

IN VITRO EVALUATION OF BACTERICIDAL AND ANTIBIOFILM ACTIVITY OF *LONICERA TATARICA* AND *VIBURNUM OPULUS* PLANT EXTRACTS ON *STAPHYLOCOCCUS* STRAINS

MARIANA VIORICA BUBULICA¹, ION ANGHEL^{4*}, ALEXANDRU MIHAI GRUMEZESCU², CRINA SAVIUC³, GEORGIANA ALINA ANGHEL^{5*}, MARIANA CARMEN CHIFIRIUC³, IRINA GHEORGHE³, VERONICA LAZAR³, ALEXANDRU POPESCU¹

¹ Faculty of Chemistry, University of Craiova, 107 Calea Bucuresti, 200515 Craiova, Romania

² Faculty of Applied Chemistry and Materials Science, "Politehnica" University of Bucharest, 1-7 Polizu Street, 011061 Bucharest, Romania

³ Faculty of Biology, University of Bucharest, Ale. Portocalelor 1-3, Bucharest, Romania

⁴ University of Medicine and Pharmacy "Carol Davila", Bucharest

⁵ ENT Clinic, "Coltea" Hospital, Bucharest, Romania

*corresponding authors: ionangheldoc@yahoo.com, dr_alina.angel@yahoo.com

Abstract

The purpose of the present work was to evaluate the bactericidal/antibiofilm efficiency of *Lonicera tatarica* (LT) and *Viburnum opulus* (VO) extracts. The LT and VO extracts were screened for their *in vitro* antibacterial activity against *Staphylococcus aureus* and *S. epidermidis* reference and clinical, multidrug resistant and biofilm producing strains, isolated from ear and wound infections. The quantitative assay of the antimicrobial activity was performed by broth microdilution method in order to establish the minimal inhibitory concentration (MIC). Subsequently, the biofilm forming ability was quantified by the microtiter assay. Our results showed that the LT and VO extracts interact differently with the growth and adherence ability of *S. aureus* and *S. epidermidis* strains. LT predominantly interfered with the bacterial growth exhibiting an inhibitory effect of similar intensity against all tested strains, while VO attenuated the ability of all tested strains to colonize the inert substratum and to form biofilms. These results are leading us to the hypothesis that *Lonicera tatarica* and *Viburnum opulus* species could be used for the development of novel antimicrobial products or strategies for fighting medical biofilms.

Rezumat

Scopul prezentului studiu a fost de a evalua eficiența bactericidă/antibiofilm a extractelor din *Lonicera tatarica* (LT) și *Viburnum opulus* (VO), față de tulpini de referință și clinice de *S. aureus* și *S. epidermidis* izolate din infecții otice și de plagă, rezistente la antibiotice și producătoare de biofilme. Analiza cantitativă a acitivității antimicrobiene a fost efectuată prin metoda microdiluțiilor pentru a stabili concentrația minimă inhibitoare

(CMI). Ulterior, a fost cuantificată capacitatea de aderență la substrat inert cu ajutorul metodei microtitrării biofilmului format pe pereții godeurilor de plastic. Rezultatele obținute au arătat că extractele din LT și VO interacționează diferit cu creșterea și capacitatea de aderență a tulpinilor *S. aureus* și *S. epidermidis*. LT interferează cu creșterea bacteriană și prezintă un efect inhibitor de intensitate similară față de toate tulpinile testate, în timp ce VO atenuează capacitatea de colonizare a substratului inert și de formare a biofilmului pentru toate tulpinile testate. Aceste rezultate demonstrează că speciile de LT și VO pot fi utilizate pentru dezvoltarea de noi produse antimicrobiene sau a unor strategii de combatere a biofilmelor medicale.

Keywords: *antibiofilm activity, Lonicera tatarica, Viburnum opulus, plant extract*

