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**Antibacterial, antifungal and anti-inflammatory activities of *Melia azedarach* ethanolic leaf extract**

## Antibacterial, antifungal and anti-inflammatory activities of *Melia azedarach* ethanolic leaf extract

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### Abstract

The aim of the present study is to examine the antibacterial, antifungal and anti-inflammatory activities of the ethanolic leaf extract of *Melia azedarach*. It was tested *in vitro* for its antibacterial and antifungal activities against *E. coli* (ATCC 8739), *Enterococcus faecalis* (Ec P07) and *Bacillus subtilis* (Bs) bacteria as well as against pathogenic fungi (*Alternaria alternata*, *Fusarium solani*, *Fusarium oxysporum* sp. *melonis*, *F. oxysporum* f. sp. *lycopersici*, *F. sambucinum* and *Botrytis cinerea*) with different techniques simultaneously with anti-inflammatory activity with carageenan method. All tested concentrations of *M. azedarach* extract showed significant antibacterial and antifungal activities with low IC<sub>50</sub> compared to ethanol 95% and a high anti-inflammatory activity compared with indomethacin as drug. Our investigation pointed that *M. azedarach* could be considered as a good medicinal agent.

## Introduction

In recent years, there are increasing hazardous effects of chemical pesticides on plant and animal health (Aktar et al., 2009) as well as the problem of drugs microorganisms' resistance have alarmed scientists to search for efficient and eco-friendly alternative ways. In fact, the problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain (Nascimento et al., 2000). Furthermore, the treatment of chronic inflammatory diseases is still problematic, since, anti-inflammatory used drugs have numerous adverse effects (Li et al., 2003). Similarly, infection due to fungal pathogens has become more frequent (Fleming et al., 2002) and affects humans as well as plants. These infections are the leading cause of death in both advanced and developing countries (Murtaza et al., 2015). This is due to the use of immunosuppressive treatments, long-term use of antibiotics and longer survival of immunocompromised individuals (Jain et al., 2010).

Therefore, an increasing demand to evaluate the antimicrobial and antifungal activities of medicinal plant is gaining ground and this for their use in green medicine as it is believed to be safer and cost less than synthetic drugs (Rajkumar and Malathi, 2015; Canli et al., 2015). In fact, plant extracts may represent an ideal solution to these problems; they could be easily tested *in vitro* (Dissanayake, 2014) and may be an alternative to chemical fungicides and bactericides (Tsair-Bor and Shang-Tzen, 2008). Among the numerous families with recognized pharmacological activities, those belonging to *Meliaceae* family deserve mention. *M. azedarach* L. - one of species belonging to *Meliaceae* family- is widespread in Tunisia as an ornamental shade tree. *M. azedarach* leaves are well-known for their ethnobotanical uses (Gopal et al., 2015).

The present investigation was designed to evaluate the antibacterial, antifungal and anti-inflammatory properties of the leaves ethanolic extract of *M. azedarach*.



## Materials and Methods

### Plant material and extraction

*M. azedarach* mature leaves powder was extracted with soxhlet apparatus as elsewhere (Akacha et al., 2016).

### Antibacterial and antifungal assays

The antibacterial and antifungal analysis were carried out using a new tool, phenotype microarrays (Biolog, USA). It offers a unique way to identify product and to infer a mode of action by which the novel inhibitor prevents microbial growth (Bochner et al., 2001). The assays are pre-filled and dried in 96-well microplates that can monitor chemical sensitivities. Cell response in each assay well is determined by the amount of color development produced by the reduction of a tetrazolium compound (a redox indicator) during cell respiration (Bochner, 2003). To identify the method of action of compounds on Gram-negative, Gram-positive bacteria and fungi, phenotype microarrays were employed to screen various sources of carbon, nitrogen, sulfur and phosphorous. The methods were done according to the phenotype microarrays procedure for *E. coli* and other Gram negative bacteria and the phenotype microarrays procedure for *B. subtilis* and other Gram-positive bacteria provided by Biolog Inc., USA. Kinetic data were analyzed with OmniLog PM software. The results were expressed by the differences of the treated bacterial cells from untreated bacterial cells.

