African Journal of Biochemistry Research

Volume 11 Number 10 October 2017

ISSN 1996-0778

Academic Journals

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Vol. 11(10), pp. 58-71, October 2017
DOI: 10.5897/AJBR2017.0955
Article Number: 7C6B58766448
ISSN 1996-0778
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African Journal of Biochemistry Research

Full Length Research Paper

Chemical composition, antibiotic promotion and *in vivo* toxicity of *Piper nigrum* and *Syzygium aromaticum* essential oil

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Received 1 August, 2017; Accepted 29 September, 2017

The antimicrobial activity of spices of human daily diet has been widely demonstrated. Yet, their capacity of promoting antibiotic activity has not been explored adequately. In this work, the chemical composition of essential oils of *Piper nigrum* and *Syzygium aromaticum* from Chad were determined and their antimicrobial effect in combination with different antibiotics towards *Staphylococcus aureus, Salmonella enteritidis* and *Salmonella typhi* were assessed. Moreover, the *in vivo* toxicity of the essential oils alone and in combination with antibiotics was performed. Results indicate that the essential oil of *P. nigrum* was mostly composed of limonene (18.59%), beta-pinene (11.51%), linalool (10.17%), alpha-pinene (9.96%), while for *S. aromaticum* essential oil, only three compounds represented about 89% of the identified compounds. They were respectively beta caryophyllene (43.63%), eugenol (42.67%) and alpha-humulene (3.73%). Regarding the antimicrobial properties, *Syzygium aromaticum* essential oil (EO) used alone was more active than *P. nigrum* EO with a minimum inhibitory concentration (MIC) of 625 ppm towards all the strains. In combination with selected antibiotics, *S. aromaticum* gave better results with less indifference association, the best being with gentamicin. This association of the different EOs with gentamicin proved not to be acutely toxic to rats after 14 days of observation at doses ten times the MIC. These results indicated that spices used in human diets can be used for the production of hybrid drugs containing antibiotic and natural substances.

Key words: *Piper nigrum*, *Syzygium aromaticum,* antibiotic promotion, *Staphylococcus aureus, Salmonella*, toxicity.

INTRODUCTION

Spices are highly used in Chadian diet and many research works indicate that some of them have biological activities (Beuchat, 1994; Shan et al., 2007). In the continuous search for natural compounds with antimicrobial properties, a more safety approach is to use substances already belonging to human diet. Bacterial infection is one of the most current causes of illness worldwide (Kuete, 2013) and especially in developing countries (Adwan et al., 2008). According to Havelaar et al. (2015), food borne diarrheal disease agents, particularly non-typhoidal *Salmonella enterica*, cause about 23000 death worldwide, with a high percentage in

Africa. In the same report, *Salmonella* Typhi was recognized as one of the major cause of food borne diseases. Regarding *Staphylococcus aureus*, Tong et al. (2015) stated that in the industrialized world, the population incidence of *S. aureus* bacteremia ranges from 10 to 30 per 100,000 person/year.

Black pepper (*Piper nigrum*) has many medicinal properties among which, antibacterial, antifungal, anti-inflamatory and antidiarrhoeal are mostly reported (Noumedem et al., 2013). Clove (*Syzygium aromaticum*) is an aromatic herb that has many useful purposes. Its essential oil has demonstrated several properties as reported by Noumedem et al. (2013). *S. aromaticum* is widely cultivated in Indonesia, Sri Lanka, Madagascar, Tanzania and Brazil. Essential oil of *S. aromaticum* and eugenol, a major component of this oil has been described to have useful antiseptic, analgesic and anaesthetic effects (Chaieb et al., 2007). Several studies have demonstrated the antimicrobial activity of *S. aromaticum* and *P. nigrum* (Pavithra and Bhagya, 2010; Shiva et al., 2013; Ayman and Mazen, 2014).

The efficiency of currently available drugs in microorganism causing toxi-infections is reducing. There is hence a need for alternative antimicrobial solutions to overcome major bacterial infections. A solution may be the synergistic association of antibiotics with antimicrobial substances from spices. Some few works have already experimented the association of plant extract with antibiotics in order to overcome antibiotic resistance. Noumedem et al. (2013) observed while studying the effect of ethanol extract of four edible spices with antibiotics on food borne pathogens, that *P. nigrum* fruits and *Telfairia occidentalis* leaves extracts had synergistic effect with selected antibiotics on 70% of target strains. Moreover, the persistence of antibiotics resistance facilitates the need for finding new therapies (Yap et al., 2014). Plant extracts due to their complex mixtures of compounds can be used alongside with antibiotics due to the synergistic effect (Yap et al., 2014; Dzotam and Kuete, 2017). The present study was hence designed to assess the efficacy of *P. nigrum* and *S. aromaticum* essential oils in association with some antibiotics to inhibit the growth of *S. aureus* and *Salmonella* species, and evaluate the toxicological properties of the combination.

MATERIALS AND METHODS

Plant materials

P. nigrum and *S. aromaticum* fruits were purchased from local market in N'Djamena (Chad) and stored in vacuum sealed bags. They were identified by the National Herbarium of Cameroon in Yaoundé, where specimens for *P. nigrum* and *S. aromaticum* fruits were respectively stored under the reference numbers 25818/SFRcam and 28524/HNC. The antibiotics: Ampicillin (AMP), ciprofloxacine (cipro), Gentamicin (Genta) and Augmentin, a mixture of Amoxicillin and clavulanic acid (Amx/AC) in a ratio 5:1 were obtained from Sigma-Aldrich, St Quentin Fallavier, France.

Animals

Wistar albino rats weighing 98 to 192 g of both sexes were used in this study. The animals were bred in the animal house of the Department of Biochemistry, University of Yaoundé I. They were placed on a semi-synthetic diet (LAVANET, Bockle, Cameroon) and given water *ad libitum*. These animals were handled according to ethical guidance of the WHO recommendation for animal welfare (World Health Organization (WHO), 1993).

Bacterial culture

Staphylococcus aureus SR196, *S. aureus* NCTC 10652, *Salmonella typhi* 15SA and *Salmonella enteritidis*, were kindly offered by the Laboratory of Food Microbiology, University of Bologna-Cesena, Italy.

Essential oils extraction

The essential oils tested were extracted from crushed fruits by hydrodistillation method using a Clevenger-type apparatus for 6 to 8 h (Lamaty et al., 1987). The obtained essential oils, separated from water by decantation, were dried over anhydrous sodium sulfate and, after filtration, the oils were quantified, recorded as percentage oil (weight/dry weight) and stored in sealed flasks at 4°C until tested and analyzed.

