In vitro-scientific evaluation on anti-*Candida albicans* activity, antioxidant properties, and phytochemical constituents with the identification of antifungal active fraction from traditional medicinal plant *Couroupita guianensis* Aubl. Flower

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**ABSTRACT**

**Background:** *Couroupita guianensis* Aubl. has many therapeutic uses in the practice of traditional medicine.

**Objective:** The current research was conducted to evaluate the anti-*Candida albicans* activity, antioxidant properties, and phytochemical constituents with the identification of antifungal active fraction from *C. guianensis* flower.

**Methods:** Anticandidal, antioxidant activities, and determination of total phenolic contents (TPCs) of *C. guianensis* flower extract were carried out. Identification of antifungal active fraction was done by using solvent partitioning technique.

**Results:** The extract inhibited *C. albicans* with a minimum inhibitory concentration value of 12.5 mg/ml. Time-kill assay suggested that *C. guianensis* flower extract had completely inhibited *C. albicans* growth and also exhibited prolonged antiyeast activity. The alterations in morphology and complete collapse of the yeast cells after 36 hours of exposure to the extract were observed through microscopic observations. Ethyl acetate fraction was considered as an active fraction on the basis of zone of inhibition by solvent partitioning technique. Phytochemical analysis of the extract showed the presence of major classes of phytochemicals alkaloids, phenolic compounds such as flavonoids, tannins, steroids, glycosides, and saponin. The extract exhibits antioxidant activity with an Inhibitory Concentration (IC₅₀) value of 93.2 ± 0.011 µg/ml in the 2,2-diphenyl-1-picrylhydrazyl assay and 46.48 ± 0.13 µg/ml in the Hydrogen peroxide scavenging activity (HPSA) assay with TPCs of 32.2 ± 0.22 mg of gallic acid equivalents/100 g of extract.

**Conclusions:** The extract of *C. guianensis* flower with good anticandidal and antioxidant activities could be an effective agent to treat the *Candida albicans* infection.

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**Introduction**

The new-fangled drug resistance to human pathogenic fungus is repetitively being reported from all over the world [1]. Nonetheless, this situation is a threat in developing as well as developed countries [2]. Resistance to the antifungal drug has taken to undesirable implication for mortality, morbidity, and healthcare in the community. In Malaysia, HIV/AIDS cases have been reported since 1986 by the Ministry of Health [3]. Since then, the number of patient with HIV positive has been increasing. In such condition, where patients’ immune system...
is compromised, infections that are opportunistic such as cryptococcosis, penicilloin, and candidiasis are common [4]. Indirectly, fungal infection holds a critical problem to health and major root for mortality worldwide. Despite the increase in the spectrum of antifungal agents, the antifungal regimen has fallen far behind bacterial chemotherapy [5]. For instance, Amphotericin B is a macrocyclic type compound and used as “gold standard” for being less toxic. It was made available in the early 60’s and prescribed up until now. Also, griseofulvin, terbinafine, and itraconazole are considered as the drug of choice for fungal infections. Unfortunately, these drugs have been withdrawn from the market as it has been replaced by new antifungal drugs [6].

Regardless of dedication to the development of new therapeutic strategies, there are only a limited number of available drugs to fight against fungal infections. Indeed, only four molecular classes that target three distinct fungal metabolic pathways are currently used in clinical practice to treat essentially fungal infections: fluoropyrimidine analogs, polyenes, azoles, and echinocandins. Therefore, a search for novel antifungal drugs selectively acting on new targets with fewer or no side effects is extremely necessary. Against this backdrop, researchers are forced to identify and explore a non-chemical, non-classical approach which is plant-based therapeutic agents that in fact are cheaper, safer, and effective antifungal drugs through systematic research are blatant [7].

Medicinal plants rich in natural sources have been used to treat mankind for various diseases since antiquity. The utilization of crude extracts of plant parts and phytochemicals for treating diseases is as old as the human species. Generally, plants produce secondary metabolite which exhibits antibacterial, antifungal, and insecticidal with minimal environmental impact and not toxic to human cells in contrast to the synthetic antifungal agent. This urged the evaluation of medicinal plants as a source of potential antifungal agent based on their usage as therapeutic agents such as Couroupita guianensis Aubl. Flower extract.

Couroupita guianensis tree is native to the tropical north-eastern South America, especially the Amazon rainforest. The flowers are pinkish red with a yellowish tinge on the outside, heavily scented. These are 3” to 5” waxy flowers growing directly on the bark of the trunk [8]. Couroupita guianensis has been referred by traditional healers to possess multifarious role, given the fact that all the parts can be utilized for medicinal application. Traditionally, the soft mass of the fruits is rubbed on the infected skin as antiseptics and to ease a toothache [9]. The juices of leaves are used to cure odontalgia, skin ailments, and shamans. Besides the leaves is also used to treat protozoal infections, stomach ache, and enteral gas formation, as antithrombotic, vitalize hair and has vasodilator properties [10,11]. Flowers are solely employed to cure scorpion poison, cold, intestinal gas formation, and stomach ache. The infusion of leaves and flower units are used for cold, abdomen ache, and pain associated with the inflammatory process [10]. Due to the emergence of antifungal drug resistance, lack of curative effect, high cost, and toxicity, a new prototype antifungal agent with antioxidant properties is needed to address this situation [12]. Hence, the present investigation was conducted to demonstrate the anti-Candida albicans activity, antioxidant properties, and phytochemical constituents of traditional medicinal plant C. guianensis.

Materials and Methods

Plant collection and extract preparation

The flowers of C. guianensis were collected from Universiti Sains Malaysia and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample was deposited (Voucher specimen: USM/HERBARIUM/11577). The flower sample was rinsed thoroughly —two to three times with running tap water, chopped, and shade dried at room temperature for 2 weeks and then homogenized to fine powder using a conventional blender for ease of extraction of active compounds. A sample of 100 g of plant powder was soaked in 500 ml (1:5) of 80% methanol at retention time (RT) (23°C ± 2°C) for 7 days. The filtrate from each extraction was concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40°C and the concentrated extract was finally poured into Petri dishes and brought to dryness at 40°C in an oven. The resultant extract paste is stored at RT in dark.

