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Research Article

Phenolic and Flavonoid Content of Black Mulberry (*Morus nigra* L.) Stem and Their Evaluation Antioxidant and Cytotoxic Profile

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Abstract

The black mulberry (*Morus nigra*) plant has been widely developed for its bioactivity as natural medicine. This study evaluated plant extracts' total phenolic and flavonoid content and their correlation to *M. nigra* stem's antioxidant activity and toxicity. Dry powder from the *M. nigra* stem was extracted by maceration with 96% ethanol to obtain a thick extract (TE) and fractionated using hexane (HF), ethyl acetate (EAF), and ethanol-aqueous (EF). The samples were analyzed for a compound using reagents and the determination of total phenolic and flavonoid content. The samples were evaluated for antioxidant activity using several parameters and their cytotoxic effects using the BSLT method. Identification of compounds in EAF was confirmed to contain phenolic, flavonoids, alkaloids, saponins, tannins, steroids, and terpenoids. EAF showed higher phenolic and flavonoid content than others. The evaluation of antioxidant activity showed that extracts and fractions from *M. nigra* stems showed the ability to reduce ions and free radicals. EF sample has activity in reducing Mo (IV) ion by TAC method of 98.82±0.53 µM/mg, indicating substantial antioxidant capacity. In addition, EAF samples showed potential activity in reducing DPPH, hydroxyl, and peroxide radicals in the β-carotene bleaching method with IC_{50} values of 12.13, 42.06, and 57.6 µg/mL, respectively. Similar activity was also seen in the cytotoxic effect of a robust EAF sample with an LC_{50} value of 16.31 µg/mL. The results show that EAF can be developed as a raw material for traditional medicine as an antioxidant and anticancer candidate with a significant flavonoid and phenolics content.

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INTRODUCTION

The black mulberry (*Morus nigra* L.) plant is one of the most commonly used *Moraceae* family of three mulberry species and has been widely used in traditional medicine. *Morus nigra* is widely distributed in tropical and subtropical climates[1-3]. *Morus nigra* has been scientifically proven for its anti-inflammatory bioactivity[3,4], antibacterial[3,5], anticancer[6], and antioxidant[6,7]. Compounds support this in *M. nigra* bark extract[8], containing flavonoids, tannins, monoterpenoids, sesquiterpenoids, quinones, phenolics, and several other secondary metabolite compounds obtained from isolation on the skin: 5'-geranyl-5,7,2',4'-tetrahydroxyflavone, mulberroside A, and mulberroside B[1,9].

Morus nigra has been widely developed for its bioactivity based on its excellent flavonoid and phenolic content[1]. Phenolics are secondary metabolites produced by most plants and are found in several plant organs, such as leaves, fruits, roots, and stems, each of which has different levels[10]. Phenolic compounds have a stable chemical structure and can stabilize free radicals. This is because phenolic compounds have hydroxyl groups that can donate hydrogen atoms or electrons to free

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radicals. Catechol and gall groups can inhibit the formation of metal-induced free radicals through the Fenton reaction of Fe^{2+} or Cu^{+} with hydrogen peroxide (H_2O_2), forming reactive hydroxyl radicals[11,12]. In addition to phenolic compounds that function as antioxidants, flavonoid compounds also have antioxidant activity in the presence of hydroxyl groups in flavonoid compounds that can inhibit free radical reactivity and are also able to chelate metal ions which can prevent the formation of free radicals so that they can damage body biomolecules[13]. Phenolic compounds and flavonoids can also act as antimicrobial, anti-inflammatory, antihepatotoxic, antitumor, and anticancer properties[13-15].

Based on this description, a study was conducted to complement the bioactivity data from previous studies of *M. nigra*. The development was carried out on the antioxidant activity of *M. nigra* stems using several test models, i.e., the total antioxidant capacity (TAC), DPPH, hydroxyl radical, and β–carotene bleaching method. The toxicity effect of extracts and fractions of the *M. nigra* stem using the Brine Shrimp Lethality Test (BSLT) method. Studies on black *M. nigra* plants' antioxidant and cytotoxic effects have been widely published. However, bioactivity information on extracts and fractions from the stem of *M. nigra* is still minimal. In this study, the bioactivity of extracts and fractions of *M. nigra* stems as antioxidants were developed using various test parameters and their cytotoxic effects using the BSLT method. This test was conducted to provide additional information on *M. nigra* stems that have not been widely reported as antioxidant candidates and their cytotoxic effects using the BSLT method. The purpose of this study can support the development of medicinal raw materials from the *M. nigra* stem as antioxidants and supportive therapy in the treatment of cancer.