Chemical composition of the spices

The composition of *S. aromaticum* and *P. nigrum* were determined through GC/MS and GC/FID while for *S. aromaticum* a confirmation of the main component overall ratio was done by head space volatile compounds analysis with a GC/MS associated with Solid Phase Molecular Extraction (SPME). The different methods are hereby synthetically presented.

The GC/FID analyses were performed on a Thermo Scientific gas chromatograph, model TRACE 1300, with a flame ionization detector equipped with two fused silica capillary columns DB-5 (30 m x 0.25 mm; film thickness 0.25 μm) (95% methyl 5% phenyl polysiloxane) and DB-Wax (30 m x 0.25 mm; film thickness 0.25 μm); N_2 was the carrier gas at 0.5 ml/min; with injection of 2 μl 10/100 diethyl ether solution, split mode 1:20; injector temperature 220°C, detector temperature 250°C; temperature program 60 to 220°C at 3°C/min, then kept at 220°C during 17 min. The linear retention indices (LRI) of the components were determined relative to the retention times of a series of *n*-alkanes with linear interpolation. The percentage composition of the EO was computed by the normalization method from the GC/FID peak areas on the DB-5 capillary column, and response factors were taken as one for all compounds.

The GC/MS analyses were performed using an Agilent 5977 apparatus MSD series equipped with two silica capillary columns HP-5 MS (5%-phenyl-methylpolysiloxane) (30 m x 250 μm; film

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thickness 0.25 µm), HP-INNOWAX fused silica column (30 m x 250 µm; film thickness 0.25 µm) interfaced with a quadrupole detector (single quadrupole acquisition Method-MS parameters report), source temperature 230°C, Quadrupole temperature 150°C; the temperature program was 60°C for 2 min, 60 to 240°C at 3°C/min, then kept at 240°C during 8 min; injector temperature was 240°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.7 ml/min; injection type, split, 20:1 (1 µl of a 10% dichloromethane solution); ionization voltage, 70 eV; electronmultiplier 1000 eV; scan range 33 to 400 amu; scan rate, 1.56 scan/s. The identification of the constituents was based on comparison of their relative retention indices with either those of authentic samples or with published data in the literature (Adams, 2007) and by matching their mass spectra with those obtained with authentic samples and/or the NIST14, FFNSC 2.L. libraries spectra.

S. aromaticum crushed fruits head space gas chromatographic analyses were done using an Agilent Technology GC/Mass 7890A/HP 5975C (Palo Alto, CA, USA). The working conditions were: injector temperature 250°C, detector temperature 280°C, EI mode (70 eV), split 1:10 capillary column Zebron ZB-5 30 m length, 0.25 mm ID and 0.25 µm film thickness (Phenomenex, Torrance, CA, USA). The temperature program was from 50 to 240°C with increment of 3°C/min, and 1 min hold at 240°C. For the head space analysis, 5 g of the crushed fruits was introduced into a 10 ml vial and hermetically sealed. After heating the sample in water bath at 30°C for 10 min with a SPME-DVB-carboxen/PDMS, 50/30 µmfiber (Supelco, Bellefonte, PA, USA) was exposed in the head space for 30 min for absorption. Subsequently, the fiber was then immediately inserted for desorption into the injector of a GC-MS for 5 min. The identification of the volatile compounds was performed using the NIST (NIST/EPA/NIH Mass spectral Library, 2005, USA) and WILEY (sixth edition, 1995, USA) and with the Kovach retention index in comparison with the laboratory database using a series of n-alkane (C6-C24) standards injected in the same column.

Antibacterial test

Bacterial strains and inoculum preparation

Bacterial strains used to assess the antimicrobial properties of the EOs were, *S. typhi 15SA*, *S. enteritidis*, *S. aureus* SR 196 and *S. aureus* NCTC10652. All these strains were maintained in 40% glycerol Mueller Hinton broth at 80°C and sub-cultured three times before use. These bacteria were inoculated in the nutrient agar (NA) at 37°C for 24 h. 2 to 3 colonies were aseptically peaked up by wire loop into physiologic saline solution. The turbidity was adjusted to 0.5 McFarland scale prepared according to the protocol previously described (CLSI, 2007).

Disk-diffusion method

A primary antibacterial screening was performed using the disk-diffusion method according to CLSI (2007). Briefly, Petri dishes containing Mueller Hinton (MH) Agar culture medium were inoculated with the bacterial inoculums previously prepared. The disks, of 6 mm diameter were impregnated with 10 µL of each essential oil and placed in the center of each plate. Petri dishes were incubated at 4°C for 2 h to allow a better diffusion of molecules. They were later incubated at 37°C for 18 to 20 h. The diameters of the inhibition zones were measured in mm. Each assay was carried out in triplicate.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC was defined as the lowest concentration of the antimicrobial agent that prevented the visible growth of a microorganism after overnight incubation (Andrews, 2001). It was determined by serial dilution method (Stokes, 1975). The serial dilution of the test compound was carried out in the Mueller Hinton broth. To each test tube, 10^5 CFU/ml of actively growing bacterial culture in log phase was inoculated. The culture tubes were incubated at 37°C for 24 h. After incubation, the tubes were checked for the growth of bacteria and MIC of that essential oil and antibiotics was determined and expressed in ppm. In order to determine the MBC, from each of the tubes with no visible growth, about 100 µL aliquot was drawn and subcultured on Mueller Hinton agar. The plates were then incubated at 37°C for 24 h. Bacterial growth on agar was observed and the concentration which led to no colony count was considered as the MBC value.

Antibiotic promotion capacity of S. aromaticum and P. nigrum EOs

The effects of interactions between essential oils (*S. aromaticum* and *P. nigrum*) and antibiotics against strains of this study were evaluated using the checkerboard technique. Determination of FIC index value is commonly used as a measurement of interactive inhibition using 96-well plates (Pillai et al., 2005; Basri et al., 2012; Wessal et al., 2015). The concentration of essential oils (*S. aromaticum* and *P. nigrum*) and antibiotics tested in seven concentrations are 0, MIC/16, MIC/8, MIC/4, MIC/2, MIC and 2MIC. Along the x-axis across the checkerboard plate, each essential oil (*S. aromaticum* or *P. nigrum*) was available in each well in the following sequence: 0, MIC/16, MIC/8, MIC/4, MIC/2, MIC and 2MIC. As for the y-axis, antibiotics were also available in each well in the same sequence at the final concentration of 0, MIC/16, MIC/8, MIC/4, MIC/2, MIC and 2MIC from the top to the bottom in decreasing concentration, in order for the well with no antibiotic and essential oil to be at the left angle. Inoculum was added to final well solution volume of 200 µL at final concentration of 6 log CFU/mL. Wells containing non inoculated MH broth, inoculated MH broth without antimicrobials and MH broth with each antimicrobial and no bacteria inoculum were used as control. The 96-well plate was then sealed and incubated at 37°C for 30 min. Experiments were carried out in triplicate. The presence of turbidity in the well indicated positive growth while no turbidity indicated negative results. The fractional inhibition concentration (FIC) index values was then calculated using the following formula:

