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Phytochemical, Antioxidant and Antimicrobial Activities of *Hevea brasiliensis* Leaves Extract

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ABSTRACT

Belonging to the Euphorbiaceae family, the Para rubber tree is formally referred to as *Hevea brasiliensis* in scientific terms. It is commonly known as an important economic commodity in Thailand because the natural rubber primarily originates from the milky latex obtained from the tree. However, the available research on the phytochemicals found in different parts of the rubber tree and their biological effects is quite restricted. This study aimed to determine the phytochemical constituents, antioxidant and antibacterial activity studies on the crude dry leaf extracts of *H. brasiliensis*. The results indicated the presence of alkaloids, anthraquinones, cardiac glycosides, coumarin, flavonoids, saponin, steroids, tannins, and terpenoids. The total phenolic content was 63.95±4.31 mgGAE/g in the ethanolic leaf extract. The ethanolic extract displayed notable effectiveness in scavenging free radicals (71.2±0.17%) at 500 µg/ml concentration and antioxidant capacity (the lowest IC_{50} value 42.57±0.91 µg/ml). The ethanol extract of the leaf of *H. brasiliensis* showed inhibition zone on all of the selected bacteria (gram-positive; *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis,* and gram-negative; *Escherichia coli, Pseudomonas aeruginosa*) at 200 mg/ml. In conclusion, the dried leaves of *H. brasiliensis* compose phytochemicals that exhibit antioxidant and antibacterial activities and possesses the potential to act as a reservoir of plant-derived antibiotics and natural antioxidants.

1. Introduction

H. brasiliensis (Will. Ex Adr. De Juss.) Muell. et Arg, the Para rubber tree or most commonly, the rubber tree belongs to the family Euphorbiaceae. The rubber tree is a perennial tropical crop that has been cultivated in Thailand since 1882. Nowadays, rubber tree is planted in many areas. It is commonly known as an important economic commodity in Thailand because the natural rubber primarily originates from the milky latex obtained from the tree. In addition, the components of rubber trees can also be used for other purposes, such as natural conservation products from wood, handicrafts from leaves and biodiesel oil from the extraction of rubber seeds (Le *et al.* 2018). Since 2014, Latex, known for its protein removal properties, has been utilized in the production of various cosmetic products (Lourith *et al.* 2014), including makeup-removal (Lourith *et al.* 2020) and hair loss treatment (Lourith *et al.* 2021). The production of rubber continues to result in the creation of various products. Farmers make use of rubber leaves that have fallen from the trees as a source of fertilizer. They collect the fallen rubber leaves and create a pile underneath the rubber tree, facilitating their decomposition into organic fertilizer. In addition, rubber leaves, which are in large numbers, are discarded uselessly, becoming valuable agricultural waste. Some scientists try to research and develop rubber and various components of rubber to seek essential sources of information that lead to value-added of rubber (Wigati *et al.* 2016).

Crude *H. brasiliensis* leaf extract also has a pharmacological activity that can inhibit the growth of various pathogenic bacteria, namely, *Escherichia coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. This may be because rubber leaf extract contains important pharmacological substances that inhibit bacterial growth, including flavonoids,

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polyphenols, tannins, polyacetylenes, terpenoids, sterols, and alkaloids (Singh and Kumar 2015). Serum from latex was effective against *Aspergillus niger*, but not *Candida albicans* (Daruliza *et al.* 2011). In addition, the rubber seed oil exhibited antioxidant properties (Chaikulab *et al.* 2017). Nevertheless, rubber planted differently may contain compounds and different pharmacological effects (Zain *et al.* 2021). Studies on the toxicity of rubber found no toxicity; for example, linamarin found in rubber seed oil showed no toxicity in rats (Salimon *et al.* 2012) and non-toxic to B16-F10 melanoma cells and 3T3-L1 cells (Chaikulab *et al.* 2017). Serum from latex was not toxic to brine shrimp (Daruliza *et al.* 2011).

However, the available research on the phytochemicals found in different parts of the rubber tree and their biological effects is quite restricted. This study aimed to determine the phytochemical constituents, antioxidant and antibacterial activity studies on the crude dry leaf extracts of *H. brasiliensis.* The phytochemicals and biological activity found in *H. brasiliensis* may significantly contribute to comprehensive analyses of the plant's various activities in the future.