MATERIALS AND METHODS

Materials

Pro analytical grade solvents such as ethanol, ethyl acetate, *n*-hexane and chemical reagents such as Na_2CO_3, Follin Ciocalteu, $AlCl_3$, ammonium molybdate, $FeSO_4$, sodium salicylate, H_2O_2, sulfuric acid, sodium phosphate, were obtained from the distributor Merck (New Practica Alkesindo) Makassar, South Sulawesi, Indonesia. DPPH reagents and standards such as gallic acid, quercetin, and beta carotene were obtained from Sigma Aldrich. *Artemia salina* leach eggs are a collection from the pharmaceutical biology laboratory of Sekolah Tinggi Ilmu Farmasi Makassar, South Sulawesi, Indonesia. *Morus nigra* stem samples were obtained from the "Kampung Sabbeta" Plantation, Soppeng Regency, South Sulawesi, Indonesia. *Morus nigra* samples were taxonomically determined by Dr. A. Marnisa, M.Si., and the specimens (094-SKAP) were documented at the Plant Anatomy and Biology Laboratory, Makassar State University, South Sulawesi, Indonesia. **Figure 1** is a documentation of the *M. nigra*.

a **b**

Figure 1. *Morus nigra* plant with stems and leaves (**a**) and shape of *M. nigra* stem (**b**).

Methods

Sample preparation

The *M. nigra* stem collected was sorted wet to separate the impurities. Furthermore, the samples were washed using running water to remove impurities still attached to the sample, then chopped and cut into small pieces to facilitate drying. After that, the samples were dried using an oven at 40°C for 3 x 24 hours. As much as 800 g of the dry *M. nigra* stem was powdered and extracted by maceration using 70% ethanol solvent three times 24 hours while stirring occasionally. The filtrate was filtered, and the same process removed the residue. The filtrate was then evaporated using a rotary vacuum evaporator to obtain a thick extract (TE). The extraction process was carried out three times to get maximum extract results, then the amount of extract obtained was calculated by calculating the % yield with the following **Equation 1**.

$$\%yield = \frac{Extract\ weight}{Dry\ sample\ weight} \times 100\% \qquad [1]$$

Liquid-liquid extraction

Ethanol extract of the *M. nigra* stem was fractionated by liquid-liquid extraction using non-polar to polar solvents: *n*-hexane, ethyl acetate, and ethanol-water (1 : 9 v/v). A total of 10 g of *M. nigra* stem extract was added to 25 mL of 70% ethanol : water (1 : 9 v/v). The mixture was put into a separating funnel and then extracted liquid-liquid with 25 mL of *n*-hexane, shaken vigorously, and allowed to stand until two phases were formed. The *n*-hexane phase was separated, and the ethanol phase was re-extracted with *n*-hexane until the solution was colorless. The ethanol residue was then added with 25 mL of ethyl acetate, shaken, allowed to form two layers, and then separated. The ethanol phase was then fractionated with the same process in ethyl acetate until the solution was colorless. The filtrate obtained from the liquid-liquid extraction of *n*-hexane and ethyl acetate was then evaporated to obtain a thick extract from the ethanol-water fraction (EF), ethyl acetate fraction (EAF), and *n*-hexane fraction (HF).

Identification of chemical compounds

The procedure identified chemical compounds in the extract and stem fraction of *M. nigra* extract and stem fraction. The phytochemical screening process in the samples was carried out using specific reagents to identify groups of phenolic compounds, flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids[16].

Total phenolic and flavonoid content analysis

The phenolic content of *M. nigra* stem extract and fraction was determined by a colorimetric method using Folin–Ciocâlteu reagent in an alkaline atmosphere, and gallic acid was used as a standard which is one of the phenolic compounds. The phenolic content was determined by varying the gallic acid stock solution (10-100 µg/mL) and 0.5 mL *M. nigra* stem fraction stock solution in a 5 mL volumetric flask. The chemical reagents were added (0.4 mL Folin–Ciocâlteu reagent and 3 mL Na_2CO_3 (7.5%w/v), then the volume was made up to 5 mL using distilled water and incubated at room temperature for 30 minutes. After incubation, the test solution mixture was measured absorbance with UV-Vis spectrophotometry at 650 nm. Flavonoids content from *M. nigra* stem extract and fraction was determined using aluminum chloride and quercetin as a standard. Determination of phenolic content was done by making a calibration curve of a standard solution of quercetin with various concentrations (10-100 µg/mL). Standard solution and *M. nigra* stem samples were reacted with 0.1 mL sodium acetate (1 M) and 0.1 mL $AlCl_3$ (10% w/v) and allowed to stand for five minutes. Then the volume was made up to 5 mL with pro-analytical ethanol and incubated for 30 minutes. After incubation, the test solution mixture was measured absorbance with UV-Vis spectrophotometry at 430 nm[15].