Σ FICI = FIC(A) + FIC(B)

Where,

$$FIC(A) = \frac{\text{MIC (A)incombination}}{\text{MIC (A) alone}}$$

$$FIC(B) = \frac{\text{MIC (B)}\textit{incombination}}{\text{MIC (B) alone}}$$

The Σ FICI values were interpreted as follows: ≤ 0.5 = synergistic; 0.5-0.75 = partial synergy; 0.76-1.0 = additive; >1.0-4.0 = indifferent (non-interactive); > 4.0 = antagonistic (Bharadwaj et al., 2003)

Toxicity test

Animal treatment and body weight measurement

Female and male Wistar albino rats were used in this experiment. The animals were randomly divided into 5 groups of 8 animals (4 females and 4 males each) and treated with the following substances diluted in 10 ml corn oil containing doses expressed in

terms of mg/kg of animal weight: group I received 10 × MIC essential oil of *S. aromaticum* (EOSa), group II 10 × MIC essential oil of (EOPn *P. nigrum*), group III 10 × MIC of the best association of EOSa and the antibiotics, groups IV 10 × the MIC of the best association of EOPn and the antibiotics. The MIC of the essential oil tested as single compound were those obtained within the best combination with antibiotics retained. Group V served as normal controls and received corn oil. The antimicrobials were given once, by gavage and normal saline 1 ml water were given after the drugs were administered to all the animals. During the 14 days observation, body weight was measured daily and expressed as percentage over initial weight per group according to the sex and treatment. After 14 days, the animals of the various groups were sacrificed and blood collected for biochemical analyses.

Biochemical analyses

Creatine and urea were analyzed using the method described by Timothy et al. (2015). Regarding the alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) assays in serum, the colorimetric test of Reitman and Frankel (1957) as published by Rodier and Mallein (1983) was used.

RESULTS AND DISCUSSION

Chemical composition

The hydrodistillation of the essential oil of *P. nigrum* and *S. aromaticum* gave an extraction rate of 1.53 and 1.87% w/w, respectively. In Table 1, the composition of the essential oils of *P. nigrum* and *S. aromaticum* are listed. Components representing less than 0.1% of the essential oil are not reported. The main compounds identified in *P. nigrum* were in order of decreasing importance: limonene (18.59%), beta-pinene (11.51%), linalool (10.17%), alpha-pinene (9.96%), α-phelladrenne (8.04%) and Delta-carene (6.13%), representing 64% of the total compounds identified. This composition quite corresponds to the intervals of compounds variability observed in literature. In fact, according to Orav et al. (2004), who analyzed the composition of *P. nigrum* of different maturity state, the most abundant compounds in pepper oils are beta-caryophyllene (1.4 to 70.4%), limonene (2.9 to 38.4%), beta-pinene (0.7 to 25.6%), delta-3-carene (1.7 to 19.0%), sabinene (0 to 12.2%), alpha-pinene (0.3 to 10.4%), eugenol (0.1 to 41.0%), terpinen-4-ol (0 to 13.2%), hedycaryol (0 to 9.1%) and beta-eudesmol (0 to 9.7%). These results were also confirmed previously by Menon et al. (2002) and Sruthi et al. (2013), who analysed cultivars from different Indian regions.

Regarding *S. aromaticum*, only three compounds represented about 89% of the identified compounds. They were respectively beta caryophyllene (43.63%), eugenol (42.67%) and alpha-humulene (3.73%). Razafimamonjison et al. (2014) observed while analysing the composition of essential oil of bud, leaf and stem essential oil composition of *S. aromaticum* from Madagascar, Indonesia and Zanzibar, that the buds were composed of eugenol between 72 and 83% while the second and third major components were eugenyl acetate (8.6 to 21.3%) and the third, beta-caryophyllene (2.8 to 8.6%). The dominance of eugenol (more than 80%) in *S. aromaticum* essential oil was also observed by Barakat (2014). These results contrast with what was observed in the current study sample. In fact, there was equilibrium between beta caryophyllene and eugenol content. These unexpected results led to suspecting sample alteration during the essential oil extraction procedure. In order to discard this eventuality, the hold bud of a new sample was analysed after crushing, using an SPME/GC-mass technique. The chemical composition of the head space of crushed *S. aromaticum* bud is presented in Table 2. The results confirmed the ratio between beta caryophyllene and eugenol observed while analysing the essential oil. The slight difference between the data obtained analysing the essential oil and the head space may be attributed to the different volatility properties of the compounds and the effect of crushing that may influence the liberation of compounds (Safrudin et al., 2015).

Antibacterial activity of the essential oil tested

The antibacterial activity of *P. nigrum* and *S. aromaticum* essential oils on *S. aureus*, *S.* Typhi and *S. enteritidis* were assessed by evaluating the MIC and MBC (Table 3). *S. aromaticum* had the same MIC (625 ppm) on all the tested strains while different MBC were observed for the same EO towards *S. aureus* (5000 ppm), *S. typhi* (2500 ppm) and *S. enteritidis* (2500 ppm). These results are in accordance with those of Xu et al. (2016) who obtained a MIC of 625 ppm on *S. aureus* ATCC 25923 using a *S. aromaticum* EO principally composed of eugenol (76.23%) and beta-caryophyllene (11.54%). Rusenova and Parvanov (2009) observed in a sample of *S. aromaticum*, a MIC of 250 ppm on *S. aureus* ATCC 25923 and *S. enteritidis*. On the other hand, Naveed et al. (2013) observed a MIC of 540 ppm both on *S. aureus* and *S. typhi* D1. Xu et al. (2016) hypothesized that *S. aromaticum* essential oil may interact with the cell wall and membrane first. On the other hand, it destroys cell wall and membranes, causing the loss of vital intracellular materials, which finally result in the bacterial death. Besides, essential oil penetrates the cytoplasmic membrane or enters inside the cell after destruction of cells structure, and then inhibits the normal synthesis of DNA and proteins that are required for bacterial growth. In the case of *P. nigrum* EO, *S. aureus* was the most sensitive in terms of MIC than the *Salmonella* species tested. There was no clear difference between the strains regarding the MBC of *P. nigrum*. The MIC was between 1250 and 2500 ppm for *S. aureus* and between 5000 and 10000 ppm for *Salmonella* species, while the MBC were about 10000 ppm or higher. Nikolic et al. (2015) observed that MIC and MBC of *P. nigrum* on *S. aureus* were

Table 1. Chemical composition of *P. nigrum* and *S. aromaticum* essential oil purchased from local market in N'Djamena (Chad) analysed using in-column techniques.