### Phenotype microarrays of compounds with bacteria

*E. coli* (ATCC 8739), *Enterococcus faecalis* (*Ec* P07) and *Bacillus subtilis* (*Bs*) were grown overnight at 37°C on nutrient agar plates. In order to prepare the control group bacteria (untreated bacteria), colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 mL of Inoculating Fluid-0a (IF-0a), in a 16 mL sterile capped glass tube. The cell density must be equaled to 42% transmittance (T) on a biolog turbidimeter. The IF-0a with Biolog Redox Dye Mix (A for *E. coli* or F for *Ec* and *Bs*) (1:5 dilution) was prepared by adding 0.9 mL of Dye A or F to 62.5 mL of IF-0a and completed the volume to 75 mL by sterile distilled water. To prepare the bacterial cell suspension, 15 mL of IF-0a with bacteria was added to 75 mL of IF-0a with Dye Mix A or F; the cell density must be 80-85% T. Later, 22 mL of this 85% T suspension were transferred to a sterile reservoir. To prepare the treated group bacteria (bacteria treated by ethanolic extract), the same previous steps were followed except the addition of ethanolic extract at different concentrations (0.001-0.1 mg/mL) instead of distilled water in IF-0a plus Dye Mix A or F. In order to prepare the bacteria cell suspension, colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 mL of inoculating Tryptic Soy Broth (TSB), in a 20

mL sterile capped glass tube. The cell density must be equaled to 80-85% transmittance (T) on a Biolog turbidimeter. The TSB with Biolog Redox Dye Mix (A for *E. coli* or F for *Ec* and *Bs*) was also prepared.

Later, 250 µL/well using Biolog multichannel pipette were added to phenotype microarrays plates as 100 µL/well bacteria cell suspension + 130 µL/well TSB with Biolog Redox Dye Mix correspondent + 20 µL/well of ethanolic extract (at different concentration 0.001-0.1 mg/mL). All the plates were incubated at 37°C in the OmniLog plate incubator and reader and were monitored for any color change of the wells. Readings were recorded for 48 hours for all phenotype microarrays plates.

### phenotype microarrays of compounds with fungi

*Alternaria alternate* and *Fusarium solani* were grown on nutrient agar plates overnight at 30°C. In order to prepare the control group fungus (untreated fungus), colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 mL of inoculating Fluid-0a (IF-0a), in a 20 mL sterile capped glass tube. The cell density must be equaled to 62% transmittance (T) on a Biolog turbidimeter. In order to prepare the fungus cell suspension, colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 16 mL of inoculating Malt extract (ME), in a 20 mL sterile capped glass tube. The cell density must be equaled to 62% transmittance (T) on a Biolog turbidimeter. The Malt extract with Biolog Redox Dye Mix F was also prepared.

Later, 250 µL/well using Biolog multichannel pipette were added to phenotype microarrays plates as 20 µL/well fungus cell suspension + 210 µL/well Malt extract with Biolog Redox Dye Mix F + 20 µL/well ethanolic extract (at different concentration 0.001-0.1 mg/mL). All the plates were incubated at 37°C in the OmniLog plate incubator and reader and were monitored for any color change of the wells. Readings were recorded for 48 hours for all phenotype microarrays plates.

### Antifungal activity with disc-methods

Ethanolic residue was dissolved in sterilized distilled water. Four plants pathogenic fungi were used for this test namely *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, and *F. sambucinum*. Targeted fungi were grown on Potato Dextrose Agar (PDA) medium amended with adequate volumes of *M. azedarach* extracts. Concentrations tested were 0.04, 0.06, 0.08 and 0.1% for ethanolic extract. Agar plugs (6 mm in diameter) removed from a fast growing fungal colony was plated in 9 cm petri dishes amended or not with the extracts tested (three plugs per plate). The colony diameter was noted after 3 days of incubation at 25°C. Control received the same volume of sterilized distilled water.

