



COMPARISON OF ANTIBACTERIAL AND ANTIOXIDANT PROPERTIES OF FRUITS AND LEAVES OF BLACKBERRY (*RUBUS PLICATUS*) AND RASPBERRY (*RUBUS IDAEUS*)

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ARTICLE INFO

Received 14. 4. 2014
Revised 7. 5. 2014
Accepted 9. 5. 2014
Published 1. 6. 2014

Regular article



ABSTRACT

Two plants – *Rubus plicatus* and *Rubus idaeus* were screened for antibacterial activity against *Staphylococcus aureus* strains, methicillin susceptible (MSSA) and methicillin resistant (MRSA), two from world collection culture and two isolated from patients. All tested extracts from fruits and leaves exhibited antibacterial activity, both against MRSA and MSSA strains. Stronger antibacterial activity had leaves extracts than fruits extracts. The strongest bacterial inhibition was observed against strains from collection culture, lower against strains directly isolated from patients' wounds. Leaf extract from *R. idaeus* exhibited stronger antibacterial activities than extracts from *R. plicatus* leaves. Antioxidant activities and phenolic content were also tested. Generally leaves of two tested plants had about 4-fold higher concentration of phenolics than fruit extracts. Leaves and fruits extracts from *R. plicatus* exhibited 2.3-fold higher concentration of phenolic compounds than *R. idaeus*. Also antioxidant activities of *R. plicatus* extracts were higher than in *R. idaeus* extracts. The present investigation expresses that plants have great potential as antimicrobial compounds against microorganisms, especially against methicillin resistant strains. Moreover these plants are a good source of antioxidants, especially leaves and fruits of *R. plicatus*, which can be used as a good exogenous sources of antioxidants in our diet.

Keywords: Blackberry, raspberry, antioxidant activity, antibacterial activity, bioscreen C

INTRODUCTION

Rubus is one of a hundred genera in the family *Rosaceae*, subfamily *Rosoideae*, tribe *Potentilleae*; there are 250 species of *Rubus* established worldwide, especially in the northern temperate zone with the majority being indigenous to Europe (Patel et al., 2004). The genus *Rubus* (raspberry, blackberry) comprises around 700 species (Gudej et al., 2004). The most common species of *Rubus* naturally occurring throughout Europe and also in Poland are blackberry (*Rubus plicatus*) and raspberry (*Rubus idaeus*) (Kurto et al., 2010; Zajac and Zajac, 2001). Extracts of the leaves and fruits of *Rubus* species have been traditionally used for their therapeutic purposes (Patel et al., 2004; Lee et al., 2012). Blackberry leaves have been used for their astringent, antidiarrhoeic, hypoglycemic activities and as an anti-inflammatory agent for the mucous membrane of the oral cavity and throat (Gudej et al., 2004). Blackberry leaves have also antiproliferative activity against cancer cells (Martini et al., 2009). Raspberry leaves (*R. idaeus* L.) have been commonly used to treat diseases of the alimentary canal, heart and the cardiovascular system. However, they are best known for their health benefits in treating fever, influenza, diabetes, menstrual pain, diarrhea and colic pain. The leaves of raspberry may also be applied externally as antibacterial, anti-inflammatory, sudorific, diuretic and choleric agents (Gudej et al., 2004). The worldwide popularity of *Rubus* fruits have increased due to the fact they are considered a healthy and nutritious food, containing many antioxidants, like phenolics, vitamin C, carotenoids, etc. (Lee et al., 2012). Antioxidants present in fruits play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against infections and degenerative diseases like cancer, cardiovascular disease, diabetes and diseases associated with ageing, like Alzheimer's disease (Grabek-Lejko and Tomczyk-Ulanowska, 2013).

Among different bacteria, *Staphylococcus aureus* is a leading cause of human bacterial infection worldwide, which has outstanding ability to acquire resistance to antibiotics, especially methicillin resistance (MRSA) *S. aureus*, which is resistant to several antibiotics (multidrug resistance). Epidemics and pandemics of antibiotic resistance *S. aureus* has arisen in the past 60 years (DeLeo et al., 2010, Otter and French, 2010). Even though pharmacological industries still produce new antibiotics, resistance to these drugs by microorganisms increases and now becoming a global concern (Nascimento et al., 2000). There is a pressing need to develop new and innovative antimicrobial agents. Among the

potential sources of new antibacterial agents, plants have long been investigated (Djeussi et al., 2013).