Preliminary phytochemical screening

Phytochemical assays were carried out on 80% methanol on the C. guianensis flower extract using standard procedures to determine the presence of flavonoids, sapopins, steroids tannins, and anthraquinone glycoside [13–15]. One mg/ml of flower extract stock solution was prepared. The test was based on the visual observation of a change in color or formation of precipitate after the addition of specific reagents.
Pharmacological activity of Couroupita guianensis Aubl. Flower

Test for tannins

Three milliliter of C. guianensis flower extract was treated with —two to three drops of 1% lead acetate and observed for yellow precipitate formation.

Test for saponins

To 6 ml of water was added 2 ml of C. guianensis flower extract and shaken vigorously. Formation of foam layer up to 1 cm showed the presence of saponins.

Test for steroids

One milliliter of C. guianensis flower extract was dissolved in 10 ml chloroform. An equal volume of concentrated sulphuric acid was added from the wall of test tube. The upper layer turns into red and the sulphuric acid layer shows a yellow with green fluorescence which indicates the presence of steroids.

Test for flavonoids

In 1 ml of C. guianensis flower extract, a drop of diluted sodium hydroxide was added. Formation of yellow color appeared which will turn colorless upon addition of few drops of dilute acid which indicates the presence of flavonoids.

Test for alkaloids

To 2–3 ml of C. guianensis flower extract, Mayer’s reagent was added. Formation of yellow precipitate indicated the presence of alkaloids.

Test for terpenoids

To 0.2 g of the C. guianensis flower extract, 2 ml of chloroform (CHCl₃) and concentrated H₂SO₄ (3 ml) were carefully added to form a layer. A reddish-brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

Test for anthraquinone glycosides (Borntrager’s test)

A small amount of C. guianensis flower extract was hydrolyzed with hydrochloric acid for few hours in a water bath. The mixture was then treated with chloroform. An equal volume of diluted ammonia solution was added. Pink color formation indicates the presence of glycosides.

Determination of total phenolic content in C. guianensis flower extract

Stock solution of C. guianensis flower extract was prepared in 1 mg/ml. Then, triplicates of the extract were prepared with 200 µl from stock solution with various concentrations (10, 20, 40, 80, 120, and 200 µg/ml). The absorbance of each concentration of the extract was recorded. The TPC of extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample. The TPC in all samples was calculated using the formula:

\[ C = \frac{cV}{m} \]

where:
- \( C \) = TPC mg GAE/g
- \( c \) = concentration of gallic acid obtained from calibration curve in mg/ml
- \( V \) = volume of extract in ml

Candida albicans

Yeast isolate used in this study was C. albicans obtained from the Microbiology Department of Universiti Sains Malaysia Hospital, Kelantan. This yeast strain was isolated from a patient. The yeast strain was stored in 50% glycerol stock at −80°C to maintain their long-term viability. For all the experiments, the yeast strain was subcultured for single colonies on Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 18 hours in an incubator (Loading Modell 100-800, Memmert, Schwabach, Germany).

Candida albicans inoculum preparation

Inoculum size is very important and has to be standardized at a certain value to obtain reliable, reproducible, and significant results. Therefore, inoculum size was standardized throughout this
study. A loop (25 µl) of yeast was aseptically obtained from a pure single colony from SDA and was suspended in 10 ml of Sabouraud dextrose broth (HiMedia, Mumbai, India). Sufficient inoculums were added until the turbidity was equivalent to 0.5 McFarland (10⁶ CFU/ml) standard (bio-Mérieux, Marcy Petoile, France).

**Disk diffusion method**

Anticandidal activity was determined by a modification of the disk diffusion method by Harris and Coote [17]. Paper disk (Advantec 90 mm, Toyo Roshi Kaisha, Ltd., Japan) with a diameter of 6 mm was sterilized by autoclaving at 121°C for 15 minutes and kept at room temperature until used. A 100 µl of mid-exponential phase yeast with the turbidity of 0.5 McFarland standard was spread onto SDA and left to dry for 1 hour at room temperature. Then, the sterile disk was placed on the surface of the plates. Sterile paper disks were impregnated with 20 µl of *C. guianensis* flower extract (100 mg/ml). An 80% methanol (v/v) was used as a negative control. Miconazole nitrate (30 µg/ml) (Duchefa Biochemie, Netherlands) was used as a positive control. The plates were incubated in an incubator (Memmert) for 18 hours at 37°C. The test was conducted in triplicate. Anticandidal activity was determined by measuring the diameter of inhibition zone around the disk.

**Minimal inhibitory concentration**

Minimal inhibitory concentration (MIC) was determined based on the 2-fold broth dilution method. The *C. guianensis* flower extract (2,000.00 mg) was dissolved in 80% methanol (10 ml) to reconstitute an extract solution of 200.00 mg/ml as stock. Subsequently, a serial dilution technique was carried out with 2.5 ml of the stock solution being transferred to a test tube containing 2.5 ml nutrient broth medium to give a concentration of 100.00 mg/ml. Next, 2.5 ml of solution from the first test tube was transferred into another second test tube containing nutrient broth medium that gave rise to a concentration of 50 mg/ml and similarly the technique was continued until a final concentration of 0.098 mg/ml was achieved. An inoculum size of 0.5 ml yeast with the turbidity of 0.5 McFarland standard was added to each test tube by maintaining the final concentration of the extract in each test tube. After 48 hours of incubation at 37°C, the tubes were examined for yeast growth. Growth was observed in those tubes where the concentration of the extract was below the inhibitory level where the broth medium turned into turbid or looks cloudy. The MIC value of the extract was taken as the lowest concentration that showed no growth or non-turbid in the test tube [18].