Introduction

The increasing occurrence of multidrug resistant, extensive drug resistant and pandrug resistant microbial strains has gradually rendered traditional antimicrobial treatment ineffective [1]. The prognosis is worsened by the formation of microbial biofilms on the biomaterials used in medicine due to their phenotypic resistance, even if the component cells tested in suspension (by the standard method) are susceptible to some antibiotics [2]. Although the potential of natural compounds of vegetal origin to be used as therapeutical remedies is known for a longtime, their use is still empirical. The problem of microbial resistance, as well as the negative impact of the chemical substances discharged in the external environment on the ecological balance has reinforced the studies concerning the characterization of the chemical structures of vegetal products and the active doses, aiming to understand their specific mechanisms of action. *Viburnum opulus* (VO) L. belongs to *Caprifoliaceae* family, *Dipsacales* order, *Magnoliopsida* class, *Asteridae* subclass. Two varieties occur naturally: var. *edule* (European Cranberry bush, spread in Europe, northern Asia and North Africa) and var. *trilobum* (American cranberry bush, distributed in western Canada and northwestern Mexico) [3,4]. It is a deciduous shrub up to 4-5m tall with oval, three-lobed leaves, serrated on the edges, 5 to 10 cm long. The white flowers bloom in late spring, on the edge of the floral cup, in a single row. The larger sterile flowers are embedding in the center the smaller and fertile ones (Figure 1 a). Fruits are red drupes with a single seed. The decorative variety (snowball) has grouped, sterile flowers (Figure 1 b). Both seeds and fruits of these species contain: fatty acid methyl esters, sterols (β -sitosterol, stigmasterol), triterpenic alcohols (lupeol) [5], oxalic acid, ascorbic acid (the fruit), citric acid (the fruit) [6], calcium oxalate crystals and valeric acid. The

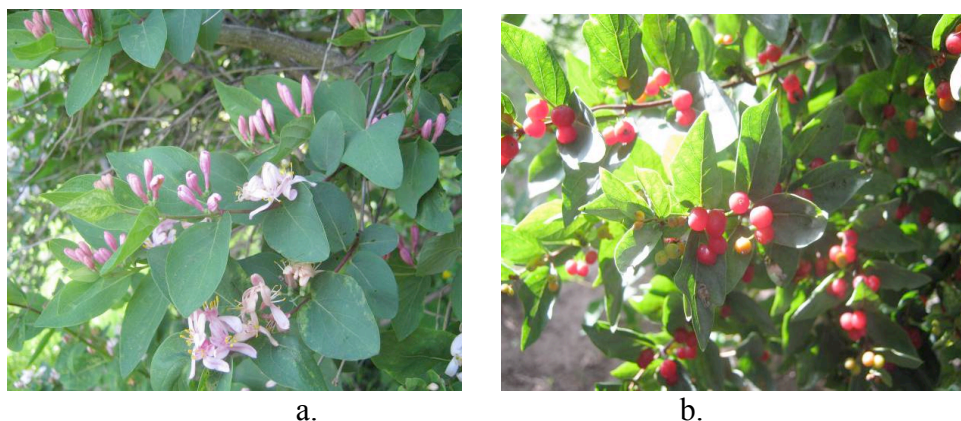
bark contains viburnine, a compound responsible for its bitter-astringent taste, tannins, valerian acid esters, flavonoids. The fruit pulp there were isolated several types of soluble polysaccharides with physiological activity [7]. The flowers contain phenolic derivatives, especially astragalin (kaempferol-3-glucoside) and peonoside (kaempferol-3,7-glucoside). The bark (*Cortex Viburni*) of *Viburnum opulus* species is used for medicinal purposes (2% infusion, decoction or hydroalcoholic extracts) in the treatment of stomach or uterine bleeding and hemorrhoids. Fruits were first used as food by Schopmeyer [8] who studied rather the properties of the seeds and by association deduced their beneficial characteristics for the human health. The fruit juice is a traditional drink in Turkey and Russia, being used in folk medicine [9].



Figure 1

Viburnum opulus flowers: a – *edule* variety b – decorative variety

Lonicera tatarica (LT) is a species of Asian origin from the North of Xinjiang province whose propagation includes large areas of central Europe, Asia, North America and Africa. It is a deciduous shrub, up to 3 m high, composed of erect shrubs. The leaves located in pairs along the shoots are 2-5 cm long, thin, ovate-elongated with 1 cm long peduncles. Pairs of white or pink flowers grow at the base of the leaves (Figure 2 a). Fruits are red, spherical in shape, of 5-6 mm diameter (Figure 2 b). There are very few literature data concerning the chemical composition of these species. The plant is currently under study for its introduction into the large group of phyto-pharmaceutical products. It is known that the stems contain nepelactone, a compound isolated from *Nepeta cataria*. LT properties are little known in Europe. In China, however, leaves and flowers are used to treat headaches, colds, flu and fever. Flower buds are used as an infusion to treat coughs, asthma and to detoxify the body.



a.

b.

Figure 2*Lonicera tatarica*: a – flowers ; b – fruits

The purpose of the present work was to evaluate the bactericidal and antibiofilm efficiency of LT and VO extracts against *Staphylococcus* sp. strains known for their emerging resistance to many classes of antibiotics and their ability to form biofilms.