Antioxidant evaluation: total antioxidant capacity (TAC) method

Total antioxidant capacity testing was carried out according to procedures from previous studies[17,18]. The antioxidant capacity of each sample was determined by the total antioxidant capacity expressed in terms of quercetin equivalent antioxidant capacity using quercetin stock solution with various concentrations (10-100 µg/mL) as a standard curve. Each concentration series of quercetin standard and sample solution was reacted with 4 mL of TAC reagent consisting of 2 mL

sulfuric acid (0.6 M), 1 mL sodium phosphate (28 mM), and 1.5 mL ammonium molybdate (1% w/v). The mixture was heated in the oven at 95°C for 10 minutes. After the incubation period, the mixture was cooled at room temperature, and then the absorbance was measured using UV-Vis spectrophotometry at a wavelength of 695 nm.

Antioxidant evaluation: DPPH radical scavenging method

The antioxidant activity of the extract and the fraction of *M. nigra* stems were measured using the DPPH radical reduction method[19]. Each extract solution and *M. nigra* stem fraction were made in series with 10-1000 µg/mL concentrations. Many volumes of extract and fraction were reacted with 1 mL of DPPH solution. The mixture was homogenized, and the final volume was 5 mL. The mixture was incubated for 30 minutes in a dark place. After the incubation period, the absorbance of the sample solution and the fraction after the reaction were measured using a visible spectrophotometer at a wavelength of 515 nm. The antioxidant activity of *M. nigra* stem extract and fraction was calculated by the percentage of inhibition using **Equation 2**, in which A_b was the absorbance of the blank without the sample and A_s was the absorbance in the presence of the sample and comparison.

$$\%inhibition = \frac{Ab-As}{Ab} \times 100\% \quad [2]$$

Antioxidant evaluation: radical hydroxyl method

The ability of extracts and stem fractions of *M. nigra* to scavenge hydroxyl radicals can be determined according to the procedure from previous studies[20,21]. About 3 mL reaction mixture contained 1 mL of 1.5 mM $FeSO_4$, 0.7 mL of 6 mM hydrogen peroxide, 0.3 mL of 20 mM sodium salicylate, and the extract and fraction of *M. nigra* stems with various concentrations (100-1000 µg/mL). The mixture was then incubated at room temperature for 10 minutes. After incubation, the absorbance of the test solution mixture was measured using UV-Vis spectrophotometry at a wavelength of 522 nm. The scavenging activity of the hydroxyl radical effect was calculated using **Equation 3**, in which A_0 was the absorbance of the blank and A_1 was the absorbance in the extract presence.

$$\%inhibition = \frac{Ab-As}{Ab} \times 100\% \quad [3]$$

Antioxidant evaluation: β-carotene bleaching method

The β-carotene emulsion was prepared using 2 mg of β-carotene powder dissolved in 0.2 mL of chloroform, evaporated, and added with 0.2 mL of linoleic acid, 2 mL of tween 20, and made up to 100 mL of distilled water. The mixture was vortexed until a transparent solution was obtained. Each extract stock solution and fraction was taken at a specific volume (10-1000 µg/mL) into a 5 mL volumetric flask, and 2 mL of the emulsion was added. Then the mixture volume was made up to 5 mL in a volumetric flask and incubated for 20 minutes at 50°C. After incubation, the absorbance was measured in the 400-500 nm wavelength range. Measurements were monitored for two hours at 30-minute intervals. Antioxidant activity was calculated based on the difference in degradation of the sample and control (emulsion only). The %inhibition of β-carotene degradation rate was calculated based on **Equation 4**, in which *a* was sample absorbance at 0 minutes, *b* was sample absorbance at 120 minutes, and *t* was time incubation (120 minutes). The IC_{50} value was determined by plotting % inhibition against each sample concentration[21,22].

$$\%inhibition = ln\frac{a}{b} \times \frac{1}{t} \times 100\% \quad [4]$$

Cytotoxic evaluation by BSLT method

The toxicity of the extract and stem fraction of *M. nigra* was conducted using the BSLT method with a slight modification[23]. In the initial step, 50-100 mg of *A. salina* eggs were soaked in seawater in an incubator for 48 hours and equipped with lights to provide lighting and an aerator that functions as an oxygen supply prevents the eggs from settling. The eggs hatched and became larvae were then taken a random number of 10 *A. salina* larvae 48 hours old and transferred to a vial container. Variations in test sample solution extract concentration and *M. nigra* stem fraction (20, 40, 60, 80, and 100 µg/mL) were put

into a vial containing *A. salina* larvae and incubated at room temperature for 24 hours under lighting. The mortality percentage of *A. salina* larvae from each concentration was calculated, and the LC_{50} value was determined using **Equation 5** to calculate the larval mortality rate.

