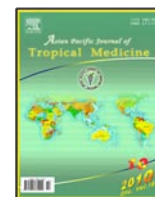




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Pharmacological activity, phytochemical analysis and toxicity of methanol extract of *Etilingera elatior* (torch ginger) flowersSubramanion Jo Thy Lachumy¹, Sreenivasan Sasidharan^{2*}, Vello Sumathy¹, Zakaria Zuraini¹¹School of Distance Education, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia²Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia

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ABSTRACT

Objective: To elucidate its pharmacological activities and medicinal potential of extract of *Etilingera elatior* (*E. elatior*). **Methods:** Phytochemical screening of the flower extract was done to determine the phytochemical in the extract. The pharmacological study included the determination of antimicrobial activity and minimum inhibitory concentration (MIC) of metabolic flower extract. The antimicrobial activity of the extract was tested against medically important bacterial, yeast and fungal strains. Apart from that, the methanolic extract of *E. elatior* flower was further tested *in vivo* toxicity using the brine shrimp lethality test. Moreover, the flower extract was qualitatively screened for their free radical scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. **Results:** The extract was effective on tested microorganisms and MIC values were in the range of 1.563–50.000 mg/mL. The brine shrimp lethality test exhibited no significant toxicity ($LC_{50} = 2.52$ mg/mL) against *Artemia salina*. The *E. elatior* flower extract with high LC_{50} value signified that this plant is not toxic to humans. While the phytochemical screening of the flower extract revealed the presence of the following compounds: flavonoids, terpenoids, saponin, tannins and carbohydrates whereas, alkaloids, anthraquinone and reducing sugars were absent. The concentration of the flower extract required for 50% inhibition of DPPH radical scavenging effect (IC_{50}) were 9.14 mg/mL and 8.08 mg/mL for butylated hydroxytoluene 8.08 mg/mL. **Conclusions:** These findings indicate that the extract of *E. elatior* flower possesses pharmacological properties and potential to develop natural products based pharmaceuticals products.

1. Introduction

Various edible plants have been used for cooking or have been eaten raw in daily life as an important therapeutic aid for various ailments. From ancient times, different parts of medicinal plants have been used to cure ailments caused by microorganisms such as bacteria, viruses and fungi. Moreover, the potential of higher plants as a source for new drugs is still largely unexplored. There is an abundant of medicinal plants throughout the world but only small amounts are investigated for its biological and pharmacological properties. Nevertheless, today there is widespread interest in drugs derived from plants. There is a wide range of medicinal plant parts which includes the

flowers, leaves, stem, fruits and roots extracts are used as powerful raw drugs possessing a variety of antimicrobial and healing properties. Discovery of new pharmaceutical agents from medicinal plants can combat the drastic increase in infectious diseases in many countries especially in rural areas and it has been used for economic reasons as well. The interest in medicinal plants reflects its recognition of the validity of many traditional claims regarding the value of natural products in health care[1]. Therefore, in order to determine the potential use of herbal medicine, it is essential to intensify the study of medicinal plants that find place in folklore[1, 2].

Etilingera is a genus of Indo-Pacific terrestrial and perennial herbs in the ginger family, Zingiberaceae, consisting of more than 100 different species native to Indonesia, Vietnam, Thailand, Malaysia and widely cultivated and neutralized in South East Asia. The commonly known species of *Etilingera* is *Etilingera elatior* (*E. elatior*) (torch ginger) is an edible plant which is

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widely used as cooking herbs or eaten raw for its medicinal properties and also used as an ingredient for local products like soap, shampoo and perfume. A total of 15 *Etilingera* species have been recorded in Peninsular Malaysia^[3]. The height of the plant is 3.6–4.7 m while all plant parts including flower bud, rhizome, stem and leaves have diverse biological and pharmacological properties with the potential to heal a wide range of illness, infections and other ailments. The showy pink flowers of *E. elatior* are very attractive and widely cultivated throughout the tropics as a spice for food flavouring and for ornamental purposes. In Peninsular Malaysia, the flower of the *E. elatior* is commonly known as *bunga kantan*, in Indonesia as *bunga kecombrang* or *honje* and in Thailand as *kaalaa*. They are commonly used as ingredients of dishes such as *laksa asam*, *nasi kerabu*, and *nasi ulam* in Peninsular Malaysia^[4]. In North Sumatra, the flower buds are used for a dish called *arisk ikan mas* (Szechuan pepper Spiced Carp). In Karo, it is known as *asam cekala*, and the flower buds are an essential ingredient of the Karo version of *sayur asam*, and are particularly suited to cooking fresh fish. In Malaysia, fruit of *E. elatior* are used traditionally to treat earache, while leaves are applied for cleaning wounds^[5]. According to Chan *et al*^[6], the leaves of *E. elatior* together with other aromatic herbs in water are used to remove body odour.

