Journal of Biological Engineering Research and Review, 2016; 3(1): 44-48
ISSN: 2349-3232 Online
DOI: 10.5281/zenodo.15337335

Research Article

Phytochemical screening and antibacterial testing of different varieties of *Morus* spp. (Mulberry)

JOB U. RAMOS*, EDEN S. DAVID, KRISTINE GRACE D. WAING

Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines 3120

****E-mail: jobramos028@gmail.com***

ARTICLE INFO:

Article History:
Received: 29/03/2016
Revised: 03/05/2016
Accepted: 12/05/2016
Available Online: 13/05/2016

Keywords:
Mulberry,
Phytochemical screening,
antibacterial testing,
Guisang you 12
Alfonso variety
phytochemical

Abstract: Mulberry (*Morus* spp.) is generally utilized in silk industries. Mulberry leaves are known as natural reservoir of botanical nutrients and show strong antibacterial activity. The presence of phytochemicals in plants is associated with therapeutic and medicinal properties. Thus, this study determined the antibacterial properties of different varieties of mulberry namely Guisang you 12, Alfonso and S54 varieties using paper disc diffusion method. Two extraction methods were used namely ethanol and hot water extraction. Results on antibacterial assay against *Escherichia coli*, showed that the ethanol extracts were active at 12 hours of incubation with S54 variety having the highest mean value of zone of inhibition of 7.80 mm, followed by Alfonso variety and Guisang you 12 variety. In the test against *Staphylococcus aureus*, ethanol extracts also showed zone of inhibition at 12 hours of incubation. Ethanol extracts of Alfonso variety showed the highest zone of inhibition with a value of 11.15 mm, followed by S54 and Guisang you 12. The phytochemical constituents were tested using test tube method in both hot water and ethanol extracts of the three *Morus* varieties. Results revealed that both extracts contain various phytochemicals that can be found in traces and/or appreciable amount. These were flavonoids, tannins, alkaloids, saponins, and terpenoids.

INTRODUCTION

Mulberries (*Morus* spp.) are generally utilized in silk and non-silk industries. In particular, the mulberry is used in silkworm (*Bombyx mori* L.) rearing and cocoon collecting, medical food utilization, ecological environment, application of livestock feed and in the culture of aesthetic enjoyment as well. Most of the mulberry varieties available now possess the characteristics of fast growth, high cutting resistance, large amounts of biological contents and abundant nutrients [1]. The mulberry leaves are known as "the natural reservoir of botanical nutrients" including a great deal of dietary fiber, multiple minerals like magnesium, zinc, iron and copper, 18 kinds of amino acids, tea polyphenol and crude protein. It also contains various vitamins, renieratene and chlorophyll, providing people with many kinds of nutrients [1]. Powdered mulberry leaves can be used as a natural pigment in contacting pastry food, the color of which is green and slashing. Traditionally, in China the mulberry plant are wholly used in any medicinal aspects, from its roots to their fruits; i.e. antioxidant, anti-diabetic, anti-inflammatory. Furthermore, its leaves were utilized and found out to have antibacterial properties. Mulberry leaves contain kuwanon G, mulberrofuran G and albanol B, all shown strong antibacterial activity ranging from 5 to 30 mg/milliliters minimum inhibitory concentrations (MIC's). Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protection from various diseases [2]. Phytochemicals are primary and secondary compounds in all plants. A wide range of phytochemicals present in plants are known to inhibit bacterial pathogens [3,4,5]. Hence, this study provided an evaluation about the phytochemicals present in the different varieties of mulberry plant and evaluated their anti-bacterial properties.

MATERIALS AND METHODS

Plant materials

The three *Morus* plant varieties were collected at Sericulture Research and Development, Don Mariano Marcos Memorial State University North Luzon Campus at Bacnotan, La Union and at "Tuklas Lunas", Department of Biological Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. These were the Alfonso variety, S54 variety and Guisang you 12 variety. Plant collection was done in the

morning to ensure that the plants are actively photosynthesizing. Mature green leaves were selected and picked by hand. Clear zip lock bags served as temporary containers before air drying and to avoid rotting.

