
Phytochemicals content, FTIR fingerprint and bioactivity of crude extract and fractions of *Mesua ferrea* L. leaves

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Abstract *Mesua ferrea* L. (Ceylon ironwood, Calophyllaceae) is a popular medicinal plant with a long history of use in South- and Southeast Asian folk medicines. In this study, the qualitative phytochemical analysis, FTIR fingerprint, as well as antimicrobial and antioxidant activities of the crude extract of *M. ferrea* leaves and its fractions were reported. The dried leaves of *M. ferrea* were extracted with ethanol by the re-maceration method. The crude extract was further partitioned in ethanol-water and ethyl acetate to obtain ethanol and ethyl acetate fractions, respectively. The identification of compounds in the extract and fractions was conducted according to the standard phytochemical screening method. Fourier-transform infrared (FTIR) spectroscopy was utilized to record the metabolites fingerprint of the extract and its fractions. The antimicrobial activity was evaluated with the disk diffusion method against *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, and *Saccharomyces cerevisiae*. The antioxidant activity assay was determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. The crude extract of *M. ferrea* leaves and its fractions contained saponins, phenolic compounds, flavonoids, and terpenoids. The FTIR data supported the presence of terpenoids and glycosides in those samples. The extract and fractions demonstrated considerable antimicrobial activity against all tested bacteria and fungi, with MIC values were 3.9-31.3 µg/ml. The ethyl acetate fraction of *M. ferrea* leaves showed the best antioxidant activity with an IC₅₀ value of 49.19 µg/ml.

Introduction

Mesua ferrea L. (Ceylon ironwood, *nagasari* in Bahasa Indonesia, Calophyllaceae) is a popular medicinal plant that can be found in many Ayurvedic formulations. The monograph of this plant's crude drug is included in the recent edition of Thai Herbal Pharmacopeia (Thai Department of Medical Sciences, 2018). *M. ferrea* is used as deodorant, diaphoretic and stimulant, as well as a brain tonic, appetizer, antiemetic, anthelmintic, aphrodisiac, diuretic and antidote (Suresh, Chandra, Meeta, & Deep,

2014). It is the identity flora of Banyumas, and traditionally is used for the treatment of leukemia, mental illness, liver disorder, dengue fever, and heart disease by people in Baturraden, Banyumas (Suparman, Diniatik, Kusumaningrum, & Yulianto, 2012). In Pidie, Nanggroe Aceh Darussalam, *M. ferrea* is used as one of the ingredients of post-natal concoction for the mothers (Viena, Yunita, Irhamni, Saudah, & Ernilasari, 2018).

The extracts, fractions, and isolates of *M. ferrea* have been evaluated for various

pharmacological activities, including antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, cytotoxic, analgesic, and anti-arthritic. Isolation of secondary metabolites of this plant guided by those aforementioned bioactivities has led to identification of several bioactive compounds, that were mainly coumarin, xanthone, and terpenoid derivatives (Chahar, Kumar, Lokesh, & Manohara, 2012; Chanda, Rakholiya, & Parekh, 2013; Hassan, Ali, Alimuzzaman, & Raihan, 2006; Jalalpure et al., 2011; Keawsaard & Kongtaweelert, 2012; Phuong & Nhat, 2015; Rawat & Upadhyaya, 2013; Roy et al., 2013; Teh, Ee, Mah, Lim, & Ahmad, 2013; Ullah, Tareq, Huq, Uddin, & Salauddin, 2013). Most of those studies subjected to *M. ferrea* originated from India, Malaysia, Thailand, and Pakistan. The report of bioactivity and metabolites profile of *M. ferrea* growing in Indonesia is still limited. In our preliminary study, the ethanol extract of this plant demonstrated antibacterial activity against *Escherichia coli* and *Bacillus subtilis* (Hartanti, Arafani, Nurlativah, & Hakim, 2017). The purposes of this research are to identify the phytochemical groups, analyze Fourier-transform infrared spectroscopy (FTIR) fingerprint, as well as evaluate antimicrobial and antioxidant activities of crude extract and fractions of *M. ferrea* leaves collected from Banyumas, Central Java, Indonesia.