2. Materials and Methods

2.1. Chemicals

All reagents and chemicals including ethanol, dimethyl sulfoxide (DMSO), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), potassium iodide, iodine, glacial acetic acid, ferric chloride, chloroform, and nutrient agar (NA) and nutrient broth (NB), Folin-Ciocalteu, Mueller Hinton agar (MH agar), DPPH (2,2-diphenyl-1 picrylhydrazyl), gallic acid, ascorbic acid and trolox were purchased from Merck (Darmstadt, Germany) in laboratory grade chemicals.

2.2. Plant Materials

Dry leaves from *H. brasiliensis*, similar to senescent foliage, may display shades of brown or tan devoid of spots or lesions. These leaves descend amid the planting rows and were gathered between January and April 2022 from a rubber plantation (2 Plots) in Paphayom district, Phatthalung province, Thailand. (Figure 1). Leaves are frequently transported within sealed containers at ambient room temperature, serving to mitigate moisture loss, physical harm, and potential contamination. Plant material was conveyed to the laboratory of the Faculty of Health and Sports Science, Thaksin University, Phatthalung campus, for additional preparation and research endeavors. The cleaned leaves were manually cut into small pieces to enhance the surface area for the extraction process. Then, small pieces of leaves were dried in a conventional oven at 40-45°C for three days (Thongmak *et al.* 2021).

2.3. Extraction of Plant Material

The ethanolic extraction process involved placing 500 g of powdered *H. brasiliensis* leaves into an extraction bottle, followed by the addition

Figure 1. A rubber plantation in Paphayom district, Phatthalung province, Thailand (A) and dry leaves of *H. brasiliensis.*

of 1,000 ml of 95% ethanol. The blend underwent a 72-hour maceration period with vigorous agitation to enhance the extraction efficiency. After this period, the mixture was filtered using Whatman No. 1 filter paper. The identical process was reiterated for 95% ethanol (1,000 ml). The filtrates were pooled and centrifuged at 5,000 rpm for 15 minutes. The filtrate was subjected to evaporation using a rotary evaporator at 40°C to eliminate surplus ethanol solvent. Stored in the refrigerator, the crude extract presented itself as a dark brown powder and gave a percentage yield of 15.12±5.05% w/w.

2.4. Phytochemical Studies

The crude leaves extract of *H. brasiliensis* was subjected to qualitative standard screening tests for secondary metabolites such as alkaloids, anthraquinones, cardiac glycosides, coumarin, flavonoids, saponin, steroids, tannins, and terpenoids according to the procedures described by Ayoola *et al.* (2008) and Siddiqui *et al.* (2009). The qualitative results were expressed as presence/positive reaction (+) and absence/negative reaction (-) of phytochemicals.

2.5. Total Phenolic Content

Total phenolic content was analyzed using the Folin–Ciocalteu colorimetric method (Majhenic *et al.* 2007) with some modifications. An aliquot of 0.5 ml of ethanolic extract (1 mg/ml) was mixed with 10% Folin–Ciocalteuphenol reagent (2.5 ml). After 5 min, 7.5% sodium carbonate (2.5 ml) was added, and the mixture was allowed to stand at 45°C for 45 min. The absorbance of the mixture was measured at 570 nm. A standard calibration curve for gallic acid in the range of 0–200 ppm was prepared in the same manner, and results were expressed as mg gallic acid equivalent (GAE) per gram of extract.

2.6. Determination of Antioxidant Activity

Ethanolic extracts had the antioxidant activity evaluated according to the free radical reduction method 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Braca *et al.* 2002). For the preparation of the DPPH stock solution, 24 mg of DPPH was dissolved in 100 ml of absolute methanol. In order to prepare the working solution, the stock solution was further diluted with absolute methanol until achieving the absorbance reading at 517 nm. A solution of the extract was created by dissolving the raw extracts in absolute methanol at a concentration of 100 mg/ml, which was subsequently diluted for analysis. The procedure involved combining 100 µl of DPPH working solution with 100 µl of the extract solution prepared at various concentrations (ranging from 3.125 to 500 µg/ml). The mixtures were agitated and left in the absence of light for 30 minutes at ambient temperature and then measured absorbance (Ab) at 517 nm using a methanol blank as reference. The reduction in absorbance reading was associated with the radical scavenging potential of the extracts. The IC_{50} values represent the concentration of the extracts needed to stabilize half of the radical population. Ascorbic acid and Trolox were used as the positive control, while the DPPH radical scavenging activity of the extracts was calculated using the relation:

$$\text{DPPH}\ (\%) = (\text{Ab (control)} - \text{Ab (sample)}) / \text{Ab (control)} \times 100$$

Where Ab (control) is the absorbance of the control, and Ab (sample) is the absorbance of the test sample.