Apart from that, there are insufficient studies on *E. elatior* flower extract and studies must be conducted on this species in order to determine its activity as medicinal plants with antimicrobial agents against various microorganisms. The present study was designed to screen the methanol extract of *E. elatior* flower against several pathogenic bacteria and fungi in order to investigate new sources of antimicrobial agents and also to report on its antioxidant properties and phytochemical screening test. This study also investigates the toxicity of the methanolic extract using the brine shrimp assay. Toxicity studies also play an important role in identification and isolation of new compounds from crude extracts^[7]. The assay is considered as useful tool for preliminary toxicity assessment of plant extract^[8,9].

2. Materials and methods

2.1. Plant collection

The flowers of *E. elatior* (pink torch ginger) were collected from Gelugor, Penang Malaysia, in April 2010 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia where the herbarium was deposited. Then the flowers were washed under running tap water and dried in oven at 50 °C. The dried flowers were homogenized to fine powder and stored in airtight bottles.

2.2. Solvent extraction

One hundred fifty grams of dried powder was extracted with 400 mL of 80% methanol (v/v) for one week. Then, it was filtrated through No.1 Whatman filter paper and the

entire extract of *E. elatior* flower was evaporated under reduced pressure using rotary evaporator. The solvent was then evaporated at 50 °C in oven to get a paste form. Then it was sealed in Petri plates and stored at room temperature for further studies. The filtrate was then re-dissolved again in 80% methanol (v/v).

2.3. Test microorganisms and growth media

The following Gram-positive and Gram-negative bacteria, yeasts and molds were used for antimicrobial activity studies: bacteria included *Staphylococcus aureus*, *Bacillus thuringiensis*, *Escherichia coli*, *Salmonella*, *Proteus mirabilis* *Micrococcus* sp. and *Bacillus subtilis*; yeast included *Candida albicans*; molds included *Aspergillus niger*. The bacterial strains were grown in Nutrient Agar (NA) plates at 37 °C, whereas the yeast and molds were grown in Sabouraud Dextrose Agar (SDA) media, respectively, at 30 °C. Then stock cultures were maintained at 4 °C.

2.4. Antimicrobial disk diffusion assay

The extract was tested for antibacterial and antifungal activities by the disk diffusion method according to the National Committee for Clinical laboratory standards^[10]. NA and Sabouraud dextrose agar (SDA) sterilized in a flask and cooled to 45–50 °C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 mL). Then NA plates, containing an inoculum size of 10⁶ colony-forming units (CFU)/mL of bacteria or 2 × 10⁵ CFU/mL yeast cells or mold spores on SDA plates, respectively, were spread on the solid plates. The filter paper discs (6 mm in diameter), individually impregnated with 25 µL of extract at concentration of 100 mg/mL, was placed on the agar plates previously inoculated with test microorganisms. Similarly, each plate carried a blank disc by adding methanol solvent alone in the center to serve as a negative control and antibiotic discs (6 mm in diameter) of 30 µg/mL of chloramphenicol (for bacteria), and 30 µg/mL of Miconazole nitrate (for fungi) was used as a positive controls. All the plates were incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for fungi. The diameters of the inhibition zones were measured in millimeters. The sensitivity of the microorganisms to the extract was determined by measuring the size of inhibitory zones on the agar surface around the discs^[10]. All the tests were performed in triplicate.

2.5. Determination of minimum inhibitory concentration (MIC)

A 16 h culture was diluted in 10 mL of distilled water with reference to the 0.5 McFarland standards to achieve inocula of approximately 10⁶ colony forming units. A serial dilution was carried out to give final concentration between 0.10–100.00 mg crude extract per mL. The tubes were inoculated with 20 µL of bacterial suspension per mL of nutrient broth, homogenized and incubated at 37 °C. Then after incubation

50 μ L was withdrawn from each tube, inoculated on agar plates and incubated at 37 °C for 24 h. The MIC value was determined at the lowest concentration of crude extract in the broth medium that inhibit the growth of the test microorganism.