Materials and methods

General

Ethanol, ethyl acetate, and distilled water were used as the solvent for extraction and fractionation of leaves of *M. ferrea*. Water, Mayer's, Liebermann - Burchard's, ferric chloride, and Shinoda's reagents were utilized for phytochemical screening of metabolites in extract and fractions of *M. ferrea* leaves. Nutrient Agar (NA), Saboroud Dextrose Agar (SDA), Saboroud Dextrose Broth (SDB), distilled water, sterile NaCl, Dimethyl Sulfoxide (DMSO),

ketoconazole and tetracycline were used for antimicrobial activity assay. *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Saccharomyces cerevisiae*, cultured by Laboratory of Microbiology and Genetics, Universitas Muhammadiyah Purwokerto, were used as the tested microorganisms. Methanol and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used for the determination of free radical scavenging activity of the extract and fractions of *M. ferrea*.

Instrumentation

UV and IR spectra were recorded on UV-Vis Spectrophotometer (Shimadzu 04782) and FTIR Spectrometer (Shimadzu IR Tracer100), respectively.

Plant materials

Leaves of *M. ferrea* were collected from Banyumas, Indonesia. The plant material was authenticated in the Laboratory of Environment Biology, Faculty of Biology, Jenderal Soedirman University, Purwokerto, Indonesia. The leaves were dried under the direct sunlight and powdered to a fine degree.

Extraction of leaves of *M. ferrea*

Powdered leaves of *M. ferrea* (150 g) were extracted with ethanol by the re-maceration method. Each maceration process was conducted with 500 ml of ethanol for 3 days. The ethanol was evaporated *in vacuo* to obtain the crude extract (Hartanti et al., 2017).

Fractionation of crude extract of leaves of *M. ferrea*

Fractionation of crude extract of *M. ferrea* followed the standard liquid-liquid extraction method with a modification (Shahraki, Khajavirad, Shafei, Mahmoudi, & Tabasi, 2016). The crude extract (10 g) was suspended in 67% ethanol in water (150 ml) and added with n-hexane (150 ml) to form two layers system. The n-hexane layer was separated and was not used in the further experiment. The ethanol layer was further added with ethyl acetate (150 ml) to

form two layers system. The ethyl acetate layer was then separated and the ethanol layer was re-fractionated with the same amount of ethyl acetate and further separated accordingly. All ethyl acetate layers were collected and evaporated *in vacuo* to obtain ethyl acetate fraction. The remained ethanol layer was evaporated *in vacuo* to obtain ethanol fraction.

Qualitative phytochemical screening of extract and fractions of M. ferrea

The presence of alkaloids, flavonoids, terpenoids, and tannins in the crude extract of *M. ferrea* leaves and its fractions was screened with the standard qualitative phytochemical screening method using Mayer's, Shinoda's, Liebermann - Burchard's, and ferric chloride reagents, respectively. The presence of saponins was identified with a standard foam test (Jaradat, Hussien, & Ali, 2015; Rawat & Upadhyaya, 2013).

Analysis of FTIR fingerprint

The spectra of extract and fractions of leaves of *M. ferrea* were recorded with ATR-FTIR method as previously reported (Topală, Tătaru, & Ducu, 2017). The analysis was conducted in the frequency regions of 4000-650 cm^{-1} and a resolution of 4 cm^{-1} .

Determination of antimicrobial activity

The antimicrobial assay of the extract of *M. ferrea* and its fractions were conducted following a method previously reported (Hartanti et al., 2017). The extract and fractions of *M. ferrea* were prepared into a series of concentrations of 1000, 500, 250; 125, 62, 32, 15, 7.8, and 3.9 $\mu\text{g/ml}$ in DMSO. DMSO was used as negative control, while tetracycline and ketoconazole were utilized as a positive control for bacteria and fungi, respectively. The microorganisms were prepared in suspension with an optical density of 0.1 at the wavelength of 600 nm. The diameters of inhibition of the growth of the tested microorganisms were measured after 24 h of incubation in a

temperature of 37°C. The lowest concentration of the extract and fractions with a diameter of inhibition zone that was statistically different from that of the negative control was assumed as the minimum inhibition concentration (MIC) of the respective samples.

Determination of DPPH Free Radical Scavenging Activity

The free radical scavenging assay was performed according to a standard method with a minor modification. DPPH was prepared in a concentration of 0.004% in methanol (Permatasari & Rohman, 2017). The extract and fractions of *M. ferrea* were prepared in concentrations of 20, 40, 60, and 80 $\mu\text{g/ml}$. One ml of DPPH solution was mixed with three ml of sample and was stand at ambient temperature for 45 min. The absorbance was subsequently recorded at a wavelength of 515.9 nm against blank of methanol. Vitamin C was used as the positive control, it was prepared in a solution in methanol with concentrations of 1, 2, 3, 4, and 5 $\mu\text{g/ml}$. Percent of inhibition of each concentration was calculated, and then the IC_{50} was determined accordingly.