2.7. Determination of Antimicrobial Activity

Five bacterial strains (Gram-positive; *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*, and Gram-negative; *Escherichia coli, Pseudomonas aeruginosa*) were obtained from the stock cultures from the bacteriology laboratory at Department of Pathology, Faculty of Medicine, Prince of Songkla University. The method used was the well diffusion dilution technique on nutrient agar plates. (Valgas *et al.* 2007). Each microbial suspension, comprising approximately 10^8 CFU/ml (as determined by a hemocytometer), was delicately spread onto Mueller-Hinton agar (MHA) using a sterile cotton swab, with a volume of 300 µl. Under sterile conditions, wells with a diameter of 6 mm were aseptically excised from the nutrient agar using sterile blue tubes. According to Suffredini *et al.* (2006), Plant extracts at concentrations below 200 mg/ml never render Gram-negative bacteria susceptible; hence, each well was filled with 100 µl of 200 mg/ml of *H. brasiliensis* ethanolic extract. The inoculation was at 37°C for 18 hours and the inhibition zone around the wells were observed. Following the incubation period, the sensitivity agar plates that had been inoculated were retrieved from the incubator. With ample illumination, the diameter of the inhibition zones surrounding the wells was measured. The calculation of the inhibition zone involved measuring the diameter around the well (in millimeters), which included the well's own diameter. The readings were taken in triplicates and the average values were tabulated (Gandhiraja *et al.* 2009).

2.8. Statistical Data Analysis

All data values were average of triplicate determination expressed with standard deviation (SD).

3. Results

The preliminary phytochemical screening of the crude leaves extract of *H. brasiliensis* showed the presence of bioactive components such as alkaloids, anthraquinones, cardiac glycosides, coumarin, flavonoids, steroids, tannins, and terpenoids, except saponin (Table 1).

As a basis, phenolic content was measured using the Folin–Ciocalteu reagent in each extract. The results were derived from a calibration curve (y = 25.315x +0.0419, R2 = 0.9958) of gallic acid (0–50 μg/ml) (Figure 2) and expressed in gallic acid equivalents (GAE) per gram dry extract weight. The content of phenolic compounds in ethanolic extract was 63.95±4.31 mg GAE/g.

This study noted that the ethanol crude extract from the leaves of *H. brasiliensis* exhibited concentration-dependent radical scavenging activities. As the concentration decreased from 500 to 3.125 μg/ml, there was an increase in absorbance, signifying greater free radical scavenging activity of the samples, as indicated by lower absorbance at 517 nm. At a concentration of 500 μg/ml, the leaf of *H. brasiliensis* demonstrated robust radical scavenging activity, displaying a DPPH inhibition percentage of (71.2±0.17%) as compared to the reference ascorbic acid (83.05%) and trolox (85.00%) at the same concentration (Figure 3). IC_{50} value gives the effective concentration required for 50% inhibition. The ethanol extract reduced DPPH radicals is evident from its low IC_{50} (42.57±0.91 μg/ml) value. While, ascorbic acid exhibited the lowest IC_{50} value (7.81 μg/ml). It is considered one of the potent naturally-occurring antioxidants within the biological system.

The leaf extract of *H. brasiliensis* demonstrated antibacterial activity, as evidenced by the observed zone of inhibition against the tested bacteria.

