DOI: 10.31276/VJSTE.2024.0026

Further insight on the potential of *Gnetum gnemon* seed, roots of *Eurycoma longifolia*, *Morinda citrifolia* leaves, *Glycine max* nut and *Citrus hystrix* leaves as medicinal herbs with high antioxidant properties

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Received 13 June 2024; revised 26 June 2024; accepted 1 July 2024

Abstract:

The antioxidant, total phenolic, and flavonoid contents of *Gnetum gnemon*, *Eurycoma longifolia*, *Morinda citrifolia*, *Glycine max*, and *Citrus hystrix* were investigated and measured. Antioxidant scavenging activity was estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. Total phenolic content (TPC) was determined via the Folin-Ciocalteu method, while antioxidant capacity was assessed using the malondialdehyde (MDA) assay. The results from the FRAP and MDA tests revealed that *C. hystrix* exhibited the highest antioxidant activity, indicating its potential as a source of natural antioxidants. Notably, the antioxidant activity of all samples displayed distinct characteristics. In the DPPH and TPC tests, *G. gnemon* showed the highest activity compared to the other samples. However, *C. hystrix* demonstrated strong antioxidant potential in both tests, ranking third in the DPPH and second in the TPC assay. In conclusion, *Citrus hystrix* was identified as the most effective sample, exhibiting the highest antioxidant activity. These findings suggest that certain medicinal herbs may serve as valuable sources of natural antioxidants.

Keywords: ***Citrus hystrix*, *Eurycoma longifolia*, *Glycine max*, *Gnetum gnemon*, *Morinda citrifolia*.**

Classification numbers: **3.2, 3.3**

1. Introduction

Herbal medicine involves the use of plants containing active ingredients for medicinal purposes, aimed at treating diseases and promoting overall health and well-being [1]. In recent years, increasing attention has been directed towards herbal medicines due to their pharmaceutical potential and natural origins [2]. According to the World Health Organization, 80% of the global population uses herbal medicines for various medicinal purposes [1]. The medicinal use of plants is largely attributed to the abundance of flavonoids and other bioactive compounds, many of which are effective in treating a wide range of diseases [3, 4]. All plant organs, including roots, stems, buds, leaves, flowers, and fruits, contain alkaloids and phytochemicals [4]. Numerous bioactive compounds are recognised for their potential health benefits, particularly in exhibiting antioxidant, anticarcinogenic, antihypertensive, antimutagenic, and angiogenesis-inhibitory activities [5].

Oxidative stress, defined as an imbalance between antioxidants and oxidants that can cause cellular damage, has been proposed as a contributing factor to ageing and various diseases in humans [6]. Heart disease, neurological disorders, cancer, and the ageing process have all been linked to oxidative stress [6]. Oxidative stress occurs when the production of free radicals, or reactive oxygen species (ROs), exceeds the body's antioxidant capacity, particularly during metabolism or other processes [7]. In modern Western medicine, maintaining the balance between antioxidants and oxidants is regarded as crucial for preserving a healthy biological system [8].

Antioxidants are substances that, even in low concentrations relative to oxidisable substrates, can significantly delay or inhibit oxidation [9]. In addition to their health benefits, antioxidants are added to food products to prevent or delay oxidation caused by free radicals when exposed to environmental factors such as air, light, and temperature [9]. Many phytochemicals in plant

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extracts exhibit antioxidant activity, making medicinal plants an ideal source of natural antioxidants [10]. The potential of plants as natural sources of antioxidants is due to the phytochemical antioxidants they produce, such as carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acids, ascorbic acids, tocopherols, and tocotrienols [10]. Commonly used antioxidants include beta-carotene, ascorbic acid, and alpha-tocopherol, which are produced by plants for their basic survival [10].

For centuries, plants, particularly herbs, have been used in traditional medicine across various cultures worldwide [2]. Herbs such as *Gnetum gnemon* (*G. gnemon*), *Eurycoma longifolia* (*E. longifolia*), *Morinda citrifolia* (*M. citrifolia*), *Glycine max* (*G. max*), and *Citrus hystrix* (*C. hystrix*) are among the most popular traditional remedies, especially in Malaysia, Indonesia, Thailand, and Vietnam, due to their pharmacological properties [2].