Materials and Methods

Plant material and aromatic water preparation

Samples were harvested from the Botanical Garden of Craiova City, Dolj County, Romania between May and June 2009. The plants were identified and voucher specimens were deposited in a herbarium. Plant material was washed with bi-distilled water to remove dust, soil particles and damaged portions and air-dried in a dark room at constant humidity. Dried samples were ground into a stainless grinder and passed through a sieve (24 meshes) to ensure homogeneity. The obtained powder was dried again in a convection oven at 45°C until no change in weight was observed. Three extractions with water for each vegetal sample were performed in microwave conditions using a reflux installation.

In vitro testing of the biological activity of LT and VO

Microbial strains

The antimicrobial activity of the investigated extracts was tested against bacterial strains recently isolated from clinical specimens as well as reference strains belonging to *S. aureus* and *S. epidermidis* species. 18 *S. aureus* and 17 *S. epidermidis* have been used, being isolated from ear and wound infections. The microbial strains were identified using the VITEK I

automatic system [10]. VITEK cards for identification and antibiotic susceptibility testing (GNS-522) were inoculated and incubated according to the manufacturer's recommendations. The results were interpreted by using software version AMS R09.1. 1.5×10^8 CFU/mL (CFU – colony forming units) or 0.5 McFarland standard adjusted suspensions, obtained from 15 – 18 h bacterial cultures developed on solid media, have been used for the *in vitro* assays.

Qualitative screening for the antimicrobial activity

The antimicrobial activity was tested on Mueller-Hinton medium (MH) recommended by the Clinical and Laboratory Standards Institute (CLSI) [11]. As for the qualitative screening, a temporal dynamic method have been chosen. Briefly, the bacterial suspensions adjusted to a density corresponding to the 0.5 McFarland nephelometric standard, were put in contact with the two extracts as well as with a antibiotic control represented by clindamycin solution, 10 µg/mL. An amount of 1 µL has been taken after 10, 15 and 30 min of contact and seeded on solid media (MH) in order to appreciate the intensity of bacterial growth.

Quantitative assay of minimal inhibitory concentration (MIC)

The MIC value was determined by twofold microdilution technique, in 96 multi-well plates. For this purpose, the aqueous plant extracts were diluted in liquid MH, the obtained concentrations ranging from 500 to 125 µL/mL. The wells have been thereafter seeded with the bacterial suspensions adjusted to a density corresponding to the 0.5 McFarland nephelometric standard, in a 1:5 vol:vol ratio. Positive (culture medium and bacterial suspensions) and negative (sterile culture medium) controls have been used. The plates were incubated for 24 h at 37°C, and MIC values were determined by the macroscopic examination of the wells [12] (as the lowest concentration of the tested extract which inhibited the visible microbial growth) and spectrophotometrically (by measuring the $A_{620 \text{ nm}}$ for the obtained microbial cultures) [13].

Microbial adherence to the plastic substratum represented by 96 multi-well plates

After the performance of the MIC protocol, the plates were emptied, washed three times with sterile saline and fixed with cold methanol for 5 minutes. The bacterial cells that adhered to the plastic wells were stained with 1% violet crystal solution for 15 min. Thereafter, the coloured biofilm was resuspended in 33% acetic acid solution. The biomass was assessed spectrophotometrically, by measuring the A_{490} for the obtained, colored suspension [14,15].

Results and Discussion

The qualitative screening demonstrated the early microbiostatic activity of both extracts against all tested strains (fig. 3), revealed by the reduced microbial growth in the presence of the aqueous extracts as compared to the untreated cultures. The antimicrobial effect was observed after 10 min of contact, but however, was less evident than the microbicidal one exhibited by the control antibiotic, clindamycin.