$$\%larval\ mortality = \frac{\sum dead\ larvae}{\sum total\ larvae} \times 100\% \qquad [5]$$

Statistical analysis

Data from the determination of phenolic levels, total flavonoids, antioxidant capacity, and BSLT were expressed in mean±SD using Microsoft Excel 15 version. Using Pearson's correlation coefficient values, data on the correlation of each sample's antioxidant activity and toxicity profile to their total phenolic and flavonoid levels were analyzed through Minitab 20 versions.

RESULTS AND DISCUSSION

The *Morus nigra* stem sample that has been obtained is then made into an extract using a maceration method based on soaking using a solvent which results in a change in concentration inside the cell and outside so that the compounds inside the plant will be illuminated with solvents. The extraction results obtained in (**Table I**) showed a small effect from the extraction process using the maceration method with a yield of 7.95%, and after the fractionation process using solvents from non-polar to polar solvents obtained yields of 3.38% (EF), 1.45% (EAF), and 0.07% (HF). The extraction results can be influenced by selecting methods and solvents used in the extraction process. The selection of solvents is based on the principle of "like dissolve like". A compound in the plant will dissolve in a solvent with the same level of polarity. The selection of extraction methods is based on the type of plant part and the level of stability of a compound that can reduce the extraction results obtained[24-26].

Table I. The yield of ethanol extract and fractions of *M. nigra* stem.

Sample	Yield (%)
Thick extract (TE)	7.95
Ethanol fraction (EF)	3.38
Ethyl acetate fraction (EAF)	1.45
Hexane fraction (HF)	0.07

The extraction and fractionation of *M. nigra* stems are then identified with the compound using a specific reagent in the test tube medium, characterized by discoloration after reagent administration. Identifying the compound showed that the extract and stem fraction of the *M. nigra* found positively contained phenolics, terpenoids, steroids, and tannins (**Table II**). However, the HF negative contains flavonoids and alkaloids, while EF and HF negative contain alkaloids and saponins. Compounds in *M. nigra* stem samples tend to be attracted to semi-polar solvents. Ethyl acetate is a semi-polar solvent that can dissolve polar and non-polar compounds[23,27]. It can be observed that the ethyl acetate fraction (EAF) has much content compared to TE, EF, and HF, including flavonoids, phenolics, alkaloids, saponins, terpenoids, steroids, and tannins.

Table II. Phytochemical screening of *M. nigra* stem extracts and stem fractions.

Compound Assay	Result obtained (conclusion)			
	TE	EF	EAF	HF
Flavonoid	Yellow (+)	Yellow (+)	Yellow (+)	Brownish-green (-)
Phenolic	Blue (+)	Blue (+)	Blue (+)	Blue (+)
Alkaloid	No reaction (-)	No reaction (-)	Brown Precipitate (+)	No reaction (-)
Saponin	Foam (+)	No foam (-)	Foam (+)	No foam (-)
Terpenoid	Red (+)	Rose-colored (+)	Red (+)	Rose-colored (+)
Steroid	Brown-green (+)	Blue-green (+)	Blue-green (+)	Blue-green (+)
Tannin	Blue (+)	Blue (+)	Blue (+)	Blue (+)

Determining phenolic and flavonoid levels is based on the discoloration in complex reactions of phenolic and flavonoids of *M. nigra* stems with specific reagents. The determination of total phenolic levels is based on the redox reaction of polyphenols from plant extracts with Folin–Ciocâlteu reagents that will form a complex reaction of phosphotungstic phosphomolybdenum and can be characterized by the presence of blue discoloration in the test mixture solution[28,29]. Based on the results obtained from determining the phenolic levels of *M. nigra* stems, EAF had higher phenolic levels of 22.21.21±0.73 mgGAE/g compared to TE of 14.63±0.344, EF of 11.10±0.44 mgGAE/g, and HF of 9.88±1.03 mgGAE/g.