In the present study, the antioxidant activity, total phenolic content, and total antioxidant capacity of five medicinal plant extracts-*G. gnemon* seed, *E. longifolia* root, *M. citrifolia* leaves, *G. max* nut, and *C. hystrix* leaves-were determined using crude and water extraction methods. Antioxidant activity was evaluated through the DPPH and FRAP radical scavenging methods. TPC was estimated using the Folin-Ciocalteu method, while antioxidant capacity was assessed using the multiple displacement amplification (MDA) method. These methods were employed to determine and correlate the antioxidant activities of the extracts.

2. Materials and methods

2.1. Extraction of G. gnemon seed, E. longifolia root, M. citrifolia leaves, G. max seed and C. hystrix leaves

The individual samples were prepared according to a previous study [11]. A total of 100 g of each sample was weighed using an electronic balance and placed in a beaker. Subsequently, 1000 ml of distilled water was added. The beaker was placed in a water bath and heated at 100ºC for 1 hour. The solution was then filtered and centrifuged at 2000 rpm for 15 minutes. The suspension was evaporated to obtain a powder, which was stored in a freezer at -20ºC until use.

2.2. Diphenyl picrylhydrazyl assay

In the diphenyl picrylhydrazyl radical scavenging assay, the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to estimate the antioxidant scavenging activity of various concentrations of the extracts [12]. Ascorbic acid (vitamin C) was used as a synthetic antioxidant, serving as a positive control [13]. Ethanol was used to prepare the DPPH solution, in which 1.7 mg of DPPH was dissolved in 10 ml of ethanol. In a microplate, 50 µl of the diluted sample extract was mixed with 100 µl of the DPPH solution, incubated in the dark for 30 minutes, and measured at 540 nm using a microplate reader [14]. The data were recorded, and the percentage of inhibition was calculated using the following formula:

$$Scavenging\ Activity\ (\%) = \frac{Accused - A\ sample}{A\ control} \text{ x } 100$$

where *A control* represents DPPH radical absorbance with ascorbic acid, and *A sample* represents DPPH radical absorbance with sample extraction.

2.3. Ferric reducing antioxidant power assay

The antioxidant capacity of iron reduction was measured using the FRAP assay [15]. The principle of this method is the reduction of iron (III)-tripyridyltriazine (Fe(III)-TPTZ) complex to the ferric form, which produces an intense blue colour in the presence of an antioxidant [16]. Ascorbic acid was used as a synthetic antioxidant, serving as a positive control, and ferrous sulfate was used as a standard to generate the standard curve [12]. A 96-well plate was loaded with 30 µl of diluted sample extract, followed by the addition of 200 µl of FRAP reagent. The mixture was briefly agitated using a microplate mixer. Absorbance was measured spectrophotometrically at 595 nm. The results for the fresh sample were expressed as moles Fe (II) equivalent per gram of sample. An increase in absorbance indicates greater reducing power [17]. The concentration range for the ferrous sulfate standard was a serial dilution from 1000 to 0 µM (1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, 1.0, 0 µM).

2.4. Total phenolic content assay

The TPC was determined using the Folin-Ciocalteu method, which measures the presence and quantity of phenolic compounds in each sample [18, 19]. Gallic acid was used to generate a standard curve, and ascorbic acid was used as a synthetic antioxidant and positive control. The standard was prepared by dissolving 0.05 mg of gallic acid in 1 ml of distilled water (dH_2O) to obtain a stock solution. The concentration range for the gallic acid standard was a serial dilution from 50 to 0 µM (50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098, 0.049, 0 µM). A total of 20 µl of the diluted sample extract, positive control, and standard were added to a 96-well plate and mixed with 10 µl of Folin-Ciocalteu reagent. After a 5-minute incubation at room temperature, 40 µl of 6% sodium carbonate (0.6 g of sodium carbonate dissolved in 10 ml distilled water) was added. The microplate was incubated for 2 hours at room temperature. The absorbance of the blue colour formed was measured at 595 nm using a microplate reader.