The MIC of the flower extract of *E. elatior* was determined by broth dilution method against *Artemia niger* (*A. niger*). The flower extract was serially diluted in Potato Dextrose Broth (PDB) to a final concentration of 0.10–100.00 mg crude extract per mL. The spore suspensions of *A. niger* was obtained from their respective 10 days stock culture, mixed with sterile distilled water to obtain a homogenous spore suspension of 1×10^8 spore/mL. Ten μ L of spore suspension was inoculated in the test tube containing PDB medium[10]. The minimum concentration at which no visible growth was observed were defined as the MICs, which expressed in mg/mL.

2.6. Toxicity testing against brine shrimp

2.6.1. Hatching shrimp

Brine shrimp eggs, *Artemia salina* (*A. salina*) were hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 L of distilled water. After 24 h incubation at room temperature (22–29 °C), the larvae was attracted to one side of the vessel with a light source and collected by pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater.

2.6.2. Brine shrimp assay

Toxicity of the extract was monitored by the brine shrimp lethality test[11]. Two mL of seawater was placed in all the bijoux bottles. A two-fold dilution of methanol extract was carried out with the artificial seawater to obtain the concentrations ranging from 50 mg/mL to 0.098 mg/mL. Potassium dichromate used as a positive control and was prepared by dissolving it in artificial seawater to obtain concentrations ranging from 0.1 to 0.9 mg/mL[12]. The last bottle was filled with sea salt water only to serve as a drug-free control or negative control. A hundred μ L of suspension of larvae containing about 10–15 larvae was added into each bottle and incubated for 24 h. The bottles were then examined and the number of dead shrimp in each bottle was counted. The total number of shrimp in each bottle was counted and recorded. The mean percentage mortality was plotted against the logarithm of concentrations. Lethal concentration (LC₅₀) was determined from the graph[13, 14].

2.7. Data analysis

The mean results of brine shrimp mortality against the logarithms of concentrations were plotted using the Microsoft Excel computer program, which also presents regression equations[15]. The regression equations were used to calculate the LC₅₀ value. Extracts giving LC₅₀ values greater than 1.0 mg/mL were considered to be nontoxic[16].

2.8. Phytochemical screening

The *E. elatior* flower extract was further subjected to determination of phytochemical constituents i.e. alkaloids, flavonoids, tannins, saponins, terpenoids, reducing sugar and anthraquinone using the methods of Evans *et al* and Parekh *et al*[17,18].

2.9. Determination of antioxidant activity

The quantitative measurement of free radical scavenging activities of the methanolic flower extract of *E. elatior* was carried out in a universal bottle[19, 20]. Each reaction mixture contained 50 μ L of test sample with concentration ranging from 0.031–2.000 mg/mL and 5 mL of 0.004% (w/v) of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) radical solution in methanol. The commercial antioxidant butylated hydroxytoluene (BHT, Sigma) was used as a positive control. The discolouration was measured at 517 nm after the incubation for 30 min in the dark condition. The 80% (v/v) methanol was used as a blank and DPPH (in 80% MeOH) used as control. Measurements were taken at least in triplicate. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A₀ was the absorbance of the control and A₁ was the absorbance of the tested sample (crude extract) in DPPH. The degree of discoloration indicates the free radical scavenging efficiency of the substances. The absorbance measurements were recorded on Spectrophotometer (HITACHI- U 1900 Spectrophotometer 200V).

3. Results

3.1. Antimicrobial activity

The data obtained from disk diffusion method was shown in Table 1. Maximum inhibitory activity was obtained against *Staphylococcus aureus* (23 mm), followed by *Bacillus thuringiensis* (21 mm), *Bacillus subtilis* (19 mm) and finally *Salmonella* sp. and *Proteus mirabilis* (18 mm). In contrast, *Escherichia coli* and *Micrococcus* sp. exhibited weak inhibition zones respectively 16 mm and 12 mm. Apart from that, antimicrobial activity of this extract was also observed against the yeast *Candida albican* (*C. albican*) (22 mm) and filamentous fungi *A. niger* (20 mm). The inhibition zone of solvent control methanol (negative control) was zero so that it was not active against all of the tested microorganisms. However, the two antibiotics 30 μ g/mL of chloramphenicol and miconazole nitrate (positive controls) were more effective than the flower extract of *E. elatior* with zones of inhibition ranging between 28 and 31 mm.

