POTENTIATION OF ANTIBIOTICS BY THE HYDROETHANOLIC EXTRACT OF *Juglans nigra* L.

ROMAN Luminița, HOSU Anamaria Delia, VASILIU Cristiana Andreea, ROMAN Horațiu, PREDAN Gențiana, CZOBOR Ilda, MIHĂESCU Grigore

Abstract. Antibacterial resistance in the case of nosocomial infections, especially in immunocompromised patients is becoming a serious concern worldwide. Moreover, the harmful effects of antibiotics in some cases can endanger the patient's life. Herbs associated with common antibiotics may not only reduce the harmful effect but would have an effect in the potentiating the antibacterial properties. Another advantage of this combination would be the avoidance of the abuse of antibiotics that may develop uncontrolled adverse reactions. *Juglans nigra*, a less studied plant, has been shown to have *in vitro* the capacity to potentiate the antibiotics with bacterial resistance.

Keywords: *Juglans nigra*, antibiotics, potentiation, MDR bacteria.

Rezumat. Potențarea antibioticelor de către extractul hidroetanolic de *Juglans nigra* L. Rezistența antibacteriană în cazul infecțiilor nosocomiale, în special care vizează pacienții imunocompromiși, este o problemă serioasă la nivel mondial. De asemenea efectele nocive ale antibioticelor pot periclita în unele situații viața pacientului. Plantele medicinale asociate cu antibioticele uzuale ar putea nu numai să reducă efectul nociv al acestora, dar ar avea un efect și în potențarea proprietăților antibacteriene. Un alt avantaj al acestei asocieri ar fi evitarea abuzului de antibiotice care pot dezvolta reacții adverse necontrolate. *J. nigra*, o plantă mai puțin studiată s-a dovedit a avea capacitatea de a potența antibiotice cu rezistență bacteriană.

Cuvinte cheie: *Juglans nigra*, antibiotics, potențare, bacterii MDR.

INTRODUCTION

Obtaining drugs from herbal extracts has started to become an area that arouses an increasing interest in conditions in which the usual antibiotics do not seem to have the targeted effect. About 25% of the drugs prescribed worldwide come from plants. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from secondary metabolism of *Juglans nigra* (RATES, 2011). Juglone occurs naturally in the leaves, roots, husks, fruit (epicarp) and bark of plants in the Juglandaceae family (STUGSTAD & DESPOTOVSKI, 2012). MIC values for juglone showed it to have moderate antifungal activity and to be as effective as certain commercially available antifungal agents (CLARK et al., 2006). The mechanism for the toxic effects of juglone is still not fully understood. The antimicrobial, antidiarrheal, anthelmintic, depurative and tonic, antihemorrhagic, hypoglycaemic, diuretic, blood purifying, and detoxifying, vascular protective, inhibitory to tumours effects have been reported as being the expected effects of juglone (STUGSTAD & DESPOTOVSKI, 2012). After we took into consideration the wide spectrum of action of the juglone against a wide pathogenic microorganisms, we have analysed *in vitro* the activity of potentiating the hydro-ethanolic extract of *Juglans nigra* of antibiotics against Gram negative bacteria isolated from nosocomial infections**.**

MATERIAL AND METHODS

1. Preparation of the hydro-ethanolic extract of *J. nigra*

Raw fruit endocarp of *J. nigra* was collected in September-October and allowed to macerate in ethyl alcohol with distilled water in a ratio of 30 g *J. nigra* / 20 ml water / 50 ml ethyl alcohol for 10 days in the dark at 4°C. After that it was purified by Whatman filter paper no. 1 and was introduced into a rotary evaporator for 10-15 minutes. This was the stock solution and was stored in an amber glass container at 4°C.

2. Phenotypic analysis of resistance MDR strains from nosocomial infections by disc-diffusion method of Kirby and Bauer

The strains isolated from urogenital infections were from patients hospitalized at Theodor Burghele Hospital, Bucharest.