Determination of flavonoid levels of *M. nigra* extracts and stem fractions was done using aluminum chloride reagents based on the colorimetric method[15,30]. The aluminum chloride method is the most commonly used in determining total flavonoid levels based on the complex reaction that occurs between aluminum chloride and the carbonyl group at C_4 and hydroxyl on the C_3 carbon chain to form a stable acid complex reaction, and sodium acetate can function in detecting the 7-hydroxyl group of the flavonoid. Based on the results obtained (**Table III**), EAF has higher levels of 9.74±0.54 mgQE/g than EF (4.97±0.18 mgQE/g), HF (5.34±0.03 mgQE/g), and TE (2.62±0.09 mgQE/g). Based on the results obtained from determining phenolic and flavonoid levels, *M. nigra* stem extracts and fractions can be candidates as antioxidants with good flavonoid and phenolic levels. This is based on the preliminary identification of extracts and fractions from *M. nigra* stems containing phenolics and flavonoids[1,2,7]. Previous report[31] also support that *M. nigra* stems exist of flavonoid and phenolic content. Some plant organs of the *M. nigra* contain flavonoids and phenolics. The total phenolic and flavonoid content of the ethanolic extract of *M. nigra* stem was also reported in the previous study, showing the ethanolic extract of the *M. nigra* stem had total phenolic and total flavonoid content of 34.87 mol GAE/mg and 0.13 mol QE/mg extract, respectively. However, the total phenolic and flavonoid content in the *M. nigra* stem fraction has not been reported.

Table III. Total phenolic and flavonoid content of extract and fraction of *M. nigra* stem.

Sample	Phenolic (mgGAE/g±SD)	Flavonoid (mgQE/g±SD)
Thick extract (TE)	14.63±0.344	2.62±0.09
Ethanol fraction (EF)	11.10±0.44	4.97±0.18
Ethyl acetate fraction (EAF)	22.21±0.73	9.74±0.54
Hexane fraction (HF)	9.88±1.03	5.34±0.03

The antioxidant activity of *M. nigra* stem extracts and fractions was measured using several methods: the TAC, DPPH, hydroxyl radical, and β-carotene bleaching method to see the potential of *M. nigra* stems as antioxidants. The antioxidant activity of a sample derived from natural materials is based on two general mechanisms: the transfer of hydrogen atoms (HAT) and the transfer of single electrons (SET) from a sample from natural materials to stabilize free radicals that have unpaired electrons[17,32]. Therefore, further investigation of the bioactivity of the extract and fraction of *M. nigra* stems as antioxidants were carried out. The antioxidant capacity of extracts and fractions of *M. nigra* stems in dampening DPPH radicals and peroxides is expressed as IC_{50}. The IC_{50} value indicates the magnitude of the concentration of each sample which can inhibit the activity of free radicals by 50%. Antioxidant strength is categorized based on the values obtained: IC_{50} values of <50 µg/mL (very strong), 50-100 µg/mL (strong), 100-200 µg/mL (medium), and >200 µg/mL (weak). Meanwhile, to describe the antioxidant capacity in its ability to reduce metal ions using the TAC method, it is determined based on its equivalence to quercetin (QEAC µM/mg sample). The higher the QEAC value, the greater the antioxidant ability of each sample to reduce ions.

The antioxidant activity was determined using the TAC method to see the potential of a sample of natural materials in reducing Mo (IV) to Mo (V). Based on the results obtained (**Figure 2**), it shows that EF has a higher reduction ability with QEAC values of 98.82±0.53 µM/mg compared to TE with 64.17±0.39 µM/mg, EAF with 60.17±0.47 µM/mg, and HF with 59.72±0.79 µM/mg. The results are based on the content of compounds in each extract, and the fraction of *M. nigra* stems that can reduce Mo (IV)[17,33,34].

