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Cite as: AIP Conference Proceedings **2347**, 020153 (2021); https://doi.org/10.1063/5.0051514
Published Online: 21 July 2021

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Effectiveness of Ethanolic and Methanolic *Morus nigra* Extracts on Microbial Strains

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Abstract. The effectiveness of *Morus nigra* as natural antimicrobial agents were assessed by the ability of the crude extract to inhibit the growth of *Escherichia coli, Staphylococcus aureus, Aspergillus brasiliensis,* and *Candida albicans*. Different parts (fruit, root, leaves and stem) of *M. nigra* were extracted by maceration using different solvents; ethanol and methanol. The antimicrobial assays were carried out by paper disc diffusion method. *M. nigra* of all part extracted in ethanol were able to inhibit the growth of test microorganisms compared to, when extracted in methanol. Root and fruit extracts of *M. nigra* were able to inhibit the test microorganisms. The analytical analysis using HPLC-DAD was able to identify the presence of flavonoids rutin, quercetin, apigenin and kaempferol in crude extracts that correspond to inhibition zones observed. Hence, the results suggested that crude extracts from *M. nigra* root and fruit have antimicrobial properties to prevent the growth of test microorganisms.

INTRODUCTION

Solvent extraction has been practice traditionally by making coffee or tea. Basically, dry leaves or seed is put into contact with hot water that takes up flavour compound and colouring agent of the plant material. Choosing a right type of solvent can determine the successful extraction of biological active compound from the prepared sample. Several properties of good solvent in plant extraction include the ease of evaporation at low heat, low toxicity, rapid physiological absorption of the extract, preservation action and inability to cause the extract to dissociate [1]. Some factors that need to be considered in choosing the right solvent are; the quantity of phytochemical to be extracted, rate of extraction, diversity of inhibitory compound, further use of the compound and toxicity of the solvent. The choices of solvent also depend on the target compound that needed to be extracted [2]. Solvents that commonly used for extracting plant material are ethanol, methanol, hexane, water, dichloromethane and acetone. During the process of growing, plants carry out metabolite and biochemical process. Various chemical substances accumulated and form chemical composition of plant. Study shown that secondary metabolite from plant extract such as flavonoid alkaloid, terpenoid, phenolic and tannins have medicinal properties. *Morus nigra* also able to produce many secondary metabolites [1]. Antimicrobial resistance of bacteria and fungi becomes a serious threat to society. New emerging microorganism that have resistance toward existing antibiotic become a reason to create new alternative way for production of antimicrobial agent. This study focuses on extraction of valuable compounds from different parts of *M. nigra*; leaves, stem, fruit and roots by maceration using ethanol and methanol as solvents. Extracted compounds are expected to have antimicrobial activities towards bacteria and fungi. The extracted valuable compounds potentially be used in pharmaceutical and agrochemical.

Proceedings of 8th International Conference on Advanced Materials Engineering & Technology (ICAMET 2020)
AIP Conf. Proc. 2347, 020153-1–020153-6; https://doi.org/10.1063/5.0051514
Published by AIP Publishing. 978-0-7354-4118-7/$30.00

MATERIAL AND METHODOLOGY

Preparation of Plant Material

M. nigra plant parts; leaves, stem, fruit and root were obtained from the University Malaysia Kelantan (UMK) Jeli campus. The samples were washed thoroughly under running distilled water to remove all soil and dirt prior to pulverize. Samples were kept at 4 °C for future use.

Extraction of Compounds from *M. nigra*

Compounds were extracted from pulverized samples using maceration method with ethanol and methanol as solvent. 40 g of samples were soaked in 400 mL of ethanol and methanol and left for 3 days at room temperature. Mixture were filtered through Whatman No. 1 filter paper and the crude extracts were dried using rotary evaporator at 40 °C for 1 hour. Dried extract were weighted and kept in a glass container for future use. Percentage yield of extract for 40 g of fine powder sample were calculated using formula $(W_2 - W_1/W_0) \times 100$, where W_2 is the weight of crude extract after removal of solvent, W_1 is the weight of crude extract with solvent and W_0 is the initial weight of powder sample.