Experimental treatments

Table 1 shows the different treatments used in the study. There were two extraction procedures used: ethanol extraction and hot water extraction. The three *Morus* plant species that were used in the study were Alfonso variety, S54 variety and Guisang you 12, a Chinese variety.

Table 1. Experimental treatments

Treatment	Treatment and Extraction Procedure
T1	Guisang you 12 (Ethanol)
T2	Guisang you 12 (Hot water)
T3	S54 variety (Ethanol)
T4	S54 variety (Hot water)
T5	Alfonso variety (Ethanol)
T6	Alfonso variety (Hot water)
C+	Streptomycin sulfate (Positive control)
C-	Distilled water (Negative control)

Plant powder and extract sample preparation

Leaves of the collected *Morus* varieties were washed thoroughly with running water. Leaf materials were air-dried under shady condition and were grinded in a blender. The ground samples were added with 80% ethanol, and distilled water for 48 hours with 1g: 5milliliters; sample: solvent ratio [6]. The ethanol extract and aqueous extract were filtered using Whatman No. 1 filter paper. The filtrates of the ethanol extraction were concentrated in rotary vacuum evaporator at 40°C, 120 rpm. All the extracts were stored in sterile amber bottles and kept in refrigerator prior to use [7].

Test organisms

Bacterial test pathogens that were used in the antibacterial activity screening of the different varieties of *Morus* plant were *S. aureus* and *E. coli* with code numbers of ATCC25923 and ATCC25922 respectively. Test organisms were obtained from the Center for Tropical Mushroom Research and Development (CTMRD), CLSU, College of Arts and Sciences, Science City of Munoz, Nueva Ecija.

Evaluation of Antibacterial Property

Preparation of inoculum

The method in "Manual on Antimicrobial Susceptibility Testing: Disk Diffusion Test" [8]Ortez (2005) with minor alterations was used as reference in inoculum preparation. Three to five well-isolated bacterial colonies having the same morphological type were selected from the cultured agar plate. A loopful of isolate was transferred into a test tube containing 10 milliliters nutrient broth. The broth culture was incubated at room temperature for 24 hours.
The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard [9]. The comparison was done visually comparing the inoculum tube and the 0.5 McFarland standard against a printed paper with a white background and contrasting black lines under sufficient light condition as shown on Figure 1.

Preparation of assay plates

Thirty eight grams of Mueller Hinton Agar was dissolved in one liter of distilled water and sterilized by autoclaving for 15 minutes at 121 ºC and cooled at 48 ºC. Twenty milliliters of sterilized media was poured at the sterilized petri plates and allowed to solidify. Sterile cotton swabs were soaked on the nutrient broth which contains inoculum and swab thoroughly on the base layer of the plate.

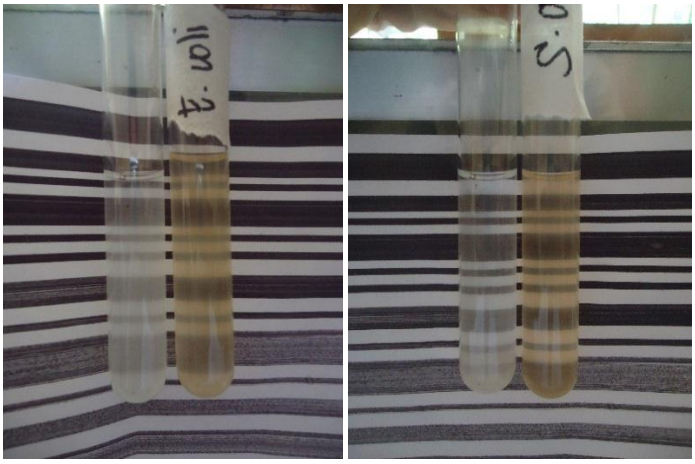

Figure 1. McFarland turbidity testing for E. coli (left) and S. aureus (right)

Preparation of paper discs

Paper discs made from Whatman no. 1 filter paper measuring approximately 6 millimeters in diameter was prepared using a paper puncher. The paper discs were placed in petri plates and then sterilized in an autoclave for 30 minutes in 15 psi at 121ºC.