Fungal growth was measured by averaging the three diameters taken at right angles for each colony. Percentage growth inhibition (%) of fungal colonies was calculated according to the following formula (Jabeen and Javaid, 2008):

$$\text{Growth inhibition (\%)} = \frac{[(\text{Growth in control} - \text{Growth in treatment}) / \text{Growth in control}] \times 100}{1}$$

#### Anti-inflammatory activity

Anti-inflammatory activity of *M. azedarach* ethanolic leaves extract and indomethacin, as standard drug, was determined by carrageenan-induced hind paw edema (Winter et al., 1962). Extract at 150 mg/kg dose (Jain et al., 2015) and indomethacin at 25 mg/kg dose were given to rats intraperitoneally. The same volume of ethanol 10% used as vehicle was given to the control group. One hour after treatment, carrageenan 0.1 mL (1%, w/v) solution in ultrapure water was subcutaneously injected into the planter surface of the right hind paw of all rats. The paw volume was measured with a plethysmometer before injection of carrageenan. Carrageenan-induced paw edema was measured at 30, 60 and 120 min. Anti-inflammatory activities of *M. azedarach* leaves ethanolic extract and indomethacin were determined by comparing their results with the ones obtained in the control group. The anti-inflammatory effect of compounds was calculated by the following equation:

$$\text{Anti-inflammatory activity (I \%)} = (1 - D/C) \times 100$$

where *D* represents the percentage difference in paw volume after extract or drug administration to the rats, and *C* represents the percentage difference of volume in the control group.

## Results

#### Antifungal and antibacterial activity

*M. azedarach* ethanolic leaves extract had shown an important antibacterial and antifungal activities with Biolog phenotype microarray technology against *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia Coli*, *Alternaria alternata* and *Fusarium solani*. Results showed a high antibacterial activity for *M. azedarach* leaves extract (IC<sub>50</sub>) compared to absolute ethanol: *E. coli* 6.6 µg/mL vs. 10.7%, *Enterococcus faecalis* 1 µg/mL vs. 61.0 % and *Bacillus subtilis* 1.3 µg/mL vs. 44.9%. While antifungal activity of *M. azedarach* ethanolic leaves extract compared with absolute ethanol was as followed *Alternaria*: 1.0 µg/mL vs. 32.5% and *Fusarium* 53 µg/mL vs. 37.8% (Figure 1, Table I).

With disc-methods, *M. azedarach* ethanolic leaves extracts exhibited a significant (p<5%) inhibitory effects on growth of tested fungal pathogens. In fact, 0.1% concentration of *M. azedarach* ethanolic leaves extract

Table I

#### Antibacterial and antifungal assays of *M. azedarach* extract with phenotype microarray technology

	Ethanolic extract (IC <sub>50</sub> µg/mL)	Ethanol (95%) I%
<i>E. coli</i>	6.6	10.7
<i>Enterococcus f</i>	1.0	61.0
<i>Bacillus S.</i>	1.3	44.9
<i>Fusarium S.</i>	53.9	37.8
<i>Alternaria A.</i>	1	32.5

was been the highest concentration that gave a mycelium growth inhibition at around 8%, 19%, 36% and 38% respectively against *F. oxysporum* f. sp. *melonis*, *F. sambucinum*, *F. oxysporum* f. sp. *lycopersici* and *B. cinerea* (Figure 2). The remaining concentrations exhibited an antifungal activities (Table II). Eventually, *F. oxysporum* f. sp. *melonis* has the greatest resistance toward *M. azedarach* leaves extract at different concentrations. Therefore, among fungal plant pathogens tested, *F. oxysporum* f. sp. *lycopersici* and *B. cinerea* were found to be more sensitive to *M. azedarach* leaf ethanolic extract than *F. oxysporum* f. sp. *melonis* and *F. sambucinum*.

