Scientific Paper

Evaluation of the antimicrobial activity of fresh extracts of *Morus alba* L. leaves

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Abstract

In the plant kingdom compounds are produced with antimicrobial properties that can be used in the control of different diseases of animals as well as plants. One of the attractive species is *Morus alba*, which has transcendental history of use in Asian traditional medicine. For such reason, the objectives of this work were to determine qualitatively the presence of phenols and flavonoids and to evaluate the antimicrobial capacity of different fresh extracts of mulberry leaves against bacterial pathogens. For the phytochemical and antimicrobial activity essays leaves from 2 varieties (tigreada and indonesia) and 2 hybrids (IZ 15/7 and IZ 64) of *M. alba* were used in a completely randomized design with one positive and three negative controls (antibiotics). Fresh extracts were used for the antimicrobial activity, which was determined by the method of perforations in agar against five microorganisms that affect the animals as well as human beings: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomona aeruginosa*, *Klebsiella pneumoniae* and *Streptococcus* β *hemolítico*. In general, the abundant presence of flavonoids and active phenols against the evaluated pathogen microorganisms without significant differences among them was observed in all the extracts. The use of *M. alba* is suggested as a healthy alternative to enrich the animal diet and the use of these extracts with pharmaceutical purposes is recommended to fight the diseases originated by the studied microorganisms.

Keywords: plant extracts, phenol, flavonoids, pathogen organisms

Introduction

Infectious diseases in animals constitute one of the main morbidity and mortality problems in developing countries. In human and veterinary medicine synthetic antibiotics play an essential role in the treatment of different diseases. However, the great diversity of antibiotics and their indiscriminate use in the treatment and prevention of bacteria-caused infections, exert a large selection pressure on the emergence and dissemination of resistance mechanisms among diverse bacteria populations (Madigan *et al*., 2014). That is why the need to search for new *treatments* of plant origin to fight different pathogen-caused diseases is enhanced, which revolutionizes the natural pharmaceutical industry.

Mulberry (*Morus alba*) is an excellent and palatable forage, besides constituting a natural source of important bioactive compounds. *M. alba* extracts are recommended as therapeutic for the treatment of several diseases, due to their antioxidant, anti-inflammatory and antimicrobial properties, among others (Huang *et al*., 2013; Chan *et al*., 2016).

The emergence of bacterial resistance has generated new interests in the search for drugs with antibacterial capacity and that is why natural products play a fundamental role because they constitute sources of bioactive molecules. For such reason, the objectives of this work were to determine qualitatively the presence of phenols and flavonoids and to evaluate the antimicrobial capacity of different fresh extracts of mulberry leaves against bacterial pathogens.

Materials and Methods

Plant material. For the phytochemical and antimicrobial activity essays fresh leaves were used from 2 varieties (tigreada and indonesia) and 2 hybrids (IZ 15/7 and IZ 64) of *M. alba* collected and referenced in the herbarium of the Pastures and Forages Research Station Indio Hatuey. Fresh extracts were used at a concentration of 1 g/mL. The leaves were previously macerated in liquid nitrogen, dissolved in sodium phosphate buffer (100 mM) and centrifuged at 5 000 rpm during 20 minutes. The supernatant was preserved cold and was used for the experiments of antimicrobial activity.

Qualitative determination of phenols and flavonoids. The metabolites were evaluated according to the procedure described by Díaz-Solares *et al*.

(2015) which consists in a non-parametric system of crosses: + + +: considerable presence; + +: noticeable presence; +: small presence; -: absence.

Microorganism strains. The bacterial strains used were: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Streptococcus agalactiae* o *Streptococcus β-hemolítico* supplied by the Provincial Center of Hygiene and Epidemiology of Matanzas province and from clinical isolates.

Cultivation media. The Mueller-Hinton (liquid and solid) medium was used, sterilized in autoclave at 121 ºC and one pressure atmosphere during 20 minutes.

Experimental design and treatments. A completely randomized experimental design was used. The extracts from the different mulberry varieties were considered as treatments and a solution of sodium phosphate buffer 100 mM was used as negative control. In the case of the positive controls, azithromycin (10 mg/mL), ciprofloxacin (10 mg/mL) and ceftazidime (10 mg/mL) were used.

