Biological activities of *Morus celtidifolia* leaf extracts

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Abstract: The aims of this research were to examine the antibacterial, cytotoxic and antiradical/antioxidant activities of the organic extracts obtained from the leaves of the medicinal plant *Morus celtidifolia* (Family: Moraceae). To evaluate its antimicrobial properties, *M. celtidifolia* was tested against the bacteria of medical importance: *Bacillus subtilis, Staphyloccocus aureus, Enterococcus faecalis, Escherichia coli, Enterobacter cloacae* and *Enterobacter aerogenes*. Cytotoxic activity was assessed by using the brine shrimp (*Artemia salina*) lethality assay and also by toxicity screening against human cancer cell lines: MCF-7 (human breast adenocarcinoma) and HeLa (cervix adenocarcinoma). The free radical-scavenging activity was determined by the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay. Results revealed that the hexanic extract has antibacterial activity only against Gram positive strains, while the methanolic extract showed better cytotoxic and antioxidant activities than the non- polar extract with a median lethal dose (LD_{50}) of 125µg/ml, 90µg/ml and 75µg/ml against *A. salina*, MCF-7 and HeLa cells respectively, and median effective concentration (EC_{50}) of 152µg/ml on radical scavenging assay. This is the first study reporting the biological activities of leaves of *Morus celtidifolia*.

Keywords: *Morus celtidifolia*; Moraceae; organic extract; medicinal plant.

INTRODUCTION

Morus is a genus of the angiosperms plants in the family of Moraceae, usually known as mulberries. They are native from temperate and subtropical regions of Asia, Africa, Europe, and America (Singhal *et al.*, 2010). *Morus* species have been widely employed in traditional medicine since ancient times. Studies carried out on differents models revealed that genus *Morus* has various and relevant biological activities, including cytoprotective, antineoplastic, antioxidants and hypoglycemic effects (Jaruchotikamol and Pannangpetch, 2013; Deepa *et al*., 2013; Nazari *et al*., 2013). Phytochemical investigations performed in the leaves of *Morus* showed the presence of phenolic acids, flavoinds, benzophenones and iminosugars (Kim *et al.*, 2012; Hunyadi *et al.,* 2013). The biological activities of the plant may be attributed to phenolic compounds. Such compounds have received attention due to their therapeutic properties, including antimicrobial and antiradical/antioxidant activities.

The World Health Organization (WHO) reports that about 80% of individuals from developed countries use traditional medicine (Akerele, 1993). For this reason, the research in medicinal plants is essential to validate their properties, efficiency and safe use. Taking this into account, the objective of this work was to evaluate the antibacterial, antiradical/antioxidant and cytotoxic activities of the leaves of *Morus celtidifolia* (Mexican mulberry), a medicinal herb used for the treatment of various diseases, ranging from preventing gyneco-obstetric disorders and pains, to the prevention of vomiting (Chino Vargas and Jacques Ríos, 1986; Castro Ramírez, 1988).

MATERIALS AND METHODS

M. celtidifolia (Moraceae) was collected in the municipality of San Pedro Garza Garcia, Nuevo León, México, during June 2011. The plant was identified by Dr. Marco Guzmán Lucio and a specimen was left in the herbarium of the Facultad de Ciencias Biológicas/ Universidad Autónoma de Nuevo León (voucher specimen: 26307).

Plant leaves were dried at room temperature and 50 g were sequentially submitted to extraction through maceration and sonication, hexane by first time and then with methanol (three sessions of 20 minutes each solvent). The plant: solvent proportion was 1:10 (weight/volume). After filtration, the hexanic and methanolic extracts were pooled separately and then the organics extracts were concentrated in vacuum until dry; all extracts were kept on 4°C until use. The yield of extracts from *M. celtidifolia* was: hexane (0.25g) and methanol (1.6g).

Clinical isolates of *Bacillus cereus, Escherichia coli, Salmonella typhi, Staphylococcus aureus, Enterobacter aerogenes, Enterobacter cloacae,* and *Salmonella typhimurium*, were tested. By using cultures of the above mentioned microorganisms, the bacterial suspension (after 18-24 h) was prepared and turbidity was adjusted to 0.5 in

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the McFarland standard, which corresponded to 10^6 colony forming units (cfu/ml) (Ripa *et al.*, 2010).

The inhibition assay tests were performed by using the antibiogram method. Petri-dishes were prepared with sterile Mueller Hinton agar. The standardized microorganism suspension was applied on the solidified culture medium by using sterile swabs. Sterile paper disks (6mm) were impregnated with 2000, 1500 and 1000µg of sample and aseptically transferred onto the inoculated agar plates and incubated. Antimicrobial activity was determined by measuring clear zones of inhibition around the crude extract test discs. The clear zones indicated the biocide effect. Gentamicin was used as positive (15µg) and the solvent as negative controls (Roy *et al*, 2011). All assays were performed in triplicate.

To determine the antiradical/antioxidant activity, 100 µl of DPPH$^{\bullet}$ (2 g/L) were mixed with 100 µl of serial dilutions of the test solution in 96-well microplates; MeOH and Trolox® were used as a negative and positive controls, respectively. The decrease in absorbance at 517 nm was measured, mean values were obtained from triplicate experiments (Viveros-Valdez *et al.*, 2008).

The brine shrimp lethality bioassay was used to determine the preliminary cytotoxic potential of the organic extracts. Working solutions from the extracts were prepared at concentrations of 1000, 100 and 10µg of extract/ml to a final volume of 5 ml of seawater. Ten brine shrimps were placed to each vial using adequate pipettes. After 24 h of exposure, the number of living and dead organisms was counted. Four replicas were used for each treatment and controls. Dimethyl sulfoxide (DMSO, no more than 1%) was used to dissolve the extracts in the assays, it was also was used as a negative control and potassium dichromate ($K_2Cr_2O_7$) as positive control (Meyer *et al.*, 1982).