#### Anti-inflammatory activity

*M. azedarach* extract was screened, for their anti-inflammatory activity by carrageenan-induced hind paw edema, using female Wister rats. The investigated extract showed a good inhibition of the carrageenan-induced paw edema evolution in comparison with indomethacin as standard drug. It was found that *M. azedarach* extract at a dose of 150 mg/kg demonstrate a highest anti-inflammatory activity at around 25% when compared with indomethacin at 32% at 10 mg/kg after 2 hours of treatment (Figure 3).

## Discussion

*M. azedarach* extract had shown important antibacterial activities against *Enterococcus faecalis* and *Bacillus subtilis* (*Bs*). Results could be due to the presence of phenolic compounds and flavonoids previously characterized (Akacha et al., 2016). The slight effect on *E. coli* bacteria is probably attributed to the higher resistance of *E. coli* (ATCC 8739) to many plant extracts (Oskay et al., 2009). The major component in the studied extract, which is the protocatechuic acid (one of metabolites of quercetin) had shown antibacterial effects in ground beef and apple juice (Chao and Yin, 2009). Similarly, Aziz et al. (1998) had proved that protocatechuic acid, among other phenolics, inhibited the growth of *E. coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Aspergillus flavus* and *Aspergillus parasiticus*. Besides, isoquercitrin; second