Piper nigrum					*Sizigium aromaticum*				
Compound	**LRI**	**LRI-Adams**	**LRI-HP5**	**Relative (%)**	**Compounds**	**LRI**	**LRI-Adams**	**LRI-HP5**	**Relative (%)**
α-Thujene	928	930	1189	0.29	Methyl salicylate	1192	1191	1195	0.12
α-Pinene	937	932	1201	9.96	α-Cubebene	1346	1348	1352	1.36
Camphene	952	954	1214	0.48	eugenol	1366	1359	1376	42.67
Sabinene	975	975	1245	4.99	(E)-β-Caryophyllene	1430	1420	1441	43.63
β-Pinene	982	979	1249	11.51	α-Humulene	1456	1454	1464	3.73
Myrcene	989	990	1261	1.92	Trans cadina-1(6),4-diene	1470	1476	1479	0.89
α-Phellandrene	1007	1002	1280	8.04	(E,E)-α-farnesene	1494	1505	1513	0.61
δ-2-Carene	1013	1002	1287	6.13	Ɣ-Cadinene	1509	1513	1517	0.08
α-Terpinene	1017	1017	1291	0.2	δ-Cadinene	1519	1523	1530	1.43
o-Cymene	1025	1026	---	0.94	Trans cadina-1,4-diene	1528	1534	1537	0.43
Limonene	1031	1029	1313	18.59	caryophyllene oxide	1583	1583	1586	0.1
(E)-β-Ocimene	1044	1050	1326	0.5					
Ɣ-Terpinene	1057	1059	1338	0.31					
Terpinolene	1087	1088	1371	0.44					
Linalool	1098	1096	1397	10.17					
α-Cubebene	1344	1348	1672	0.78					
α-Copaene	1373	1376	1707	4.91					
β-Elemene	1386	1390	1424	2.12					
α-Gurjumene	1407	1409	1743	1.07					
(E)-β-caryophyllene	1418	1419	1758	3.64					
α-Trans-Bergamotene	1427	1434	1772	0.72					
6,9-Guaiadiene	1443	1444	1780	0.48					
α-Humulene	1451	1454	1794	1.77					
Y-Muurolene	1463	1479	1812	0.15					
Germacrene D	1479	1485	1831	6.16					
Ɣ-amorphene	1484	1495	1835	0.73					
α-Selinene	1492	1498	1843	0.7					
β-bisabolene	1497	1505	1857	0.41					
(E)-Y-Bisabolene	1521	1531	1882	0.14					
germacrene B	1530	1561	1911	0.05					
Allo aromadendrene epoxide	1548	1641	2004	0.49					
cis- Thujopsenic acid	1880	1864	2229	0.14					

LRI-HP-5, Linear retention index on apolar (HP-5) column; LRI, linear retention index on polar (DB-Wax) column; RI-Adams, retention indices from Adams (2007).

Table 2. GC-mass associated with solid phase molecular extraction (SPME) chemical identification of crushed *S. aromaticum* head space compounds.

Compound name	Area (%)
Copaene	1.28
Beta-caryophyllene	37.44
Alpha-caryophyllene	4.30
Benzyl acetate	0.24
Trans, beta-ocimene	0.18
Delta-cadinene	0.34
Gamma-cadinene	0.04
Methyl salicylate	0.42
Eugenol	55.76

Table 3. Sensitivity of *S. aureus*, *S. typhi* and *S. enteritidis* to *S. aromaticum* and *P. nigrum* essential oil assessed by disk diffusion method.

Strain	Inhibition diameter (mm)					
	***S. aromaticum* (ppm)**			***P. nigrum* (ppm)**		
	10000	**5000**	**2500**	**10000**	**5000**	**2500**
Staphylococcus aureus SR196	19	13	10	14	12	8
Staphylococcus aureus NCTC 10652	19	18	10	15	13	7
Salmonella typhi 15SA	16	16	9	12	11	7
Salmonella enteritidis	18	17	11	14	11	7

respectively, 3750 and 5000 ppm. The mechanism of action of *P. nigrum* is similar to many other plants essential oils. Briefly, they disrupt the bacteria membrane destabilizing the cell activities related to energy conversion, structural macromolecule production, nutrients processing and hence induce growth reduction or microbial death (Oussalah et al., 2006; Swamy et al., 2016). The major activity of *S. aromaticum* as compared to *P. nigrum* is mainly due to the presence of eugenol that has largely been studied and in which literature has been recently reviewed by Marchese et al. (2017). This compound has demonstrated a large spectrum antibacterial and antifungal effect, as well as antioxidant and anti-inflammatory activity. Beta-caryophyllene also contributes to the antimicrobial properties of *S. aromaticum* with a broad effect on Gram positive bacteria, as reported by Xiong et al. (2013). Regarding the main components of *P. nigrum*, most terpenes do not possess high inherent antimicrobial activity against many Gram-negative pathogens (Bagamboula et al., 2004; Nazzaro et al., 2013). Dorman and Deans (2000) also reported that other terpenes, such as limonene, α-pinene, β-pinene, γ-terpinene δ-3-carene, (+)-sabinene and α-terpinene showed a very low or no antimicrobial activity against 25 genera of bacteria. On the contrary, limonene has been reported as having good antimicrobial properties on Gram negative bacteria with the cell envelope as target (Espina et al., 2013; Vuuren and Viljoen, 2007).

Type of association of *S. aromaticum* and *P. nigrum* with different antibiotics on the inhibition of selected strains

The possible synergistic effect of each essential oil when combined with selected antibiotics was assessed. The checkerboard method was used and results are reported in Tables 4 and 5 for *P. nigrum* and *S. aromaticum* essential oils, respectively. Regarding *P. nigrum* EO, all the association with Amoxicilline/clavulanic acid (AMX/AC), Ampicillin (AMP), Ciprofloxacin (Cipro) and Gentamicin (Genta) were synergistic towards the strain *S. aureus* SR196. On another strain of the same species, *S. aureus* NCTC 10652 *P. nigrum* was additive with Cipro, synergistic with Genta and indifferent with AMX/AC and AMP. On *Salmonella* typhi 155A, *P. nigrum* has a synergistic association with AMX/AC and Cipro while it was additive with AMP and Genta. On the other hand, *P. nigrum* EO was synergistic with Cipro and Genta, additive with AMP and indifferent with AMC/AC. In general, it can be said that Cipro and Genta were generally additive or synergistic with *P. nigrum* EO towards all the tested strains.

