

MALAYSIAN MEDICINAL PLANTS (*Euphorbia milii*) AS A DRUG ALTERNATIVE SOURCE FOR ANTI-GOUT THERAPY

(Tumbuhan Perubatan Malaysia (*Euphorbia milii*) sebagai Sumber
Ubat Alternatif untuk Terapi Anti-gout)

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Abstract

Gout has been detected as amongst the oldest disease since the 5th Century BC and to date it is arising. This disease occurs due to excess production of uric acid that leads to the formation of crystals around the joints. It may become worse when permanent tissue damage is involved. Nowadays, many advance treatments are found. However, it may be very costly. One of the cheapest alternatives is a synthetic drug called allopurinol, which has negative side effect for long term consumption. *Euphorbia milii* (Euphorbiaceae) was chosen as the sample due to various secondary metabolites being reported which might be beneficial for treatment of gout. Quantitative analysis of total phenolic content and xanthine oxidase inhibitory assay were done to the crude extracts of Malaysian *E. milii*, followed by IC₅₀ to determine the concentration needed to reduce 50% of uric acid production. The result showed that methanol leaves extract of *E. milii* had the highest total phenolic content (0.77 ± 0.02 mg QAE/g of sample), which made this extract a potent uric acid inhibitor (Half maximal inhibitory concentration, IC₅₀ = 0.0864 mM). This value had significantly reduced uric acid production by 65.6%. Therefore, methanolic leaves extract that was subjected to GC-MS and hexadecanoic acid and has anti-gout properties, was discovered in the crude extract. In conclusion, the Malaysian *E. milii* plant has potential to be developed as a new drug which might be beneficial for the treatment of gout.

Keywords: *Euphorbiaceae*, Malaysian plant, quantitative analysis, anti-gout, uric acid

Abstrak

Gout telah dikesan sebagai antara penyakit tertua sejak abad ke-5 SM dan ia terus wujud hingga kini. Penyakit ini berlaku akibat pengeluaran asid urik berlebihan yang menyebabkan pembentukan kristal di sekeliling sendi. Ia mungkin menjadi lebih teruk apabila melibatkan kerosakan kekal pada tisu. Kini, banyak rawatan awal telah ditemui. Walau bagaimanapun, ia mungkin sangat mahal. Salah satu alternatif yang paling murah ialah ubat sintetik bernama allopurinol yang mempunyai kesan sampingan negatif untuk penggunaan jangka panjang. *Euphorbia milii* (Euphorbiaceae) telah dipilih sebagai sampel kerana pelbagai metabolit sekunder yang mungkin bermanfaat untuk rawatan gout telah dilaporkan. Analisis kuantitatif jumlah kandungan fenolik dan ujian perencatan xanthine oxidase telah dilakukan terhadap ekstrak mentah *E. milii* Malaysia diikuti dengan IC₅₀ untuk menentukan

kepekatan yang perlu untuk mengurangkan 50% daripada pengeluaran asid urik. Hasilnya, ekstrak metanol daun *E. milii* menunjukkan jumlah kandungan fenolik yang tertinggi (0.77 ± 0.02 mg QAE/g sampel) yang menjadikan ekstrak ini berpotensi sebagai perencat asid urik (Kepekatan perencatan separuh maksimum, $IC_{50} = 0.0864$ mM). Nilai ini mengurangkan pengeluaran asid urik dengan ketara sebanyak 65.6%. Oleh itu, ekstrak metanol daun kemudiannya tertakluk kepada GC-MS dan asid heksadekanoik serta mempunyai sifat anti-gout, telah dikenal pasti wujud dalam ekstrak mentah. Kesimpulannya, tumbuhan *E. milii* Malaysia ini berpotensi untuk dibangunkan sebagai satu ubat baru yang mungkin bermanfaat untuk rawatan gout.

