50} of XOI (Figure 3), the concentration of allopurinol needed to inhibit XO at 50% was 6.94 ± 0.32 µg/mL, while that of leaves pellet of *Euphorbia hirta* was 9.40 ± 0.15 µg/mL. This indicated that allopurinol required lesser dose than the leaves pellet extract do to inhibit XO at 50% inhibition response, which was expected because allopurinol has a structure similar to that of purines and pyrimidines. This means that it has to compete with xanthine for the metabolism process, hence, leading to a decrease in uric acid production [19]. The low dose of leaves pellet needed for half-maximal inhibitory on XO also ascertained that the leaves pellet may have contained compounds that were capable of inhibiting XO. The structure of flavonoids and its role as an XO inhibitor was studied, revealing that the hydroxyl groups at C-5 and C-7, as well as the presence of double bond between the C-2 and C-3 structures, had contributed to the high inhibitory activity of XO [5].

For the IC_{50} of DPPH free radical inhibition (Figure 4), the IC_{50} of gallic acid (3.47 ± 0.47 µg/ml) was lower than that of the leaves pellet (10.56 ± 0.58 µg/mL). This confirmed that only a small concentration of gallic acid required in order to inhibit the free radicals at 50%. However, gallic acid, which is known to be a pure synthetic antioxidant, usually displays lower IC_{50} values compared with that of other extracts. The small value of IC_{50} required by the leaves pellet also substantiated its anti-oxidative effect. Previous research has established that *Euphorbia hirta* has an antioxidant effect by demonstrating its dose-dependent reducing power due to the presence of phenolic compounds, such as flavonoids [18, 20].

Correlation study

The coefficient correlation between xanthine oxidase inhibition and DPPH free radical inhibition activities was determined at 0.9212, which indicated a strong correlation due to the value being very close to 1.000 (indicative of positively correlated). This confirmed that when the antioxidant activity increased, the XO inhibitory activity also increased as shown in Figure 5. The presence of flavonoids, such as quercetin and myricetin, is known to have effects on XO, as well as on the additional superoxide scavenging activity, which could have resulted in the antioxidant effect [21].

Kinetic study of enzyme

DMSO was used as the negative control, in which its V_{max} in the assay demonstrated the highest maximum reaction rate at 0.063 abs/min; whereas allopurinol as the positive control in this assay displayed the lowest maximum reaction rate, V_{max}, at 0.020 abs/min (Table 1 and Figure 6). This is followed by the leaves pellet of *Euphorbia hirta* at 0.050 abs/min. The high V_{max} value of DMSO indicated a higher content of uric acid in comparison to that of allopurinol and the leaves pellet of *Euphorbia hirta* extract; while the low V_{max} value of allopurinol indicated a low formation of uric acid and low detection of its content via the UV-Vis spectrophotometer. The low formation of uric acid by allopurinol and leaves pellet of *Euphorbia hirta* extract ascertained that there was an inhibition mechanism by the compounds that prevented XO from forming the uric acid. The K_m values of allopurinol, leaves pellet of *Euphorbia hirta*, and DMSO were 11.618, 11.618, and 11.614 µM, respectively. This shows that there were no changes in the K_m values of the extracts. The screening of kinetic parameters of the extracts exhibited a mixed-inhibition mode by the leaves pellet of *Euphorbia hirta* extract, as the non-competitiveness of inhibition mode will only lower the V_{max} values but with no influence on the K_m values [22].