Table 1. Phytochemical screening of ethanolic extract of *H. brasiliensis*

Phytochemical test	Leaves extract of *H. brasiliensis*
Alkaloids	+
Anthraquinones	+
Cardiac glycosides	+
Coumarin	+
Flavonoids	+
Saponin	-
Steroids	+
Tannins	+
Terpenoids	+

Key (+) means presence of phytochemical and (−) means absence of phytochemical means

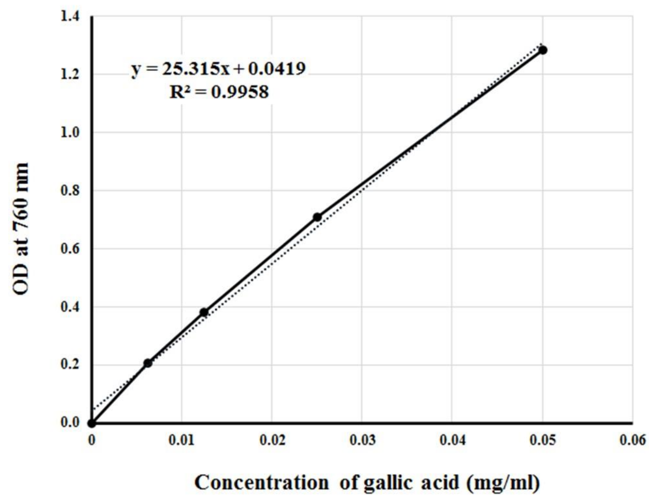

Figure 2. Standard curve of gallic acid standard at 760 nm

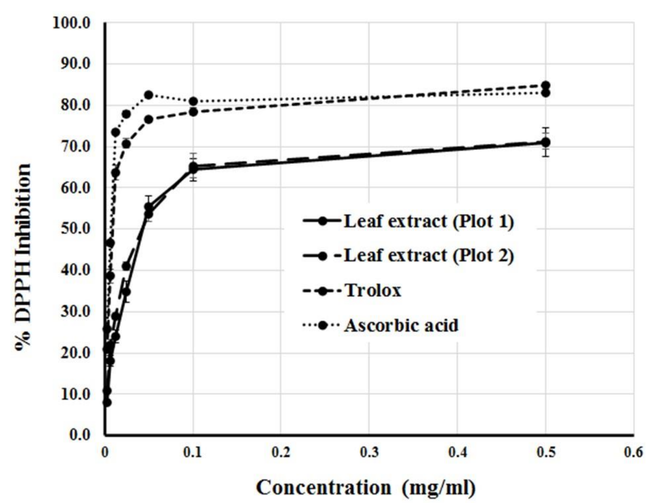

Figure 3. DPPH radical scavenging activities of various concentrations of H. brasiliensis leaf extracts (Plot 1 and Plot 2), and positive control (Ascorbic acid and Trolox). Data is represented as Mean ± SD, n=3

The average value of the zone of inhibition (mm) toward *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli*, and *Pseudomonas aeruginosa* bacterium was shown in Table 2. The results revealed that ethanolic extract showed inhibition against all tested bacterial strains and compared to two reference antibiotic drugs; vancomycin and gentamicin on the same pathogen.

4. Discussion

4.1. Phytochemical Composition

This study and Singh and Kumar (2015), observed that *H. brasiliensis* extracts revealed the presence of all phytochemical compounds while saponins were absent. The same type of solvent (ethanol) was used to extract the plant and all the extracts showed negative saponin tests, indicating that *H. brasiliensis* might contain little or no saponin content. Saponin was examined in the water extraction of *H. brasiliensis* leaf (Singh and Kumar 2015). Typically, saponins exhibit high polarity, are chemically and thermally unstable, lack volatility, and are commonly present in plants in low concentrations (Li *et al.* 2006). Thus, saponins provided the highest yields in plant extraction when polar solvents were employed (Majinda 2012).

According to Singh and Kumar (2015), this study reported that the ethanolic extract of *H. brasiliensis* did not contain anthraquinones and triterpenoids. The lack of anthraquinones and triterpenoids in certain extracts could be attributed to the unsuitability of ethanol as the extraction solvent for these types of compounds. The environmental condition can affect the different types and number of phytochemical compounds present in the plants. Conditions like heightened sunlight exposure, nutrient-deprived soil, pest infestation, and drought-induced stress can lead to heightened production and accumulation of secondary compounds within the plant (Selmar and Kleinwächter 2013).

Overall, the current study elucidated that *H. brasiliensis* exhibited a diverse range of secondary metabolites that played a significant role in its pharmacological activities, operating through multiple mechanisms. This result may contribute many significant ways for various studies in a truth complete manner to the various activities of the plant in the future.