Kata kunci: *Euphorbiaceae*, tumbuhan Malaysia, analisis kuantitatif, anti-gout, asid urik

Introduction

Gout has been alarming worldwide and it is now the most common type of inflammatory arthropathy in adults. This disease is caused by excess uric acid levels in blood due to oxidative stress that increases the production of xanthine oxidase incorporation to increase the uric acid production. This condition results in the deposition of urate (as monosodium urate monohydrate) crystals around joints, which causes an acute inflammatory response and induces a permanent tissue damage that is characterized by the appearance of ulceration of the joint cartilage, marginal osteophytosis, erosive lesions and chronic inflammation of synovial membrane [1, 2]. The most common urate lowering treatment is the development of xanthine oxidase (XO) inhibitor. Allopurinol is one of the best and the most widely used as XO inhibitor. However, prolonged usage of Allopurinol can cause negative side effects to the patient such as nephrolithiasis, hypersensitivity reaction, Stevens-Johnson syndrome, renal toxicity, allergic reactions, and fatal liver necrosis [3].

Flavonoids are secondary metabolites that are responsible to give color to plants. In medicinal aspect, it can also act as anti-oxidative, anti-inflammatory, anti-mutagenic, antifungal, antiviral, antibacterial activity and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme function [4]. Moreover, flavonoids are known to inhibit a number of enzymes such as phosphodiesterase, Ca^{2+} ATPase, aldosereductase, lipoxygenase, cyclooxygenase [5], and human P450 enzymes. Recently, studies on flavonoids against xanthine oxidase (XO) had become a trend. The study by Yuan et al. [6] revealed that different structure of flavonoids would affect the binding process which affected the inhibition of xanthine oxidase. Research by Suyun Lin et al. [7] proposed that amongst the 20 flavonoids used in their project can be categorized into three groups based on activeness. The first group was composed of the most active flavonoids with low α values (≤ 10), which were chrysin and genistein (α values

of 0.49 and 0.59, respectively). The first group had α values of below 1, which indicated that only compounds in small amount was needed to inhibit XO as compared to allopurinol. Additionally, quercetin ($\alpha = 9.76$) and kaempferol ($\alpha = 5.76$) were less active than apigenin ($\alpha = 1.20$). The second group was referred as flavonoids that expressed intermediate activity ($10 < \alpha \leq 50$). Most of them were flavonols, such as morin, myricetin, diosmetin, isorhamnetin, and hyperin. In the last group ($\alpha > 50$), as compared to rutin ($\alpha = 57.29$) and baicalin ($\alpha = 50.13$), naringenin and silybin, exhibited much weaker inhibition ($\alpha = 140.4$ and 189.6, respectively). Nonetheless, phloretin, curcumin, and epicatechin did not even show XO inhibition activity [8, 9].

Therefore, this study is designed to identify potential xanthine oxidase inhibitor properties contained in *Euphorbia milii* Malaysian species as it is rich in natural bioactive compound. *Euphorbia milii* Des Moul. ex Boiss var. *hislopii* is a decorative, cactus-like plant that contains milky sap and the branches and stems are covered with thick sharp black thorns. This plant is native to Inselberge, which is located in the central Plateau of Madagascar, Africa and it is also found in tropical and subtropical countries [10]. Phytochemical studies of methanolic extract of *E. milii* by Delgado et al. [11] revealed the presence of β -sitosterol, cycloartenol, β -amyirin acetate, lupeol, euphol, and flavonoid. Meanwhile, there were two triterpenoids (Taraxerol and 28-hydroxyfriedelan-1, 3-dione-29-oic acid), one flavone (Quercetin 3-O-(2''-O-galloyl)-a-L-arabinofuranoside) and two phenolic compounds (7,7'-dihydroxy, 8, 6'-bicoumarin and 9-acetyl-3',4'-dimethoxy dehydroconiferyl-3-alcohol) from the red flowers of *E. milii* that were isolated by Kamurthy et al. [12]. Based on literature review, *E. milii* was expected to show positive results towards the XO inhibitor properties which might give benefits as a safer approach for the prevention and treatment of gout [13]. Table 1 shows the taxonomic hierarchy of *E. milii* and its origin. Meanwhile, Figure 1 shows the *E. milii* plant used.