**Minimum fungicidal concentration**

To determine the minimum fungicidal concentration (MFC) value, all the tubes used in the MIC study which did not show any visible growth of the yeast after the incubation period were diluted (1:4) in fresh Potato Dextrose Broth (PDB) before subcultured on the surface of the freshly prepared Potato Dextrose Agar (PDA) plates and incubated at 37°C for 48 hours. The MFC was recorded as the lowest concentration of the extract that did not permit any visible fungus colony growth on the appropriate agar plate after the period of incubation [19].

**Time-kill study**

The time killing study of *C. guianensis* flower extract against *C. albicans* was conducted with 1/2, 1, and 2 times MIC over time whereby a growth profile curve was plotted [20]. A 16-hour culture was harvested by centrifugation, washed twice with phosphate saline, and re-suspended in phosphate saline. The suspension was adjusted using the McFarland standard and was then further diluted in phosphate saline to achieve an approximation of 10⁷ colonies forming unit (CFU/ml). *Couroupita guianensis* flower extract was added to aliquots of 25 ml PDB in 50 ml Erlenmeyer flask and was placed in a water bath at 37°C with amounts corresponding to the concentration of 1/2, 1, and 2 times of MIC value (12.5 mg/ml) upon the addition of the inoculums. Free medium without extract was used as a control. Next, 100 µl of *C. albicans* inoculum was added to all Erlenmeyer flasks. After the addition of the inoculums, 1 ml portion was removed from Erlenmeyer flask and the growth of *C. albicans* was monitored using this portion by measuring the optical density by using UV/spectrophotometer at 540 nm (UV-9100, Ruili Co, China). The growth of *C. albicans* was measured at every 4 hours throughout 48 hours by the above method. After that, a graph was plotted to determine the effect of *C. guianensis* flower extract on the growth profile of *C. albicans*.

**Morphological changes of *C. albicans* after treatment with *C. guianensis* flower extract**

The morphological change of *C. albicans* treated with *C. guianensis* flower extract was observed with a scanning electron microscope (SEM).
**Preparation of the antifungal agent from plant extract**

MIC value (12.5 mg/ml) was used as the concentration of treatments for *C. guianensis*. The fungal sample was harvested for electron microscopic observation. For treatment purpose, the *C. albicans* was inoculated in 10 ml PDB and then incubated at 25°C for 2 days. The final density of the fungal suspension was adjusted with phosphate saline to achieve approximately $1 \times 10^6$ CFU/ml, inoculated on PDA plate and incubated at 37°C for 6 hours. Two ml of *C. guianensis* flower extract at the concentration of MIC was then dropped on the inoculated agar and was further incubated for 48 hours at the same incubation temperature. A 50% methanol treated culture was taken as a control. A small block of *C. albicans* containing agar was cut and withdrawn from the inoculated plates at 0, 12, 24, 36, and 48 hours intervals of extract treatment, after which the plates are sealed with paraffilm and stored at 4°C before being processed for the SEM (FESEM LEO Supra 50VP, Carl Zeiss, and Germany) viewing [21].

**Preparation of the sample for SEM viewing**

A segment between 5 and 10 mm was obtained from the treated plate for SEM examination. A double-stick adhesive tab was used to place the specimen on a planchette. The subsequent process is carried out in a fume hood. The planchette was secure in a Petri dish and a vial containing 2% osmium tetroxide was placed in a deserted area of the plate. Latterly, the plate was parafilmed, and vapor fixation of the sample proceeded for 1 hour. After 1 hour, the planchette was subjected to slushy nitrogen (−210°C) and shifted to "peltier-cooled" stage of freeze dryer (Emitech K750) for 10 hours. The freeze dried sample is then coated with 5–10 nm gold prior to SEM viewing under following conditions: $L = SE1$, Working Distance (WD) = 21 mm, and Extra High Tension (EHT) = 10.0 kV to study the effect of the extract on *C. albicans* cell [21].

**Solvent partitioning (Liquid–liquid extraction)**

The *C. guianensis* flower extract was dissolved in 90% methanol before further partitioned in hexane: methanol: water (100:90:10 v/v/v) and yield hexane fraction. Subsequently, the aqueous layer formed was further partitioned into ethyl acetate (100 ml) to yield ethyl acetate fraction. Consequently, the aqueous layer formed was further partitioned with butanol (100 ml) and yielded butanol fractionation. The remaining aqueous layer was collected as water fraction. The entire fraction was evaporated to dryness in the rotary evaporator.

Each fraction was tested for their antifungal activity using the disk diffusion assay. The most active fractions were further analyzed using gas chromatography/mass spectrometry (GC/MS).

**Gas chromatography/mass spectrometry**

The GC/MS analysis of the ethyl acetate fraction from *C. guianensis* flower extract was performed using an Agilent 6890N series II gas chromatograph interfaced with an Agilent 5973 series quadrupole mass spectrometer (Palo Alto, CA) and equipped with an autosampler; Agilent 7673A. Helium gas (99.999%) was used as a carrier gas with a constant flow rate of ±1 ml/minute. Mass transfer line and injector temperature were set at 220°C and 290°C, respectively. The temperature set for oven was from 50°C to 150°C at 3°C/minute, then held isothermal for 10 minutes and finally raised to 250°C at 10°C/minute. The ethyl acetate crude fractions were diluted with methanol solvent into 10 mg/ml. The diluted samples (1 µl) were injected in the split mode with split ratio 120:1. The delay time was 2 minutes and the total running time was 120 minutes. The relative percentage of the chemical ingredients in ethyl acetate fractions from *C. guianensis* flower extract was expressed as percentage by peak area normalization. The relative percentage amount of each component was deliberated by comparing its average peak area to the total area. Software used to handle mass spectra and chromatograms was a GC-MS solution version 2.53.