On solid media Mueller-Hinton (MH) agar, they were inoculated in a cloth with a suspension (0.5 McFarland standard) of the pathogen bacterial culture. Then, they were placed on filter paper disks (6 mm) impregnated with a known concentration of an antimicrobial compound (in the vicinity of the disc producing a higher concentration of antibiotic that decreases as the distance increases. The plates were incubated at 37°C for 16-18 hours. There occur the simultaneous growth of the bacteria and diffusion of the antimicrobial compounds. The point at which critical mass is reached is demonstrated by a sharply marginated circle of bacterial growth around the disk. The concentration of antimicrobial compound at this margin is called the critical concentration and is approximately equal to the minimum inhibitory concentration obtained in broth dilution susceptibility tests. The current interpretation standards can be found in the CLSI (Clinical Laboratory Standards Institute) 2009 (HUDZIKI, 2013).

3. Analysis of interactions between the extract of *J. nigra* and the antibiotics

The interactions between the extract of *J. nigra* and the antibiotics were determined using disc-diffusion method. Thus, on the solid media Mueller-Hinton agar seeded with the bacterial suspension, the disks impregnated with antibiotics was pipetted with 10 mg extract of *J. nigra*. The fractional inhibitory concentration was derived from the concentrations of the *J. nigra* extract and the antibiotics in combination permitting no visible growth of the test microorganisms in the Mueller-Hinton agar after incubation for 24 h at 37°C. Minimum inhibitory concentration (MIC) was made by the method of serial microdilution in a liquid medium BHI (Hearth Infusion Broth) in deepwell plates 96 (Eppendorf tubes of 550 ml) following the protocol published in a recent article (ROMAN et. al., 2015).

RESULTS AND DISCUSSION

Following the analysis of the antibiotic and extract of *J. nigra* resistance via disk-diffusion method resulted in many situations of synergism that can be observed in Fig. 1.