2.5. Malondialdehyde assay

In this study, the formation of lipid peroxides was measured using egg yolk homogenates as a lipid-rich medium with a modified thiobarbituric acid-reactive substance (TBARS) assay [20]. A 10 ml egg yolk solution was prepared by mixing 1 ml of egg yolk with 9 ml of distilled water. Falcon tubes containing the sample extract, normal control, positive control, and negative control were filled with 500 µl of diluted egg yolk. In addition, 50 µl of 52 mM ferric chloride ($FeCl_3$) (141 mg of ferric chloride dissolved in 10 ml of distilled water) was added to all tubes except the normal control. The test tubes were incubated at 37ºC for 1 hour. Following incubation, 100 µl of each sample was transferred to a new Falcon tube and mixed with 900 µl of distilled water or 1000 µl of TBARS reagent. The samples were incubated at 95ºC for 30 minutes and centrifuged at 4000 rpm for 15 minutes. A total of 200 µl of the sample supernatant was then added to a 96-well plate and measured at 540 nm using a microplate reader.

3. Results and discussion

The free radical DPPH produces a violet solution in ethanol and is stable at room temperature [19]. When antioxidants in the samples absorb hydrogen, the colour changes from dark purple to yellow, indicating antioxidant activity. The DPPH method is a rapid and straightforward way to evaluate antioxidants [19]. Fig. 1 shows that the percentage of DPPH inhibition in all samples is lower than that of the positive control, ascorbic acid. A higher inhibition percentage indicates a greater presence of antioxidants, leading to the reduction of DPPH's colour [21]. The degree of decolourisation provides insight into the samples' scavenging ability [21]. *G. gnemon* exhibited the highest DPPH inhibition, followed by *M. citrifolia*, *C. hystrix*, *E. longifolia*, and *G. max*, which showed the lowest inhibition.

G. gnemon is commonly cultivated for its leaves, flowers, and seeds [22]. The seeds of *G. gnemon* (GSE) are known for their health benefits, containing bioactive compounds with antioxidant and pharmacological properties [23]. GSE has demonstrated antimicrobial, antiallergic, antiangiogenic, and antitumor effects [24, 25]. It has also been shown to reduce serum uric acid levels in humans, with no serious adverse effects [24, 26]. Resveratrol in GSE plays a significant role as a free radical scavenger and antioxidant [27]. The extract of *G. gnemon* has been reported to scavenge DPPH radicals even after 5 hours of experimentation, suggesting that the delayed oxidative activity may be due to the donation of additional electrons to the single DPPH radical [28]. *M. citrifolia* extract showed the second-highest DPPH inhibition.

M. citrifolia is a tropical plant native to Asia, particularly Polynesia, where it has been used for almost 2000 years to treat conditions such as diabetes, hypertension, cancer, and eye diseases [29]. In recent decades, *M. citrifolia* has become a significant source of natural medicines, with various parts of the plant-including fruits, leaves, bark, and roots-exhibiting therapeutic properties [30]. *M. citrifolia* fruits contain antioxidants such as beta-carotene, terpenoids, alkaloids, ascorbic acid, and polyphenols like flavonoids [31]. The antioxidant activity of these compounds can also be assessed using the FRAP assay (Fig. 2).

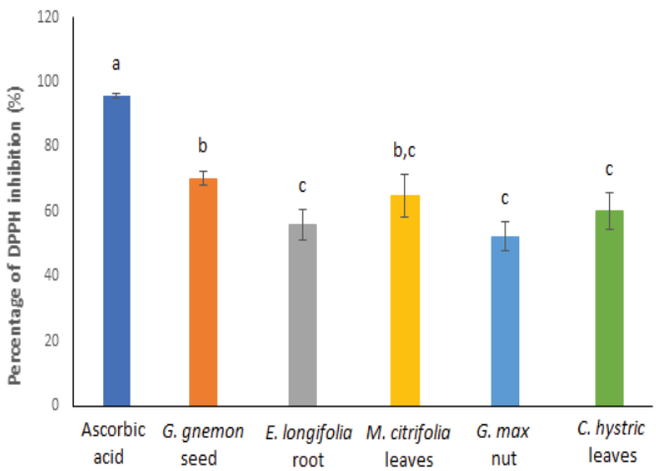

Fig. 1. The inhibition percentage of diphenyl picrylhydrazyl free radical scavenging of extracts compared to positive control (ascorbic acid). Results are expressed as mean±S.D. Bars with different letters are considered significantly different (p<0.05).