Table 4. Minimal inhibition (MIC) and bactericidal (MBC) concentrations of *S. aromaticum* and *P. nigrum* essential oil on *S. aureus*, *S. typhi* and *S. enteritidis* strains

Strain	Minimal inhibition concentration / Minimal bactericidal concentration (ppm)					
	Antibiotics				Essential oils	
	AMX/AC	AMP	Cipro	Genta	*S. aromaticum*	*P. nigrum*
Staphylococcus aureus SR196	0.78/1.56	1.95/3.91	0.78/1.56	6.25/6.25	625/5000	1250/>10000
Staphylococcus aureus NCTC 10652	0.78/0.78	1.95/3.91	0.39/0.78	6.25/6.25	625/5000	2500/10000
Salmonella typhi 15SA	0.78/0.78	1.95/3.91	0.39/0.39	3.12/3.12	625/2500	10000/>10000
Salmonella enteritidis	0.78/0.78	1.95/3.71	0.78/0.78	6.25/6.25	625/2500	5000/10000

AMX/AC = Amoxicilline/ clavulanique acid; AMP = Ampicillin; Cipro = Ciprofloxacin; Genta = Gentamicin.

Table 5. Minimum inhibition concentration (MIC) of *S. aromaticum* essential oil (EOSa) alone and in combination with different antibiotics and the type of interaction of the combinations on *S. aureus*, *S. typhi* and *S. enteritidis* strains

Strain	Combination of *S. aromaticum* and antibiotics					
	Antibacterial	MIC(ppm		FIC (ppm)		Outcome
		Alone	Combination	FIC	FICI	
Staphylococcus aureus SR196	EOSa	625	78.12	0.125	0.25	Synergistic
	AMX/AC	0.78	0.0975	0.125		
	EOSa	625	156	0.25	0.5	Synergistic
	AMP	1.95	0.48	0.25		
	EOSa	625	78.12	0.125	0.25	Synergistic
	Cipro	0.78	0.097	0.125		
	EOSa	625	78.12	0.125	0.25	Synergistic
	Genta	6.25	0.78	0.125		
Staphylococcus aureus NCTC 10652	EOSa	625	312.5	0.5	1	Additive
	AMX/AC	0.78	0.39	0.5		
	EOSa	625	625	1	2	Indifferent
	AMP	1.95	1.95	1		
	EOSa	625	312.5	0.5	1	Additive
	Cipro	0.39	0.195	0.5		
	EOSa	625	78.12	0.125	0.25	Synergistic
	Genta	6.25	0.78	0.125		
Salmonella typhi 15SA	EOSa	625	39.6	0.063	0.12	Synergistic
	AMX/AC	0.78	0.048	0.063		
	EOSa	625	19.53	0.031	0.62	Partially Synergistic
	AMP	1.95	0.061	0.031		
	EOSa	625	156.25	0.25	0.5	Synergistic
	Cipro	0.39	0.097	0.25		
	EOSa	625	312.5	0.5	1	Additive
	Genta	3.12	1.56	0.5		
Salmonella enteritidis	EOSa	625	312.5	0.5	1	Additive
	AMX/AC	0.78	0.39	0.5		
	EOSa	625	312.5	0.5	1	Additive
	AMP	1.95	0.975	0.5		
	EOSa	625	312.5	0.5	1	Additive
	Cipro	0.78	0.39	0.5		
	EOSa	625	78.12	0.125	0.25	Synergistic
	Genta	6.25	0.78	0.125		

AMX/AC = Amoxicilline/ clavulanic acid; AMP = Ampicillin; Cipro = Ciprofloxacin; Genta = Gentamicin.

Table 6. Minimum inhibition concentration (MIC) of *P. nigrum* essential oil (EOPn) alone and in combination with different antibiotics and the type of interaction of the combinations on *S. aureus*, *S. typhi* and *S. enteritidis* strains.

Strain	Combination *P. nigrum* and antibiotics					
	Antibacterial	MIC (ppm)		FIC (ppm)		Outcome
		Alone	Combination	FIC	FICI	
Staphylococcus aureus SR196	*EOPn*	*1250*	*39.6*	*0.031*	0.062	Synergistic
	AMX/AC	0.78	0.024	0.031		
	EOPn	1250	156.25	0.125	0.25	Synergistic
	AMP	1.25	0.24	0.125		
	EOPn	1250	312.5	0.25	0.5	Synergistic
	Cipro	0.78	0.195	0.25		
	EOPn	1250	156.25	0.125	0.25	Synergistic
	Genta	6.25	0.78	0.125		
Staphylococcus aureus NCTC 10652	EOPn	2500	2500	1	2	Indifferent
	AMX/AC	0.78	2500	1		
	EOPn	2500	2500	1	2	Indifferent
	AMP	1.95	1.95	1		
	EOPn	2500	1250	0.5	1	Additive
	Cipro	0.39	0.195	0.5		
	EOPn	2500	312.5	0.125	0.25	Synergistic
	Genta	6.25	0.78	0.125		
Salmonella typhi 15SA	EOPn	10000	625	0.0625	0.125	Synergistic
	AMX/AC	0.78	0.048	0.0625		
	EOPn	10000	5000	0.5	1	Additive
	AMP	1.95	0.975	0.5		
	EOPn	10000	2500	0.25	0.5	Synergistic
	Cipro	0.39	0.0975	0.25		
	EOPn	10000	5000	0.5	1	Additive
	Genta	3.12	1.56	0.5		
Salmonella enteritidis	EOPn	5000	5000	1	2	Indifferent
	AMX/AC	0.78	0.78	1		
	EOPn	5000	2500	0.5	1	Additive
	AMP	1.95	0.975	0.5		
	EOPn	5000	156.25	0.031	0.061	Synergistic
	Cipro	0.78	0.0243	0.031		
	EOPn	5000	1250	0.25	0.5	Synergistic
	Genta	6.25	1.56	0.25		

AMX/AC = Amoxicilline/clavulanic acid; AMP = ampicillin; Cipro = ciprofloxacin; Genta = gentamicin.