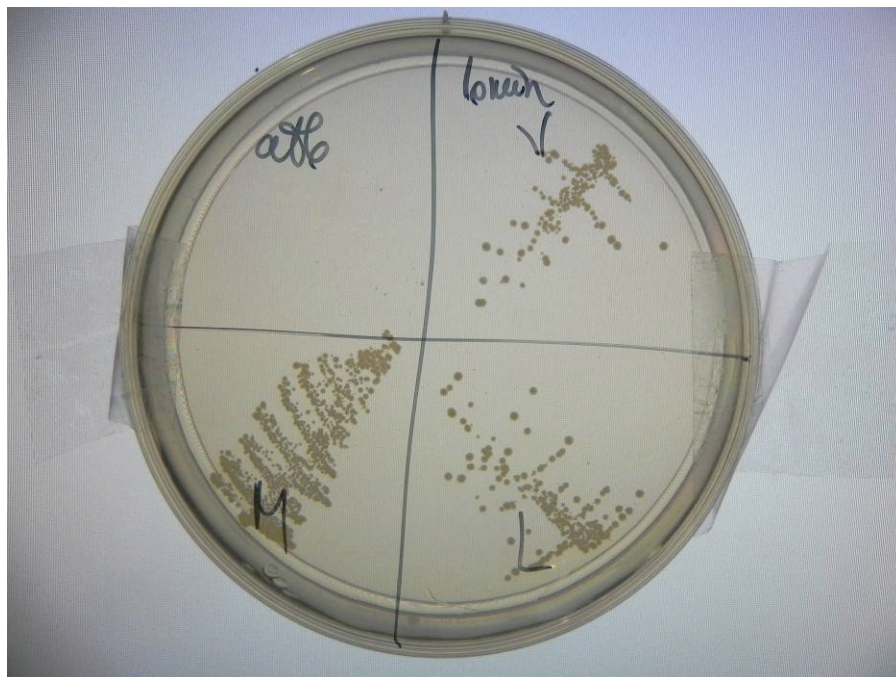
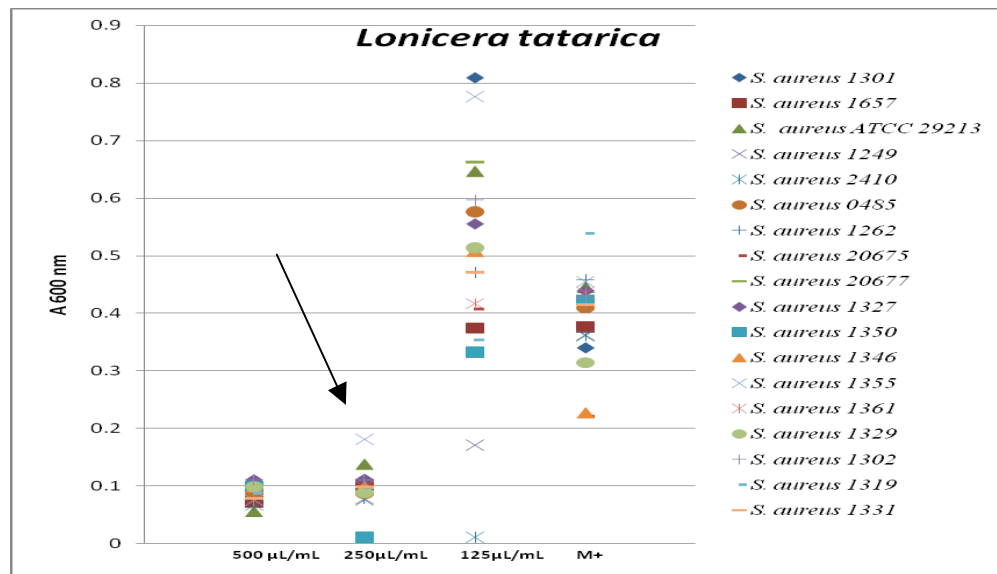