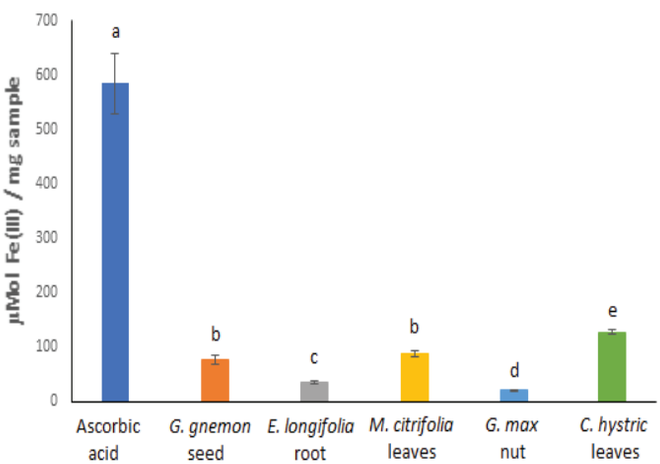

Fig. 2. The ferric reducing antioxidant power value of extracts compared to positive control (ascorbic acid). Results are expressed as mean±S.D. Bars with different letters are considered significantly different (p<0.05).

Figure 2 shows that all samples exhibited lower FRAP values than the positive control. In the FRAP test, the colour changes from green to blue depending on the reducing power of the compounds present [32, 33]. The ability to donate hydrogen atoms is crucial for the reducing power of antioxidants, as the reducing power of the extracts is concentration-dependent [34]. Reduction occurs when antioxidants donate hydrogen atoms, converting the ferricyanide complex (Fe^{3+}) to iron (Fe^{2+}) [35]. *C. hystrix* exhibited the highest FRAP value, followed by *M. citrifolia*, *G. gnemon*, *E. longifolia*, and *G. max*, which showed the lowest value, indicating that *C. hystrix* has a high antioxidant activity.

C. hystrix originates from Indonesia and has spread to countries such as Malaysia, Thailand, and other tropical regions of Asia [36]. Traditionally, *C. hystrix* has been used to treat fever, high blood pressure, abdominal pain, diarrhoea in infants, colds, and stomach disorders [37, 38]. The antioxidant activity of *C. hystrix* may be associated with its phenolic content. Previous studies have shown that increased antioxidant activity correlates with high phenolic content.

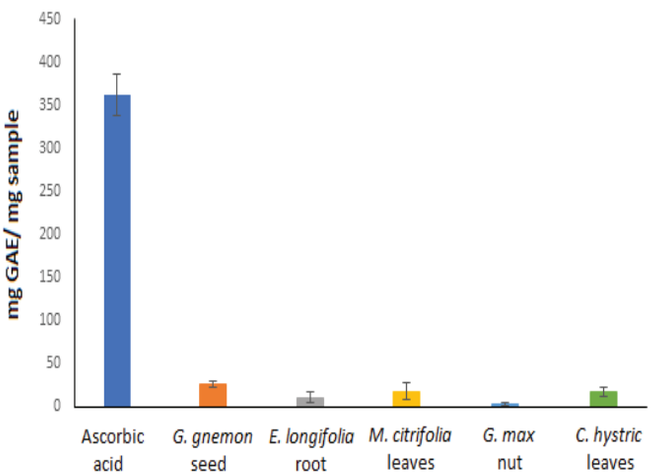

Fig. 3. Total phenolic content level of all extracts compared to positive control (ascorbic acid). Results are expressed as mean±S.D. Bars with different letters are considered significantly different (p<0.05).

The TPC assay, commonly used alongside DPPH and FRAP assays, provides additional information on the phenolic content of plant extracts [14]. Fig. 3 shows that all samples had lower TPC values than the positive control, ascorbic acid. *G. gnemon* exhibited the highest TPC level, followed by *M. citrifolia*, *C. hystrix*, *E. longifolia*, and *G. max*, which showed the lowest absorbance. The TPC assay measures phenolic compounds such as flavonoids, lignins, and tannins. Antioxidant activity in plants is influenced by variations in the structure of phenolic compounds, including their hydroxylation and methylation patterns, which may extend the lag time by interacting with lipoproteins [39]. The TPC assay correlates with DPPH scavenging activity, as higher antioxidant activity in the DPPH assay often reflects higher phenolic content [14]. Both *E. longifolia* and *G. max* showed lower TPC values, which corresponded to their lower DPPH inhibition and FRAP values.