The aim of our work was to compare antioxidant activities between two most popular and naturally present in European forests *Rubus* species: *R. plicatus* (blackberry) and *R. idaeus* (raspberry). These abilities were measured in fruit and leaves extracts. According to our knowledge there are many articles about most common *Rubus* species, such as *R. idaeus* and *R. fruticosus*. The *R. fruticosus* refers not to a single species, but is used in the aggregate sense, comprising some 2000 described European species. This is due to difficulty to distinguish *Rubus* species by their fruits, many species arose as a result of hybridization and apomixis. That's why in medicine and food processing blackberries are under one name *R. fruticosus*. However, in Poland there are over 100 species of blackberries, among them *R. plicatus* occurs most frequently, also in Europe. According to our knowledge there are no data on *R. plicatus* abilities and in this work there are described for the first time. Another goal of this work is to detect antibacterial activity of tested plants against *S. aureus* strains – two of them taken from international collections and two isolated in local hospital from patients' wounds. Among them are methicillin resistant (MRSA) strains. In most articles antibacterial activities were analyzed against *S. aureus* strains from world collections, but only few described antibacterial activities against strains isolated from patients.

MATERIAL AND METHODS

Preparation of samples

Fruits and leaves of *Rubus idaeus* (raspberry) and *Rubus plicatus* (blackberry) were collected from several shrubs and identified during 2011 year in the Southern-Eastern part of Poland, then mixed together. Plants were identified by plant taxonomist Professor Krzysztof Oklejewicz.

Fruits were lyophilized, grinded, then 5g of dried fruits were immersed in 150 ml of extraction solution, composed of methanol and water (50:50, v/v). The duration of the extraction was 24h, with agitation 200 rpm at room temperature. Extract was collected and left at 4°C and another part of extraction solution (100 ml) was added. Extraction was conducted over 12 hours, at the same conditions as above. Extracts were combined and made up with methanol solution to final volume 250 ml. Freshly prepared extracts were used for antioxidant and phenolic

content determination. For antibacterial determination the crude extracts were concentrated using a rotary evaporator under reduced pressure (40°C) (Rotavapor R-215, BÜCHI, Flawil, Switzerland) to a final volume of 100 ml.

Leaves were dried at 105°C and 1 g of leaves were grinded and extracted with 80% of methanol at 100°C in an accelerated solvent extractor (Speed Extractor E-916 BÜCHI, Flawil, Switzerland) at a working pressure of 100 bar. Extracts were taken and were concentrated under reduced pressure and temperature 45°C (Rotavapor R-215, BÜCHI, Flawil, Switzerland) to final volume of 20 ml. Extracts were immediately used for analysis. For antibacterial activities all extracts were filtered using a filter 0.2 µm.

Antimicrobial activity

Two bacterial strains: *Staphylococcus aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 43300 (MRSA) and also two bacterial strains isolated from patients' wounds in the local hospital: named "1" and "2" were used for antibacterial assay. Strains from patients were identified by the multiplex PCR method described below.

Antibacterial activities were determined by growth rates obtained from the Bioscreen C analyzer. Overnight cultures in Mueller-Hinton broth medium were used. Optical densities of bacterial cultures were measured using a UV-VIS Helios Lambda spectrophotometer. For inoculation, bacterial biomass was added in appropriate volume, that the starting cell density distributed to Bioscreen C microplates after appropriate dilutions with plant extracts was $OD_{600nm}=0,1$ in total volume of 200 µl. 100 µl double-concentrated LB medium was mixed with 100 µl of appropriately diluted leaf or fruit extracts (4x diluted). Incubations were performed at 37°C and absorbance readings were taken every one hour. Operation of the Bioscreen C and collection of turbidity measurements (OD_{600nm}) were computer automated with EZ Experiment. Data were collected and exported to Microsoft Excell spreadsheets.