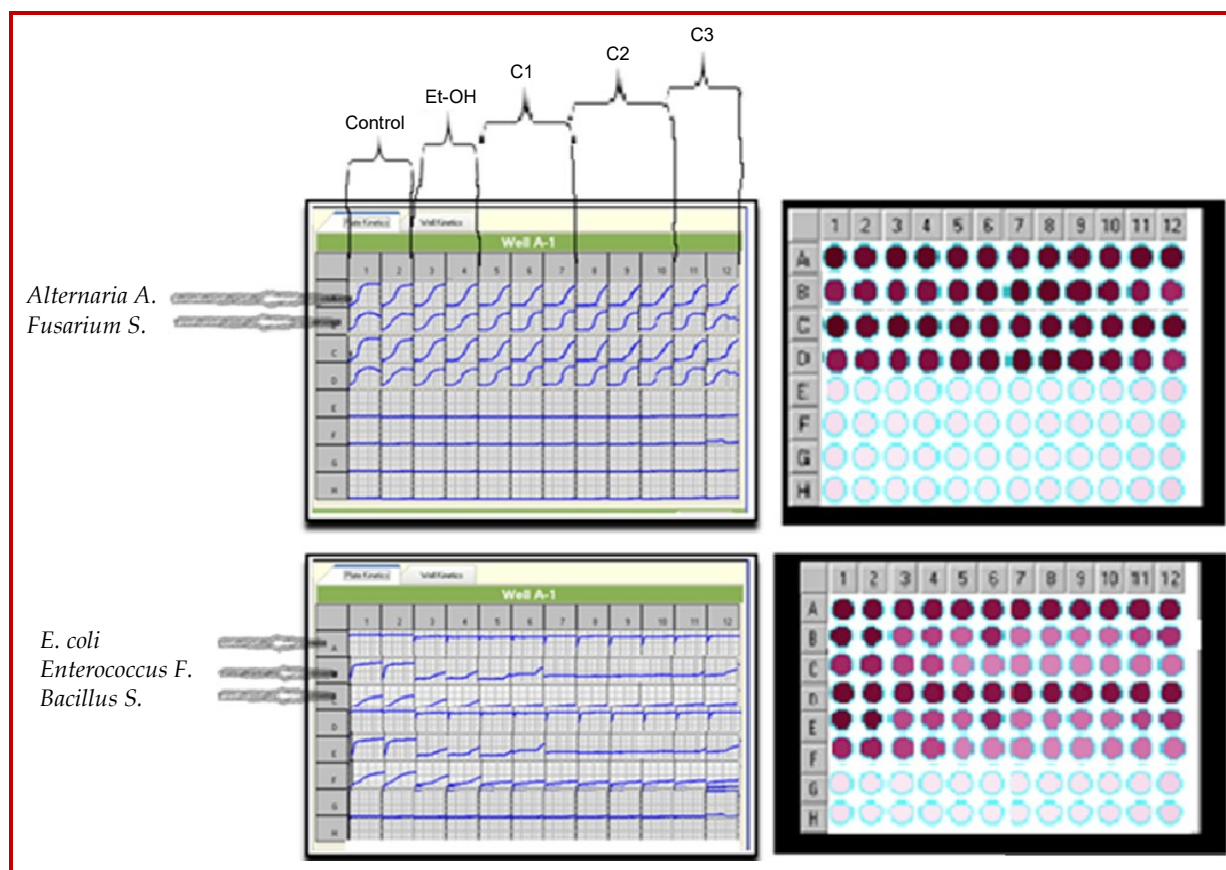


Figure 1: Photo of antibacterial and antifungal assays of *M. azedarach* leaves extract with Biolog phenotype microarray technology

predominant component in studied ethanolic extract possess fungicidal action exerted by disturbing the membrane of cells as investigated by Yun et al. (2015).

Other phenolics present- even in lower doses- in our extract could contribute to the observed fungicidal and microbial effects such as gallic acid which had been proven to possess bactericidal and antifungal activity against *F. solani* (Nguyen et al., 2015) and *Candida* species (Alves et al., 2014). Therefore, syringic acid- known to have antimicrobial activity and fungitoxicity- as proven against *Gano derma boninense* by Chong et al. (2012) could result in observed antimicrobial effects. Similarly, chlorogenic acid also developed antifungal (Vandal et al., 2015) and antimicrobial effects on both Gram-positive and Gram-negative bacteria (Suárez-Quiroz et al., 2013).

Previous GC-MS analysis of the ethanolic extract revealed the presence of mainly stearic, palmitic, palmitoleic, then oleic and linoleic fatty acids. Indeed, stearic, palmitic, linoleic and oleic acids are known to be potential antibacterial and antifungal agents (McGaw et al., 2002; Seidel and Taylor, 2004). Moreover, oleic acid was reported to possess antimicrobial activity against human pathogens (Kabara et al., 1972). Furthermore, the antifungal activities exhibited by linolenic, linoleic

and oleic acids were investigated by Walters et al. (2004). Also, the antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum* was previously demonstrated by Dilika et al. (2000) as well as synergistic effect between the two fatty acids. A further, palmitoleic acid exhibited the greatest antibacterial activity against meat spoilage organisms according to a study conducted by Ouattara et al. (1997).

In the current study, *F. oxysporum* f. sp. *Lycopersici* and *B. cinerea* were found to be more sensitive to *M. azedarach* leaves extracts than *F. oxysporum* f. sp. *melonis*. This result is interesting since *B. cinerea* is a necrotrophic fungal pathogen that attacks over 200 different plant species (Elad, 1997) and has become resistant to chemical pesticides (Bardas et al., 2010).

Our results are in line with the findings of some earlier workers reporting antibacterial/antifungal effects of *M. azedarach* alcoholic leaves extracts against many fungal species such as *Ascochyta rabiei* (Jabeen et al. 2011), *Aspergillus flavus*, *Diaporthe phaseolorum* var. *meridionales*, *F. oxysporum*, *F. solani*, *F. verticillioides*, and *Sclerotinia sclerotiorum* (Carpinella et al., 2003). More recently, Neycee et al. (2012) had shown that *M. azedarach* leaf extract effect on *Sclerotium* spp., *F.*