Data analysis

All data were obtained from triplicate work and expressed as mean \pm standard deviation (SD) using Microsoft Excel (Microsoft Inc., USA). Mean of separation of the diameter of the inhibitory zone of crude extract and its fractions against a given microorganism was analyzed with ANOVA followed with Duncan's test to determine the MIC of the respective samples. Mean of separation of IC_{50} were analyzed with ANOVA followed with Duncan's test at p-value <0.05. All the statistical analysis was performed with SPSS ver. 20.

Results and discussions

The extraction of dried leaves of *M. ferrea* produced a dark green crude extract with a total yield of 39.84%. After the fractionation process, the yield of ethyl acetate and ethanol fractions

were 9.6 and 20.5%, respectively. Ethyl acetate fraction was in dark green color, while the ethanol fraction was brownish.

The phytochemical screening of crude extract of *M. ferrea* leaves and its fractions demonstrated that they contained saponins, phenolic compounds, flavonoids, and terpenoids (Table 1). The known bioactive metabolites of *M. ferrea* are including isolodene, xanthenes, and coumarins (Asif et al., 2016; Chahar et al., 2012; Chukaew et al., 2019; Roy et al., 2013; Teh et al., 2011, 2013; Verotta, Lovaglio, Vidari, & Neric, 2004). An anti-inflammatory flavonoid, mesuaferrin A, was isolated from this plant (Chaithanya, Gopalakrishnan, Hagos, & Rao,

2018). Various polyphenol compounds and flavonoids, including gallic acid, ellagic acid, coumaric acid, vanillic acid, rutin, quercetin, myricetin, and kaempferol, were identified in ethanol and chloroform extracts of *M. ferrea* barks (Rajesh, Manjunatha, Krishna, & Swamy, 2013). Essential oils of *M. ferrea* leaves was mainly constituted of sesquiterpenes including trans-caryophyllene, β -caryophyllene oxide, and α -humulene (Keawsaard & Kongtaweelert, 2012). There are no saponins isolated from *M. ferrea* to date, hence further explorations to find out bioactive compounds from this group are needed to be conducted.

Table 1. Phytochemicals content of crude extract and fractions of *M. ferrea* leaves.

No	Metabolite	Reagent/ method	Positive Result	Result		
				Crude extract	Ethanol fraction	Ethyl acetate fraction
1	Alkaloids	Meyer's	formation of a white precipitate	(-)	(-)	(-)
2	Saponins	foam	formation of stable foam	(+)	(+)	(+)
3	Phenolics	FeCl ₃	formation of dark green color	(+)	(+)	(+)
4	Flavonoids	Shinoda's	formation of yellow color	(+)	(+)	(+)
5	Terpenoids	Liebermann - Burchard's	formation of reddish brown color in organic phase	(+)	(+)	(+)

The FTIR fingerprint of crude extract of leaves of *M. ferrea* and its fractions is depicted in Figure 1. Table 2 presents the different FTIR absorption bands for 3 tested samples. The assignments of both stretching and bending vibrations at eight regions of fingerprint were localized between 700 and 3600 cm⁻¹ (Zavoi et al., 2011). However, there are no signals observed in region 8 (3350-3600 cm⁻¹) that represents OH stretching vibrations of water, alcohols, phenols, carbohydrates, or peroxides in all three samples.

Region 1 corresponding to C-H out-of-plane bending vibrations was specific to terpenoids. Vibrations in this region observed in extract and both fractions supported the phytochemistry screening result, that all three samples containing terpenoids. A strong peak in regions 2 correspond to C-O stretching of sugar moiety in glucosides was also observed in all samples. A weak peak was also observed in region 7 that represented the presence of lipid (Topală et al., 2017).