Concerning *S. aromaticum* EO (Table 5), the association with antibiotics gave in general, more positive results than that with *P. nigrum* EO (Table 6). In particular, the only association resulting in indifference was observed with *S. aromaticum* EO and AMX/AC on *S. aureus* NCTC10652. All the associations of tested antibiotics and *S. aromaticum* EO were synergistic on *S. aureus* SR196, while on *S. enteritidis*, they were mainly additive except for the association of *S. aromaticus* EO and Genta. From a general point of view, the association of *S. aromaticum* at 312.5 ppm and Gentamicin at 1.56 ppm can be considered as dose active on all the strain tested. Regarding the combination of *P. nigrum* with antibiotics, the association with ciprofloxacin and gentamicin proved to be the best among those tested. *P. nigrum* at 2500 ppm combined with ciprofloxacin at 0.0975 ppm and *P. nigrum* at 5000 ppm combined with gentamicin at 1.56 ppm were concentrations that could be suitable for all strains. According to Brown (1986), Clavulanic acid, a beta lactamase inhibitor from a streptomycete in combination with amoxicillin was the outcome of the approach that deals with association of

antibiotics with new antimicrobials to overcome strain resistance. Ampicillin and Amoxicillin are beta-lactamides that act by inhibiting the synthesis of cell wall while ciprofloxacin and gentamicine inhibit protein synthesis. Even though EOs sometimes are not much effective on bacterial when used alone, the new approach to use them in combination with antibiotics has been shown to enhance the antibiotic activity (Gibbons et al., 2003; Yap et al., 2014). In the case of synergistic activity between the essential oil and the antibiotics, this may result in the reduction of the antimicrobial concentration and its adverse effects (Yap et al., 2014). According to Yap et al. (2014), most antibiotics need to be transported across the cell membrane and the overproduction of protein pumps at the bacterial membrane will facilitate the resistance of strain by pumping out the antibiotics. The inhibition of bacterial efflux pump by EO is hence a probable mechanism underlying the synergic and additive effects with the antibiotics (Lorenzi et al., 2009). Cell wall and membrane disturbance is also a means of increasing the ability of antibiotics to assess the cell wall enzymes and organs within the cytoplasm (Hemaiswarya and Doble, 2009; Veras et al., 2012).

Toxicity test

For a better comparison, the same antibiotic was used in combination with the two essential oils, at the MIC obtained in combination with the essential oil. In this regard, the association of *S. aromaticum* at 312.5 ppm, Gentamicin at 1.56 ppm and *P. nigrum* at 5000 ppm combined with gentamicin at 1.56 ppm increased by ten-fold and converted into mg/kg of animal weight. Figure 1A and B reported the mean body weight percentage variation of female and male rats during the experimental period. It was observed that irrespective of the sex and the treatment, an increase in weight of the same amplitude was observed with no statistical differences ($p<0.05$) between the test groups. Weight increase is an indication of growth. The reasonable homogeneous increasing trend of weight in all the rats groups can be taken as an indication of low impact of different treatments on animal feeding and health. Njayou et al. (2010) observed that body weight may increase or decrease with relation to sex, absence of toxicity or induced anorexia. In this work, no toxicity and induced anorexia was observed. The effect of 10 times the MIC of essential oils alone and in combination with gentamicine as well as the control, on the serum transaminases (ALAT and ASAT) is presented in Figure 2A and B. It was observed that irrespective of the high dose treatments, the ASAT and ALAT values were in general not significantly different among animals that received different test treatments and the control after 14 days.

The only significant difference observed was the reduction of the serum ALAT in female rats after administration of the high dose of *P. nigrum* combined with gentamicin as compared to the control group. Hepatic necrosis and oxidation have always been assessed through the measurement of markers like ASAT and ALAT (Tala et al., 2015; Olaleye et al., 2014; Bidie et al., 2010). In case of hepatotoxicity, the ALAT and ASAT increased simultaneously in the serum (Tala et al., 2015). In this work, no statistically significant increase of ASAT and ALAT in rats exposed to 10 times the active dose of *P. nigrum* or *S. aromaticum* EOs in combination with gentamicine was observed with respect to the control. This absence of transaminases activities increase is hence an indication that the excess do not damage the liver. The significant reduction of ALAT in females after administration of the combination *P. nigrum* and gentamicine with respect to the control experiment is an indication of an antioxidant protective effect of thecombination toward natural occurring oxidations. Regarding serum creatinine and urea, no significant differences ($p<0.05$) were observed between the test groups and between each test group, and the control not treated (Figure 3A and B). The increase of creatinine and urea, in the serum is an indication of renal function disorder. Among these two indicators, creatinine acts when renal dysfunction is significant (Odutola, 1992; Timothy et al., 2015). No significant differences were observed between serum creatinine and urea levels in the control and in rats groups exposed to the maximized active compounds, implying no renal alteration.

Conclusion

The results obtained in this work suggested that *P. nigrum* EO has good synergistic activity in combination with gentamicin and ciprofloxacin, while *S. aromaticum* EO promotes well, the activity of gentamicin. No toxic effects were observed in renal and hepatic functions after feeding rats with 10 times the MICs observed during the association of the essential oils and gentamicin. These EOs can hence be used to reduce the active dose of antibiotics during the fight against *S. aureus*, and *Salmonella* species without any induced toxicity effect. Moreover, it appears important to assess the synergistic effect of the combination tested as it may depend on the ratio of the two drugs. These results are a contribution towards the designing of future phyto-antibiotic hybrid drugs.

CONFICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge the contributions of Prof