Furthermore based on the initial antimicrobial screening assay, the susceptible strains were selected for further studies to determine the concentration effect and MIC

Table 1Antimicrobial activity (zone of inhibition and MIC) of *E. elatior* flower extract compared with commercial antibiotic.

| Microorganisms | Zone of inhibition (mm) | | | MIC of the extract (mg/mL) ^a | |
|----------------|-------------------------------|---------------------------|--------------------|---|--------|
| | Extract(100 mg/mL) | Chloramphenicol(30 μg/mL) | Micanazole nitrate | | |
| Bacteria | | | | | |
| | <i>Staphylococcus aureus</i> | 23 | 28 | ND | 1.563 |
| | <i>Bacillus thuringiensis</i> | 21 | 31 | ND | 6.250 |
| | <i>Escherichia coli</i> | 16 | 30 | ND | 12.500 |
| | <i>Salmonella</i> sp. | 18 | 29 | ND | 12.500 |
| | <i>Micrococcus</i> sp. | 12 | 32 | ND | 50.000 |
| | <i>Bacillus subtilis</i> | 19 | 30 | ND | 25.000 |
| | <i>Proteus mirabilis</i> | 18 | 30 | ND | 25.000 |
| Yeast | <i>C. albican</i> | 22 | ND | 24 | 3.125 |
| Fungi | <i>A. niger</i> | 20 | ND | 23 | 1.563 |

^aThe values (average of triplicate) are diameter of zone of inhibition at 100 mg/mL extract and Broth dilution method, mean value $n = 3$.

values. The MIC values against all the tested strains ranged from 3.125– 50.000 mg/mL (Table 1) and were active against bacteria such as *Staphylococcus aureus*, *Bacillus thuringiensis* and fungal strains such as *Candida albicans*(*C. albicans*) and *A. niger* with MIC values of 1.563 mg/mL to 6.250 mg/mL. Meanwhile, the MIC value ranged from 12.500– 50.000 mg/mL for bacterial stains such as *Salmonella* sp., *Bacillus subtilis*, *Proteus mirabilis* and *Escherichia coli*.

3.2. Toxicity study

The result of the brine shrimp lethality bioassay showed the extract to be none toxic to brine shrimp (LC_{50} : 2.52 mg/mL at 24 hours) (Figure 1). Potassium dichromate served as the positive control. The LC_{50} value for the positive control at 24 hours was 0.4 mg/mL (Figure 2). Figure 3 showed the extract treated *A. salina*.

3.3. Phytochemical screening

The phytochemical screening of the flower extract of *E. elatior* shows the presence of the flavonoids, terpenoids, saponins, tannins, cabohydrates and absence of alkaloids, reducing sugars, anthraquinone in the extract.

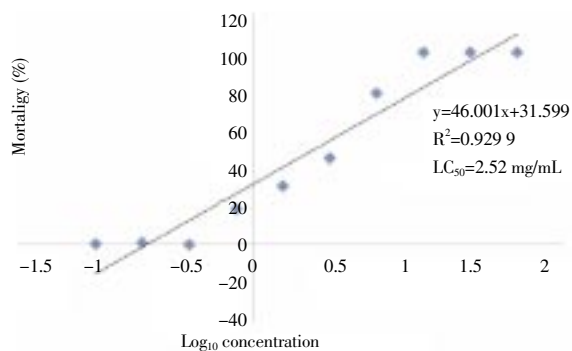


Figure 1. The toxicity effects of the *E. elatior* flower extract using brine shrimp lethality assay after 24 hours.

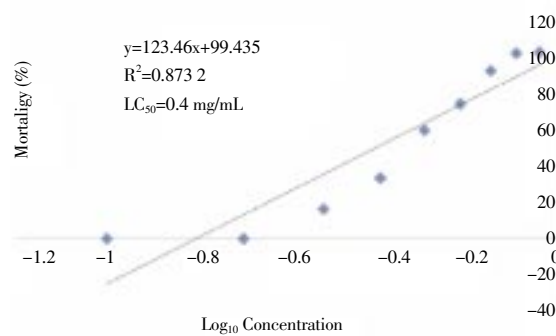


Figure 2. The toxicity effects of the potassium dichromate using brine shrimp lethality assay after 24 hours.



Figure 3. Light microscope micrograph of the *E. elatior* flower extract treated *A. salina*.