Screening of antibacterial activity

The disc diffusion test method based on "Manual on Antimicrobial Susceptibility Testing: Disk Diffusion Test" [8] was used for the antibacterial property test. Five filter paper discs of approximately 6 millimeters in diameter were impregnated with each treatment together with the two treatments as positive control (Streptomycin 10µg) and negative control (distilled water). The paper discs were oven dried for one hour at 40 °C. The plates were incubated at room temperature and diameters of the zone of inhibition were measured in millimeters (mm) using a digital vernier caliper after 12, 24, 36 and 48 hours of incubation.

Phytochemical screening

Examination of different phytochemical constituents were carried out on the different extracts of *Morus* varieties following the standard methods as described in [10] Laboratory Manual for the UNESCO (1986) utilizing the point scale + (present in trace amount) ,++ (present in appreciable amount) and - (absence of phytochemical). The different phytochemical components tested were the following: tannins, steroids, saponins, flavonoids, terpenoids, cardiac glycosides, and alkaloids.

Test for tannins

In five milliliters of the extract, 1% lead acetate was added. When yellow precipitate was formed, it indicated the presence of tannins [11].

Test for steroids

Two milliliters of acetic anhydride were added to a 5 milliliters extract of plant sample with 2 milliliters of H_2SO_4. Presence of steroid was indicated by violet to blue or green precipitate [12].

Test for saponins

Plant extract was diluted with 20 milliliters of distilled water and was agitated for 15 minutes in a test tube. Presence of saponins was observed with the formed 1cm layer of foam [12].

Test for flavonoids

In one milliliter of the extract, few drops of dilute sodium hydroxide were added. An intense yellow color was observed indicating the presence of flavonoids [11].

Test for terpenoids

Five milliliters extract of plant sample were added with 2 milliliters $CHCl_3$ in a test tube. Then, 3 milliliters of H_2SO_4 were added carefully to the mixture to form a layer. The formation of the reddish to brown color on the interface indicate the presence of terpenoids [12].

Test for cardiac glycosides

One milliliters of concentrated H_2SO_4 was prepared in a test tube. Five milliliters of the extract from the plant sample were mixed with 2 milliliters of glacial HCH_3CO_2 containing one drop of $FeCl_3$. The mixture was added carefully to 1 milliliter of concentrated H_2SO_4 so that the concentrated H_2SO_4 was underneath the mixture. The appearance of brown ring indicated the presence of cardiac glycosides [13].

Test for alkaloids

Five milliliters of the extract was prepared, then 200 milliliters of 10% HCH_3CO_2 in C_2H_5OH were added. The mixture was filtered and the extract was allowed to become concentrated in a water bath until it reached one fourth of the original volume. Then, concentrated NH_4OH was added. Formation of the white precipitate or turbidity indicated the presence of alkaloids [13].

RESULT AND DISCUSSION

Results showed that the antibacterial property of the plant extracts against *E. coli* was only observed after 12 hours of incubation. As seen on Figure 2 the clear area encircling the disc is the zone of inhibition of each extracts against *E. coli*, with S54 variety ethanol extract with the highest zone of inhibition of 7.80 mm and Alfonso variety hot water extract with the lowest (6.80 mm) (Table 2).

Meanwhile, on the antibacterial screening against *S. aureus*, after 12 hours of incubation all of the treatments showed inhibitions against the said pathogen, as shown on Figure 3. With such, ethanol extracts of Alfonso variety shows the highest value of zone of inhibition with a total mean value of 11.15 mm, followed by S54 variety with 10.89 mm and Guisang you 12 variety 9.08 mm (Table 2).

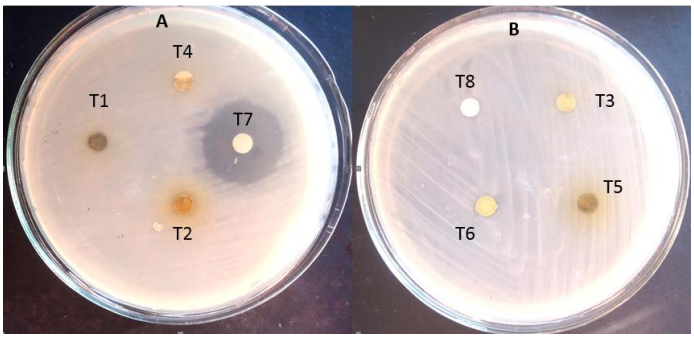

Figure 2. Zone of inhibition treatments against E. coli from 12 hours of incubation. A: (T2) Guisang You 12 HWE), (T6) Alfonso HWE, (T7) positive control (Steptomycin Sulfate. B: (T8) negative control (distilled water). B. (T1) Guisang You 12 EE, (T3) S54 EE, (T4) S54 HWE and (T5) Alfonso EE