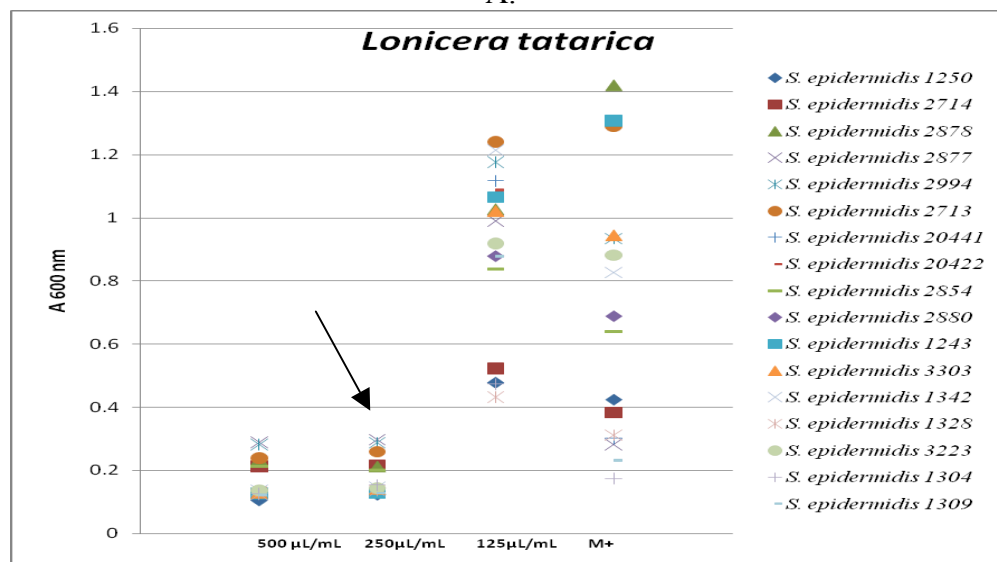
Figure 3

Qualitative screening of the antimicrobial activity, showing the absence of the microbial growth in the presence of clindamycin (atb) and the reduced microbial growth in the presence of LT (L) and VO (V) plant extracts, as compared to the untreated culture (M)

The LO extract exhibited the same antimicrobial activity against all tested strains, with a MIC value of 250 $\mu\text{L/mL}$. The VO extract antimicrobial activity was clearly lower than that of LTV (Fig. 4 A, D), the MIC value being situated around the value of 125 $\mu\text{L/mL}$ for *S. epidermidis* strains (Fig. 4 D). In exchange, the tested concentrations of VO aqueous extract seemed to stimulate the growth of *S. aureus* strains, probably due to the ability of this species to metabolize some compounds present in the respective extract, using them as additional and easily accessible nutrients (Fig. 4 C).



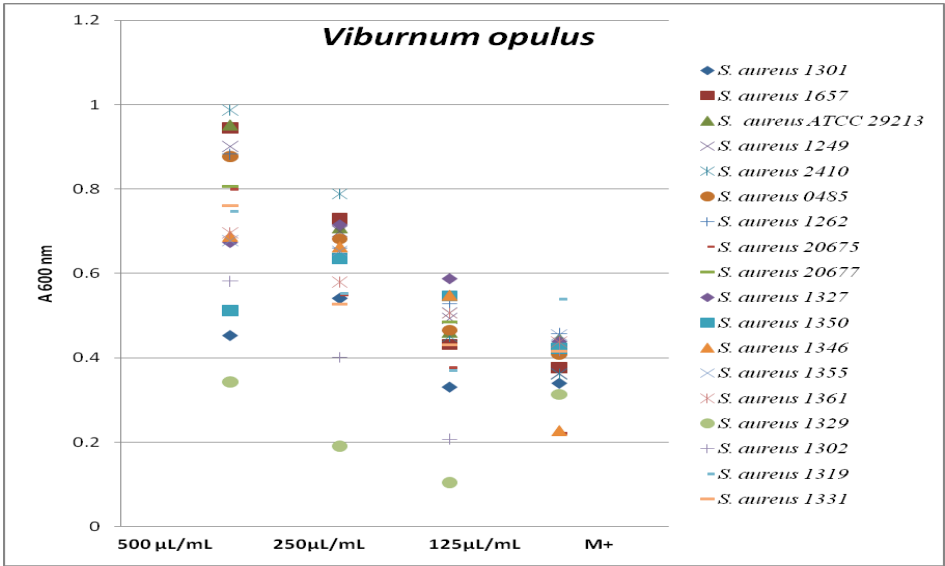
A.



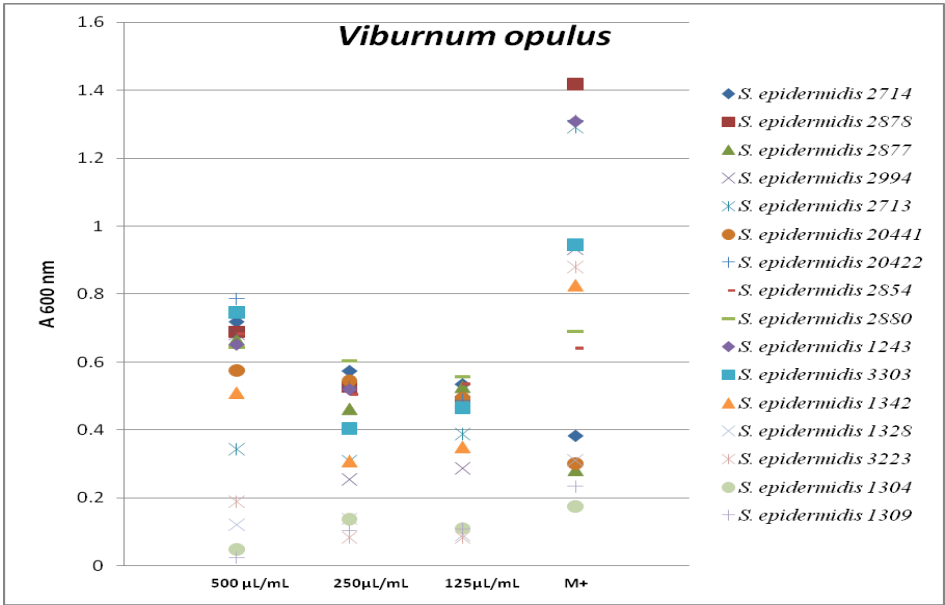
B.