Antimicrobial Analyses

The antimicrobial activities of methanolic and ethanolic *M. nigra* crude extracts were tested against Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, fungi *Aspergillus brasiliensis* and mold *Candida albicans* using disc diffusion assay according to Kirby and Bauer method. Test microbial plates were prepared as follows; *S. aureus* and *E. coli* starter cultures were grown in Nutrient Broth at 37 °C for 24 hours and inoculated on the Mueller Hinton agar using sterile cotton swab. *C. albicans* was grown in Sabouraud Dextrose Broth at 28 °C for 48 hours and inoculated on the Sabouraud Dextrose Agar (SDA) using sterile cotton swab. *A. brasiliensis* was cultured on SDA and incubated at 25 °C as a lawn. 2 mL of sterile distilled water was used to scrape off the *A. brasiliensis* spores and 20 µL of the spore suspension was used to spread the SDA agar. 10 µL of the ethanolic and methanolic crude extracts were impregnated on a 6 mm diameter sterile paper disc. The discs were then air-dried in laminar flow for few minutes before transferred onto the inoculated test microbial plates using sterile forcep. Positive controls; Trimethoprim (50 mg/mL) was used for *S. aureus* and *E. coli,* cycloheximide (1 mg/mL) for *A. brasiliensis* and fluconazole (30 mg/mL) for *C. albicans* assay plates, respectively. Whereas, ethanol and methanol as negative control respectively. Bacterial assay plates were incubated at 37 °C overnight, whereas *A. brasiliensis* and *C. albicans* assay plates were incubated at 25 and 28 °C for 2 days, respectively. The formations of inhibition zones were observed daily and recorded. All test were conducted in triplicate.

Quantitative High-Performance Liquid Chromatography Analysis

The quantitative analysis of main phenolic compounds in the ethanolic and methanolic *M. nigra* crude extracts were performed using high-performance liquid chromatography (HPLC) system, equipped with diode array detection (DAD), quaternary pump, online degasser and a C18 reverse-phase column Eclipse plus (4.5 nm × 150 nm; particle size 3.5 µm). DAD was adjusted to 251 nm, 320 nm, and 365 nm with a column temperature at 40 °C. A gradient elution was used with the mobile phase consist of A (95% formic acid) and B (5% acetonitrile) as follows: 0-17 min, A-B (95:5 v/v); 17-21 min, A-B (75:25 v/v); 21-26 min, A-B (50:50 v/v); 26-28 min, (95:5 v/v). The flow rate was kept at 0.75 mL/min with the injection volume of 10 µL. The standard compounds such as rutin, quercetin, quercitrin, apigenin and kaempferol were used to profile various extracts in *M. nigra*. 200 µg/mL of each standard was prepared in HPLC grade methanol. The peak identification was based on the retention time of standards used, presented in the chromatogram. All chromatographic data were recorded and analyzed.

RESULTS AND DISCUSSIONS

Crude Extract Yield

M. nigra leaves extracted in both ethanol and methanol solvents show higher percentage yield compared to root and stem. The highest percentage yield is methanolic root extract at 12.40%. The percentage yield of ethanolic extract from leaves is 7.83% and methanolic extract from leaves is 7.93% (Table 1). The variation in extraction yields might be linked to the different chemical nature of the extractable compounds as well as the polarity of the solvent used. Yield and composition of the plant were influenced by several parameters such as extraction method, temperature, type of solvent used and duration of the extraction process. In this study, ethanol and methanol were selected based on the solubility of target compound in the *M. nigra*. A high yield of extract was observed when ethanol and methanol used in the *M. nigra* extraction process [3]. This might due to the high polarity of the solvents [4]. The most important task in solvent extraction is to find a suitable solvent that can extract the target compound from the plant material. Thus, solvent or solubility screening through prediction method or experiment-based is necessary [5].

TABLE 1. The percentage yield of *M. nigra* crude extracts.