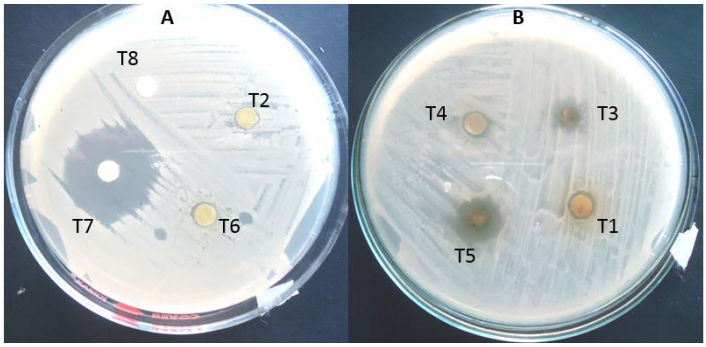

Figure 3. Zone of inhibition treatments against S. aureus from 12 hours of incubation. A: (T2) Guisang You 12 HWE), (T6) Alfonso HWE, (T7) positive control (Steptomycin Sulfate B and (T8) negative control (distilled water). B: (T1) Guisang You 12 EE, (T3) S54 EE, (T4) S54 HWE and (T5) Alfonso EE

Based on the results of study of [14] Omidiran et al. (2012), the cultivars of *M. alba* namely S14 and S34 showed zones of inhibition against *E. coli*, S14 with mean value of 9 mm and

S34 with 8 mm. Others studies supported the antibacterial property of mulberry leaves against *E. coli*. [15]Yigit and Yigit (2009) studied the antibacterial property of Black Mulberry using methanol extraction and water extraction method, wherein, both extracts showed total mean value of 10 mm.
Likewise, in line with the study of [15] Yigit and Yigit (2009), methanol leaf extracts of *M. nigra* (black mulberry) showed 18 mm and the water extracts showed 15 mm value of zone of inhibition against *S. aureus.* On the work of [14] Omidiran et al. (2012) S14 and S34 variety of *M. alba* showed zone of inhibition both with the total mean value of 8 mm. Kuwanon C, Morusin, , Sanggenon B and D, bioactive molecules from *Morus* bark, exhibit strong antimicrobial activity against *B. subtilis, Mycobacterium smegmatis*, *S. aureus, Streptococcus faecalis,* and some molds species [16].
As noticed, all ethanol extracts had the highest antibacterial property among the test plants. The following observations may be correlated to the nature of biologically active components whose activity can be increased in the presence of ethanol. This is also due to the stronger extraction capacity of ethanol that produced more important number of active constituents responsible for antibacterial activity of the mulberry extracts [17].

Table 2. Measured zone of inhibition (mm) of the different treatments against *E. coli* and *S. aureus* after 12, 24, 36, and 48 hours of incubation using hot water and ethanol extract

S.N.	Treatments	*Escherichia coli*				*Staphylococcus aureus*			
		Hours of Incubation				Hours of Incubation			
		12	24	36	48	12	24	36	48
1	Guisang you 12 EE	7.39^{b}	6.00^{b}	6.00^{b}	6.00^{b}	9.08^{bc}	7.37^{b}	7.09^{b}	6.82^{b}
2	Guisang you 12 HWE	7.07^{bc}	6.00^{b}	6.00^{b}	6.00^{b}	7.54^{bc}	6.33^{b}	6.00^{b}	6.00^{b}
3	S54 Variety EE	7.80^{b}	6.00^{b}	6.00^{b}	6.00^{b}	10.89^{b}	7.62^{b}	6.87^{b}	6.54^{b}
4	S54 Variety HWE	6.78^{bc}	6.00^{b}	6.00^{b}	6.00^{b}	8.31^{bc}	6.93^{b}	6.57^{b}	6.26^{b}
5	Alfonso Variety EE	7.56^{b}	6.00^{b}	6.00^{b}	6.00^{b}	11.15^{b}	7.00^{b}	6.79^{b}	6.66^{b}
6	Alfonso Variety HWE	6.80^{bc}	6.00^{b}	6.00^{b}	6.00^{b}	7.76^{bc}	6.00^{b}	6.00^{b}	6.00^{b}
7	Streptomycin Sulfate (+) control	27.21^{a}	23.71^{a}	23.5^{a}	22.36^{a}	31.24^{a}	26.4^{a}	23.35^{a}	22.1^{a}
8	Distilled Water (-) control	6.00^{c}	6.00^{b}	6.00^{b}	6.00^{b}	6.00^{c}	6.00^{b}	6.00^{b}	6.00^{b}