MCF-7 (human breast adenocarcinoma) and HeLa (cervix adenocarcinoma) neoplastic cell lines were used to determine the cytotoxicity of the extracts. Cell cultures were grown in Eagle´s Minimum Essential Medium supplemented with 10% fetal bovine serum, penicillin [100 U/ml], streptomycin [100µg/ml] and incubated in an atmosphere of 5% CO_2 at 37°C. The cell viability was measured after treatments by reduction of WST-1, a sodium tetrazolium salt (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H. Ten microliters of WST-1 were added to each well and after 90 min of incubation. The absorbance was measured at 450 nm (Ishiyama *et al.*, 1996).

The half maximal inhibitory concentration (IC_{50}) was calculated from the log-dose inhibition growth curve obtained by a nonlinear regression algorithm. Taxol and 1% DMSO were used as positive and negative controls respectively. The results are presented as the mean ± SD.

RESULTS

Table 1 displays the biological effects of the crude organic extracts prepared from the *M. celtidifolia* leaves. The methanolic extract exhibited such an effect only against *Enterococcus faecalis* and *Staphylococcus aureus* with a dose-response relationship observed on the analyzed concentrations. The hexanic extract displayed lethal effects exclusively over Gram-positive bacteria, with *S. aureus* showing the highest sensitivity to this extract. While the methanolic extract showed better cytotoxic and antioxidant activities than the non- polar extract.

DISCUSSION

Recent investigation (Rahman *et al.*, 2013) suggests that a plant extract is suitable for prospection studies when it exhibits inhibition zones ≤ 2000 µg/disk, meaning that the methanolic and hexanic extracts obtained from *M. celtidifolia* could be considered as bactericides. Previous studies have brought to light the antimicrobial effect of organic extracts derived from different species of the *Morus* genus (Fukai *et al.*, 2005; Kuete *et al.*, 2009; Wang *et al.*, 2012). Our results show that the non-polar extract of *M. celtidifolia* possesses the highest bactericidal efficiency, which is also in accordance to what was stated by Zhi-ming *et al.* (2011), who observed that the hexanic extract obtained from *M. alba* leaves inhibited the growth of *S. aureus*, presenting inhibition rings close to 11 mm in diameter. The same study reports null effect over *E. coli*, which corresponds as well to our results.

The *M. celtidifolia* extracts were submitted to the *Artemia salina* toxicity assay, a technique that has been widely employed to assess the toxicity of organic extracts derived from medicinal plants. Both extracts exhibited toxicity over the *A. salina* larvae, which suggest that they possess molecules of biological interest, given that the assay possess widespread acceptance as a screening method in the searching for molecules with antineoplastic activity because to the correlation between them and tumor-cell cytotoxicity assays (Meyer *et al.*, 1982). In this context, extracts with LD_{50} <1000µg/ml are considered active.

Cytotoxicity over HeLa and MCF-7 cells was also determined (table 1). The methanolic extract of *M. celtidifolia* presented the highest anti-proliferative effect over the aforementioned cell lines. Previous studies have shown the anti-proliferative effect over diverse cancer cell lines displayed by different *Morus* species, against the human cancer cell lines BGC-823 (gastric cancer), HeLa (cervical adenocarcinoma), MCF-7 (breast denocarcinoma), HepG2, and Hep3B (hepatocarcinomas) (Naowaratwattana *et al.*, 2010; Dat *et al.*, 2010).

Table 1: Biological activities of organic of crude extracts of *Morus celtidifolia* leaf

Organism /Test	Gram	Zone of Inhibition (μg/disk)						
		Methanolic Extract			Hexanic Extract			Control*
		2000	1500	1000	2000	1500	1000	15
Bacillus subtilis	G+	-	-	-	14±1	8±1	-	24±2
Staphyloccocus aureus	G+	14±3	11±2	8±1	15±3	10±3	-	26±2
Enterococcus faecalis	G+	13±3	-	-	12±3	-	-	28±4
Escherichia coli	G-	-	-	-	-	-	-	20±3
Enterobacter cloacae	G-	-	-	-	-	-	-	22±4
Enterobacter aerogenes	G-	-	-	-	-	-	-	15±3
	Median Lethal or Efective Dosis (μg/ml)							
	Methanolic Extract			Hexanic Extract			Control*	
Artemia salina	125±8			167.3±18			18±4	
MCF-7	90±13			150±23			0.5±0.1	
HeLa	75±10			96±17			0.9±0.2	
DPPH	152±15			>200			11±2	

*Gentamycin was used as positive control in bacteria; $K_2Cr_2O_7$ was used in *A. salina* assays; Taxol in cytotoxicity tests, and Trolox in DPPH antioxidant effect.

It was observed, on the other hand, that *M. celtidifolia* extracts possess only slight effectivity as DPPH radical scavengers, which is in contrast to data shown by *Morus* species originating from other regions (Iqbal *et al.*, 2012; Khan *et al.*, 2013)

CONCLUSION

In recent years the bioprospection efforts surrounding the *Morus* genus have intensified, due in no small measure to their ingrained use in traditional medicine practices. Taking this as a starting point, the present paper report for the first time the biological activity of organic extracts obtained from the leaves of the Mexican mulberry (*M. celtidifolia),* which have the capability to inhibit Gram-positive bacterial growth, as well as an interesting cytotoxic effect. This, in turn, opens up the possibility of following these findings with a systematic study in order to identify the molecules responsible for the encountered biological activities.

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