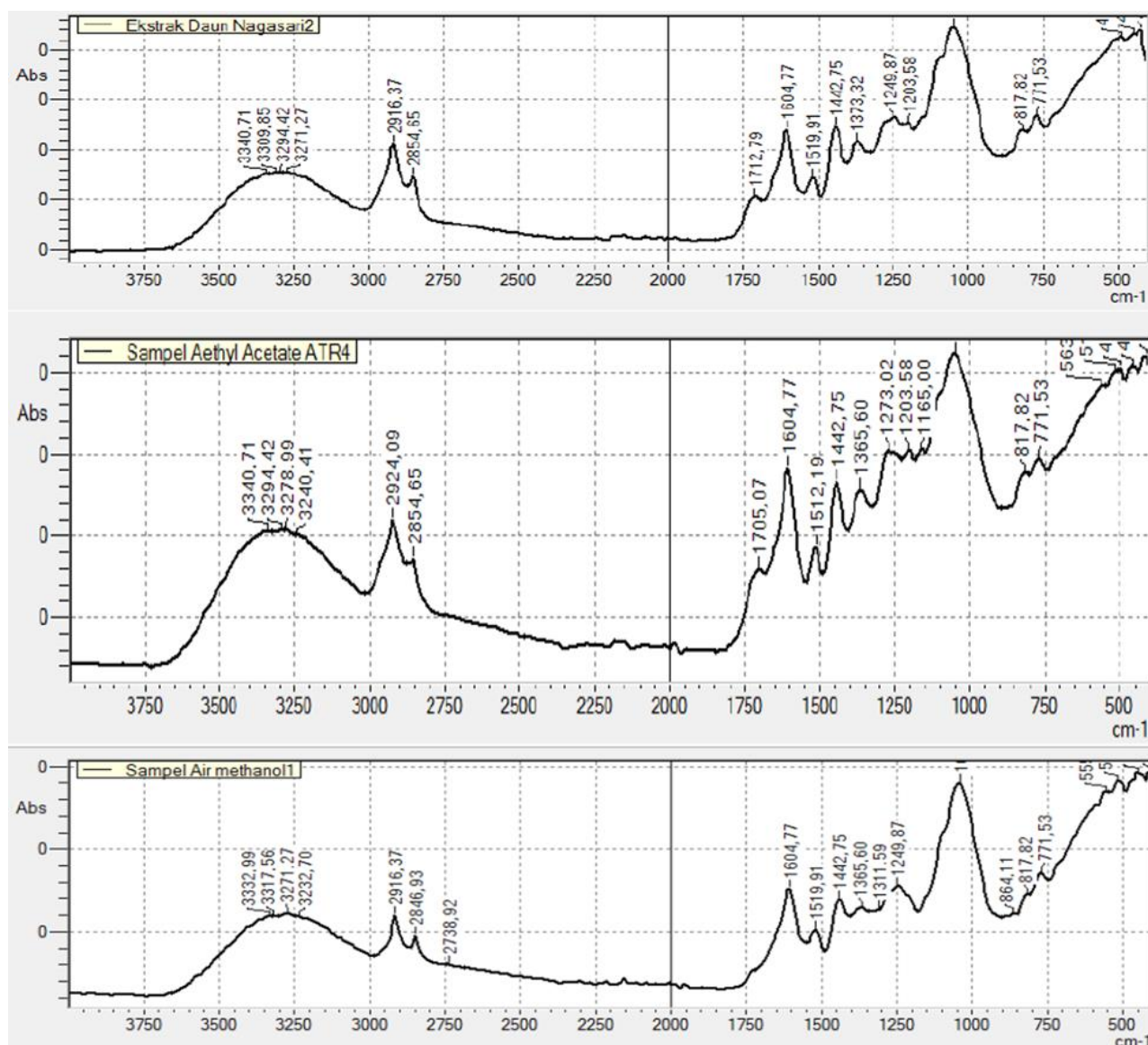


Figure 1. The representative FTIR fingerprint of crude extract and fractions of *M. ferrea* leaves.

Table 2. Comparison of FT-IR absorption bands and the vibration assignments of crude extract and fractions of *M. ferrea* leaves.