**Antioxidant assays**

2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay

The *in vitro* determination of antioxidant activity was done according to the method described by Hatano et al. [22]. Five mg/ml stock solution of *C. guianensis* flower extract was prepared, and distributed into six different concentrations (10, 20, 40, 80, 120, and 200 µg/ml) in triplicates, by adding up the volume in each tube to 300 µl with distilled water. A control tube was prepared with 300 µl of distilled water. 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.1 mM) solution was prepared by dissolving 3.9 mg of DPPH in 100 ml methanol and stirred overnight at 4°C. To each 0.5 ml extract solution, 2.5 ml of 76 0.1 mM DPPH solution was added, which was prepared freshly. This sample was vigorously shaken using vortex machine and left in dark for 60 minutes at room temperature. It is important to note that DPPH is reactive with light and may affect the readings of absorbance. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm using a spectrophotometer.
The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

\[
\% \text{RSA} = \left( \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \right) \times 100\%
\]

Where A control is the absorbance of the solution without the extract and A sample is the absorbance of the solution containing extract with different concentration. Ascorbic acid was used as standard and a triplicate was performed.

**Hydrogen peroxide assay**

Hydrogen peroxide scavenging activity of the *C. guianensis* flower extract was determined by using the method described by Jayaprakash et al. [23]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (pH 7.4). Various concentrations of flower extract (10–200 µg/ml) in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution. The absorbance of \( \text{H}_2\text{O}_2 \) was determined after 10 minutes, measured at 230 nm against a blank solution that contained extracts in phosphate buffer without \( \text{H}_2\text{O}_2 \). The experiment was carried out in triplicate and the percentage of \( \text{H}_2\text{O}_2 \) scavenging of the flower extract was calculated using the equation:

\[
\% \text{scavenged (H}_2\text{O}_2) = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100\%
\]

**Statistical analysis**

Experimental results are expressed as means ± standard deviation. All measurements were replicated three times. The data were analyzed by an analysis of variance and t-test. \( P \) values less than 5% were considered statistically significant (\( p < 0.05 \)). The IC\(_{50}\) values were calculated from the linear regression analysis.

**Results**

**Preliminary phytochemical screening**

The phytochemical analyses of *C. guianensis* flower extract by the qualitative method are presented in Table 1. The presence or absence of the phytochemical was indicated with a positive and negative sign. The intensity of the present compounds was designated as “+,” “++,” and “+++.” The results showed that the *C. guianensis* flower extract contains a wide range of phytoconstituents including alkaloids, flavonoids, saponins, steroids, tannins, anthraquinone glycoside, and terpenoids. The *C. guianensis* flower extract was rich in tannins, flavonoids, and terpenoids and was indicated with “+++” sign. Moderate amount of alkaloids and saponins were found to be the constituent of

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Results</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+++</td>
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<tr>
<td>Saponins</td>
<td>++</td>
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<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+++</td>
</tr>
<tr>
<td>Anthraquinone glycoside</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+++</td>
</tr>
</tbody>
</table>

(+): weak positive test (if the reagent has slight color opacity).

(++) positive test (if the reagent produces observable color intensity).

(+++) test strongly positive (if the reagent produces heavy color intensity).

*C. guianensis* flower extract which was represented with “++” sign in Table 1. As for anthraquinone glycoside and steroids, insignificant amount was present and which was represented with “+” sign.

**Total phenolic content**

The TPC in the *C. guianensis* flower extract was measured using the Folin–Ciocalteu’s reagent and the result was expressed in terms of gallic acid equivalent. Gallic acid was used as a standard and the calibration curve was prepared with the range of concentration from 10 to 200 µg/l. The standard curve equation obtained was \( y = 0.0052x \), \( R^2 = 0.9993 \) (Fig. 1). The absorbance value obtained for *C. guianensis* flower extract at the concentration of 100 µg/ml was substituted in the standard curve equation. The TPC of *C. guianensis* flower extract was found to be 32.2 ± 0.22 mg of GAE/100 g of extract.

**Anticandidal activity**

*Couroupita guianensis* flower extract showed a good inhibitory activity against *C. albicans* at 100 mg/ml. Therefore, the MIC value was determined in this study with a maximum concentration of 100 mg/ml.

**Minimum inhibitory concentration and Minimum fungicidal concentration**

The MIC value for *C. guianensis* flower extract was depicted in Figure 2. The MIC values confirmed the existence of inhibitory effects on *C. albicans* tested in the study, with MIC value of 12.5 mg/ml for the *C. guianensis* flower extract. There is no visible growth of *C. albicans* observed in test tube with the *C. guianensis* flower extract with the concentration of 100.0, 50.0, 25.0, and 12.5 mg/ml and therefore, the MIC value was determined as 12.5 mg/ml concentration indicating the lowest concentration which inhibits the growth of *C. albicans*.
Pharmacological activity of *Couroupita guianensis* Aubl. Flower

The minimal fungicidal concentration of the *C. guianensis* flower extract was determined by pipetting out 0.1 ml yeast culture from the mixture obtained in the determination of MIC tubes (100, 50, 25, and 12.5 mg/ml) which did not show any growth and subcultured on to PDA agar and incubated at 37°C for 48 hours. The concentration of which there was no single colony was determined and recorded as 25 mg/ml of *C. guianensis* flower extract. It was noted that the *C. guianensis* flower extract MFC value (25 mg/ml) was 2-fold higher than MIC value (12.5 mg/ml).

**Figure 1.** Standard calibration curve (gallic acid) for the quantification of total phenolic content in *Couroupita guianensis* flower extract.

**Figure 2.** Minimum Inhibitory Concentrations (MIC) in mg/ml of the *Couroupita guianensis* flower extract against *Candida albicans* by broth dilution method. (A) 100 mg/mL, (B) 50.0 mg/mL, (C) 25.0 mg/mL, (D) 12.5 mg/mL, (E) 6.25 mg/mL, (F) 3.125 mg/mL, (G) 1.56 mg/mL, (H) 0.78 mg/mL.

The minimal fungicidal effect of the *C. guianensis* flower extract was determined by pipetting out 0.1 ml yeast culture from the mixture obtained in the determination of MIC tubes (100, 50, 25, and 12.5 mg/ml) which did not show any growth and subcultured on to PDA agar and incubated at 37°C for 48 hours. The concentration of which there was no single colony was determined and recorded as 25 mg/ml of *C. guianensis* flower extract. It was noted that the *C. guianensis* flower extract MFC value (25 mg/ml) was 2-fold higher than MIC value (12.5 mg/ml).