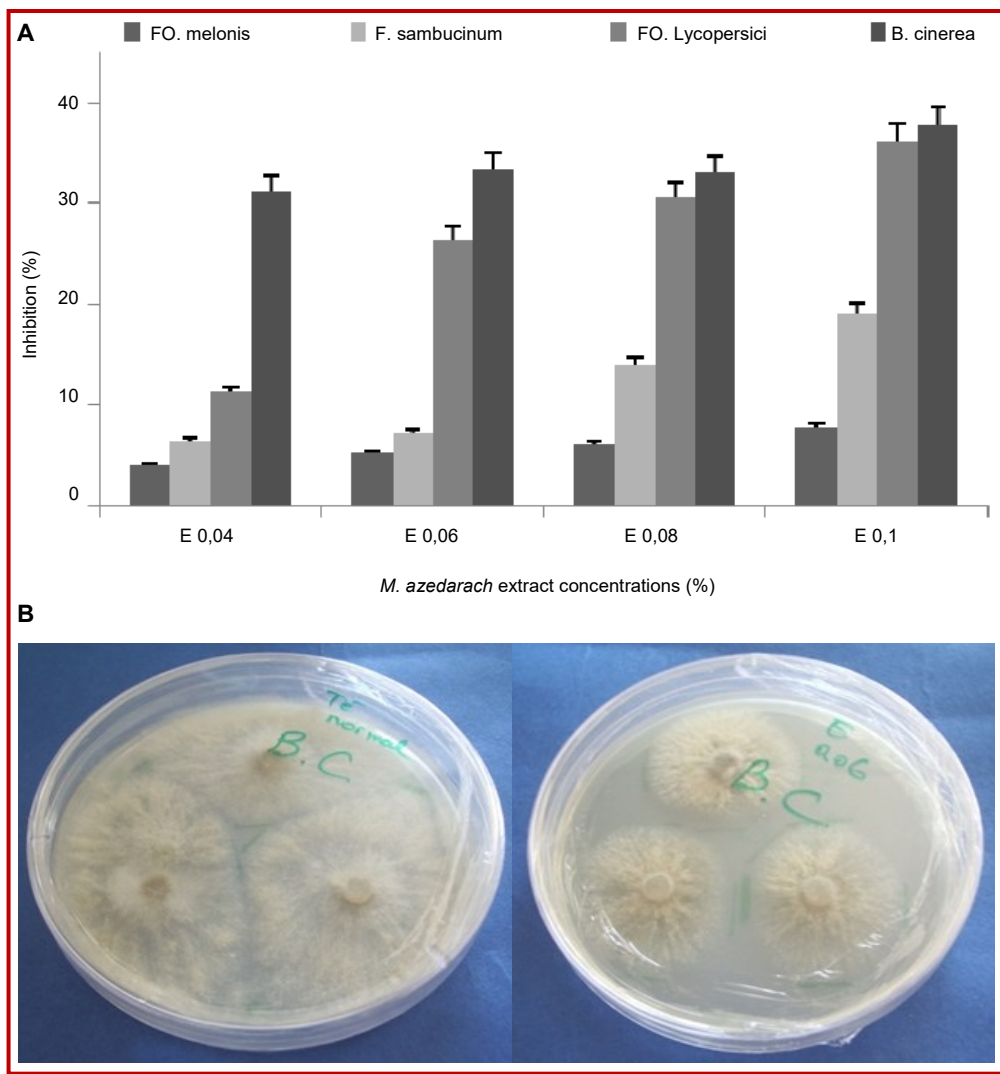


Figure 2: Antifungal activity of *Melia azedarach* ethanolic leaves extract. A: Antifungal activity against *FO. melonis*, *F. sambucinum*, *FO. Lycopersici* and *B. cinerea* with disc-methods at the concentrations 0.04, 0.06, 0.08 and 0.1%; B: Photo of antifungal assays of *M. azedarach* leaves extract with disc method

Table II				
Antifungal assays of <i>M. azedarach</i> leaves extract with disc method				
Fungi	<i>M. azedarach</i> ethanolic extract concentration (%)			
	0.02	0.04	0.06	0.1
	Inhibition (%)			
<i>F.O. melonis</i>	3.9 ± 0.2	5.2 ± 0.2	6.1 ± 0.3	7.8 ± 0.4
<i>F. sambucinum</i>	6.4 ± 0.3	7.2 ± 0.3	14 ± 0.7	19.1 ± 0.9
<i>F. O. Lycopersici</i>	11.2 ± 0.6	26.4 ± 1.3	30.5 ± 1.5	36.1 ± 1.8
<i>Botrytis cinerea</i>	31.1 ± 1.5	33.3 ± 1.7	33.0 ± 1.6	37.7 ± 1.9