Table 1. Taxonomic hierarchy of *Euphorbia milii* [13]

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Family	Euphorbiaceae
Genus	<i>Euphorbia</i>
Scientific Name	<i>Euphorbia milii</i> Des Moul
Others Name	Christ plant, Christ thrown (Brazil) Chrown of Thorn (Malaysia)
Origin	Plateau of Madagascar, Africa



Figure 1. *Euphorbia milii* plant (flowers and leaves)

Materials and Methods

Plant identification

Plant material (flowers and leaves) were collected from Kota Tinggi, Johor and identified by Tuan Haji Muhamad Razali Salam, a senior lab assistant of Biological laboratory, Faculty of Science and Marine Environment, Universiti Malaysia Terengganu.

Sample preparation

Each sample was washed and dried at room temperature until it was completely dried. About 40 g of dried flower samples and 40 g of dried leaf samples were ground and extracted by using three different solvents with increasing polarities (hexane, ethyl acetate, and methanol) at room temperature. Then, each extract was concentrated by using a rotary evaporator to obtain the crude extracts.

Determination of total phenolic content

This test was performed by using Folin–Ciocalteu method that was developed by Chun and his teams with some modifications in 2013 [14]. A calibration curve was established by using gallic acid (0.01 to 0.05 mg/mL) as standard references plotted. TPC was revealed as mg GAE/ g sample [15].

Preparation of calibration curve of standard uric acid

Standard uric acid (1 mM) was prepared by dissolving 1.69 mg of uric acid with a few drops of 0.2 M sodium hydroxide (NaOH) and added with distilled water until the calibration mark of 10 mL volumetric flask. Then, a series of concentrations of uric acid solution were prepared (0.01 mM to 0.2 mM) and the absorbance was recorded by using UV-Vis spectrophotometer (Shimadzu, UV- Vis 1800) at a wavelength of 295 nm.

Xanthine oxidase inhibitory activity

This test was carried out by referring to the method reported by Boumerfeg et al. [16] with some modification. The reaction mixture consisted of 1500 μ L phosphate buffer solution, 300 μ L of distilled water, 300 μ L of 0.1 mg/mL extract solution and 300 μ L of enzyme solution. After 5 min of incubation, 600 μ L of xanthine solution was added to the mixture. The absorbance of the kinetic assay was measured immediately by using UV-Vis spectrophotometer (Shimadzu, UV- Vis 1800) at 295 nm at an interval of 2 min for 30 min. The blank was prepared by using the same method but the enzyme was replaced with a buffer solution.

A standard reaction mixture was prepared in the same way as the sample reaction mixture. However, the extracts solution was replaced with 0.1 mg/mL of

positive control of Allopurinol. Meanwhile, for negative control (blank) the extracts solution was replaced with distilled water. The percentage of XOI assay was calculated by using the following formula:

Concentration of uric acid produced for every 2 min of reaction (G),

$$(G) = \frac{(D-E)}{F} \quad (\text{eq. 1})$$

where, D is absorbance of each sample, E is blank absorbance of each sample and F is slope from the calibration curve of standard uric acid.

Production rate of uric acid (activity of enzyme) (A) is,

$$(A) = \frac{B}{2} \quad (\text{eq. 2})$$

where, B is slope (gradient) from G for 30 min of reaction.

Percentage of inhibition is,

$$\% \text{ inhibition} = \left(1 - \left(\frac{C}{A}\right)\right) \times 100 \quad (\text{eq. 3})$$

where, A is absorbance of control at 30 min and C is absorbance of sample at 30 min.

IC₅₀ evaluation for methanol leaves extract on XOI activity

The sample and positive control of Allopurinol were repeated as the xanthine oxidase inhibitory activity method. However, five different concentrations (0.02 mg/mL - 0.1 mg/mL) were used to evaluate the inhibitory percentage at 50%. The equation was calculated by using natural Log dose inhibition curve.

Gas chromatography - mass spectroscopy (GC-MS) analysis

Methanolic leaves extract of *E. milii* was subjected to GC-MS analysis by using GCMS-QP2010 Ultra (Shimadzu Co., Japan) equipped with SLB™ – 5ms (Fused Silica Capillary Column; 30m x 0.25mm x 0.25µm film). Electron impact mode of 70 electron volt (eV) was used for ionization in the GC-MS analysis. The

analysis was carried out by injecting 1µL of sample with split ratio of 10 with inert helium gas (99.99%) as carrier gas. The initial oven temperature was maintained at 50°C for 1 min, then increased up to 320 °C and held for 5 min. The injection was performed on the split-less mode with the injector port temperature maintained at 300 °C. The chromatograms were recorded at range 50 600 m/z MS - 600 m/z MS scan mode and the compounds were identified by comparing the molecular weight, intensity of peak and peak mass with NIST library and HMDB library.