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Time-kill study

The growth profile study of *C. albicans* treated with 1/2 MIC (6.25 mg/ml), MIC (12.5 mg/ml), 2 MIC (25.0 mg/ml) concentration of *C. guianensis* flower extract, and untreated control group are shown in Figure 3. The growth profile of *C. albicans* in the presence of various MIC concentrations of *C. guianensis* flower extract was studied to evaluate the ability of the extract to eradicate *C. albicans* growth *in vitro*. In the case of 1- and 2-fold MIC concentrations, the *C. guianensis* flower extract inhibited the yeast growth within 4 hours and subsequent regrowth was not seen. However, subsequent regrowth was seen in *C. albicans* treated with 1/2 MIC concentrations of *C. guianensis* flower extract. The flower extract of *C. guianensis* exhibited a concentration and time-dependent killing profile. This observation confirmed the fungicidal effect of the *C. guianensis* flower extract on *C. albicans* at the concentration with MIC value.

Scanning electron microscope

The morphological features of photomicrographs by the untreated and *C. guianensis* flower extract treated *C. albicans* at various incubations time were shown in Figure 4. Untreated or control cells of *C. albicans* (Fig. 4A) show many regular spherical or oval in shape cells with smooth cell wall and some cells undergoing budding stage. After 12 hours of exposure to the *C. guianensis* flower extract as shown in Figure 4B; the formation of viscous material and a small number of cells presented with cavitation was witnessed. Figure 4C displays 24 hours treated cell with rough and wrinkled bodies, cells appear to be elongated and tend to form a clustered group of cells. After 36 hours of exposure (Fig. 4D), shrunken and sign of cell ruptures begins to be visible. A complete disruption of *C. albicans* cell wall with a rough, irregular, excessive shrinkage surface morphology, and vesicular formation were observed at 48 hours (Fig. 4E).

Solvent partitioning (Liquid–liquid extraction)

The crude *C. guianensis* flower extract was partitioned with various solvent systems, namely, hexane, ethyl acetate, and butanol and yielded hexane, ethyl acetate, butanol, and the aqueous fractions which were evaporated and weighed. The anticandidal activity of all the four fractions of *C. guianensis* was performed at the concentration of 50 mg/ml and the zone of inhibition was compared among all four fractions to determine the best active fraction for anticandidal activity as shown in Figure 5. Each fraction tested against *C. albicans* exhibited different diameter for the zone of inhibition against with the ethyl acetate fraction that showed a higher anticandidal activity.

GC–MS analysis

Interpretation of mass spectrum GC–MS for the ethyl acetate fraction of *C. guianensis* flower extract...
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**Figure 4.** Scanning Electron Micrograph of the untreated and *Couroupita guianensis* flower extract treated cells of *Candida albicans*. A, Control cells of *C. albicans*. B, 12 h; C, 24 h; D, 36 h and E, 36 h of *C. albicans* cells treated with 12.5 mg/mL of *C. guianensis* flower extra.

**Figure 5.** Antifungal activity of partition fractions of *Couroupita guianensis* flower against *Candida albicans* by disc diffusion method; (1) negative control (methanol), (2) positive control (miconazole nitrate (30 µg/mL)), (3) ethyl acetate fraction, (4) butanol fraction, (5) water fraction, (6) hexane fraction. Each fraction was tested at 50 mg/mL.
was conducted using the database of National Institute Standard and Technique (NIST). The spectrum of the unidentified component was compared with the spectrum of the known components stored in the NIST library. Qualitative analyses of ethyl acetate fractions of *C. guianensis* flower by using GC–MS showed the presence of 25 compounds in ethyl acetate fraction of *C. guianensis* flower extract. Figure 6 shows the gas chromatogram of ethyl acetate fraction of *C. guianensis* flower extract. The active principle, an area of the peak in concentration (%), and RT are presented in Table 2.

The identified compounds are: methyl tetradecanoate, tetradecanoic acid, pentadecanoic acid, 14-methyl-, n-hexadecanoic acid, heptadecanoic acid, 9,12-octadecadienoic acid (Z,Z)-, octadecanoic acid, 9,12-octadecadienoic acid (Z,Z)-, 9H-carbazole, 2-methyl-, benzamide, 2,3,4,5-tetrafluoro-N-(3-methylthio-1,2,4-triazol-5-yl)-, 11-eicosenoic acid, eicosanoic acid, 2,5-diphenyltetrazole, docosanoic acid, 1-propene, 3-(2-cyclopentenyl)-2-methyl-1,1-diphenyl-, (2,3-diphenylcyclopropyl)methyl phenyl sulfoxide, trans-, 1-propene, 3-(2-cyclopentenyl)-2-m ethyl-1,1-diphenyl-, methadone N-oxide, borinic acid, 2,6,10,14,18,22-tetracosenoic acid, vitamin E, stigmastera-7,16-dien-3-ol, (3.beta.,5.alpha.), beta-amyrin, alpha-amyrin, 9,19-cyclolanost-25-en-3-ol, 24-methyl-, and (3.beta.,24S)-.

**Free radical scavenging ability on 2,2-diphenyl-2-picrylhydrazyl**

The DPPH radical scavenging ability of the *C. guianensis* flower extract was recorded in terms of % inhibition as shown in Table 3 with the gallic acid as standard reference. The inhibition rate shows the capacity of the *C. guianensis* flower extract to reduce the absorption of the DPPH free radicals. The result obtained for DPPH free RSA of *C. guianensis* flower extract was in a concentration dependent manner by which the activity or the inhibition percentage gradually increased with concentration. The IC$_{50}$ value was calculated from linear regression analysis and the value obtained for *C. guianensis* extract was 93.2 ± 0.011 µg/ml, and for the standard gallic acid was 32.31 ± 0.08 µg/ml. The results of this study indicate *C. guianensis* flower extract has a noticeable scavenging effect on DPPH radicals.