M. nigra crude extract	Percentage Yield (%) / Solvent	
	Ethanol	Methanol
Leaves	7.83	7.93
Stem	5.63	3.93
Fruit	8.13	7.38
Root	4.56	12.40

Antimicrobial Analyses

M. nigra extracts show high to low efficacy against *E. coli*, *S. aureus*, *A. brasiliensis*, and *C. albicans* when extracted using ethanol compared to methanol (Table 2). It also inhibits the growth of *E. coli*, but no activity on *S. aureus*. Largest inhibition zone (40 mm) was observed when *M. nigra* leaves ethanolic extract tested on *C. albicans* Fig. 1. Red arrow indicates zone of inhibition, test sample (T), positive control (+), and negative control (-).

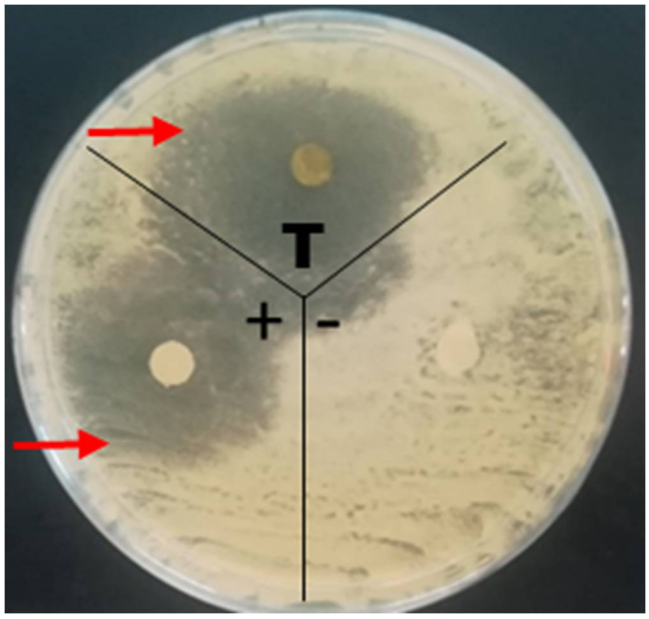

FIGURE 1. The inhibition zone observed on *M. nigra* ethanloic extract against *C. albicans*.

Ethanolic extract of *M. nigra* stem, fruit and root inhibit growth of all test microorganisms, with higher inhibition zone compared to crude extract in methanol. Antimicrobial activities against *E. coli* and *S. aureus* were observed in *M. nigra* fruit when extracted with ethanol [4, 6]. Not much research on antifungal activities on *M. nigra*, but reported that *M. nigra* fruit had anticandidal activity toward *C. albicans* [4]. In this study, the *M. nigra* extracted using both ethanol and methanol show various antimicrobial efficacies ranging from no activity to high inhibitory towards test microorganisms. This could be due to several factors such as extraction method, solvent used, solubility of the compound and the plant parts. Selection of solvents is based on a database that contains only information about the legislation of solvents in different fields of application, exposure limits, and solvent prices [5]. Previous studies show that *M. nigra* had secondary metabolites consist of phenolic acids and flavonoids [3,6,7]. The antimicrobial activity observed in the extract is due to the higher content of phenolic acids and flavonoids, such as presence of quercetin [8], kaempferol and rutin [6] in *M. nigra*. Flavonoids are known to retard the growth of microorganism by inhibiting their nucleic acid synthesis, energy metabolism and cytoplasmic membrane function [9]. Each plant part plays an important role as some part does have more flavonoid compared to other plant part that contributed to the quantity and spreading of antimicrobial agent in different parts of *M. nigra*.

TABLE 2. Average zone of inhibition (mm) of different parts of *M. nigra* crude extract against test microorganisms using paper disc diffusion method. (-) indicates no inhibition zone.