Identification of bacterial strains isolated from patients

DNA isolation

Total DNA was isolated from 5 ml of LB broth culture grown overnight for all the bacterial strains used in the study. *Extractme* DNA bacterial kit (Blirt, Poland) was used for DNA extraction. DNA samples were used for PCR reaction.

PCR amplification

Primers for detection of the bacterial *mecA* gene, and *nuc* gene were used, giving products of 533 bp, and 270 bp, respectively. Primers used for multiplex PCR are listed in Table 1 (Louie et al., 2002). Multiplex PCR was performed in a 20µl volume with 1×PCR buffer (Fermentas), 3 mM MgCl₂, a 200 µM concentration of each deoxynucleoside triphosphate, 2.5 U of Taq polymerase (Fermentas), 0.2 µM each of the *mec* and *nuc* primers with 1 µl of template DNA (as prepared above). Thermocycling conditions in a GeneAmp 9600 thermocycler (Applied Biosystems) were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 35s, 55°C for 35s, and 72°C for 60s, with a final extension at 72°C for 10 min. PCR products were visualized as below. Electrophoresis at 100 V for 30 min was performed to separate the PCR amplicons on a 1% 1×TBE (Tris-borate-EDTA)-agarose gel. Gels were then stained with ethidium bromide and photographed under UV illumination. Multiplex PCR was also performed from each positive control – MRSA and MSSA *S. aureus* strains.

Table 1 Primers used for multiplex PCR detection of staphylococci and their methicillin resistance

Primer	Sequence (5'→3')	Product size (bp)
<i>mecA</i> -1	AAAATCGATGGTAAAGGTTGGC	533
<i>mecA</i> -2	AGTTCTGCAGTACCGGATTTGC	
<i>nuc</i> -1	GCGATTGATGGTGATACGGTT	270
<i>nuc</i> -2	AGCCAAGCCTTGACGAATAAAGC	

Estimation of total polyphenol content

Total polyphenol content was measured using Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Plant extracts 50 µl were mixed with 450 µl of water, then 2 ml of 7.5% sodium carbonate and 2.5 ml of Folin-Ciocalteu reagent were added. After 5 minutes of incubation at room temperature the absorbance of the resulting blue color was measured at Spectrophotometer Helios Lambda UV-VIS at 750 nm. For standard curve gallic acid was used. The results were expressed as mg of gallic acid equivalents (GAE) per 1g of dry weight (dw).

Ferric reducing/antioxidant power (FRAP) assay

A manual assay was used based upon the methodology of Benzie and Strain (1999). FRAP reagent was freshly prepared with 1 mM 2,4,6-tripyridyl-2-triazine

(TPTZ), 2 mM ferric chloride in 0.25 M sodium acetate buffer, pH 3.6. A 200 µl of analyzed extracts were added to 1.8 ml of FRAP reagent and mixed. After incubation at room temperature for 10 minutes, absorbance at 593 nm was determined against a water blank. Standard curve was prepared using different concentrations of Trolox. Results were expressed as µmoles of Trolox per 1g of dry weight (dw).

ABTS - radical cation decolorization assay

The TAC (total antioxidant capacity) was estimated by 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation decolorization assay (ABTS^{•+}). A modified method of Re et al. (1999) was used. A fresh solution of 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation (ABTS^{•+}) was prepared by dissolving 19.5 mg of ABTS and 3.3 mg of dipotassiumperoxodisulfate in 7 mL of 0.1 mol/L phosphate buffer, pH 7.4. The solution was stored for 16 hours in the dark for completion of the reaction. The ABTS^{•+} solution was then diluted in the 0.1 mol/L phosphate buffer to obtain an absorbance of about 1.0 at 414 nm. Aliquots (20 µL) of measured samples were added to 980 µL of ABTS^{•+} solution and mixed thoroughly. The decrease in the absorbance of the mixture was measured in a spectrophotometer at 414 nm exactly 10 seconds and 3 minutes after mixing the sample with the ABTS^{•+} solution. Parallel calibration curves were produced, at various concentrations of Trolox. The results were expressed as µmoles of Trolox per 1 g of dry weight (dw).