Results and Discussion

In this research, the flowers and leaves of *Euphorbia milii* were selected as samples and underwent an extraction method by using three different solvents (hexane, ethyl acetate and methanol) to obtain the crude extracts. Then, the crude extracts underwent a total phenolic content test to determine the presence of phenolic compounds from the crude extracts.

Methanol leaves extract had the highest phenolic content (0.77 ± 0.02 mg GAE/g of sample), followed by methanol flowers extract (0.51 ± 0.03 mg GAE/g of sample), ethyl acetate leaves extract (0.17 ± 0.00 mg GAE/g of sample) and ethyl acetate flowers extract (0.07 ± 0.00 mg GAE/g of sample). Nonetheless, the lowest phenolic content was discovered in the hexane extracts; leaves (0.04 ± 0.01 mg GAE/g of sample) and flowers (0.05 ± 0.00 mg GAE/g of sample). Another study which used *E. splendida* also revealed that the methanolic extract showed the highest total phenolic content, which was 270.74 ± 0.005 mg GAE / g as compared to ethyl acetate extract (208.54 0.005 mg GAE / g sample) [17].

Referring to the plant parts, the highest phenolic content was contributed by leaves with 0.98 mg QAE/g of sample and followed by flowers (0.63 mg GAE/g of sample). A significant result was reported by Abu Arra et. al. [18], in which the leaves extract of *E. hirta* showed the highest amount of phenolic content (206.17 ± 1.95 mg GAE/g) as compared to flowers, roots and stems crude extracts. Figure 2 summarizes the estimation of phenolic contents in different crude extracts of *E. milii*.

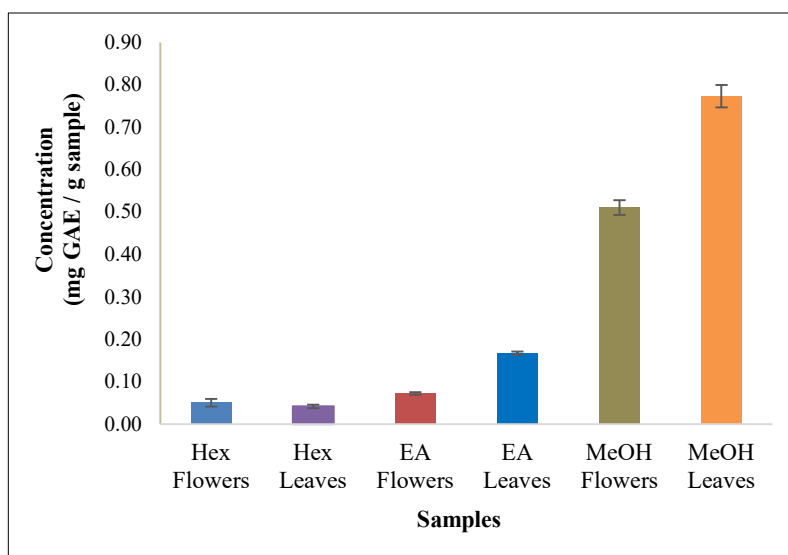


Figure 2. Estimation of total phenolic content in different crude extracts of *E. milii*

Xanthine oxidase inhibitor was tested on the ethyl acetate and methanol crude extracts which contain the highest phenolic and flavonoid content to identify the behavior of crude extracts in reducing the uric acid production [19, 20]. The reducing potential was measured by calculating the percentage of xanthine oxidase inhibition by using the absorbance recorded by UV-Vis spectrophotometer at wavelength 295 nm. Methanolic leaves extract showed the highest percentage of xanthine oxidase inhibition ($66.5 \pm 0.4\%$), followed ethyl acetate leaves ($33.3 \pm 4.3\%$), methanol flowers ($29.3 \pm 4.5\%$) and lastly ethyl acetate flowers ($12.2 \pm 1.4\%$). The standard allopurinol had $98.3 \pm 1.3\%$ XO inhibition. The results obtained were equivalence with the total phenolic and flavonoids content in *E. milii*. This result was in agreement with other researchers, in which it indicated that XO inhibition activity could be linked to the content and nature of flavonoids and phenolic compounds present in the extract [21]. Figure

3 shows the percentage of xanthine oxidase inhibition of the four crude extracts of *E. milii* and standard allopurinol.