Essay of antibacterial activity. The bacteria preserved in glycerol at 30 % were inoculated in the liquid Mueller-Hinton medium and were incubated at 37 ºC in agitation (100 rpm) during 18 h. afterwards a suspension was prepared for each of the bacterial strains at 0,5 of the Mc Farland scale. The microorganisms were inoculated in the agar medium, previously melted down and maintained at 45 ºC. The growth in lawn occurred at a final concentration of approximately 10^8 cells/mL. After the medium solidification, 6 perforations of 0,8 cm diameter were made, where 100 µL of the mulberry extract, as well as of the negative and positive controls, were put. Ten repetitions were made per treatment for each microorganism. The 10 Petri dishes per microbial group were incubated at 37 ºC during 24 h. Then, the diameter of the microorganism growth inhibition halo was measured and the percentage of the relative inhibitory effect was calculated with regards to the positive control, as expressed in the following equation:

$$\%\ \text{inhibitory effect} = \frac{\text{mean of the diameter of the inhib.halo of the extract}}{\text{mean of the diameter of the inhib.halo positive control}} \times 100$$

Statistical analysis. The fulfillment of the assumptions of variance homogeneity (Levene's test) and normal distribution (modified Shapiro Wilk's test) was tested in the data of the halo diameter, and when it did not fulfill these requisites a non-parametric variance analysis was made through the Kruskal-Wallis test with 5 % significance. To determine the differences among the mean ranges of each treatment, paired comparisons were carried out through the Mann-Whitney U-test with Bonferroni adjustment. The statistical pack Infostat, version 1.1, was used (InfoStat, 2002).

Results and Discussion

The fresh extracts from the 2 varieties and 2 hybrids had considerable presence of flavonoids. In the case of phenols in tigreada and IZ 64 they were abundant and in indonesia and IZ15/7, noticeable (table 1).

Secondary metabolites have been associated to important physiological functions in the plants, specifically to the defense against herbivores and microbial pathogens (Buchanan *et al*., 2000). The pharmacological properties of plant extracts are ascribed to the high content of phenolic compounds, which are in turn related to antioxidant and antimicrobial activities (Al-Abd *et al*., 2015).

Recently, Chan *et al*. (2016) reported around 34 different flavonoids and 11 phenolic compounds in the *M. alba* leaves. Moracins constitute an important source of anti-infective agents, because they have a marked antibacterial and fungicide activity (Naik *et al*., 2015).

The antimicrobial activity of the *M. alba* varieties and hybrids against the Gram-positive bacterium *S. β hemolítico* are shown in table 2. The inhibition halos of the varieties and hybrids did not show significant differences among them, but they did differ from the controls, except with azithromycin which was similar to the variety tigreada and the hybrid IZ 15/7. Nevertheless, the inhibition among the controls was significantly different, which proved that antibiotics do not act with the same effectiveness. All the extracts showed activity over 50 % of inhibitory effect if compared with

Table 1. Metabolites present in aqueous extracts of *M. alba* leaves

Metabolite	Tigreada	Indonesia	IZ 15/7	IZ 64
Phenols	+++	++	++	+++
Flavonoids	+++	+++	+++	+++

Table 2. Antimicrobial activity against *S. β hemolítico*.

Treatment	Inhibition halo (mm) (mean ± SD)	Inhibition (%)		
		Control I	Control II	Control III
Tigreada	9,38[cd] ± 0,52	92,00	71,40	37,68
Indonesia	9,06[d] ± 0,42	88,94	69,02	36,42
IZ 15/7	9,25[cd] ± 0,46	90,78	70,45	37,18
IZ 64	9,13[d] ± 0,35	89,55	69,50	36,68
Control I (Azithromycin)	10,19[c] ± 0,66	-	-	-
Control II (Ciprofloxacin)	13,13[b] ± 1,02	-	-	-
Control III (Ceftazidime)	24,88[a] ± 1,93	-	-	-

Means in column followed by different letters indicate significant differences for $p \leq 0,05$

azithromycin and ciprofloxacin, which proves the bactericidal potential of the mulberry extracts to fight this pathogen.