Figure 4 A-B

A-B. The results of quantitative determination of minimal inhibitory concentration (MIC) of VO and LT aqueous extracts against the *S. aureus* and *S. epidermidis* tested strains by serial microdilution technique



C.



D.

Figure 4 C-D
C-D. The results of quantitative determination of minimal inhibitory concentration (MIC) of VO and LT aqueous extracts against the *S. aureus* and *S. epidermidis* tested strains by serial microdilution technique

The LT plant extract slightly inhibited the adherence of *S. aureus* strains at the highest tested concentration of 500 $\mu\text{L/mL}$, and that of *S. epidermidis* strains at 500 and 250 $\mu\text{L/mL}$ concentrations (Fig. 5A,B). Surprisingly, despite its low antibacterial activity, the VO extract exhibited a stronger antibiofilm effect, manifested on the entire range of tested concentrations against all bacterial strains, irrespective to their species (Fig. 5 C, D).

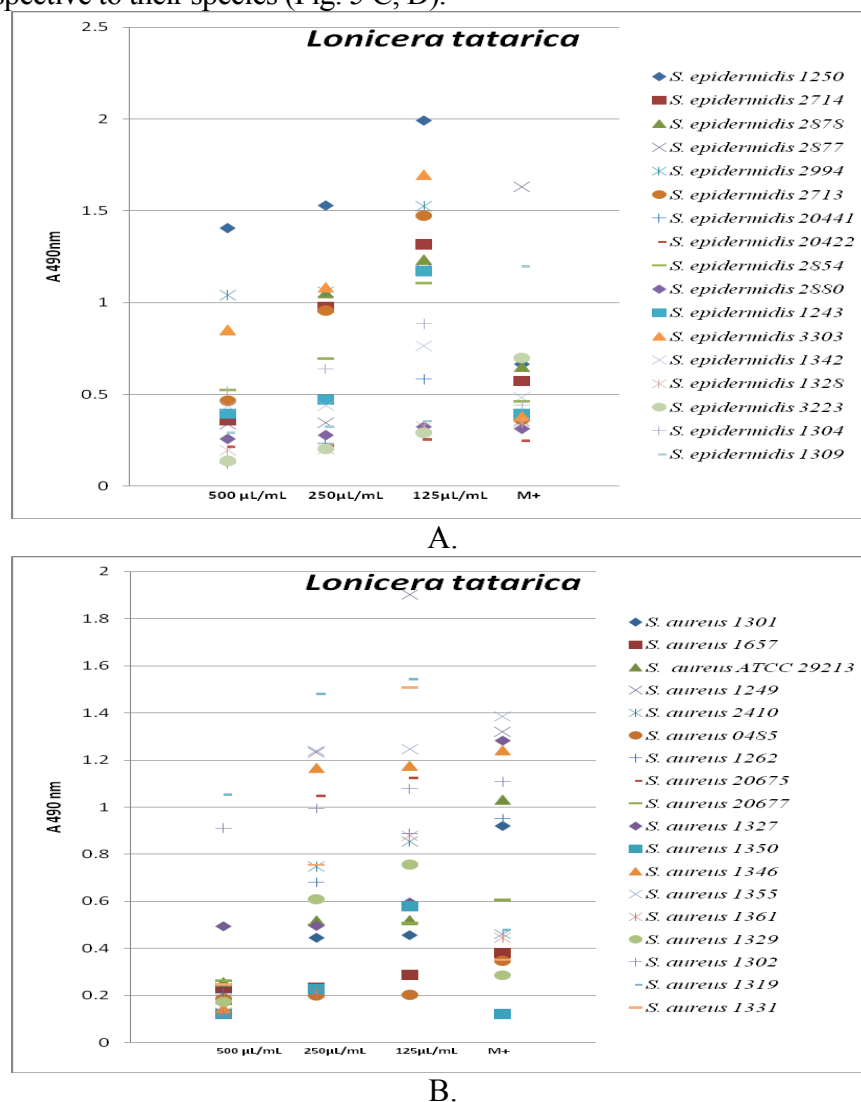
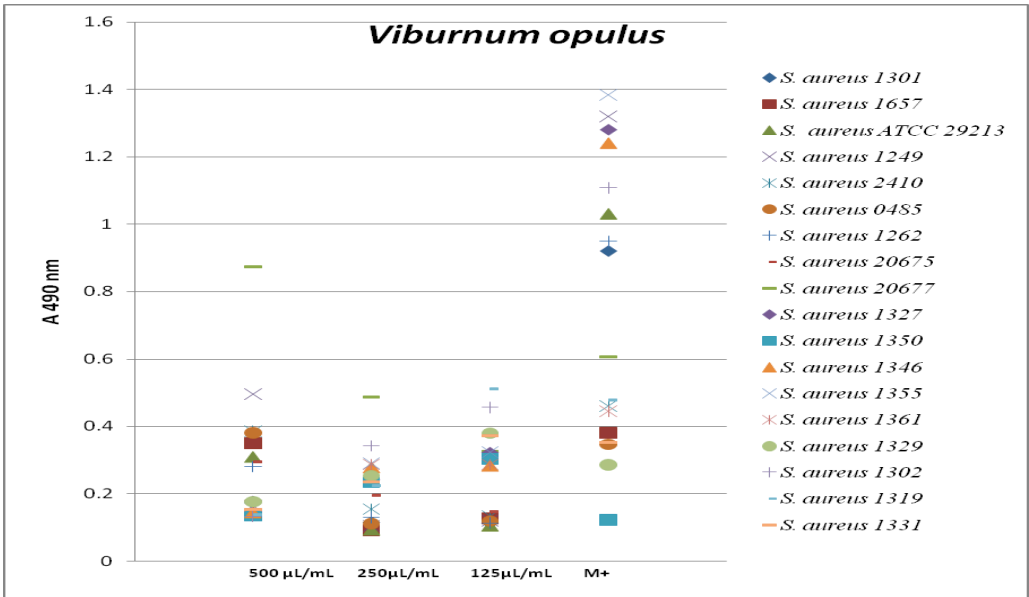
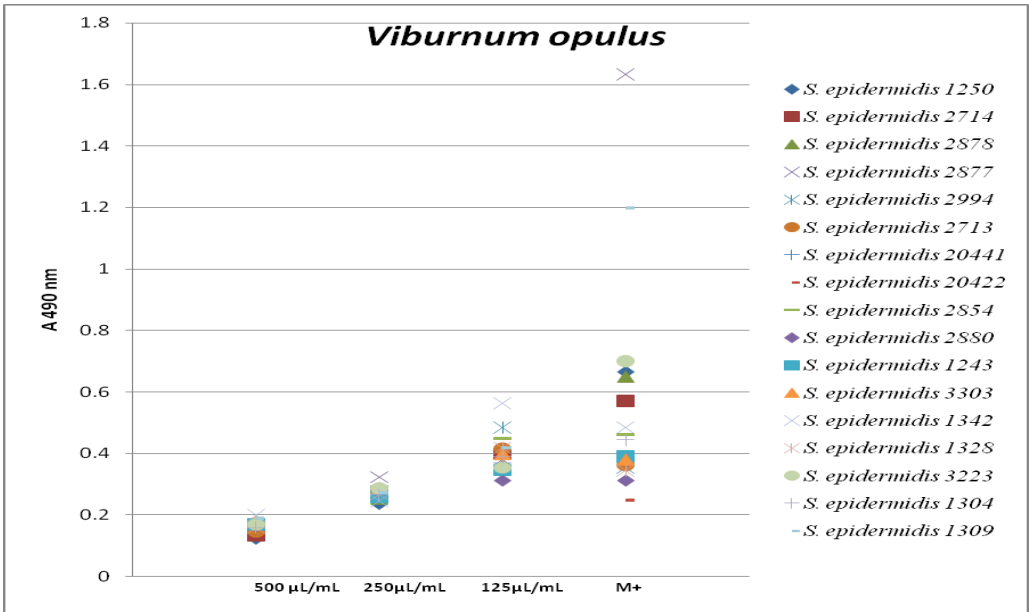


Figure 5 A-B

A-B. The influence of the LT and VO extracts on the *S. aureus* and *S. epidermidis* bacterial strains adherence to the plastic substratum represented by 96 multiwell plates



C.



D.