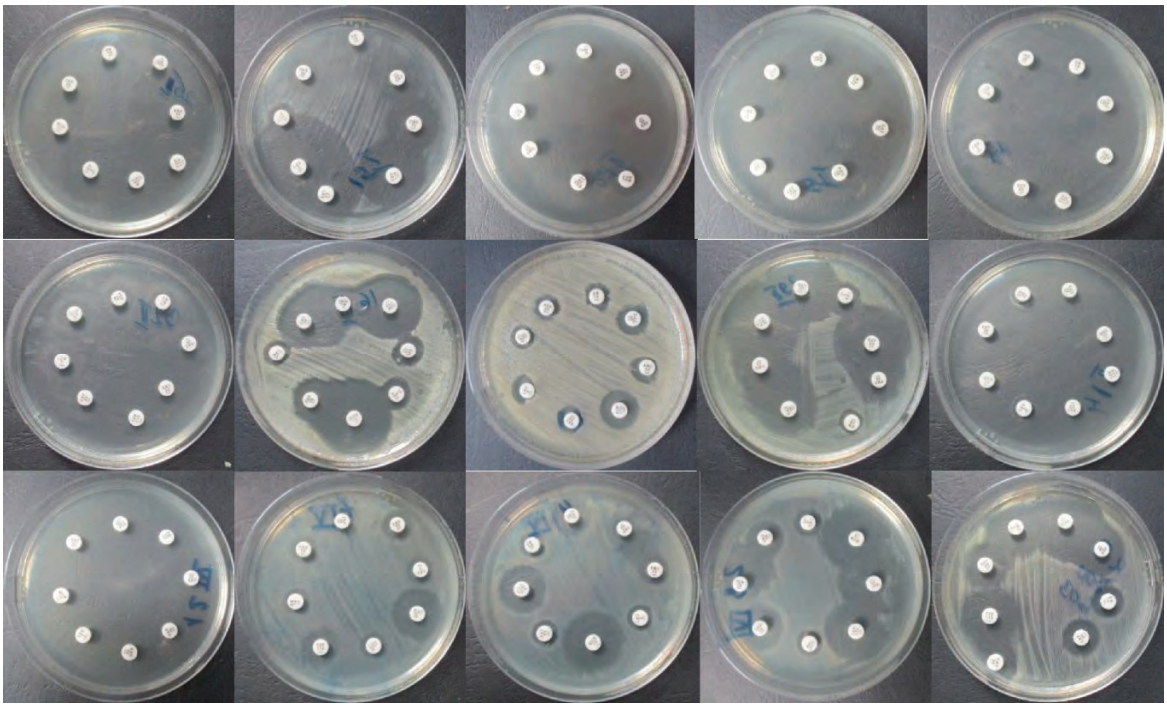

Figure 1. The potentiation of antibiotics by *J. nigra* extract. Inhibition zones of bacterial growth are marked by clear areas that appear around the paper disks impregnated with antibiotic and extract of *J. nigra* (*A. faecalis*$_{II25}$, *K. pneumoniae*$_{I15}$, *P. aeruginosa*$_{II27}$, *A. baumannii*$_{I5}$, *P. mirabilis*$_{I1}$, *K. pneumoniae*$_{I6}$, *K. pneumoniae*$_{III8}$, *K. pneumoniae*$_{II29}$, *E. coli*$_{III16}$, *P. aeruginosa*$_{I4}$, *K. pneumoniae*$_{I12}$, *E. coli*$_{IV7}$, *K. pneumoniae*$_{IV4}$, *E. coli*$_{IV23}$, *K. pneumoniae*$_{ATCC\ 700603}$. (original).

The antibiogram by disc diffusion method was performed by measuring inhibition zones around the antibiotic impregnated disks and the results were summarized in Table 1.

Table 1. Antibiogram by disc diffusion method.

Strain	Average zones of inhibition (±0.5 mm) of antibiotics							
	CN_{10}	FEP_{30}	SXT_{25}	LEV_5	TZP_{110}	AMC_{30}	CAZ_{30}	MEM_{10}
K. pneumoniae$_{II29}$	0	12	25	10	20	20	14	25
K. pneumoniae$_{III8}$	0	10	0	0	14	10	10	14
E. coli$_{IV23}$	0	20	0	0	25	14	25	25
E. coli$_{III16}$	0	0	0	14	25	0	20	25
A. baumannii$_{I5}$	0	0	10	12	12	10	0	0
P. mirabilis$_{II32}$	0	0	0	0	12	14	0	0
P. mirabilis$_{I1}$	0	0	14	0	14	12	0	0
P. aeruginosa$_{II27}$	0	0	12	10	0	0	0	10
A. faecalis$_{II25}$	0	0	14	12	10	14	0	0
K. pneumoniae$_{I12}$	14	14	20	0	0	0	0	0
P. aeruginosa$_{I4}$	0	0	14	0	12	14	0	0
K. pneumoniae$_{II30}$	0	0	0	0	25	32	0	0
K. pneumoniae$_{I6}$	20	30	14	18	25	25	20	30
K. pneumoniae$_{ATCC\ 700603}$	12	20	19	25	25	20	18	32
E. coli$_{IV28}$	0	14	14	12	18	12	12	14
K. pneumoniae$_{IV4}$	0	12	0	0	14	0	20	14
E. coli$_{IV7}$	0	10	0	0	12	12	25	20
E. coli$_{I\ ATCC\ 25922}$	25	30	30	30	30	30	30	30

The synergism between the antibiotic and *J. nigra* extract has been calculated by measuring the inhibition zone with graded ruler and centralized in Table 2.

Table 2. Synergism between the antibiotic and *J. nigra* extract.