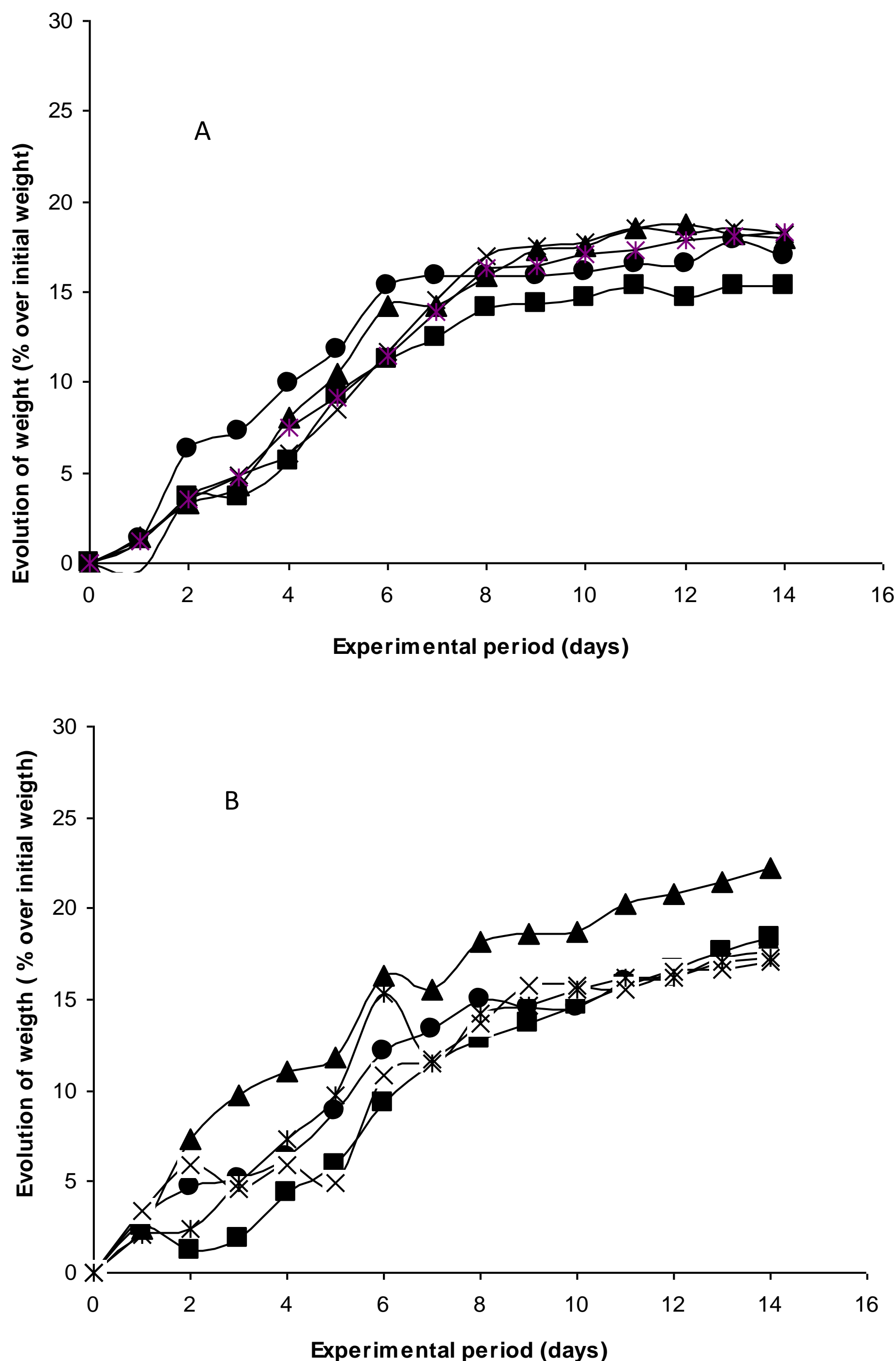

Figure 1. Percentage increase of body weight of different groups during the experimental period after administration of different drugs in female (A) and male (B) rats. Stars (Control), black squares (group that received 50000 mg/kg of body weight of *P. nigrum* EO), triangle (combination *S. aromaticum*/Gentamicine at 3125 and 15.6 mg, respectively, per kg of body weight), cross (combination *P .nigrum*/Gentamicine at 50000 and 15.6 mg, respectively per kg of body weight), Black circles (EO of *S. aromaticum* at 3125 mg/kg of body weight).

Chantale Menut of the l'IBMM, Université de Montpellier, équipe Glyco et nanovecteur pour le ciblage thérapeutique (France) for the essential oil analyses, and Pr Rosalba Lanciotti and Dr Chiara Montanari of the space of the