Region	Assignment (Topală et al., 2017)	The frequency of measured peaks (cm ⁻¹)		
		Crude extract	Ethanol fraction	Ethyl acetate fraction
Region 7 (2800-2900 cm ⁻¹)	C-H stretching vibrations specific to CH ₃ and CH ₂	2854.65;	2846.93;	2854.65;
		2916.37	2916.37	2924.09
Region 6 (1600-1760 cm ⁻¹)	C=O stretching vibration, Amide I C-N stretching, COO ⁻ anti-symmetric stretching	1604.77;	1604,77	1604.77;
		1712.79	1705.07	1705.07
Region 5 (1500-1600 cm ⁻¹)	Amide II N-H deformation and aromatic domain	1519.91	1519.91	1512.19

Region	Assignment (Topalá et al., 2017)	The frequency of measured peaks (cm ⁻¹)		
		Crude extract	Ethanol fraction	Ethyl acetate fraction
Region 4 (1300-1450 cm ⁻¹)	C-O stretching vibrations (amide) and C-C stretching from phenyl groups, COO symmetric stretching, CH ₂ bending, CH ₂ symmetric bending	1373.32; 1442.75	1311.59; 1365.60; 1442.75	1365.60; 1442.75
Region 3 (1150-1270 cm ⁻¹)	C-O stretching	1203.58; 1249.87	1249.87	1165.0; 1203.58
Region 2 (997-1140 cm ⁻¹)	C-O stretching, C-C stretching of carbohydrates	1049.28	1041.56	1049.28
Region 1 (< 1000 cm ⁻¹)	C-H out-of-plane bending vibrations	817.82; 711.53	864.11; 817.82; 771.53	817.82; 711.53

In this study, MIC was determined statistically. The lowest concentration of the extract and fractions with a significant difference of diameter of inhibition zone from that of the negative control was assigned to be the MIC of the tested samples. The inhibitory zones resulted from various concentrations of crude extract of leaves of *M. ferrea* and its fractions are presented in Table 3. Their MICs are presented in Figure 2.

At the higher concentration (500 and 1000 µg/ml), crude extract and fractions of leaves of *M. ferrea* demonstrated a better inhibitory activity against *E. coli* compared to the positive control, tetracycline. The crude extract showed an excellent inhibitory activity, with MIC value of 3.9 µg/ml. However, the fractionation process decreased this activity, resulted in the lower MICs of the fractions compared to that of the crude extract. In other hand, crude extract and fractions of leaves of *M. ferrea* at all given concentrations were less potent compared to the positive control against *B. subtilis*. The MIC of the crude extract was significantly higher than those of fractions. Hence, the fractionation process of the crude extract increased its antibacterial activity against *B. subtilis*.

These results showed that the crude extract of *M. ferrea* leaves was more potent against *E. coli* than against *B. subtilis*. It is in accordance with the available antibacterial activity data of this plant. For example, methanol extract of *M. ferrea* seeds was more potent against *E. coli* than against *B. subtilis*, with their respective MIC were 10 and 80 µg/ml (Rawat & Upadhyaya, 2013). The similar result was reported, that the MIC of methanol extract of whole flowers of *M. ferrea* towards *E. coli* (10 µg/ml) was lower than that of *Bacillus* spp. (80 µg/ml) (Mazumder, Dastidar, Basu, Mazumder, & Singh, 2004). Another report mentioned that the diameter of the inhibition zone of 10% ethanolic extract of barks of *M. ferrea* against *E. coli* was significantly higher than that of *B. subtilis* (Phuong & Nhat, 2015).

Table 3. The diameter of inhibitory zones of crude extract and its fractions of *M. ferrea* leaves against tested microorganisms