RESULTS AND DISCUSSION

The phenolic composition and antioxidant properties of many medicinal plants, including blackberries (*R. fruticosus*) and raspberries have been evaluated (Radovanovic et al. 2013, Gudej and Tomczyk 2004, Sariburun et al. 2010) but these properties of other species of *Rubus* (like *R. plicatus*) are still in some extent unexplored. Table 2 presents the total phenolic content (TPC) expressed as mg gallic acid per 1 g of dried mass determined in fruit and leaves extracts. The higher TPC was determined in leaves extracts, than in fruit extracts, almost four times more. Phenolic content in *R. plicatus* leaves extracts are twice higher than in *R. idaeus* leaves. Similar results were marked for fruit extracts. In literature, no data were found on the phenolic content of the investigated *R. plicatus*, so results obtained in this work can't be compared and, according to our knowledge, are reported for the first time. Nevertheless, obtained results can be compared with other blackberry species described by other authors. Ördögh et al. (2010) found that the content of TPC in *R. idaeus* juices is about 3 times lower than in *R. fruticosus* fruits and the values achieved were 21.52 and 61.24 mg of gallic acid per 1 g of dry mass, respectively. The similar correlation was observed in our study, but generally content of TPC in tested fruits was about twice lower. But similar phenolic content was observed by Wang and Lin (2000) for raspberry fruits (around 13 mg of gallic acid/g dry mass). According to these authors there is no difference between TPC of ripe raspberry and blackberry fruits, which is not in line with our results. According to Buřičová et al. (2011) TPC content in blackberry leaves (*R. fruticosus*) is higher than in *R. idaeus* leaves, but the differences are not as significant as we received. It may be due to the fact, that *R. fruticosus* refers not to a single species, but is used in the aggregate sense, comprising some 2000 described European species and there is impossible to determine which species were present in the analyzed sample. TPC of blackberry fruits naturally present in Northern part of America, like *R. laciniatus* and *R. occidentalis* varied from 30.94 mg gallic acid/g dry mass to 57.65 mg gallic acid/g dry mass, respectively. But the level of TPC for raspberries was 36.93 mg gallic acid/g dry mass (Wada and Ou, 2002). These results confirm the necessity for detecting phenolic content, antioxidant activities in different blackberry species, because concentration of these substances may be very different between species. In the future other species naturally occurring in polish forests should be detected in order to find the blackberry species with the highest antioxidant and antibacterial activities, which could be cultivated. Compared with blackberries grown in tropical climates, like tropical highland blackberry (*R. adenotrichus*), *R. plicatus* growing in temperate climate presents similar phenolic contents, around 30 mg gallic acid/g dry mass (Acosta – Montoya et al., 2010). TPC of leaves extracts of *R. plicatus* was higher (115.92 mg GAE/g dw) than that found by Gawron – Gzella et al. (2012) for three different native blackberries species present in polish forests and ranged from 70 to 87 mg GAE/g.

The antioxidant activity of the leaf and fruits extracts were assessed by two spectrophotometric methods: ABTS and FRAP. The results are expressed in Table 2. The highest level of antioxidants determined by FRAP were detected for *R. plicatus* leaves extracts. Concentration of antioxidants in *R. idaeus* leaves extracts is twice lower than in *R. plicatus* leaves. Fruits of blackberry have more than twice antioxidants than raspberry fruits. The differences between antioxidants in leaves and fruits are comparable to phenolic content. Generally leaves of these two tested plants possess more than twice antioxidants than fruits. Comparing results obtained by ABTS method in leaves extracts we can see similar correlation as for FRAP method. *R. plicatus* leaves extracts possess more than twice more antioxidants, both fast-reacting and slow-reacting than *R. idaeus*

leaves. But in fruits, the differences in antioxidants concentration between these analyzed species are not so huge. Fruits of *R. plicatus* have around 40-50% more slow-reacting and fast-reacting antioxidants than *R. idaeus* fruits. Similar H-ORAC activity were determined in tropical blackberry fruits (*R. adenotrichus*), which is very interesting, because many other blackberry species growing in temperate climates have lower antioxidant activities than tropical blackberries (Acosta – Montoya *et al.*, 2010). Higher ORAC values for thornless blackberry fruits than raspberry fruits were also detected by Wang and Lin (2000), but differences are smaller (about 30%) than in our work. According to Deighton *et al.* (2000) antioxidant activities of raspberries determined by FRAP are lower than of 8 other tested blackberries, and higher than other 9 blackberries species. Pantelidis *et al.* (2007) determined FRAP values between 77.7 and 169 μmol ascorbic acid/g dw for *R. idaeus* cultivars and 113.6 and 169 μmol ascorbic acid/g