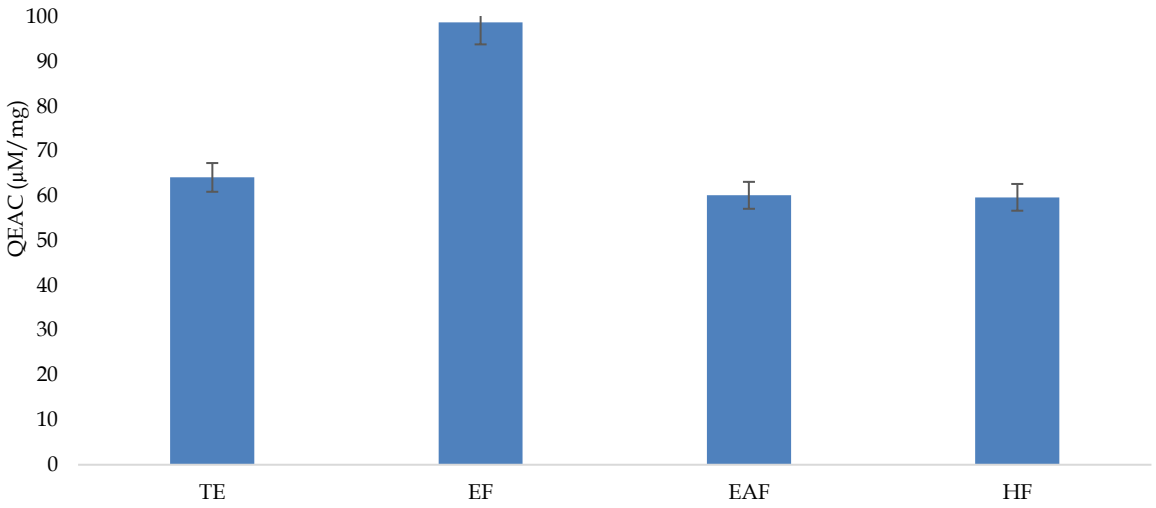

Figure 2. The antioxidant activity of *M. nigra* using TAC Assay from thick extract (**TE**), ethanol fraction (**EF**), ethyl acetate fraction (**EAF**), and hexane fraction (**HF**).

Based on the results of testing antioxidant activity using the DPPH cation radical suppression method (**Table IV**) that EAF and TE have better antioxidant activity with IC_{50} values of 12.13 µg/mL (very strong) and 29.38 µg/mL (very strong), compared to EF and HF with IC_{50} values of 203.01 µg/mL (weak) and 88.72 µg/mL (strong). The DPPH method, which is a cation radical that is widely used as an initial identification to see the potential of biological material samples as antioxidants with a mechanism based on the ability of a sample of natural materials to donate hydrogen atoms or electrons to DPPH cation radicals, which will form stable bonds and can be evaluated by the change in color from purple to yellow along with an increase in concentration[19,35]. Pham *et al.*[31] also obtained similar results in testing the antioxidant activity of *M. nigra* stem ethanol extract in reducing DPPH radicals. The antioxidant activity produced has a very strong activity. The results of the antioxidant activity have the same action found in this study. However, assays using fractions of *M. nigra* stems have not been widely reported.

Table IV. The antioxidant activity of *M. nigra* stem using the DPPH, hydroxyl radicals, and β-carotene bleaching method.

Sample	Antioxidant activity (IC_{50})		
	DPPH (µg/mL±SD)	Hydroxyl radicals (µg/mL±SD)	B-carotene bleaching (µg/mL±SD)
Thick extract (TE)	29.38±1.94	42.06±0.60	63.01±0.84
Ethanol fraction (EF)	203.01±0.40	82.50±0.91	126.25±0.63
Ethyl acetate fraction (EAF)	12.13±1.23	78.27±0.48	57.6±0.05
Hexane fraction (HF)	88.72±1.14	167.07±1.54	83.17±0.55

Based on the test results of extracts and fractions from *M. nigra* stems, TE has antioxidant activity in suppressing hydroxyl radicals with IC_{50} values of 42.06 µg/mL (very strong), while EF and EAF have antioxidant activity with IC_{50} values of 82.50 µg/mL (strong) and 78.27 µg/mL, (strong) respectively. However, HF has antioxidant activity with moderate activity category (167.07 µg/mL). The results showed that the extract and fraction of the *M. nigra* stem had activity in dampening hydroxyl radicals by 50%. The testing of antioxidant activity using the hydroxyl radical method is based on the ability of a sample to reduce hydroxyl radicals. Hydroxyl radicals can be produced in the body in the presence of hydrogen peroxide H_2O_2 which will react with (Fe^{2+} or Cu^{+}) through a Fenton reaction and produce $OH^{\bullet}$ radicals. Hydrogen peroxide can be made in the body from $O^{2\bullet}$ exposure radicals that react with the enzyme superoxide dismutase and produce H_2O_2[12,21,36].
The antioxidant activity was also tested using the β–carotene bleaching method, which is based on the ability of a sample to inhibit lipid peroxide radicals. The principle of the β–carotene bleaching method is to inhibit the degradation rate of β-carotene caused by linoleic acid, which is an unsaturated fatty acid that will be oxidized during the incubation process at 50°C to a hydroperoxide and will attack the chromophore group of β-carotene so that fading occurs color in β-carotene[21,37]. Based on the results obtained from testing the antioxidant activity of the extract and stem fraction of *M. nigra*, it was shown

that TE, EAF, and HF could reduce lipid peroxide radicals with IC_{50} values of 63.01, 57.6, and 83.17 µg/mL, respectively, with strong category. Meanwhile, EF has moderate activity in inhibiting lipid peroxide radicals. This is based on the principle that "like dissolves like" compounds with non-polar properties can inhibit lipid peroxides from attacking the β-carotene chromophore group in the lipid phase of the β-carotene emulsion.