Data are mean ± SEM

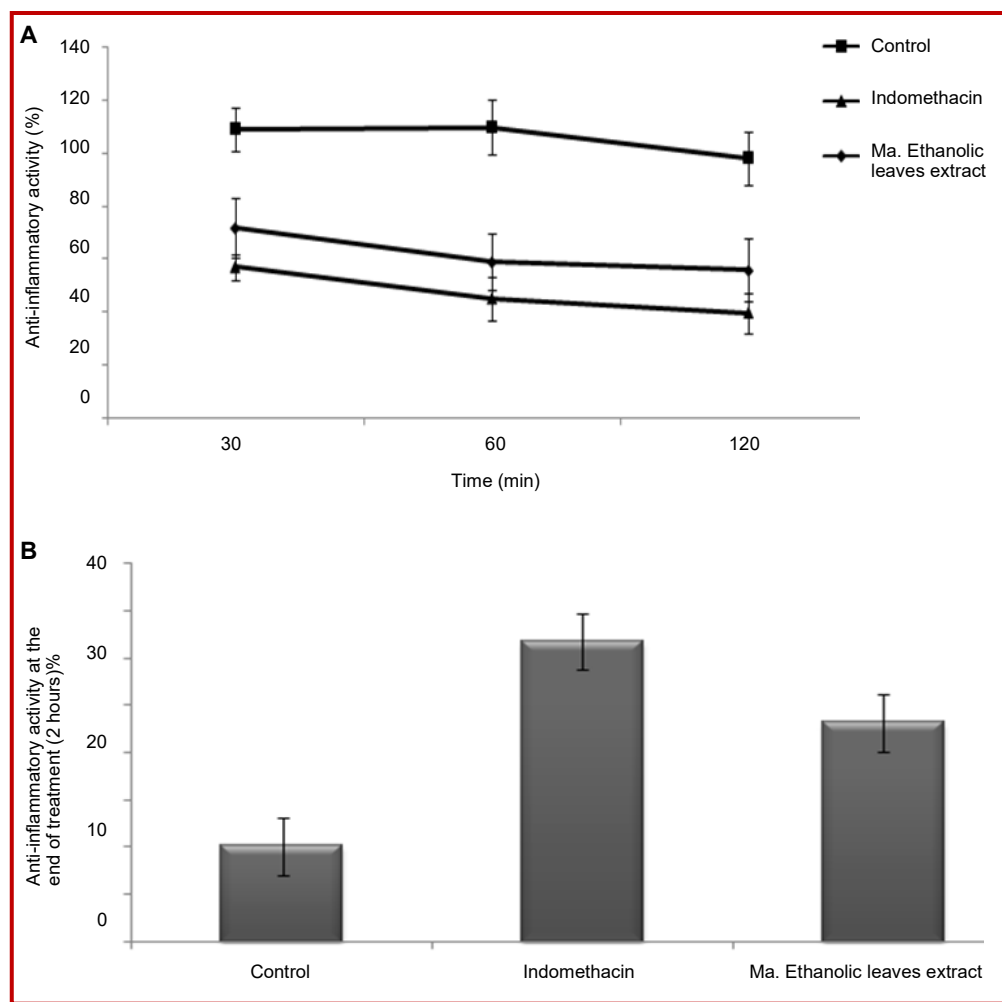


Figure 3: Anti-inflammatory effect of *Melia azedarach* leaves extract with carrageenan induced paw edema. A: Inhibition of the carrageenan-induced paw edema evolution during the treatment; B: percentage of anti-inflammatory activity at the end of treatment

*oxysporum*, and *Rhizoctonia solani* were not significant. However, Sen and Batra (2012) stated that *M. azedarach* leaf extracts were effective against some bacteria such as *B. cereus*, *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and fungi namely *Aspergillus niger*, *A. flavus*, *F. oxysporum*, and *Rhizopus stolonifer*.