dw for *Rubus fruticosus* cultivars. As it can be seen above, it is very difficult to compare the results obtained in this work with those available in the literature, because researchers use different methods to estimate antioxidant capacities, use different standards, incubation time, concentrations, different cultivars of plants. Moreover, various factors such as variety, growing conditions, maturity, season, geographic origin, fertilizer, soil type, storage conditions, and amount of sunlight received, among others, might be responsible for the observed differences (Al-Farsi *et al.*, 2005). It was, however confirmed that higher antioxidant activities displayed fruits and leaves of blackberries than raspberries, and analyzed *R. plicatus* have similar or even higher antioxidant and phenolic content than many blackberries cultivars described in the literature (Pantelidis *et al.*, 2007; Acosta – Montoya *et al.*, 2010; Gawron – Gzella *et al.*, 2012).

Table 2 Antioxidant activity – FRAP values (μmol Trolox/g); ABTS (μmol Trolox/g) and phenolic content (mg gallic acid/g)

Plants	FRAP μmol Trolox/g (dw)	ABTS(10 sek) μmol Trolox/g (dw)	ABTS (3 min) μmol Trolox/g (dw)	Phenolic content mg gallic acid/g (dw)
Fruits				
<i>Rubus idaeus</i>	165.94±0.4	48.49±2.9	93.11±3.6	13.58±0.9
<i>Rubus plicatus</i>	365± 19.5	68.87±5.1	146.32±7.6	32.10±0.5
Leaves				
<i>Rubus idaeus</i>	387.5±27.8	685±7.0	1269.72±17.4	50.45±9.4
<i>Rubus plicatus</i>	805.36±51.3	1518.74±139.5	2606.02±352.2	115.92±3.5

Results obtained after 10 seconds – reflects the concentration of fast-reacting antioxidants.

Results obtained after 3 minutes – reflects the concentration of slow-reacting antioxidants.

Data are means \pm standard deviation of three independent determinations.

Antibacterial activities of *Rubus* extracts

Multiplex PCR

For identification of *S. aureus* strains and detection of methicillin resistance a multiplex PCR assay to detect the *mec A* gene (responsible for the true methicillin resistance in staphylococci) and the *nuc* gene (found only in *S. aureus* and not in coagulase-negative staphylococci) was used (Louie *et al.*, 2002). Among two staphylococci strains isolated from patients' wounds, one (nr 1) was methicillin susceptible, only product of the *nuc* gene was amplified in this probe (Fig. 1, lane 4). The second one strain (nr 2) was methicillin resistant. On the agarose gel two products of the *mec A* and *nuc* genes are seen (Fig 1, lane 5). As a control two strains from ATCC collection were used: *S. aureus* 43300 MRSA (Fig 1, lane 3) and *S. aureus* 25923 MSSA (Fig 1, lane 2). All four strains were used in the antibacterial activity of *Rubus* leaves and fruits extracts.

The obtained results demonstrates that all tested extracts have antibacterial activity against *S. aureus* strains, but differences between blackberries and raspberries are not so huge.