Based on the result obtained, methanolic leaves extract and allopurinol were chosen to undergo IC_{50} test by using different concentrations ($0 \mu\text{g/mL}$ - $100 \mu\text{g/mL}$). The value of IC_{50} was obtained by using natural Log dose inhibition curve of a series of different sample concentrations against percent inhibition. Methanolic leaves extract showed a significant XO inhibitory activity with an IC_{50} value of $86.7 \mu\text{g/mL}$ (Figure 3). However, its activity was low and remained not as good as allopurinol with IC_{50} , $4.18 \mu\text{g/mL}$. The obvious gap of activity between the extract and allopurinol due to the concentration of active flavonoid and phenolic compounds in the extract was very low as compared to the mass of pure allopurinol compound.

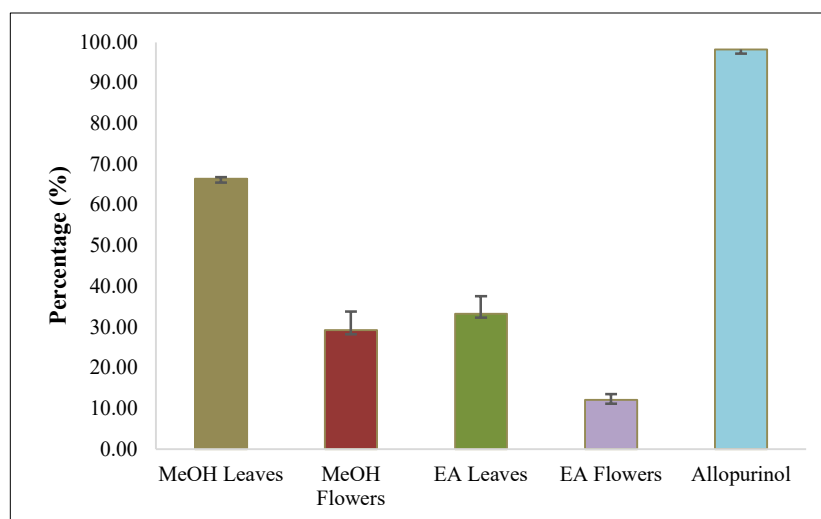


Figure 3. Percentage of xanthine oxidase inhibition of the four crude extracts of *E. milii* and the standard allopurinol