3.4. Radical scavenging (antioxidant) activity

The scavenging effects of the flower extract and BHT on DPPH radicals was 76.26% and 86.77% at 1.0 mg/mL, respectively. The methanol flower extract exhibited a significant dose dependent inhibition of DPPH activity with 50% inhibition (IC_{50}) at concentration of 9.14 mg/mL and IC_{50} value of BHT was 8.08 mg/mL.

4. Discussion

Plants are an important source of potential useful bioactive compounds for the development of new therapeutic agents. There are many reports available on the antibacterial, antiviral, antifungal properties of plants^[21–23]. Thus, these observations have helped in developing new drugs for the therapeutic use in human beings. However, not many studies are available on the antibacterial or antifungal property of *E. elatiora*. Hence, here we report the pharmacological activities of *E. elatiora*.

In the present study, we have investigated the potential of the of flower extract of *E. elatiora* as a source for antimicrobial and antifungal agents. The results in this study revealed that the methanolic extract of flower of *E. elatior* possesses great *in vitro* potential for antimicrobial activity to varying degrees against all microorganisms tested. The data obtained from disk diffusion method indicates that Gram-positive bacteria are more susceptible than Gram-negative bacteria towards the flower extract. These differences could be due to the nature and level of antimicrobial agents present in the flower extract, their mode of action and the typical differences in the cell wall between the strains^[24]. Our results also showed that some of the susceptible strains selected have a high range of MIC values. This could be possible due to the fact that the compounds responsible for displaying their antimicrobial activities that present in the flower extract at different concentrations and were not enough to exhibit their antimicrobial action at a low concentration of extract^[25].

In toxicity evaluation of plant extracts by the brine shrimp bioassay, an LC₅₀ value of more than 1 mg/mL is considered non-toxic^[11]. Therefore, they can be classified as biologically safety compounds with pharmaceutical properties. In our study, the *E. elatior* flower extract exhibited no significant toxicity against brine shrimp with an LC₅₀ value 2.52 mg/mL (24 h). Thus, this signified that *E. elatior* flower might not be toxic to *A. salina*. *E. elatior* flower extract was not toxic against brine shrimp therefore it can be used as an antimicrobial agent in known dosage with further *in vivo* evaluations. The brine shrimp bioassay can be useful as a quick, simple and low cost test for predicting the toxicity of the plant extract in order to consider the safety of the therapeutic agents to human beings^[26].

E. elatior flower has been used in traditional medicine and main flavouring ingredient in cooking since ancient times in many countries. This could be due to the presence of the compounds as revealed in this study. Apart from that, the presence of saponins has abundant of medicinal use as expectorants, and for treatment excessive salivation, chlorosis and migraines^[27]. Furthermore, it can be used as Ayurvedic medicine for removing body odour. Presence of tannins also has wide variety of usage as antibacterial, antiviral and antiparasitic^[28–30]. Both flavonoids and tannins are phenolic compounds that act as primary antioxidants

or free radicals scavengers^[31]. Apart from that, since the flower has a strong-smelling and pungent tasting thus it has been widely used as main ingredient in cooking, traditional medicinal use and also in local products. Moreover, the presence of this chemical compound further confirmed that the flower extract use as a traditional remedy for headaches or stomach aches, and is commonly applied externally to relieve itching and treat skin problems and also the biological activities observed in this study.

The DPPH test provides information on the reactivity of the test compounds with stable free radicals. The DPPH assay is often used to evaluate the ability of antioxidants to scavenge the free radical from test samples. Whereby, the free radicals cause biological damage through oxidative stress and such process lead to many disorders like neurodegenerative diseases, cancer and AIDS^[32]. Therefore, DPPH assay is an effective method to measure their scavenging power. The principle of the DPPH assay is based on the colour changes from purple (DPPH solution) to yellow^[33]. The colour changes can be measured quantitatively by spectrophotometer absorbance at 517 nm. There is no significant difference between the flower extract and the commercial antioxidant (BHT) thus, this further confirm that *E. elatior* flower extract is a comparable antioxidant to BHT. In addition, this finding will help to develop new drugs for medicinal uses such against aging and other diseases related to radical mechanisms^[34–37].

The results of the present investigation clearly indicate that the flower extract of *E. elatior* possesses broad spectrum antibacterial and antifungal activity. Also revealed that, it has good antioxidant properties and possessing all the necessary phytochemical constituents. In addition *E. elatior* flower is not toxic to *A. salina*. Moreover, this plant extract has been widely used as main ingredient in traditional medicine. Hence, *E. elatior* flower extract could be of considerable interest to the development of new drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

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