The flavonoid Kuwanon G, isolated from the bark of the *M. alba* root showed good activity against cariogenic bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguis* and *Porphyromonas gingivalis* which cause periodontitis (Park *et al*., 2003); this showed the action that mulberry metabolites can exert on the species of this pathogenic genus.

In the case of *S. aureus* (table 3) all the extracts showed inhibitory activity and their inhibition halos did not significantly differ among themselves. When it was compared with ceftazidime specifically such inhibitory activity was low (around 30 %), which turned out to be interesting for a third-generation cephalosporin, as in the case of this wide-spectrum antibiotic. These results, at a glance, could seem contradictory when dealing with a Gram-positive bacterium. According to Chouna *et al*. (2009), the microorganisms that belong to this group tend to be more susceptible because their cell wall is less complex and lack effective filtration against large molecules and due to the size of the pores in their cell wall they are less selective. However, this fact could be explained taking as starting point the report by Tavares (2000), who stated that there are resistance mechanisms by *S. aureus* which are related to the activation of a cell wall synthesis, with hyper-production of penicillin-binding cells.

The enzyme enoyl-ACP reductase has become a referent for the development of antibacterial products because it is essential for the growth of bacteria and shows 4 isoforms (FabI, FabK, FabL and FavV). The chalcomoracin present in the methanol extracts of *M. alba* has a strong inhibitory capacity against FabI of *S. aureus*, while moracin C shows a moderate effect, which proves the antibacterial property of mulberry (Kim *et al*., 2012).

The extracts showed antimicrobial activity against the Gram-negative bacteria *K. pneumoniae* close to 50 % if it is compared with ceftazidime (table 4). In spite of being a bacterium characterized by its

Table **3**. Antimicrobial activity against *S. aureus*.

Treatment	Inhibition halo (mm) (mean ± SD)	Inhibition (**%**)		
		Control I	Control II	Control III
Tigreada	9,25[d] ± 0,46	63,53	46,69	32,03
Indonesia	9,25[d] ± 0,71	63,53	46,69	32,03
IZ 15/7	9,19[d] ± 0,53	63,10	46,38	31,81
IZ 64	9,50[cd] ± 0,58	65,25	47,96	32,89
Control I (Azithromycin)	14,56[bc] ± 2,25	-	-	-
Control II (Ciprofloxacin)	19,81[b] ± 1,42	-	-	-
Control III (Ceftazidime)	28,88[a] ± 3,01	-	-	-

Means in column followed by different letters indicate significant differences for $p \leq 0,05$

Table 4. Antimicrobial activity against *K. pneumoniae*.

Treatment	Inhibition halo (mm) (mean ± SD)	Inhibition (%)		
		Control I	Control II	Control III
Tigreada	15,63[b] ± 1,30	95,39	93,96	51,98
Indonesia	16,88[b] ± 1,46	103,02	101,47	56,14
IZ 15/7	15,38[b] ± 1,69	93,86	92,45	51,15
IZ 64	15,88[b] ± 1,89	96,92	95,46	52,81
Control I (Azithromycin)	16,38[b] ± 3,30	-	-	-
Control II (Ciprofloxacin)	16,63[b] ± 2,50	-	-	-
Control III (Ceftazidime)	30,06[a] ± 1,29	-	-	-

Means in column followed by different letters indicate significant differences for $p \leq 0,05$

marked resistance to multiple antibiotics (Yi *et al*., 2003), there were no significant differences among the varieties and hybrids; the inhibition halos were statistically similar to the antibiotics azithromycin and ciprofloxacin.

K. pneumoniae is part of the habitual intestinal flora and of the oral cavity and it is capable of causing infection of the urinary tract and pneumonia in healthy people. Most infections caused by this microorganism are acquired in the hospital or occur in patients weakened by underlying diseases. According to Ko *et al*. (2002), *Klebsiella* occupies the second position in the incidence of appearance of bacteremia by Gram-negative bacteria, only surpassed by *E. coli*.