M. nigra crude extract	Solvent	Diameter zone of inhibition (mm)			
		E. coli	*S. aureus*	*A. brasiliensis*	*C. albicans*
Leaves	Ethanol	10.0	-	-	40.0
	Methanol	-	-	-	-
Stem	Ethanol	12.0	12.0	20.0	11.0
	Methanol	8.0	12.0	10.0	-
Fruit	Ethanol	12.0	11.0	22.0	16.0
	Methanol	9.0	-	-	-
Root	Ethanol	10.0	13.0	14.0	10.0
	Methanol	-	-	-	15.0
Positive control	Ethanol	26.0	21.0	12.0	30.0
	Methanol	26.0	21.0	11.0	30.0
Negative control	Ethanol	-	-	-	-
	Methanol	-	-	-	-

Compound Analysis

In this study, the reference standards that were used are rutin, quercitrin, quercetrin, apigenin, and kaempferol and the chromatograms were evaluated by comparing the retention time of the reference standard with extracts. The flavonoids rutin, quercetin, apigenin, and kaempferol were identified in both alcoholic extracts of *M. nigra* when compared to the reference standards used (Table 3 and 4). HPLC chromatograms (at 245 nm, 320 nm, and 365 nm) show the presence of flavonoids with different peaks and retention times which correspond to different types of compounds. Only rutin presents in *M. nigra* leaves ethanol and methanol extracts, however, *M. nigra* leaves contain high concentration of total flavonoid compound (TFC) that indicates more flavonoid can be found in leaves [3]. This result relate with the antimicrobial assay, which shows activity against *E. coli* only. Different results might due to several factors such as the extraction method, temperature and period. Other parts of *M. nigra* extracts show the presence of more compounds that correlate to the antimicrobial assay which shows extracts from *M. nigra* fruit, root and stems were able to inhibit more compared to the leaves. Previous study also confirmed that fruit of *M. nigra* able to inhibit various gram-positive and gram-negative bacteria [10].

Presence of rutin and quercetin were observed in both ethanol and methanol *M. nigra* fruit extracts. The area under the curve of rutin in *M. nigra* fruit ethanol extract is 61.13 mAU, which is lower compared to rutin when extracted using methanol with the value of 67.84 mAU. This indicates that rutin selectively be extracted by methanol. Although methanol relatively more efficient in extracting rutin, ethanol serves more advantages, as it is bio-solvent and degradable thus choosing ethanol to extract rutin from fruit might be good choices of solvent. Ethanol extract has slightly higher area under curve with 25.45 mAU compared to methanol with 24.45 mAU. *M. nigra* root however displays different results in which, three compounds including quercetin, apigenin, and kaempferol present in both ethanol and methanol extracts. This shows that *M. nigra* root contain more flavonoid compared to other parts. Rodrigues et al., (2019) reported 20 flavonoids were found in *M. nigra* including quercetin, kaempferol, and isorhamnetin.

Rutin, quercetin, and apigenin were present in *M. nigra* stem ethanol and methanol extracts. Methanol extracts seem to have greater area under the curve (more than 50 mAU) for all three flavonoids, whereas area under the curve for ethanol extract was less than 30 mAU. This shows that rutin, quercetin, and apigenin were efficiently extracted using methanol. The highest flavonoid amounts were quantified in hydro-methanolic and aqueous of stem and leaves of Morus [12]. Aqueous alcohol solvent is also proved to be a greater solvent compared to absolute alcohol [3]. Rutin, quercetin, apigenin, and kaempferol are flavonoid known to have an antimicrobial activity, which can be exerted in three ways: directly kill the bacteria, synergistically activate the antibiotics, and constrict the bacterial pathogenicity. Apigenin that is flavonoid in subclass flavones has known to have antibacterial properties, potentially be used for development of antibacterial drugs [13], whereas rutin is widely studied for antimicrobial activity against various strains of bacteria and fungi and synergistically enhanced antibacterial activity of other flavonoids against *Bacillus cereus* and *Salmonella enteritidis* [14].

TABLE 3. Flavonoids extracted from different parts of *M. nigra* using ethanol as solvent.

Plant parts	**Fruit**		**Root**		**Leaves**		**Stem**	
Standard	Rt	Auc	Rt	Auc	Rt	Auc	Rt	Auc
Rutin	16.25	61.13	ND	ND	16.25	92.52	16.28	27.35
Quercitrin	ND	ND	ND	ND	ND	ND	ND	ND
Quercetin	22.77	25.45	22.78	58.15	ND	ND	22.81	28.34
Apigenin	ND	ND	23.85	183.83	ND	ND	23.81	26.62
Kaempferol	ND	ND	24.00	118.24	ND	ND	ND	ND

TABLE 4. Flavonoids extracted from different parts of *M. nigra* using methanol as solvent.