4.2. Total Phenolic

Phenolic compounds are plant constituents primarily characterized by their redox properties, which account for their antioxidant activity (Soobrattee 2005). The presence of hydroxyl groups in plant extracts enables effective scavenging of free radicals. According to Zain *et al.* (2021), Among the various parts of *H. brasiliensis*, the leaf contains the highest quantity of phenolic compounds, whereas the seed has the lowest. The plant leaf extracts exhibited a range of total phenolic content, spanning from 0.003 to 0.020 mg GAE/ml. The amount of phenolic compounds in the plant varied depending on the different locations where it grew, indicating that the plant's growth locations played a significant role in determining the levels of these compounds. The plant experiences varying levels of stressors, including factors like soil composition, elevated temperatures, and nutritional deficiencies (Selmar and Kleinwachter 2013).

4.3. Antioxidant Activity of Ethanol Crude Extract

The antioxidant activity of the ethanol crude extract of the leaf of *H. brasiliensis* was assessed using a DPPH assay. DPPH is a stable purple free radical characterized by an absorption band at 517 nm. It can transition to a yellow state by accepting electrons from antioxidants (Munteanu and Apetrei 2021). The antioxidants in the plant extracts were assessed for their ability to reduce DPPH radicals. Various concentrations of the extract led to the transition from a purple hue in the DPPH solution to yellow. DPPH assay is very simple and can test many samples in a short period. Especially, this

Table 2. Antibacterial activity of ethanolic extract of *H. brasiliensis* against pathogens at 200 mg/ml concentration

Sample	Inhibition zone (mm)				
	S. aureus	*S. epidermidis*	*B. subtilis*	*E. coli*	*P. aeruginosa*
200 mg/ml Extract	11.67±0.5	11.33±1.15	11.00±1.00	10.33±0.58	10.67±0.58
30 μg/ml Vancomycin	32.00±0.3	30.00±0.3	32.00±0.1	ND	ND
30 μg/ml Gentamicin	ND	ND	ND	25.00±0.1	23.00±0.11

ND: Not determined

approach exhibits ample sensitivity to evaluate antioxidant levels even at low concentrations (Hseu *et al.* 2008). Free radicals produced in our body have been linked to developing and progressing several diseases, including inflammation, atherosclerosis, multiple sclerosis, stroke, heart diseases, diabetes mellitus, cancer, Parkinson's disease, Alzheimer's disease and ischemic conditions (Forman and Zhang 2021). Plants bioactive compounds, are the important source of active natural products, especially secondary metabolites, such as alkaloids, terpenoids, polyphenols, flavonoids, and tannins that can inhibit or suppress the adverse effect of these free radicals (Kasote *et al.* 2015). Polyphenols share a common chemical structure, known as the phenolic group, which aids them in scavenging hydroxyl radicals and counteracting free radicals (Francenia Santos-Sánchez 2019). The leaf extracts of *H. brasiliensis* may owe their radical scavenging abilities to the presence of phytochemicals such as alkaloids, terpenoids, flavonoids, phenols, tannins, and coumarin.

4.4. Antibacterial Activity

In this present study, the leaf extract of H. brasiliensis showed broad-spectrum antibiotic activities. The previous study indicated that *H. brasiliensis* extract possesses antimicrobial activities against *E.coli, K. pneumoniae*, and *P. aeruginosa* (Singh and Kumar 2015). Numerous secondary metabolites from plants and their derivatives have been recognized as potential antimicrobial agents. Notably, alkaloids and polyphenols have demonstrated robust antimicrobial properties in studies (Othman *et al.* 2019). Tannins (Scalbert 1991) Tannins display antimicrobial properties by causing the precipitation of microbial proteins, making them inaccessible to bacteria (Banso 2009). Flavonoids have antibacterial activity via various mechanism of actions, such as inhibition of nucleic acid synthesis, disruption of cytoplasmic membrane function, suppression of energy metabolism, hinderance of attachment, and prevention of biofilm formation (Xie *et al.* 2015). The antimicrobial effects observed in the leaf extracts of *H. brasiliensis* may be attributed to the existence of secondary metabolites such as alkaloids, tannins, flavonoids, and terpenoids.

In summary, this study illustrates that the *H. brasiliensis* extract holds promise as a source of both natural antioxidants and antibiotic compounds. This study offers initial insights, suggesting that additional antioxidant and antimicrobial investigations may be required to identify, isolate, and refine the bioactive compounds for potential use in complementary and alternative medicine for treating various diseases. Furthermore, these findings will be advantageous for others as a reference for pharmaceutical product development.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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