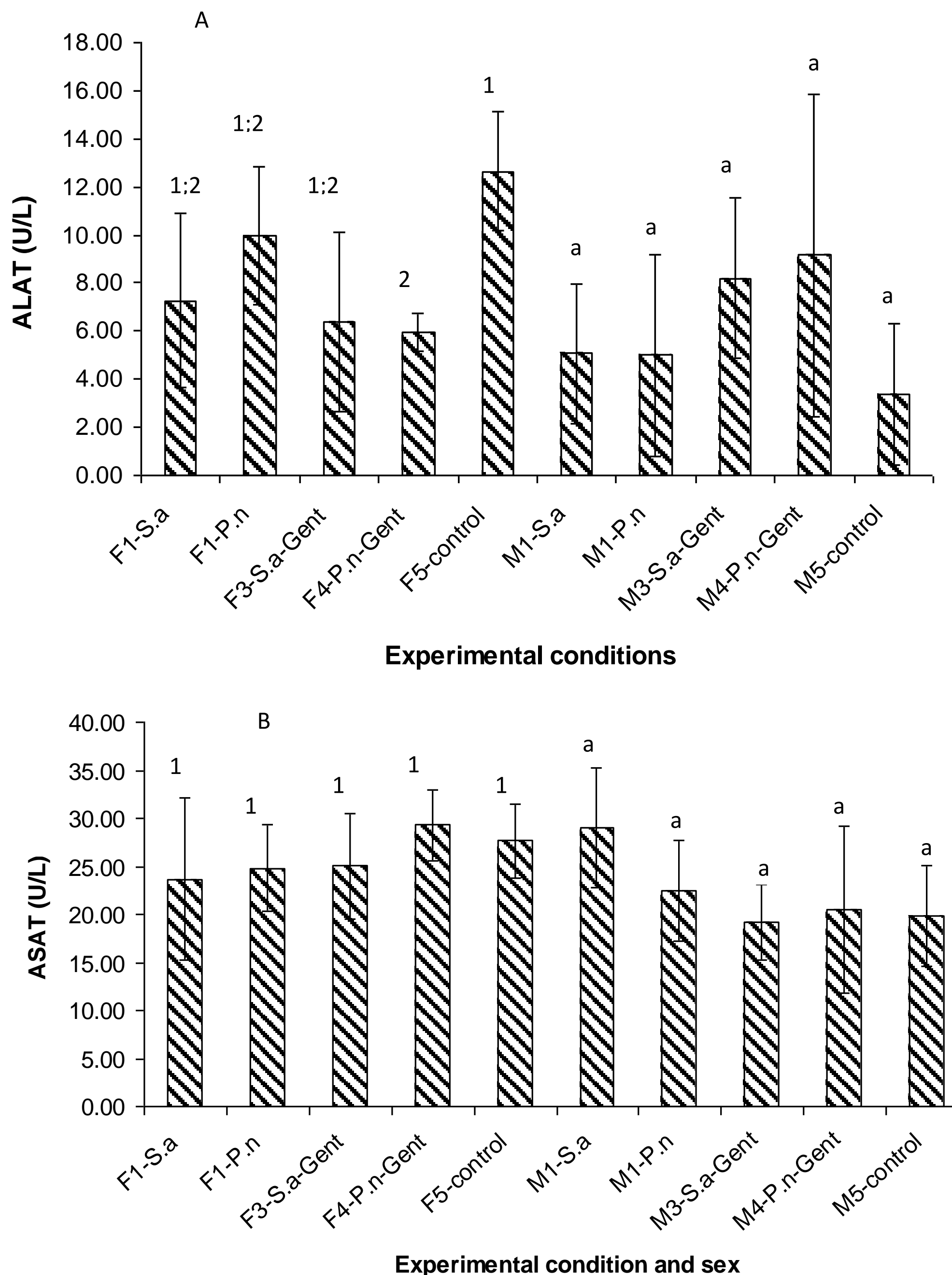

Figure 2. Variation of ALAT (A) and ASAT (B) in different groups during 14 days experimental period after administration of 10 times the MIC obtained by associating the EO with gentamicin for each essential oil (Sa; Pn) or during the combination of each essential oil with gentamicin (S.a-Gent; P.n-Gent) in female (F) and male (M) rats. S.a, group that received 3125 mg/kg of body weight of *S. aromaticum* EO; P.n, group that received 50000 mg/kg of body weight of *P. nigrum* EO; (S.a-Gent) group that received an association of EO of *Syzygium aromaticum* and gentamicin of 3125 and 15.6 mg respectively per Kg of body weight; P.n-Gent, group that received an association of EO of *Piper nigrum* and gentamicin of 50000 and 15.6 mg respectively per Kg of body weight; control, group that received only corn oil. Female groups with the same numbers are not statistically different, while male groups with the same letters are not statistically different.

S. aromaticum crushed fruits. Laboratory of Food Microbiology, University of Bologna-Italy for facilities offered for the SPME analyses of head space of the *S. aromaticum* crushed fruits.

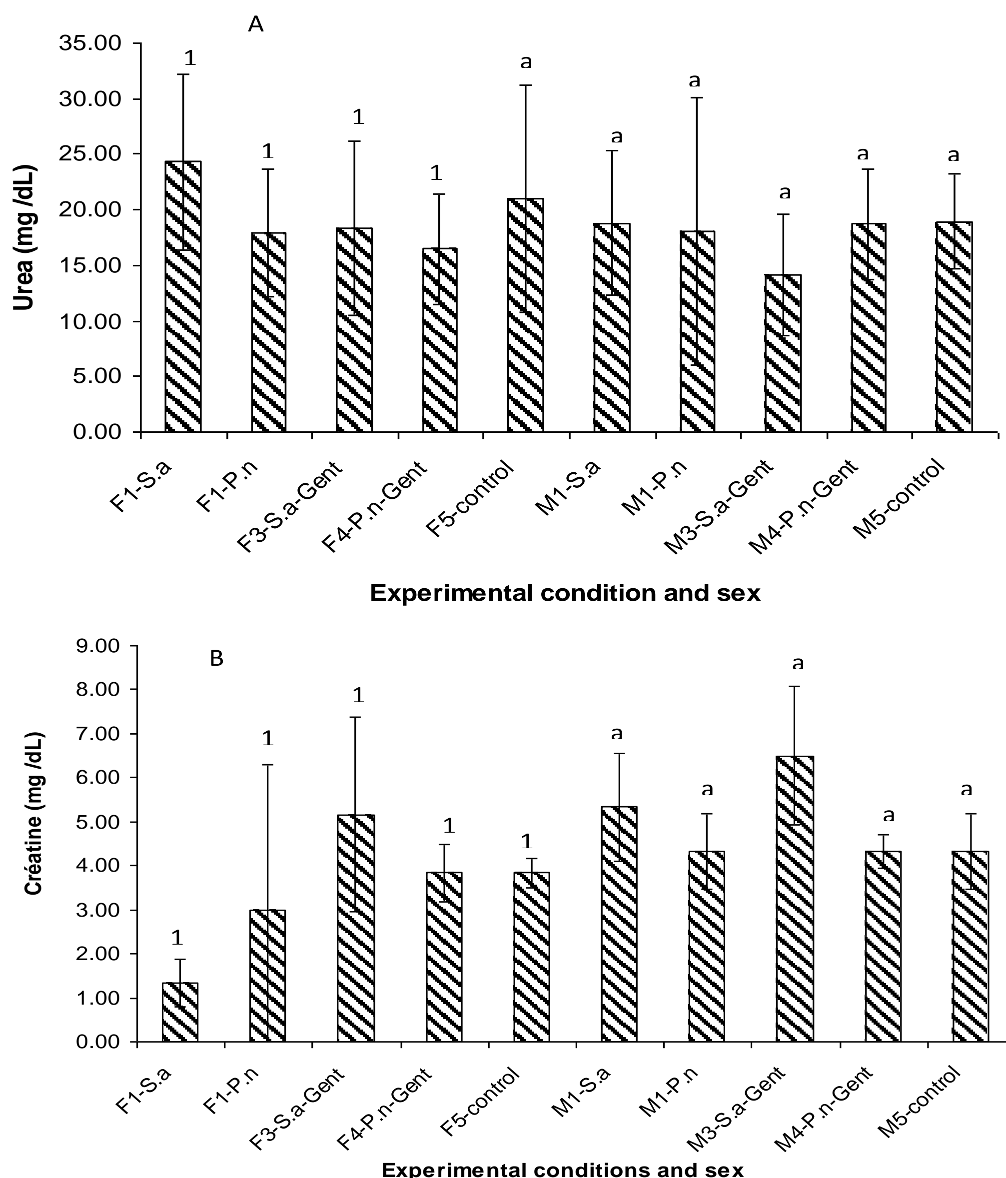

Figure 3. Variation of serum urea (A) and creatinine (B) in different groups during 14 days experimental period after administration of 10 times the MIC obtained by associating the EO with gentamicin for each essential oil (Sa; Pn) or during the combination of each essential oil with Gentamicin (S.a-Gent; P.n-Gent) in female (F) and male (M) rats. (S.a) group that received 3125 mg/kg of body weight of *Syzygium aromaticum* EO; (P.n) group that received 50000 mg/kg of body weight of *Piper nigrum* EO; (S.a-Gent) group that received an association of EO of *S. aromaticum* and gentamicin of 3125 mg and 15.6 mg respectively per Kg of body weight; (P.n-Gent) group that received an association of EO of *P. nigrum* and gentamicin of 50000 and 15.6 mg respectively per kilogram of body weight; (control)group that received only corn oil. Female groups with the same numbers are not statistically different, while male groups with the same letters are not statistically different.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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