Group	The diameter of the inhibitory zone (mm) against															
	<i>E. coli</i>				<i>B. subtilis</i>				<i>C. albicans</i>				<i>S. cerevisiae</i>			
	Crude extract	Ethanol fraction	Ethyl acetate fraction	Crude extract	Ethanol fraction	Ethyl acetate fraction	Crude extract	Ethanol fraction	Ethyl acetate fraction	Crude extract	Ethanol fraction	Ethyl acetate fraction	Crude extract	Ethanol fraction	Ethyl acetate fraction	
Control (-)	6.9±0.20	6.9±0.20	6.9±0.20	6.7±0.60	6.7±0.60	6.7±0.60	2.6±0.04	2.6±0.04	2.6±0.04	0.4±0.03	0.4±0.03	0.4±0.03	0.4±0.03	0.4±0.03	0.4±0.03	
Control (+)	18.2±2.90	18.2±2.90	18.2±2.90	16.0±0.29	16.0±0.29	16.0±0.29	32.0±0.18	32.0±0.18	32.0±0.18	36.2±0.04	36.2±0.04	36.2±0.04	36.2±0.04	36.2±0.04	36.2±0.04	
1000 µg/ml	25.0±0*	24.3±0.51*	24.6±0.50*	22.4±3.14*	12.7±0.60*	12.3±1.10*	22.6±0.01*	20.1±0.02*	18.3±0.02*	23.3±0.03*	20.5±0.05*	20.2±0.02*	20.2±0.02*	20.2±0.02*	20.2±0.02*	
500 µg/ml	21.3±0.58*	20.0±0*	23.0±1.70*	20.4±5.57*	11.7±0.60*	10.6±1.10*	20.2±0.02*	17.6±0.02*	17.2±0.02*	19.1±0.02*	1.83±0.05*	18.2±0.00*	18.2±0.00*	18.2±0.00*	18.2±0.00*	
250 µg/ml	18.0±0*	15.3±4.00*	20.6±0.50*	18.1±3.52*	11.0±0*	10.6±0.50*	18.8±0.02*	17.2±0.04*	16.2±0.02*	18.1±0.00*	17.3±0.02*	17.1±0.02*	17.1±0.02*	17.1±0.02*	17.1±0.02*	
125 µg/ml	17.0±0*	12.6±0.50*	17.3±0.37*	17.7±3.93*	12.2±2.40*	12.3±0.50*	17.7±0.06*	15.8±0.02*	14.5±0.04*	17.5±0.02*	16.4±0.04*	16.1±0.01*	16.1±0.01*	16.1±0.01*	16.1±0.01*	
62.5 µg/ml	16.1±1.01*	20.0±0*	14.8±0.36*	15.3±4.62*	10.4±0.30*	10.6±0.50*	10.2±0.88*	10.3±0.89*	8.7±0.75*	15.4±0.04*	15.2±0.01*	14.5±0.04*	14.5±0.04*	14.5±0.04*	14.5±0.04*	
31.3 µg/ml	15.7±0.58*	10.3±0.46*	11.6±0.35*	13.4±3.37*	11.2±1.60*	10.6±0.50*	13.3±0.05*	12.1±0.05*	12.2±0.02*	14.5±0.02*	14.3±0.01*	14.2±0.02*	14.2±0.02*	14.2±0.02*	14.2±0.02*	
15.6 µg/ml	13.7±3.29*	8.0±0.20	9.3±1.50	12.0±0.06*	9.4±1.20*	10.0±0*	12.0±0.08*	10.5±0.05*	11.3±0.02*	13.3±0.01*	13.0±0.01*	12.7±0.01*	12.7±0.01*	12.7±0.01*	12.7±0.01*	
7.8 µg/ml	16.0±0*	12.6±0.50	9.6±0.50	11.3±0.58	9.3±0.60*	9.6±0.50*	11.1±0.11*	9.6±0.50*	10.1±0.02*	12.5±0.01*	12.1±0.01*	12.1±0.01*	12.1±0.01*	12.1±0.01*	12.1±0.01*	
3.9 µg/ml	14.0±0*	10.6±0.25	9.0±0.26	10.0±0.00	7.0±0.56	3.8±0.53	10.1±0.07	6.2±0.02	8.2±0.02	10.8±0.07*	10.3±0.02*	10.0±0.00*	10.0±0.00*	10.0±0.00*	10.0±0.00*	

Note: * showed that the diameter of the inhibitory zone of the given group was significantly different from that of negative control against the same tested microorganisms, the analysis was conducted at the level of confidence of 0.95.

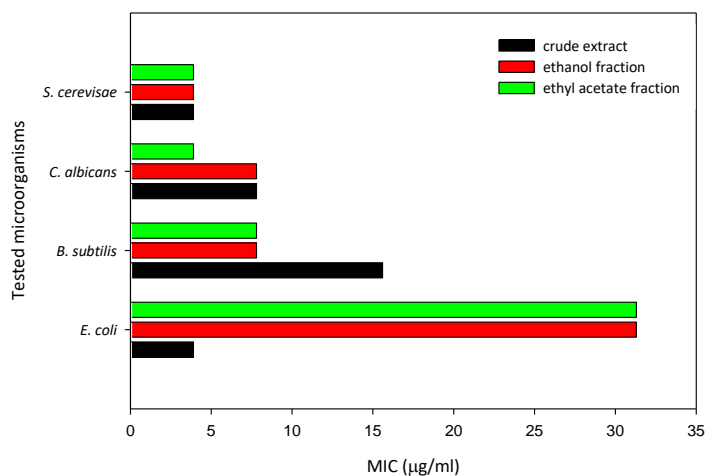


Figure 2. The MIC values of crude extract and fractions of of *M. ferrea* leaves against tested microorganisms.