Figure 5 C-D

C-D. The influence of the LT and VO extracts on the *S. aureus* and *S. epidermidis* bacterial strains adherence to the plastic substratum represented by 96 multiwell plates

Conclusions

Our results showed that the *Lonicera tatarica* (LT) and *Viburnum opulus* (VO) extracts influence differently the growth and adherence ability of *S. aureus* and *S. epidermidis* strains. LT predominantly interfered with the bacterial growth exhibiting an inhibitory effect of similar intensity against all tested strains, while VO attenuated the ability of all tested strains to colonize the inert substratum and to form biofilms. These results are leading us to the hypothesis that *Lonicera tatarica* and *Viburnum opulus* species could be used for the development of novel antimicrobial products or strategies for fighting medical biofilms.

References

1. Flagas M.E., Karageorgopoulos D.E., Pandrug resistance (PDR) extensive drug resistance (XDR), and multidrug resistance (MDR) among gram-negative bacilli: need for international harmonization in terminology, *Clin. Infect. Dis.*, 2008, 46:1121-1122.
2. Donald R.M., Costerton J.W., Biofilms, Survival mechanisms of clinically relevant microorganisms, *Clinical Microbiol. Rev.*, 2002, 15:164-193.
3. Bown D., Encyclopaedia of Herbs and their Uses. Dorling Kindersley, Londra, 1995.
4. Moerman D., Native American Ethnobotany. Timber Press, Oregon, 1998.
5. Karimova A. R., Yunusova S. G., Maslennikov S. I., Galkin E. G., Yunusov T. S., Shereshovets V. V., Yunusov M. S., Lipids, lipophilic components, and biologically active fractions of *Viburnum opulus* l. seeds, *Chemistry of Natural Compounds*, 2000, 35:560-564.
6. Cam M., Hisil Y., Kusu A., Organic acid, phenolic content, and antioxidant capacity of fruit flesh and seed of *Viburnum opulus*, *Chemistry of Natural Compounds*, 2007, 43:460-461.
7. Ovodova, R.G., Golovchenko, V.V., Popov, S.V., Shashkov, A.S. & Ovodov, Y.S., The isolation, preliminary structural studies, and physiological activity of water-soluble polysaccharides from the squeezed berries of snowball tree *Viburnum opulus*, *Bioorganicheskaya Khimiya*, 2000, 26:61-67.
8. Schopmeyer, C.S. Seeds of Woody Plants in the United States. US Department of Agriculture, Washington DC, USA, 1974.
9. Soylak, M., Elci, L.; Saracoglu, S., Divrikli, U., Chemical analysis of fruit juice of European cranberrybush (*Viburnum opulus*) from Kayseri – Turkey, *Asian J. Chem.*, 2002, 14:135-138.
10. Saviuc C., Grumezescu A.M., Oprea E., Radulescu V., Dascalu L., Chifiriuc M. C., Bucur M., Banu O., Lazar V., Antifungal activity of some vegetal extracts on *Candida* biofilms developed on inert substratum, *Biointerface Res. in App. Chem.*, 2011, 1:15-23.
11. Banu O., Bleotu C., Chifiriuc M.C., Savu B., Stanciu G., Antal C., Alexandrescu M., Lazăr V., Virulence factors of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains involved in the etiology of cardiovascular infections, *Biointerface Res. in App. Chem.*, 2011, 1:72-77.
12. Limban C., Missir A.V., Chirita I. C., Neagu A. F., Draghici C., Chifiriuc M.C., Synthesis of new thiourea scaffold compounds, *Rev. Chim.*, 2011, 62:168.
13. Panus E., Chifiriuc M.C., Banu O., Mitache M., Bleotu C., Rosoiu N., Lazăr V., Comparative study of resistance and virulence markers in *Escherichia coli* strains isolated from hospital surfaces, clinical specimens and drinking/ marine waters, *Biointerface Res. in App. Chem.*, 2011, 1:24-30.

14. Saviuc C., Grumezescu A.M., Oprea E., Radulescu V., Dascalu L., Chifiriuc M.C., Bucur M., Banu O. , Lazar V., Antifungal activity of some vegetal extracts on *Candida* biofilms developed on inert substratum, *Biointerface Res. in App. Chem.*, 2011, 1:15-23.
15. Saviuc C., Grumezescu A.M., Holban A., Chifiriuc C., Mihaiescu D., Lazar V., Hybrid nanostructured material for biomedical applications, *Biointerface Res. in App. Chem.*, 2011, 1:64-71.

Manuscript received: July 26th 2011