Plant part	**Fruit**		**Root**		**Leaves**		**Stem**	
Standard	Rt	Auc	Rt	Auc	Rt	Auc	Rt	Auc
Rutin	16.28	67.84	ND	ND	16.29	299.54	16.28	53.07
Quercitrin	ND	ND	ND	ND	ND	ND	ND	ND
Quercetin	22.80	24.66	22.94	171.09	ND	ND	22.70	50.69
Apigenin	ND	ND	23.87	133.58	ND	ND	23.80	90.10
Kaempferol	ND	ND	24.00	156.10	ND	ND	ND	ND

CONCLUSIONS

The medicinal value of natural herb of the plant is due to the active constituents. It is generally advantages to extract the active constituents to formulate a control doses form of that active constituents rather than using the bulk quantity. Several factors must be considered when selecting extraction process and the solvent used. Only the active, desired constituents should be extracted from the plant material, which means that a high selectivity is required. We managed to extract flavonoid compounds from M. nigra using maceration method with ethanol and methanol as solvents. The compounds extracted show antimicrobial activities on *E. coli*, *S. aureus*, *A. brasiliensis* and *C. albicans*. Results presented may contribute to the development of natural product discoveries.

ACKNOWLEDGMENTS

The authors thank Faculty of AgroBased Industry, Universiti Malaysia Kelantan for the laboratory facilities and SIRIM Berhad for the microbial strains. This project was funded by Fundamental Research Grant Scheme (FRGS) with grant number: R/FRGS/A07.00/01121A/003/2016/000367 from the Ministry of Higher Education (MOHE) Malaysia.

REFERENCES

1. T. Prashant, K. Bimlesh, K. Mandeep, K. Gurpreet, and K. Harleen, Internationale Pharmaceutica Sciencia 1(1), 98–106 (2011).
2. N. S. Ncube, A. J. Afolayan and A. I. Okoh, Current Methods and Future Trends 7(12), 1797–1806 (2008).

3. F. Anwar, S. Kanwal, G. Shabir and A. Hassan, Int. J. Pharmacol 11, 757-765 (2015).
4. M. A. Minhas, A. Begum, S. Hamid, M. Babar, R. Ilyas, S. Ali and S. Andleeb, Pakistan Journal of Zoology 48(5), 1381–1388 (2016).
5. D. Bergs, J. Merz, A. Delp, M. Joehnck, G. Martin and G. Schembecker, Chem. Eng. Technol. 36, 1739-1748 (2013).
6. C. Hu, Y. Wansha, C. Guo, M. Shuai, X. Zhonghuai and H. Ningjia, Molecules 23(1), 4 (2017).
7. S. Iqbal, U. Younas, K. W. Chan, R. A. Sarfraz and K. Uddin, Int J Mol Sci. 13(6), 6651-64 (2012).
8. P. Agata, W. Oleszek and B. Alessandra, J Agric Food Chem 56(9), 3377-80 (2008).
9. B. Thiem and O. Goslinska, Fitoterapia 75, 93–95 (2004).
10. N. Khalid, S.A. Fawad, and I. Ahmed, Pakistan J. Botany 43(SI), 91-96 (2011).
11. Rodrigues et al., (2019) E. L. Rodrigues, G. Marcelino, G. T. Silva, P. S. Figueiredo, W. S. Garcez, J. Corsino, R. C. A. Guimarães and K. C. Freitas, Int J Mol Sci. 14;20(2):301 (2019).
12. I. Thabti, W. Elfalleh, N. Tlili, M. Ziadi, M. G. Campos and A. Ferchichi, International Journal of Food Properties 17:4, 842-854 (2014).
13. H. B. Nayaka, R. L. Londonkar, M. K. Umesh and A. Tukappa, Int. J. Bacteriol. 6, 90-111 (2014).
14. A. Ganeshpurkar and A. K. Saluja, Saudi Pharm J. 25(2):149-164 (2017).