Strain	Average zones of inhibition (±0.5 mm) of hydro-ethanolic extract of *J. nigra* (JN) alone and MIC(µg/ml), combination of antibiotics and JN and the degree of potentiation of the antibiotic (A, S, I)								
	JN area and MIC (g/ml)	**CN_{10} +JN**	**FEP_{30} +JN**	**SXT_{25} +JN**	**LEV_{5} +JN**	**TZP_{110} +JN**	**AMC_{30} +JN**	**CAZ_{30} +JN**	**MEM_{10} +JN**
K. pneumoniae$_{II29}$	14 (250)	10 (S)	22 (S)	32 (S)	12 (S)	32 (S)	22 (S)	28 (S)	32 (S)
K. pneumoniae$_{III8}$	17 (125)	12 (S)	14 (S)	14 (S)	14 (S)	18 (S)	13 (S)	14 (S)	18 (S)
E. coli$_{IV23}$	16 (125)	14 (S)	25 (S)	32 (S)	14 (S)	32 (S)	25 (S)	28 (S)	32 (S)
E. coli$_{III16}$	20 (15.625)	12 (S)	18 (S)	14 (S)	13 (S)	32 (S)	20 (S)	25 (S)	32 (S)
A. baumannii$_{I5}$	12 (250)	14 (S)	20 (S)	30 (S)	25 (S)	32 (S)	32 (S)	12 (S)	14 (S)
P. mirabilis$_{II32}$	16 (125)	12 (S)	12 (S)	14 (S)	14 (S)	32 (S)	32 (S)	12 (S)	14 (S)
P. mirabilis$_{I1}$	18 (31.25)	7 (S)	12 (S)	20 (S)	12 (S)	30 (S)	30 (S)	12 (S)	12 (S)
P. aeruginosa$_{II27}$	17 (31.25)	12 (S)	12 (S)	32 (S)	32 (S)	32 (S)	32 (S)	12 (S)	14 (S)
A. faecalis$_{II25}$	12 (250)	14 (S)	14 (S)	25 (S)	25 (S)	32 (S)	32 (S)	14 (S)	12 (S)
K. pneumoniae$_{I12}$	16 (125)	14 (I)	14 (S)	20 (S)	30 (S)	25 (S)	20 (S)	18 (S)	14 (S)
P. aeruginosa$_{I4}$	20 (15.625)	14 (S)	0 (I)	32 (S)	0 (I)	32 (S)	30 (S)	0 (I)	14 (S)
K. pneumoniae$_{II30}$	18 (31.25)	0 (I)	0 (I)	0 (I)	0 (I)	30 (S)	32 (S)	0 (I)	0 (I)
K. pneumoniae$_{I6}$	14 (250)	12 (A)	32 (S)	30 (S)	32 (S)	32 (S)	32 (S)	20 (I)	30 (I)
K. pn$_{ATCC\ 700603}$	17 (125)	14 (S)	20 (I)	25 (S)	30 (S)	25 (S)	20 (I)	18 (I)	32 (I)
E. coli$_{IV28}$	21 (15.625)	7 (S)	20 (S)	25 (S)	20 (S)	25 (S)	18 (S)	18 (S)	21 (S)
K. pneumoniae$_{IV4}$	12 (250)	7 (S)	12 (I)	7 (S)	7 (S)	22 (S)	10 (S)	20 (I)	18 (S)
E. coli$_{IV7}$	16 (125)	7 (S)	10 (I)	10 (S)	12 (S)	12 (I)	12 (I)	10 (A)	20 (I)
E. coli$_{I\ ATCC\ 25922}$	14 (250)	30 (S)	30 (I)	30 (S)	30 (I)	30 (I)	30 (I)	30 (I)	30 (I)