Table 3 shows the different results of phytochemicals present in hot water extracts and ethanol extracts of the different *Morus* varieties. Saponins, steroids, cardiac glycosides and terpenoids showed negative results and only flavonoids, tannins and alkaloids showed positive results for hot water extracts. Meanwhile, steroids and cardiac glycosides were absent on the ethanol extracts and only flavonoids, tannins, terpenoids, saponins and alkaloids showed significant content.

Table 3. Phytochemicals analysis of hot water extracts and ethanol extracts of the different *Morus* varieties.

Phytochemicals	**Guisang You 12**		**S54 Variety**		**Alfonso Variety**	
	HWE	EE	HWE	EE	HWE	EE
Flavonoids	++	+	++	+	++	+
Tannins	++	++	++	++	++	++
Alkaloids	++	+	++	+	++	+
Terpenoids	-	+	-	+	-	+
Saponins	-	+	-	+	-	+
Cardiac Glycosides	-	-	-	-	-	-
Steroids	-	-	-	-	-	-

(+) present on trace amounts; (++) present on appreciable amount; (-) absent

Saponins, steroids, cardiac glycosides and terpenoids showed negative results and only flavonoids, tannins and alkaloids showed positive results for hot water extracts. Meanwhile, steroids and cardiac glycosides were absent on the ethanol extracts and only flavonoids, tannins, terpenoids, saponins and alkaloids showed significant content.
On the study of [14]Omidiran et al. (2012), the *M. alba* varieties namely S34 and S14 showed presence of flavonoids with 2.815% and 2.14 %. Wherein, Mulberrofuran C, a new 2-aryl benzofuran derivative along with six known flavonoids are present on *M. bombycis koldz* as stated by [18]Kumar and Chauhan (2008).
Root bark of *M. nigra* Linn. or black mulberry was reported to have tannins as stated by [18]Kumar and Chauhan (2008). Results of the study of [14]Omidiran et al. (2012), showed that *M. alba* varieties, S14 and S34 also contain tannins.
Furthermore, [14]Omidiran et al. (2012) stated that, S14 and S34 varieties of *M. alba* varieties contain alkaloids of 0.830% (S14) and 0.825% (S34). This support the study of [18]Kumar and Chauhan (2008) that *M. alba* contains sanggenon, an alkaloid found at the root bark of white mulberry which inhibited plaque formation and *M. nigra* that contains Deoxyjirimycin (DJM) an alkaloid which said to bioactive against AIDS virus.
As seen on Table 3, only ethanol extracts showed presence of saponins. White mulberry varieties such as S14 and S34 were found to have saponins with 4.915% and 5.005% respectively [14](Omidiran et al., 2012).

CONCLUSION

Antibacterial assay revealed that extracts of Guisang You 12, S54 and Alfonso varieties have potential antibacterial activity against *E. coli* and *S. aureus.* Ethanol extracts of the three

Morus varieties showed inhibitory effect against *E. coli* while only ethanol extracts of S54 and Alfonso varieties showed inhibitory effect against *S. aureus*.
Phytochemicals that were present in traces and/or appreciable amount were found in both extracts of the three *Morus* varieties. These were flavonoids, tannins, alkaloids, saponins and terpenoids.

ACKNOWLEDGEMENT

The authors would like to offer particular thanks to Dr. Cristeta F. Gapuz of Sericulture Research and Development Institute, Don Mariano Marcos Memorial State University, Bacnotan La Union, for giving permission for collecting samples on the said institution.