The antifungal activity of extract and fractions of leaves of *M. ferrea* were lower than that of ketoconazole, the control positive used in this study. However, their MICs were considerably low. The most potent sample against *C. albicans* was the ethyl acetate fraction, while the crude extract and ethanol fraction shared the same MIC value. *S. cerevisiae* was the most sensitive microorganisms to all sample tested, with the same MIC value of 3.9 µg/ml (Table 3 and Figure 2). Compared to the MIC of these samples to bacteria, their MIC against fungi were lower. Our result described the antifungal activity of *M. ferrea* for the first time. Previously, studies demonstrated that the seeds and flowers of this plant did not possess antifungal activity. Extracts of *M. ferrea* seed collected from Rajkot, India demonstrated no antifungal activity against strains of *Candida* spp. and *Cryptococcus* spp. (Chanda et al., 2013). The lack of inhibition of the growth of *C. albicans*, *Aspergillus niger*, and *S. cerevisiae* by dichloromethane-methanol (1:1 v/v) extract of *M. ferrea* flowers was also reported (Kumar, Chauhan, Padh, & Rajani, 2006). These different results might be due to the different metabolites in the tested *M. ferrea* samples, related to the parts of the plants that were used as well as the geographic origin where the plants were grown. The evidence of the effect of growing site of a given medicinal plant to its metabolites profile has been documented (Zhao et al., 2012).

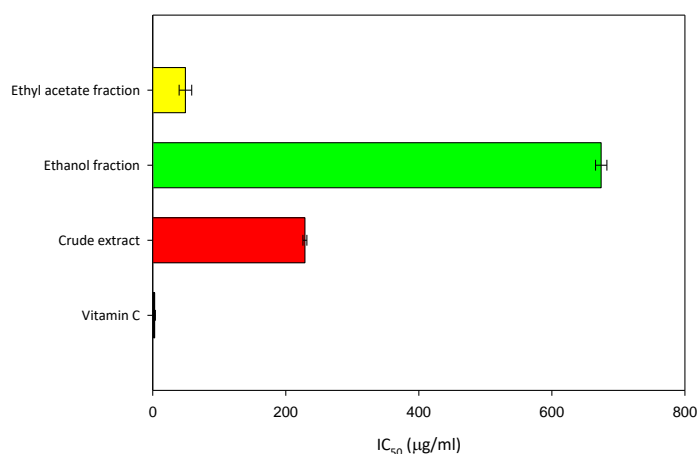


Figure 3. IC₅₀ of crude extract of leaves of *M. ferrea* and its fractions in scavenging free radical of DPPH. EtOH and EtOAc are the ethanol and ethyl acetate fractions of the extract of *M. ferrea* leaves, respectively.

Ethyl acetate fraction of *M. ferrea* leaves demonstrated the strongest free radical scavenging activity with an IC₅₀ value of 49.19 µg/ml, while crude extract and ethanol fraction had a much weaker activity. It indicated that the antioxidant metabolites of *M. ferrea* leaves were those with relative intermediate to lower polarity (semipolar compounds). However, the antioxidant activity of ethyl acetate fraction was significantly lower compared to vitamin C (Figure 3). Our finding supported the available reports on the antioxidant potency of this plant. The IC₅₀ values of hexane, ethyl acetate, and methanol extracts of *M. ferrea* barks collected from Visakhapatnam District, India, were 237.40, 67.35, and 103.66 µg/ml, respectively (Chaitanya et al., 2015). Chloroform and ethanol extracts of *M. ferrea* barks also demonstrated a significant in vitro antioxidant activity tested on erythrocytes, Hb and DNA (Rajesh et al., 2013). In addition, methanolic extract of *M. ferrea* flowers exhibited in-vivo antioxidant activity in Balb/c mice (Garg, Sharma, Ranjan, & Attri, 2009).

Conclusions

The crude extract of *M. ferrea* leaves and its ethanol and ethyl acetate fractions contained saponins, phenolic compounds, flavonoids, and terpenoids. The presence of terpenoids and glycosides in those samples was also supported by the FTIR data. Those secondary metabolites might be responsible for a considerable antibacterial, antifungal, and free radical scavenging activity demonstrated by the crude extract and the fractions.

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