E. longifolia is a popular tropical medicinal plant in Southeast Asia, especially in Malaysia, Vietnam, Java, Sumatra, and Thailand [40]. Known as "Tongkat Ali" in Malaysia, this plant has been used as a general health tonic to improve physical and mental energy and quality of life [41]. The root extract is primarily known for boosting testosterone levels in men [42]. Both *E. longifolia* and *G. max* offer various health benefits and high nutritional value due to their content of isoflavones, saponins, phytic acids, phytosterols, trypsin inhibitors, and bioactive peptides [43]. *G. max* has been shown to reduce the risk of non-communicable diseases (NCDs) such as cancer and cardiovascular diseases, as well as to delay lipid oxidation [44]. The extracts from *E. longifolia* and *G. max* were further tested for their ability to reduce lipid peroxidation.

In addition, assessing the ability of a plant extract to reduce lipid peroxidation is considered one of the most reliable ways to evaluate its antioxidant capacity under real-life conditions. Lipid peroxidation is a marker of oxidative damage in various organisms and results in the formation of derivatives such as malondialdehyde (MDA), which can be used to quantify the extent of damage in plant cells [45]. The MDA assay has become a preferred method for estimating lipid peroxidation, although other techniques, such as high-performance liquid chromatography or gas chromatography, can directly detect the presence of MDA [46]. Techniques like the TBARS test are more commonly used due to their simplicity and speed, offering a relatively straightforward and rapid spectrophotometric method that requires minimal sample preparation [44]. TBARS is a by-product of polyunsaturated fatty acid oxidation and forms a pink chromogen with a maximum absorbance of 532 nm when it reacts with two molecules of thiobarbituric acid (TBA) [46].

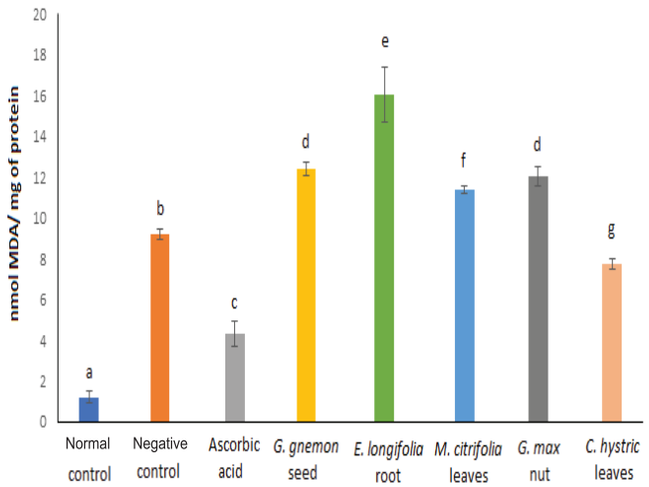

Fig. 4. Malondialdehyde level after treatment of samples. Results are expressed as mean±S.D. The bar with different alphabet is considered as significantly different ($p<0.05$).

Figure 4 shows that *C. hystrix* had the lowest absorbance value compared to the other samples and the negative control, indicating strong inhibition of oxidative activity. *M. citrifolia* showed the second-lowest values, followed by *G. max*, *G. gnemon*, and *E. longifolia*, which exhibited the highest MDA levels [15, 16]. These results indicate that all samples possess moderate or lower antioxidant capacity.

4. Conclusions

In summary, across the DPPH, FRAP, TPC, and MDA assays, *C. hystrix* consistently showed the highest antioxidant activity, suggesting that it contains bioactive compounds with significant antioxidant potential. Additionally, the extract was effective in reducing MDA concentrations compared to the negative control. The DPPH and TPC assays demonstrated that *G. gnemon* had the highest antioxidant activity among the samples. Further research is necessary to identify the bioactive components and explore the molecular pathways involved in antioxidant activity, as well as to conduct in vivo studies to determine efficacy before proceeding to clinical trials.

CRediT author statement

N.H. Mohd Kamal: Methodology, Data analysis, Writing; C.R. Muhammad Danial: Methodology, Writing; Z.Z. Zurain Syahira: Experimentation; M.Z. Zaridah: Writing; Chee Beng Jin: Writing; M. Azman: Experimentation; S.H. Andik Nurul: Experimentation.

ACKNOWLEDGEMENTS

This research was financially supported by the RMK11 Project, Natural Product Division, Cell Signaling and *In Vivo* Research Laboratory, Forest Research Institute Malaysia.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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