**Hydrogen peroxide radical scavenging activity**

The scavenging ability of *C. guianensis* flower extract on hydrogen peroxide is shown in Figure 7 by comparing with the gallic acid as standard. The *C. guianensis* flower extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. A linear regression curve was used to calculate IC$_{50}$ values. The IC$_{50}$ for *C. guianensis* flower extract for scavenging of hydrogen peroxide was 46.48 ± 0.13 µg/ml compared ($p < 0.05$) to standard...
Pharmacological activity of Couroupita guianensis Aubl. Flower

gallic acid which was 33.12 ± 0.03 µg/ml. The regression correlation ($R^2$) was measured for $C. guianensis$ flower extract and the standard gallic acid and $R^2$ was found to be 0.9895 and 0.9336, respectively. It can be inferred from the findings that $C. guianensis$ flower extract possesses the ability to inhibit oxidation by virtue of the presence of phenolic compounds.

Discussion

Phytochemical analysis

Phytochemical screening of $C. guianensis$ flower extract revealed the presence of various bioactive compounds such as alkaloids, flavonoids, saponins, steroids, tannins, anthraquinone glycoside, and terpenoids which have been linked to antifungal activity [24]. It is therefore possible that these compounds may be responsible for the excellent antifungal properties, which was exhibited by $C. guianensis$ flower extract. The phytochemical screening results showed that the tannin, flavonoids, terpenoids, moderate amount of saponins, steroids, and anthraquinone were present in the $C. guianensis$ flower extract and may be responsible for the observed good antifungal properties, which was exhibited by $C. guianensis$ flower extract.

Table 2. Total ionic chromatogram of ethyl acetate fraction of $C. guianensis$ flower extract with RT and peak area.

<table>
<thead>
<tr>
<th>Peak</th>
<th>$R^t$</th>
<th>Area (%)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.66</td>
<td>0.50</td>
<td>Methyl tetradecanoate</td>
</tr>
<tr>
<td>2</td>
<td>9.91</td>
<td>0.79</td>
<td>Tetradecanoic acid</td>
</tr>
<tr>
<td>3</td>
<td>10.78</td>
<td>11.95</td>
<td>Pentadecanoic acid, 14-methyl-, me 100727 005129-60-2 99 thyl ester</td>
</tr>
<tr>
<td>4</td>
<td>11.10</td>
<td>17.11</td>
<td>n-Hexadecanoic acid</td>
</tr>
<tr>
<td>5</td>
<td>11.24</td>
<td>0.50</td>
<td>Heptadecanoic acid</td>
</tr>
<tr>
<td>6</td>
<td>11.64</td>
<td>15.89</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
</tr>
<tr>
<td>7</td>
<td>11.73</td>
<td>2.42</td>
<td>Octadecanoic acid</td>
</tr>
<tr>
<td>8</td>
<td>11.91</td>
<td>15.53</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
</tr>
<tr>
<td>9</td>
<td>12.33</td>
<td>0.64</td>
<td>9H-Carbazole, 2-methyl-</td>
</tr>
<tr>
<td>10</td>
<td>12.33</td>
<td>0.42</td>
<td>Benzamide, 2,3,4,5-tetrafluoro-N- (3-methylthio-1,2,4-triazol-5-yl)-</td>
</tr>
<tr>
<td>11</td>
<td>12.50</td>
<td>0.81</td>
<td>11-eicosanoic acid</td>
</tr>
<tr>
<td>12</td>
<td>12.60</td>
<td>1.33</td>
<td>Eicosanoic acid</td>
</tr>
<tr>
<td>13</td>
<td>13.36</td>
<td>1.00</td>
<td>2,5-diphenyltetrazole</td>
</tr>
<tr>
<td>14</td>
<td>13.56</td>
<td>0.88</td>
<td>Docosanoic acid</td>
</tr>
<tr>
<td>15</td>
<td>13.93</td>
<td>1.19</td>
<td>1-propene, 3-(2-cyclopentenyl)-2-m ethyl-1,1-diphenyl-</td>
</tr>
<tr>
<td>16</td>
<td>14.01</td>
<td>1.88</td>
<td>(2,3-diphenylcyclopropyl)methyl ph enyl sulfoxide, trans-</td>
</tr>
<tr>
<td>17</td>
<td>14.05</td>
<td>0.54</td>
<td>1-propene, 3-(2-cyclopentenyl)-2-methyl-1,1-diphenyl-</td>
</tr>
<tr>
<td>18</td>
<td>14.10</td>
<td>0.92</td>
<td>Methadone N-oxide</td>
</tr>
<tr>
<td>19</td>
<td>14.65</td>
<td>0.72</td>
<td>Borinic acid</td>
</tr>
<tr>
<td>20</td>
<td>15.69</td>
<td>4.26</td>
<td>2,6,10,14,18,22-tetracosahexaene,23-hexamethylyl, (all-E)-</td>
</tr>
<tr>
<td>21</td>
<td>19.55</td>
<td>2.93</td>
<td>Vitamin E</td>
</tr>
<tr>
<td>22</td>
<td>23.33</td>
<td>3.55</td>
<td>Stigmasta-7,16-dien-3-ol, (3.beta.,5.alpha.)-</td>
</tr>
<tr>
<td>23</td>
<td>24.23</td>
<td>5.11</td>
<td>Beta.-amyrin</td>
</tr>
<tr>
<td>24</td>
<td>25.38</td>
<td>5.49</td>
<td>Alpha.-amyrin</td>
</tr>
<tr>
<td>25</td>
<td>26.86</td>
<td>3.63</td>
<td>9,19-cyclolanost-25-en-3-ol, 24-methyl-</td>
</tr>
</tbody>
</table>

Table 3. Evaluation of DPPH free radical scavenging activity of Couroupita guianensis flower extract.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Couroupita guianensis</th>
<th>Standard (gallic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.6953 ± 0.11*</td>
<td>12.46 ± 0.03</td>
</tr>
<tr>
<td>20</td>
<td>26.4788 ± 0.09*</td>
<td>24.19 ± 0.02</td>
</tr>
<tr>
<td>40</td>
<td>30.6103 ± 0.08*</td>
<td>52.64 ± 0.01</td>
</tr>
<tr>
<td>80</td>
<td>41.97183 ± 0.09*</td>
<td>97.21 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td>51.07981 ± 0.13*</td>
<td>98.24 ± 0.03</td>
</tr>
<tr>
<td>200</td>
<td>71.06103 ± 0.09*</td>
<td>98.67 ± 0.04</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM; *statistically significant compared to standard gallic acid ($P < 0.05$).
disrupting the membrane integrity of the cells of fungal [36]. Steroid or sterol and anthraquinone are the derivatives of terpenoids and flavonoids, respectively. These compounds indirectly attribute to the exertion of antifungal properties of C. guainensis flower extract as flavonoids and terpenoids possess good antifungal properties [37].