Toxicity testing using the BLST method can provide an initial picture of the activity of compounds contained in the extract and fractions of *M. nigra* stems that have toxic effects. This effect allows for the development of anti-cancer compounds in *M. nigra* stems. A sample will be categorized as toxic if it has an LC_{50} value of >30 µg/mL and less than 1000 g/mL with a less toxic category[23]. Based on the results of the toxic effect test (**Figure 3**), EAF has a high toxic effect with an LC_{50} value of 16.31 µg/mL compared to TE with 40.42 µg/mL, EF with 95.01 µg/mL, and HF with 54.27 µg/mL. The results are related to the content of compounds in the extracted sample and the *M. nigra* stem fraction. The flavonoids in the extracts and fractions will interfere with the digestive organs of *A. salina* larvae causing them to be unable to recognize their food and starve to death[23,38]. Based on the previous determination of flavonoid levels, EAF had higher flavonoid levels. Therefore, the results of testing the toxicity effect with the BSLT method, EAF, have a high toxic effect.

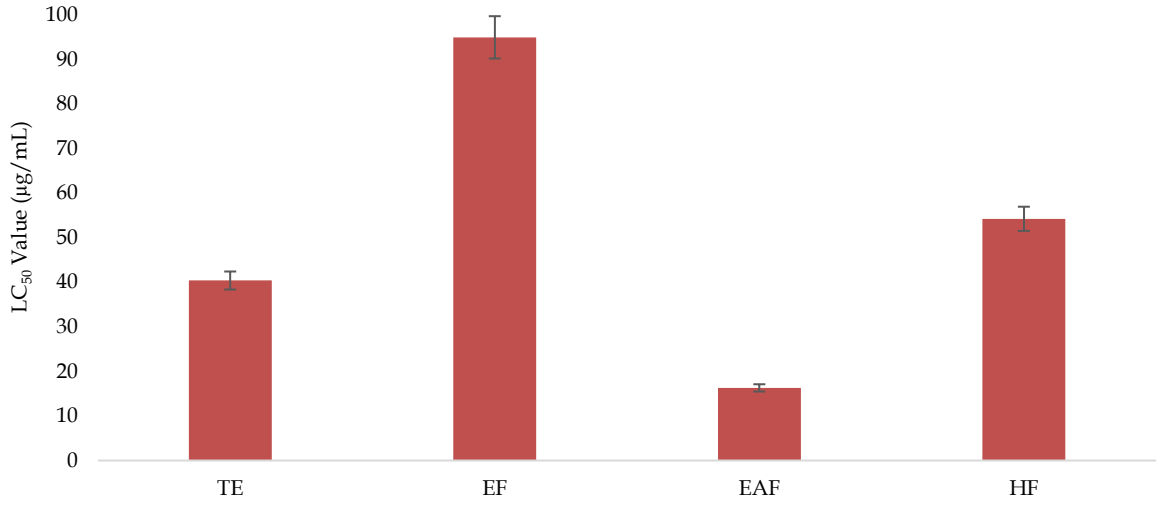

Figure 3. Toxicity test of extract and fraction of *M. nigra* stem using BSLT method from thick extract (**TE**), ethanol fraction (**EF**), ethyl acetate fraction (**EAF**), and hexane fraction (**HF**).

The presence of phenolic and flavonoids from extracts and stem fractions of *M. nigra* has contributed to its biological activity. **Figure 4** shows the relationship between the extract's total phenolic and flavonoid levels and the stem fraction of *M. nigra* on its activity as an antioxidant and its toxic effect, which has been determined based on the simple linear Pearson's correlation. In this study, it was seen that there was a significant negative correlation (R = 0.724 to 0.785) between the total phenolic content of the extract and *M. nigra* stem fraction on the antioxidant activity of the DPPH, β-carotene bleaching, hydroxyl radical, and toxic effects using the BSLT method. This shows that the biological activity of each extract and fraction is influenced by total phenolic compounds >70%, and other compounds affect the rest. However, the TAC method gives a weak negative correlation with R = 0.451. Similar results showed a strong to robust negative correlation (R = 0.736 to 0.822) on total flavonoid levels on antioxidant activity using DPPH, β-carotene bleaching, hydroxyl radicals, and their toxic effects. The correlation value of R = 0.736 to 0.822 for the total flavonoid content indicated that >70% to 82.2% of the biological activity was influenced by flavonoids in each sample. However, the TAC method positively correlated with moderate category (R = 0.504) between total flavonoids and antioxidant levels. This study shows that phenolics and flavonoids influence the extract's antioxidant activity, toxic effects, and the stem fraction of *M. nigra*. This information can allow researchers to develop *M. nigra* stems by searching for active compounds with medicinal properties.