COMPETING INTERESTS: The authors have declared that no competing interests exist.

REFERENCES

1. Reference Book for Human Resource Development Cooperation Program in the Framework of China Aid Seminar on Sericulture Production and Management for Asian Countries Guangzhou, China 2014.
2. Yadav R, Agarwala M. Phytochemical analysis of some medicinal plants. J. Phytol, 2011. 3(12): 10-14.
3. Cowan, MM. Plants products as antimicrobial agents. Clin. Microbiol. Rev. 1999. 12: 564-582.
4. Medina AL, Lucero ME, Holguin FO, Estell RE, Posakony JF, Simon J, O'Connell M. Composition and antimicrobial activity of *Anemopsis californica* leaf oil. J. Agri. Food Chem. 2005. 53:8694-8698.
5. Romero CD, Choph SE, Buck G, Martinez E, Garcia M, Bixby L. Antibacterial properties of common herbal remedies of the southwest. J. Ethnopharmacol. 2005. 99:253-257.
6. Arunkumar S, Muthuselvam M. Analysis of phytochemical constituents and antimicrobial activities of *Aloe vera* L. against clinical pathogens. World Journal of Agricultural Sciences. 2009. 5:572 -576
7. Penicilla G, Magno C. Antibacterial activity of extracts of twelve common medicinal plants from the Philippines. J. Med. Plants Res. 2011. 5(16):3975-3981.
8. Ortez JH. Manual on antimicrobial susceptibility testing: Disk diffusion testing. 2005. ISBN 1-55581-349-6.
9. Islam MA, Alam MM, Choudhury ME, Kobayashi N, Ahmed MU. Determination of minimum inhibitory concentration (Mic) of cloxacillin for selected isolates of methicillin-resistant *Staphylococcus Aureus* (Mrsa) with their antibiogram 1. Bangl. J. Vet. Med. 2008. 6 (1): 121–126.
10. Laboratory Manual for the UNESCO. Sponsored workshop on the phytochemical, microbiological, and pharmacological screening of medicinal plants. 1986. May 26-31. Department of Chemistry, U.P. Diliman.
11. Poongothai A, Sreena KP, Sreejith K, Uthiralingam M, Annapoorani S. Preliminary phytochemicals screening of *Ficus racemosa* Linn. bark. Int. J. Pharma Biol. Sci. 2011. 2(2): 431-434.
12. Sofowora A. The state of medicinal plants research in Nigeria. Book Review. Economic Botany. 1988. 42(2):241-241
13. Trease GE, Evan WC. "Pharmacognosy", Textbook of pharmacognosy, 12th (Ed) Balliers Tindall, London. 1983. 343-384.
14. Omidiran MO, Baiyewu RA, Ademola IT, Fakorede OC, Toyinbo EO, Adewumi OJ, Adekunle EA. Phytochemical analysis, nutritional composition and antimicrobial activities of white mulberry (*Morus alba*). Pak. J. Nutr. 2012. 11(5):456-460.
15. Yigit D, Yigit N. Antibacterial activity of black mulberry *(Morus nigra*) fruits and leaves. Journal of the Institute of Science and Technology. 2009. 1(1):39-47
16. NomuraT, Fukai T, Hano Y, Yoshizawa S, Suganuma M, Fujiki H. Chemistry and anti-tumor promoting activity of *Morus* flavonoids. Prog. Clin. Biol. Res. 1988. 280, 267-281.
17. Bhattacharjee I, Chandra G. Bactericidal activities of some common herbs in India; Pharm. Biol. 2007. 45: 350-354.
18. Kumar VR, Chauhan S. Mulberry: Life enhancer. J. Med. Plants Res. 2008.2(10):271-278

About Author

Job U. Ramos developed an early interest and curiosity in science and research; he established an early appreciation of nature and science. He took his undergraduate course, Bachelor of Science in Biology, which is categorized as Center of Excellence's of Commission on Higher Education (CHED) in one of the top universities in the Philippines, Central Luzon State University (CLSU). He finished his bachelor's degree at the year 2016, a month after his graduation; Job Ramos is currently working as a Science Research Analyst in a collaborated project of the Philippine Rice Research Institute (PRRI) and Korean International Cooperation Agency (KOICA).