Legend: Abbreviations: S=Synergy; I=Indifference; A=Antagonism

The minimum inhibitory concentration (MCI) of the hydro-ethanolic extract of *J. nigra* ranged between 250 and 15,625 µg / ml. Inhibition zones of *J. nigra* extract ranged between 12 and 21 ± 0.5 mm. In combination with the TZP (piperacillin-tazobactam) these zones of inhibition were significantly increased; in 89% of the cases, it is observed the synergism in the interaction of the antibiotic and the extract of *J. nigra* and only 11% of the cases are characterized by indifference, having no significance in combination with the antibiotic (*E. coli*$_{IV7\ and}$ *E. coli*$_{I\ ATCC\ 25922}$). A remarkable interaction is also observed in the combination with AMC (amoxicillin-clavulanic acid); in 83% of the cases it is observed the synergism while 17% are characterized by indifference, without having any influence on the antibiotic. In combination with the CN (gentamicin), it has been observed one case of antagonism in the interaction between the antibiotic and extract (*K. pneumoniae*$_{I6}$) and in the combination with the CAZ (ceftazidime) and the extract of *J. nigra*, in the presence of a single strain (*E. coli*$_{IV7}$). It is noteworthy that in 9 cases where the antibiotic did not show any growth of the inhibition zone, the interaction of the antibiotic with the extract did not have any influence on the growth of the inhibition zone. In 49 cases, in which the antibiotic has not submitted any growth of the inhibition zone, in combination with the extract, the inhibition zone had a growth with values between 7 and 18 mm; the greatest interaction by potentiating the antibiotic was registered in combination with CAZ (*K. pneumoniae*$_{I12}$), LEV (levofloxacin) in case of *K. pneumoniae*$_{I12}$, SXT (sulfamethoxazole) in case of *E. coli*$_{IV23}$ and FEP (cefepime) in *A. baumannii*$_{I5}$. MEM (meropenem) belongs to the class carbapenems, antibiotics with large spectrum, being administered in circumstances where bacterial infections do not respond to other antibiotics. In interaction of the MEM and *J. nigra* extract, in 72% of cases it was observed the synergism and in 28% of the cases the interaction between the extract and the MEM did not have any influence on the inhibition zone. The action mechanisms of compounds of plants against bacterial growth are less understood. Their synergism with some antibiotics in certain situations, it may suggest their compatibility with the mode of action of antibiotics. The vegetal extracts have multiple action targets in the microbial cell, which represents an advantage of their use as antimicrobial agents with synergistic activity (BENNETTE & WALLSGROVE, 1994).

CONCLUSIONS

Hydro-ethanolic extracts of *J. nigra* in combination with antibiotics had an enhanced effect against resistant bacteria, by potentiating the antibiotics. This study has demonstrated the *in vitro* antibacterial activity of the extract *J. nigra* against antibiotic resistant strains. The association of the antibiotic and *J. nigra* extract had a greater effect against the most studied bacteria. *J. nigra* is a plant with multiple therapeutic properties that deserve to be studied and used in the pharmaceutical industry in order to obtain a new antibacterial synthetic substance with large spectrum of action.

ACKNOWLEDGEMENS

We express our gratitude to Professor Coordinator, Mihăescu Grigore, Professor Carmen Mariana Chifiriuc director of the Research Institute of the Faculty of Biology, Dr. Camelia Tudor, head of the laboratory of ”Theodor Burghele” Hospital, Bucharest.

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Roman Luminița, Vasiliu Cristiana Andreea,
Predan Genţiana, Czobor Ilda, Mihăescu Grigore
Faculty of Biology, University of Bucharest, Romania.
Email: luminitaroman9@yahoo.com; cristianavasiliu@yahoo.com;
ggentiana@yahoo.com; ilda.czobor@yahoo.com; grigoremihaescu2006@yahoo.com

Hosu Anamaria Delia
Faculty of Chemistry and Chemical Engineering,
Babeș-Bolyai University, Cluj-Napoca, Romania.
Email: hosuanamaria@yahoo.com

Roman Horațiu
Faculty of Geology, University of Bucharest, Romania.
Email: horace_the_horace@yahoo.com

Received: March 31, 2016
Accepted: August 12, 2016