These differences in bioactivities of *M. azedarach* extracts between reports could result from the differences in chemical composition between species naturalized in different regions (Gottlieb et al., 2001; Szewczuk et al., 2003; Orhan et al., 2012). Based on phytochemical investigation of Tunisian *M. azedarach* leaves performed in previous work (Akacha et al., 2016) and taking into account the chemical profile, we can speculate that observed bactericide/antifungal effects may result partly from the action of chlorogenic acids as previously shown by Ling et al. (2013). In fact, chlorogenic and caffeic acids exhibit inhibitory effects against conidial germination and growth of *F. oxysporum* f. sp. *niveum* where the target pathogen is found to be much more

susceptible to chlorogenic acid than to caffeic acid. Furthermore, protocatechic acid as shown by Nguyen et al. (2015) to display potent antifungal activity against *B. cinerea* and *R. solani* and is considered as a promising alternative to chemical fungicides for *B. cinerea* control in strawberry crops.

Besides, observed antifungal potency may be due to the presence of linoleic in the extract (Abdelillah et al., 2013).

Ethanollic *M. azedarach* leaves extract had shown high anti-inflammatory effect. This result corroborates with the findings of previous workers who investigate anti-inflammatory activity of *M. azedarach* flowers (Sumathi et al., 2014), roots (Vishnukanta, 2010) and seeds (Khadse and Kakde, 2014).

Besides, Aouadia et al. (2013) proved that *M. azedarach* water and aqueous ethanol (50%) leaf extracts exerted strong anti-inflammatory effects by inhibiting human monoacylglycerol lipase.

Observed anti-inflammatory effect might result of elementary and/or synergic action of present components in *M. azedarach* leaves extract such as gallic acid. In fact, the latter was found to possess anti-inflammatory activity towards zymosan-induced acute food pad swelling in mice (Kroes et al., 1992) and using human basophils (KU812 cells) which are crucial effector cells in allergic inflammation. Kim et al. (2006) explain this capacity by the aptitude of gallic acid to inhibit histamine release and pro-inflammatory cytokine production in mast cells. A further, structure-activity relationship analysis showed that the o-dihydroxy group of gallic acid is important for the inhibitory activity *in vitro* (Kroes et al., 1992). Also, catechins possess anti-inflammatory effects (Reto et al., 2014) as well as chlorogenic acid (dos Santos et al., 2006; Shin et al., 2015). The latter significantly inhibited not only nitric oxide production but also the expression of inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 (COX-2). Chlorogenic acid also attenuated pro-inflammatory cytokines and other inflammation-related markers such as interleukin-6 in a dose-dependent manner (Hwang et al., 2014).

Similarly, sinapic acid has anti-inflammatory activity by eventual suppression of the expressions of iNOS and COX-2, tumor necrosis factor (TNF)-alpha, and interleukin (IL)-1beta and this via nuclear factor-kappa B (NF-kappaB) inactivation (Yun et al., 2008). Furthermore, syringic acid possesses anti-obesity, anti-inflammatory and anti-steatotic effects via the regulation of lipid metabolic and inflammatory genes (Ham et al., 2016). Also, protocatechic acid has shown anti-inflammatory effect (Lin et al., 2009). Treatment with protocatechic acid inhibits significantly different biological parameters like hind paw edema, granuloma exudates formation, and arthritis index in carrageenan edema, cotton pellet granuloma, and Freund's adjuvant arthritis, respectively. The biochemical parameters like glutathione, superoxide dismutase, catalase, lipid peroxidation and nitric oxide in edematous occurring during different types of inflammation were either significantly restored or inhibited with protocatechic acid pretreatment (Lende et al., 2011). In addition, isoquercitrin anti-inflammatory effect was investigated in a murine model of asthma. It was an effective eosinophilic inflammation suppressors, suggesting a potential for treating allergies (Rogerio et al., 2007). Finally, isorhamnetin was proved to have anti-inflammatory properties (Boesch-Saadatmandi et al., 2011; Chirumbolo, 2014).

## Conclusion

This work provide scientific evidence for the traditional uses of *M. azedarach* as anti-inflammatory, antibacterial and antifungal agents.

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## Conflict of Interest

Authors declared no conflict of interest

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