Total phenolic content

Phenols are important plant phytochemical because of their action as primary antioxidants and scavenging capability on free radicals due to the presence of hydroxyl groups. They play an important role in counteracting the free radicals, quenching singlet’s and triplets oxygen [38]. Phenolic compound has a unique chemical structure responsible for free RSA. The mechanism of action of phenolic compound relies on the accepting or donating electron(s) to eliminate the unpaired condition of the free radicals. The antioxidant capacity (AOC) of a compound relies greatly on the number and location of hydroxyl groups [39]. According to Koleva et al. [40], antioxidant capacity increases with TPC and there is a linear correlation between the phenolic content and antioxidant activity. In this study, the phenolic content in C. guianensis flower was moderately high (32.2 mg GAE/100 g of extract) and was determined to correlate between the content of phenolic compound in a plant with the antioxidant activity [41]. Most medicinal plant with potential pharmacological effect has TPC ranged from 30 to 200 mg GAE/100 g of extract [13]. The method engaged in this study to evaluate the content of phenolic is roughly proportional to the number of phenolic hydroxyl groups in a given extract, but for reducing or scavenging capacity, it is enhanced when two phenolic hydroxyl groups are oriented ortho or para. Moreover, it was reported that different phenolic compounds have different responses in this analysis [42].

Anticandidal activity

The results expressed from the disk diffusion method are qualitative data where the obtained results gave an initial idea of the anticandidal activity of C. guianensis flower extract. The highest concentration of 100 mg/ml was applied for the screening of the anticandidal activity in this study since the crude extract of C. guianensis flower was used. C. guianensis flower extract exhibited a favorable anti-yeast activity against C. albicans with a MIC and MFC value of 12.5 and 25.0 mg/ml, respectively. The MIC value is the lowest concentration that completely inhibits any visible fungal growth [43]. Meanwhile, the MFC is a determinant for inhibited growth (static) or no-growth (cidal) after incubation. The observed anticandidal activity may be attributed
to the rich plant content of active compounds which is an important source of microbicides. The MFC value (25 mg/ml) obtained in this study was 2-fold higher than the MIC value (12.5 mg/ml). This finding suggests that the *C. guianensis* flower extract was fungistatic at lower concentration and fungicidal at higher concentration. Moreover, the crude plant extract, which has fewer or no side effects with MIC values between 2.5 and 15 mg/ml, has good potential to be the candidate extract to obtain new antifungal compounds [44,45]. Therefore, the findings from this study suggest that the *C. guianensis* flower extract may be a potential lead extract for the isolation of novel antican didal compound(s).

**Growth profile study**

The presence of the active component in the *C. guianensis* flower extract may act synergistically to produce good antifungal effect as observed in the growth profile study [46]. Moreover, the observed antifungal activity may also be credited with the high percentage of phenols group in the *C. guianensis* flower extract. Various reports in the literature are in agreement that the antifungal activity of a particular plant is mainly attributed to the phytochemicals such as tannins, alkaloids, terpenoids, flavonoids, and saponins [47]. The time-killing study revealed prolonged antican didal activity when *C. albicans* was exposed to *C. guianensis* flower extract at 0.5 MIC, MIC, and 2 MIC for 48 hours. The findings of this study also clearly indicated the potential of *C. guianensis* flower extract to be developed as a therapeutic agent against *C. albicans* infection. To verify this hypothesis, *C. albicans* cells (untreated and extract treated) were observed through SEM techniques.

**Scanning electron microscope**

Microscopy was employed to obtain detailed information about the in situ ultrastructural changes of *C. albicans* caused by *C. guianensis* flower extract. The most important structure that enhances the pathogenicity of *C. albicans* is the cell wall. Adhesion of *C. albicans* to the host cell is the prerequisite for colonization and an essential step in establishing an infection. Therefore the succession of antican didal activity of a potential medicinal plant extract is by acting on the few layers of the cell wall and penetration of the cell wall. The interaction of the bioactive compounds in the plant with the fungal cell aids the breakage of the cell wall [48]. Hence, the extracellular morphological changes of flower extract treated with *C. albicans* were observed by using SEM. The untreated cells were elongated and showed few daughter cells budding out of the parent cells. The microscopic examination of *C. albicans* using SEM showed that the cells treated with *C. guianensis* flower extract decreased in size, appeared irregular in shape with cell wall modifications, and clear depressions on the cell surface with holes. Interestingly, the exposure of *C. albicans* cells to *C. guianensis* flower extract increased the disruption of the cell wall and cell membrane structures.

About 90% of the *C. albicans* cell wall is carbohydrate [48]. There are three basic constituents that make up the cell walls polysaccharide. First is the polymers of glucose containing β-1,3 and β-1,6 linkages, second is the unbranched polymers of N-acetyl-D-glucosamine containing β-1,4 bonds (chitin), and the third one is polymers of manose protein and 1%–7% of lipids. It can be postulated that the *C. guianensis* flower extract could possibly be acting upon one or more of the cell wall constituent which results in detaching or breaking the cell wall structure and encounter the *C. albicans* infection which warrants further study. These SEM micrographs study confirmed the evidence of antican didal potential of *C. guianensis* flower extract.