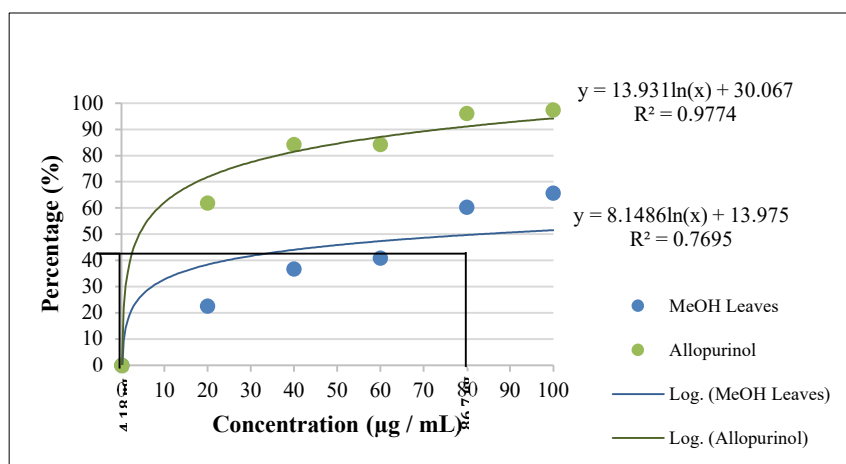


Figure 4. Percentage inhibition of IC₅₀

Therefore, methanolic leaves extract of *E. milii* was subjected to GC-MS in order to trace or identify phytochemicals that exist in the sample. The chromatogram obtained (Figure 5) revealed the presence of hexadecanoic acid [RT: 32.923, Peak Area (%): 14.79%, Molecular Formula: C₁₇H₃₄O₂] that possess anti-gout behavior. Hexadecanoic acid (Figure. 6) or also known as palmitic acid is one of the most common saturated fatty acid chains that can be found naturally in plant. Various studies had proven that hexadecanoic acid can act as an antifungal, antioxidant, antimalarial or a potent antimicrobial agent and inhibit the production of uric acid [22, 23]. According to Li and his team [24], oxidative stress played a crucial role in the pathogenesis of gout and the effective treatment was by lowering the

oxidative stress. This was supported by Zamudio-Cuevas et al. [25], monosodium urate (MSU) crystals were shown to induce renal cell apoptosis through a mechanism which involved reactive oxygen species (ROS) generation and led to loss of cartilage repair and tissue damage observed in gouty patients. In part of that, Trevisan et al. [26], suggesting that oxidative stress (OS) was responsible for acute gout attack due to overproduction of OS traced. In fact, Masuoka et al. [27], have identified the possible mechanism pathway which relate the compounds that are superoxide radical antioxidants and XO activity inhibitor. Hexadecanoic acid was identified as able to induce the formation of hydrogen peroxide, H₂O₂ (source of oxygen-derived free radical) [28, 29]. In referring to *Euphorbia* species, the

leaves of *E. cuneata* [30], essential oil from dried leaves of *E. hirta* Linn [31, 32] and the culture cell of *E. tirucalli* and *E. milii* showed the presence of

hexadecanoic acid as one of their phytochemicals. Table 2 shows a summary of the data obtained from the GC-MS and biological activity of hexadecanoic acid.

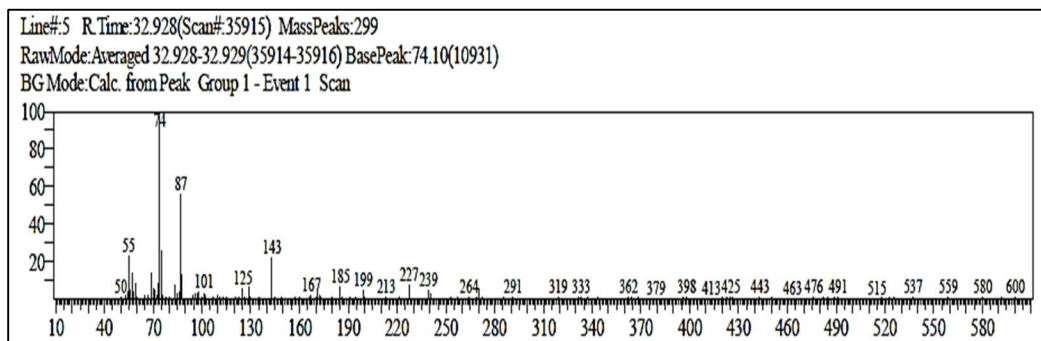


Figure 5. Mass spectra of hexadecanoic acid contained in methanolic leaves extract of *E. milii*

Figure 6. The molecular structure of hexadecanoic acid

Table 2. Name, retention time, percentage area, molecular weight, molecular formula, and biological activity of hexadecanoic acid

No	Name of Compound	Retention Time	Percentage Area (%)	Molecular Weight	Molecular Formula	Biological Activity
1.	Hexadecanoic acid	32.923	23.88	270	C ₁₇ H ₃₄ O ₂	Antifungal, antioxidant, antimalarial, potent antimicrobial agent, inhibit production of uric acid [22, 23]

Conclusion

This study revealed that the methanolic leaves extract of *E. milii* possess anti-gout properties because it significantly reduced uric acid production and had higher xanthine oxidase inhibitor as compared to other crude extracts. Moreover, the estimation of phenolic contents contained in the methanolic leaves extract was the highest amongst others. Therefore, the methanolic leaves extract of *E. milii* had higher potential to be used as a new medicine in the treatment of gout.

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