Tables 5 and 6 show the antimicrobial activity of the mulberry varieties and hybrids against *E. coli* and *P. aeruginosa*, respectively. In the case of these pathogens, the percentages of the inhibitory effect of all the extracts with regards to the antibiotic ceftazidime were below 50 % and did not show significant differences among them. Ceftazidime shows high degree of stability in the presence of beta-lactamases (penicillinases and cephalosporinases) and for such reason it has high activity against and large number of Gram-negative bacteria, including penicillinase-producing strains and a great number of Enterobacteriaceae. Besides, it is the cephalosporin of higher activity against *P. aeruginosa* (Lister *et al*., 2009; Vila and Marco, 2010). Thus, the detected activities of the extracts exposed to *E. coli* can be considered efficacious, because they are over 70 % when compared with other antibiotics (azithromycin and ciprofloxacin) and the inhibition halos did not differ significantly among them. In the case of *P. aeruginosa*, no significant differences were observed either among the inhibition halos of all the extracts, but differences from the controls were observed. The hybrid IZ 15/7 did not differ from controls I and II (azithromycin and ciprofloxacin) and it was the one with the best inhibitory activity.

Table 5. Antimicrobial activity against *E. coli*.

Treatment	Inhibition halo (mm) (mean ± SD)	Inhibition (%)		
		Control I	Control II	Control III
Tigreada	9,25[c] ± 0,38	99,57	82,44	28,63
Indonesia	8,75[c] ± 0,46	94,19	77,99	27,08
IZ 15/7	9,38[c] ± 0,58	100,91	83,56	29,02
IZ 64	9,13[c] ± 0,38	98,22	81,33	28,24
Control I (Azithromycin)	9,29[c] ± 0,64	-	-	-
Control II (Ciprofloxacin)	11,22[b] ± 1,05	-	-	-
Control III (Ceftazidime)	32,31[a] ± 2,52	-	-	-

Means in column followed by different letters indicate significant differences for $p \leq 0,05$

Table 6. Antimicrobial activity against *P. aeruginosa*.

Treatment	Inhibition halo (mm) (mean ± SD)	Inhibition (%)		
		Control I	Control II	Control III
Tigreada	$8{,}94^{c} \pm 0{,}32$	55,86	46,60	31,04
Indonesia	$8{,}63^{c} \pm 0{,}95$	53,91	44,97	29,96
IZ 15/7	$9{,}75^{bc} \pm 0{,}29$	60,94	50,83	33,87
IZ 64	$8{,}31^{c} \pm 0{,}53$	51,95	43,34	28,87
Control I (Azithromycin)	$16{,}00^{b} \pm 2{,}46$	-	-	-
Control II (Ciprofloxacin)	$19{,}18^{b} \pm 1{,}94$	-	-	-
Control III (Ceftazidime)	$28{,}79^{a} \pm 1{,}81$	-	-	-

Means in column followed by different letters indicate significant differences for $p \leq 0{,}05$

It must be emphasized that the ethanol extracts of mulberry leaves collected in Brazil showed antimicrobial activity against *S. aureus*, *P. aeruginosa*, *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *Aspergillus flavus* (de Oliveira *et al*., 2015).

The antimicrobial activity of mulberry extracts can be ascribed to the presence of phenols and flavonoids, which have multiple biological effects, including the antioxidant, healing and antimicrobial effects (Aditya *et al*., 2012). Phenolic compounds can cause disruption of the cell membrane, protein binding, enzyme inhibition and synthesis of new proteins, as well as the formation of disulfide bridges and intercalation with the cell wall and/or DNA (Niño *et al*., 2012; Oh *et al*., 2013).

Conclusions

In all the extracts a high content of phenols and flavonoids was observed. The varieties and hybrids showed antimicrobial activity against the five bacterial pathogens. In all the cases the inhibition was close to or over 30 %. The microorganism *K. pneumoniae* was the most sensitive to the mulberry extracts with an inhibitory percentage of approximately 50 % compared with the third-generation antibiotic ceftazidime.

Recommendations

The study of these extracts with pharmaceutical purposes to fight the diseases caused by microorganisms of medical relevance, for animals as well as humans, is recommended. In addition, the use of *M. alba* as a healthy alternative to enrich the animal diet is suggested.

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