**Solvent partitioning (liquid–liquid extraction) and GC/MS analysis for antifungal active fraction identification**

Bioassay-guided fractionation is widely used in the isolation and identification of the bioactive compound from plant extracts [49]. Hence in this study, this method was employed to identify antifungal active fraction from *C. guianensis* flower extract. Fractionation of compounds from the crude form of medicinal plant is important in the search of bioactive principle(s) from organic fractions. This method is based on the differential solubility of compounds in the crude extract between two different solvents employed. The chloroform was initially used as solvent in the fractionation process, but due to the reported toxicity it led to discontinuing of its use and in favor, ethyl acetate was employed in this study [50]. The bioassay-guided (antifungal activity) fractionation revealed that ethyl acetate showed a higher zone of inhibition compared to butanol. To reveal the presence of the bioactive component in the antifungal ethyl acetate fraction of *C. guianensis* flower was further analyzed with GC/MS. The Benzamide, 2,3,4,5-tetraflouro-N(-3-methyl-thilthio-1,2,4-triazol-5-yl), 9,12-Octadecadienoic acid (ZZ), Octadecadienoic acid, Pentadecanoic acid, 14-methyl-, Squalene, and Tetradecanoic acid were identified as possible antifungal agents in
C. guianensis flower extract active fraction. Therefore, it is possible that these active components in C. guianensis flower extract was mainly responsible for the observed anticanidal effects in this study especially against the C. albicans that might warrant further detail studies.

Antioxidant studies

One of the most common sources of free radicals in the human biological system is generated during infection [51] of pathogenic microorganisms. Therefore, in this study, the antioxidant activity of the C. guianensis flower extract was studied besides the anticanidal activity, to evaluate the ability of the flower extract to counteract the adverse effects of free radicals after the post-infections which lead to various life-threatening health problems [52,53]. Since the possible toxicity of the synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have been increasingly reported, the attention of seeking for a new source of antioxidant has been directed to natural antioxidant [54]. Consequently, in this study, the antioxidant activity of the C. guianensis flower extract was evaluated as a natural antioxidant. Plants such as C. guianensis have almost boundless ability to synthesize secondary metabolites that have been reported to possess remarkable antioxidant activities. Hence, in the current study, the phytochemical screening of C. guianensis flower extract is also done to determine whether the antioxidant phytochemicals are responsible for the observed anticanidal and AOC of the C. guianensis flower extract.

DPPH assay and hydrogen peroxide assay

AOC assays have been developed on the basis of the chemical reaction mechanisms involved. In general, single electron transfer is used to measure an antioxidant’s reducing capacity, and the hydrogen atom transfer is for quantifying the hydrogen atom donating capacity. In this study, the antioxidant activity of C. guianensis flower extract was evaluated by two different methods based on these two mechanisms [55]. Single electron transfer-based assays quantify the capability of a compound (antioxidant) to donate an electron to reduce radicals. Single electron transfer-based assays take after the redox titration in classical chemical analysis and can be defined by the following electron-transfer (redox) reaction:

\[
\text{Free radicals} + e^{-} \text{ (antioxidant)} \rightarrow \text{reduced free radicals} + \text{oxidized antioxidant}
\]

The most common single electron transfer is DPPH assay [55]. Assays based on hydrogen atom transfer measures the ability of an antioxidant to scavenge free-radicals by donating hydrogen atom. In most hydrogen atom transfer based methods, the free radicals will remove the antioxidants, which becomes radical itself. One of the important hydrogen atom transfer assays is hydrogen peroxide assay [56].

DPPH is a stable free radical and their mechanism of action is limited to extracellular compartment. The donation of electrons by antioxidant to the DPPH radicals made a resultant change from purple to yellow in the solution. As DPPH receive one electron in the presence of antioxidant or free radical scavenger, the absorption reduces and results in the decolorization. This reaction is stoichiometric with the respect to a number of electrons gained [57]. Hydrogen peroxide itself is not mostly reactive with biologically important molecules but is an intracellular precursor for formation of hydroxyl radicals which are poisonous to the cell. Hydrogen peroxide can inactivate a number of enzymes directly since it can cross the cell membrane rapidly. Once hydrogen peroxide enters the living cell, it is converted into free radical called hydroxyl radicals (• OH), reacts with biomolecules, causes tissue damage and cell death [53]. In both scavenging assays carried out in this study, the results showed a strong ability of C. guianensis flower extract to scavenge the DPPH and hydrogen peroxide free radicals with IC\(_{50}\) values of 93.2 ± 0.011 µg/ml and 46.48 ± 0.13 µg/ml. According to Kumaran and Karunakaran [58], a standard antioxidant such as quercetin on DPPH assay and hydrogen peroxide gives a value between 10 and 35 µg/ml.

The result of this study supports the claim that there is no correlation between the TPC and total antioxidant potential. As reported, phenolic compound attributes to great scavenging assets due to the presence of special active group known as a hydroxyl group (OH) [59]. The Folin–Ciocalteu phenol reagent used to quantify the TPC present in an extract is specific only to polyphenols containing hydroxyl as their active group, leaving behind amino substitute phenol (NH\(_2\)) and hydroxyl substitute phenol (OH) by which a major factor for excellent scavenging activity. The phenolic compounds in the C. guianensis flower extract are powerful chain breaking antioxidants.
because of their scavenging ability associated their active groups [60]. It is proven that the antioxidant activity is not directly dependent on absolute measurement of the phenolic content as mention in Folin–Ciocalteu, which only measures the presence of hydroxyl group but it also dependent on different structure types of phenolic compound and that has a role in antioxidant capacities [61].

**Conclusion**

The present study clearly demonstrated that *C. guianensis* flower extract exhibited good antifungal and antioxidant activities. Ethyl acetate fraction from *C. guianensis* flower extract was the most effective agents for antifungal activity. The antifungal and antioxidant activities in *C. guianensis* flower extract may contributed by the presence of various phytochemical in the extract which was support by phytochemical analysis in this study. These findings provide promising baseline information for the potential use of *C. guianensis* flower in the treatment of oxidative damage and infections associated with the studied microorganisms.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**


Pharmacological activity of *Couroupita guianensis* Aubl. Flower