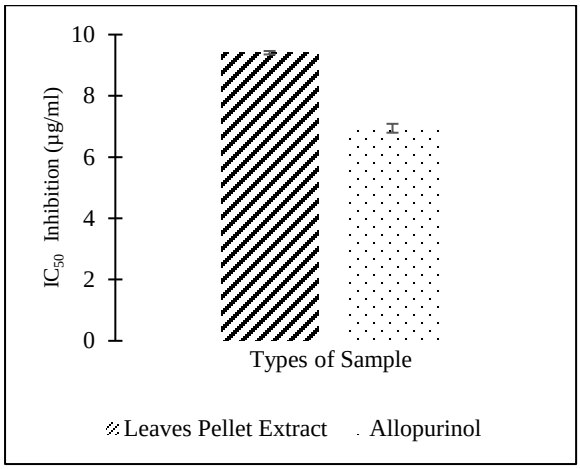

Figure 3. IC_{50} of xanthine oxidase

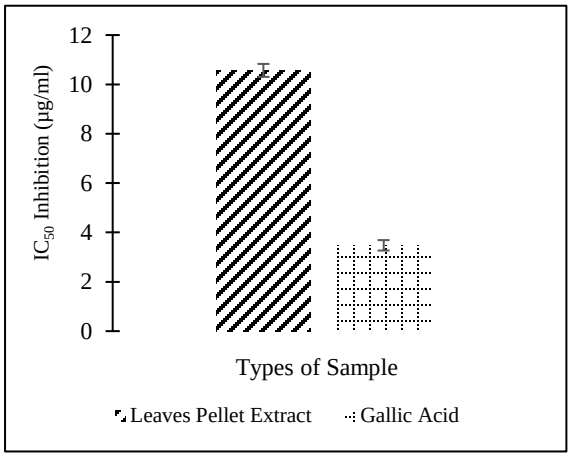

Figure 4. IC_{50} of DPPH

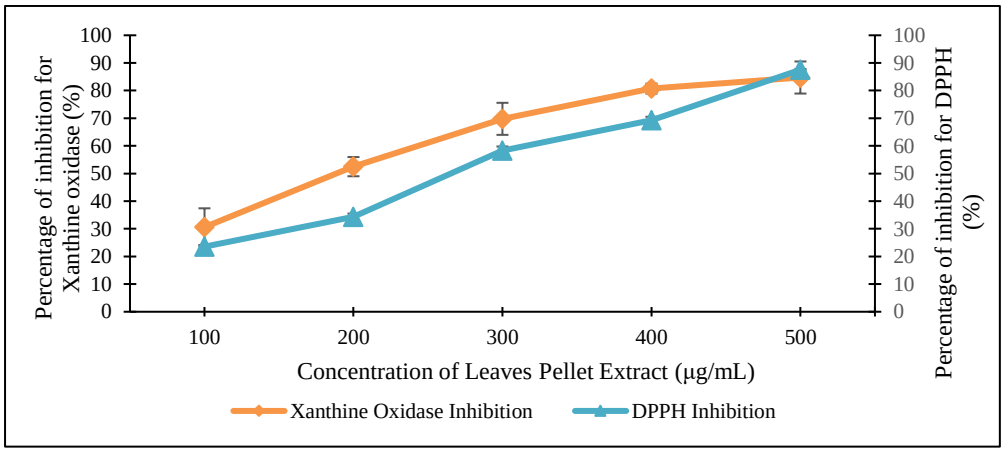

Figure 5. Correlation graph between xanthine oxidase inhibition (XOI) and free radical inhibition of the leaves pellet extract

Table 1. Enzyme Kinetic Parameters

Sample	V_{max} (abs/min)	K_m (µM)
Leaves pellet of *Euphorbia hirta* extract	0.0503	11.6182
Allopurinol (positive control)	0.0207	11.6187
Dimethyl sulfoxide (negative control)	0.0631	11.6140

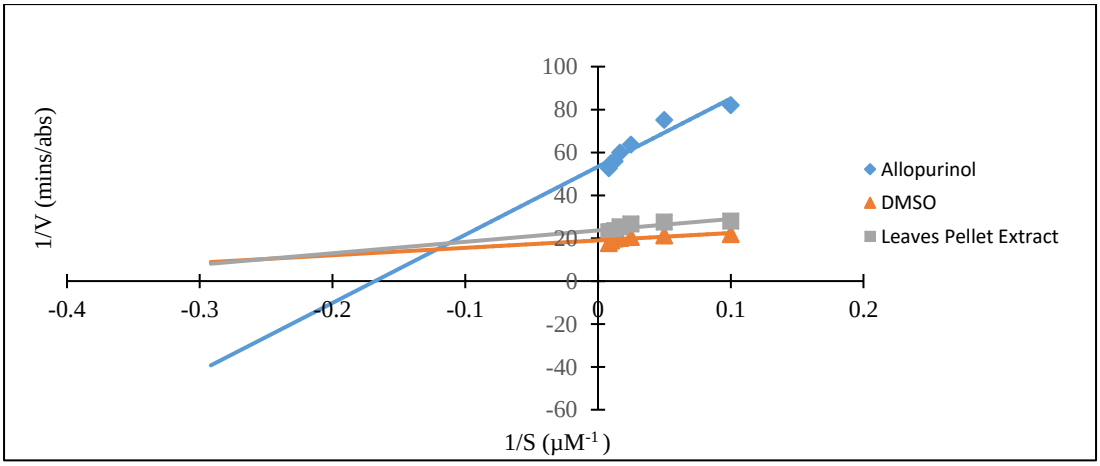

Figure 6. Lineweaver-Burk Plot

Conclusion

This study reveals that the leaves pellet of *Euphorbia hirta* indicated the highest free radical inhibition (87.95 ± 0.006%) and IC_{50} of the leaves pellet. The IC_{50} of the leaves pellet was 10.56 ± 0.58 µg/mL, while that of the gallic acid was 3.4779 ± 0.4736 µg/mL. Meanwhile, the IC_{50} values of the leaves pellet with allopurinol and gallic acid were 9.40 ± 0.13 and 6.94 ± 0.32 µg/mL, respectively. The close values between both assays showed great ability of the extract in inhibiting XO. The correlation value between both effects was 0.921, which is very close to 1.0; thus, this proved the direct relationship of both effects. From the Lineweaver-Burk plot and enzyme kinetic parameters obtained, the leaves pellet of *Euphorbia hirta* also signified a mixed-inhibition mode by comparing allopurinol and the absence of inhibitors. These results have substantiated the potential application of the *Euphorbia hirta* plant as both a xanthine oxidase inhibitor and a free radical inhibitor.

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