It can be said that higher antibacterial potential have leaves extracts and lower is observed for fruits extracts. Leaf extracts exhibited lower antibacterial activities against staphylococci strains isolated directly from patients. Similar situation can be observed in fruit extract against MRSA strains. Antibacterial activities of fruit extracts are similar when we compare MSSA *S. aureus* strains. In this case inhibition of growth was around 40%. The lowest antibacterial activity was observed for fruits extracts against MRSA staphylococci isolated from patient. In the literature, we can find data of antibacterial activities of medicinal plants against different bacteria, also against *S. aureus* (Vatpák *et al.*, 2014, Wendakoon *et al.*, 2011; Riaz *et al.* 2011; Velićanski *et al.*, 2012; Kacaniová *et al.*, 2014; Fatrcová-Šramková *et al.*, 2013). It is very important, that analyzed extracts can be also used against MRSA strains. Wendakoon *et al.* (2011) also described different medicinal plants with very high antibacterial activities against MRSA *S. aureus* strains. Inhibition of staphylococci by the juices of *R. fruticosus* and *R. idaeus* extracts were confirmed also by Ördögh *et al.* (2010). Moreover these authors didn't notice correlation between antioxidant capacity and antibacterial activities, suggesting that not only phenolic antioxidants are responsible for the antimicrobial effect. Lack of real correlation between antioxidants and antibacterial activities was detected also in our work. Quite huge differences of antioxidants and phenolic content can be observed between blackberry and raspberry fruits and leaves, but there is slightly difference in antibacterial activities of these extracts (Figure 2, 3). Differences can be only seen between leaves and fruits extracts, emphasizing that leaves extracts have stronger antibacterial activities.

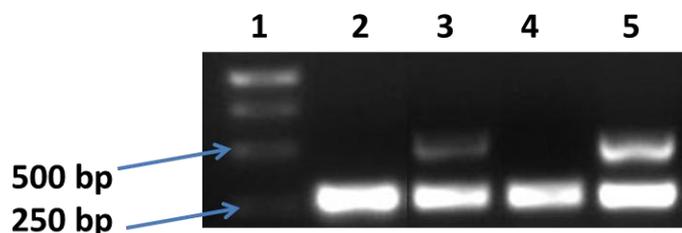


Figure 1 Agarose gel showing PCR-amplified products of the *mecA* and *nuc* genes

Lanes: 1 - DNA ladder; Lane 2 – MSSA *S. aureus* ATCC 25923 control (showing one amplification product *nuc* at 270 bp); Lane 3 – MRSA *S. aureus* ATCC 43300 control (showing two amplification products *mecA* at 533 bp, and *nuc* at 270 bp); Lane 4 – *S. aureus* nr 1 from patient with only *nuc* product – methicillin sensitive; Lane 5 – *S. aureus* nr 2 from patient, with *mecA* and *nuc* products, methicillin resistant.

Plant extracts constitute important sources of biologically active compounds that may show significant antimicrobial properties. In this work *R. idaeus* and *R. plicatus* leaf and fruit extracts demonstrated antibacterial activity against four *S. aureus* strains. The antibacterial activity of the leaves extract are depicted in Fig. 2. The antibacterial activity of fruits extracts are depicted in Fig. 3.