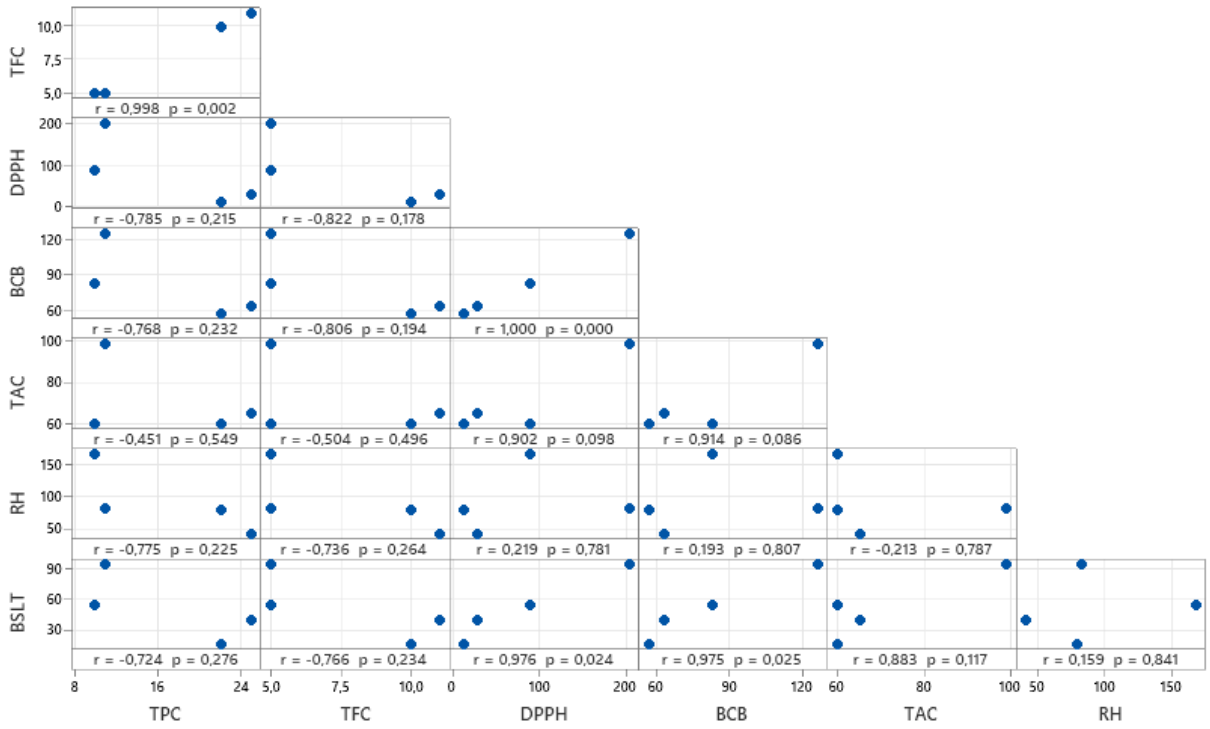

Figure 4. Simple linear Pearson's correlation p-value between total phenolic and flavonoid toward bioactivity assay of extract and fraction of *M. nigra* stem. TPC: Total phenolic content; TFC: Total flavonoid content; DPPH: DPPH method; BCB: β-carotene bleaching; TAC: total antioxidant capacity; RH: hydroxyl radical; BSLT: BSLT method

CONCLUSION

Based on the results of this study to see the antioxidant and toxicity profile of the extract and fraction of the *M. nigra* stem. The ethyl acetate fraction of *M. nigra* stem can be further developed as an antioxidant with its activity in reducing DPPH radicals (powerful category), hydroxyl radicals (strong category), and lipid peroxide radicals by β-carotene bleaching method (strong category) and can be developed as an anticancer with toxic effects on EAF by LC_{50} value of 16.31 µg/mL (very toxic category) which was evaluated using the BSLT method.

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AUTHORS' CONTRIBUTION

Concept design and research development (**SN** and **N**), Extraction procedure, phytochemical composition, and determination of phenolic and flavonoid assay (**FJS** and **M**), Antioxidant evaluation design and data analysis (FJS and SN), Cytotoxic effect testing using the BSLT approach analyzed by **M** and **K**. **N** and **K** performed the statistical analysis. Manuscript preparation, editing, and review process (**SN**, **FJS**, **N**, and **M**).

DATA AVAILABILITY

The data are available from the author Syamsu Nur upon request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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