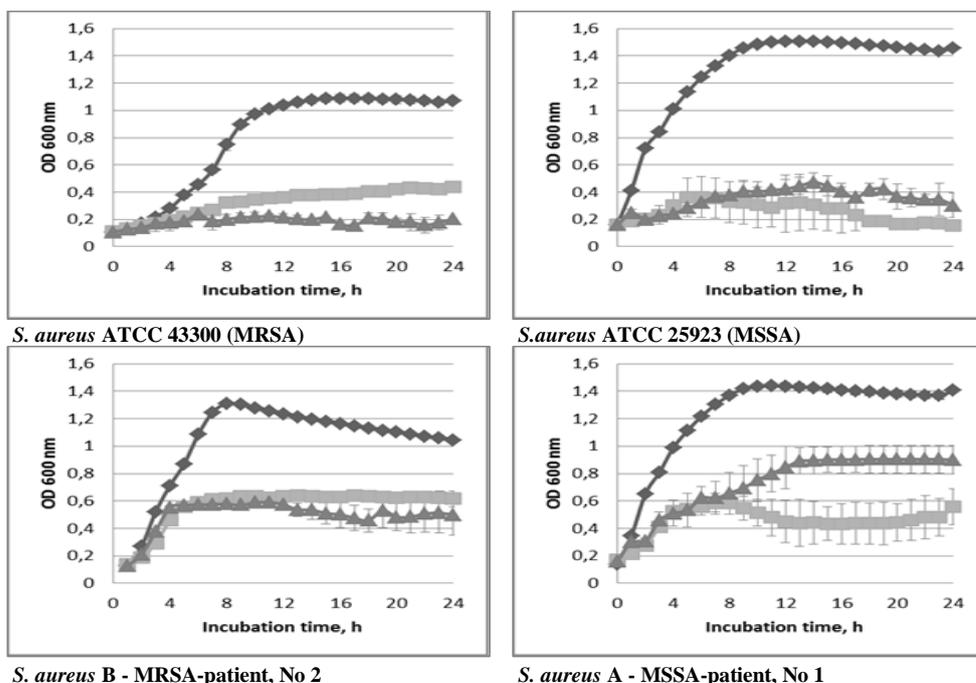


Figure 2 Antibacterial activities of leaves extracts against *S. aureus* strains
 Legend: rhombus – positive control *S. aureus* without leaves extract; triangle – *R. idaeus* extract; square – *R. plicatus* extract.

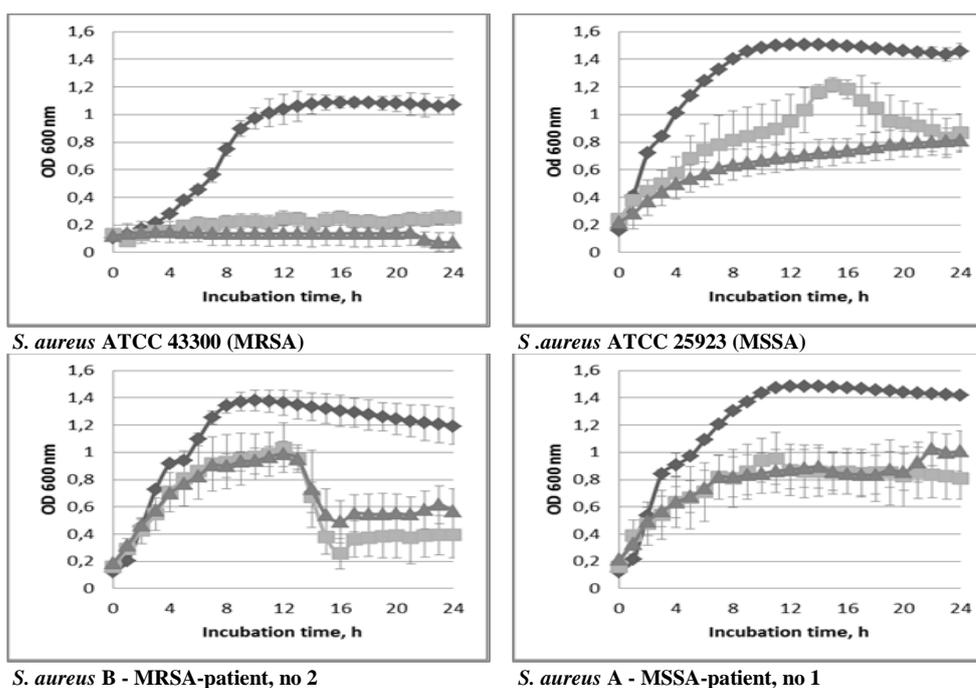


Figure 3 Antibacterial activities of fruits extracts against the *S. aureus* strains
 Legend: rhombus – positive control *S. aureus* without leaves extract; triangle – *R. idaeus* extract; square – *R. plicatus* extract.

CONCLUSION

Our results for the first time showed antioxidant and antibacterial activities of *R. plicatus* leaves and fruits in comparison with well-known raspberry fruits and leaves. It was demonstrated that blackberries extracts have higher antioxidant activities than raspberries. And among other blackberries cultivars described in the literature has very high antioxidant and phenolic content. These blackberries and raspberries if added to our diet could constitute a good source of antioxidants. Moreover they exhibit antibacterial activities against *S. aureus* strains, those which are susceptible and those which are resistant to methicillin, and with such antimicrobial properties, can be of great significance in therapeutic treatments. It would be interesting to analyze other blackberry cultivars naturally present in polish forests and find these with highest antibacterial and antioxidant capacities and use them for cultivation.

Acknowledgments: We are grateful to Professor Krzysztof Oklejewicz from University of Rzeszow, Poland, for his help in collecting and identification of *Rubus* samples.

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