In vitro assessment of antibiotic-resistance reversal of a methanol extract from *Rosa canina* L

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Abstract

Rosa canina (RC, the Dog Rose) is used in European folk medicine for the treatment of various human ailments. The crude methanol extract of this botanical was tested against multidrug-resistant (MDR) bacterial strains including methicillin-resistant *Staphylococcus aureus* SA1199B, EMRSA-16 and XU212 harbouring NorA, PBP2a and TetK resistance mechanisms, respectively, as well as *S. aureus* ATCC25923, a standard antimicrobial–susceptible laboratory strain. Inhibition of the conjugal transfer of plasmids PKM101 and TP114 by the RC extract was also evaluated. The RC extract demonstrated a mild to poor antibacterial activity against the panel of bacteria having MIC values ranging from 256 to >512 mg/L, but strongly potentiated tetracycline activity (64-fold) against XU212, a tetracycline-effluxing and resistant strain. Furthermore, the extract showed moderate capacity to inhibit the conjugal transfer of TP114 and PKM101; transfer frequencies were between 40% and 45%. Cytotoxicity analysis of the RC extract against HepG2 cells line showed the IC_{50} >500µg/mL and thus was considered non-toxic toward human cells. Phytochemical characterisation of the extracts was performed by the assessment of total phenolic content (RC: 60.86 mg TAE/g) and by HPLC fingerprint determination. The results from this study provide new mechanistic evidence justifying, at least in part, the traditional use of this extract. However, the inhibition of bacterial plasmid conjugation opens the possibility of combination therapies to overcome antibiotic-resistance.

Keywords: *Rosa canina*; resistance modulator; anti-plasmid; MDR bacteria; cytotoxicity.

1. Introduction

The rapid spread of multidrug-resistant (MDR) bacteria has posed a serious global health threat over the last few decades and continues to constitute a major problem in the treatment of hospital- and community-acquired infections [1]. As a consequence, treatment of infections caused by MDR bacteria strains is becoming increasingly difficult to manage with existing antibiotics. This is in part due to the ability of bacteria to evolve mechanisms that thwart antimicrobial action [2]. Diverse strains of *Staphylococcus aureus* such as SA-1199B (NorA), XU-212 (TetK and PBP2a), RN-4220 (MsrA), EMRSA-15 and -16 (PBP2a) are prominent for their high level of resistance to antibiotics via efflux mechanisms and altered target site [3, 4]. Recent studies have suggested that the horizontal transmission of genes conferring antibiotic resistance may be the dominant force behind the growing problem of antibiotic resistance [5]. For instance, dissemination of extended-spectrum beta-lactamase (ESBL) genes, conferring resistance to third-generation cephalosporins and other β-lactams, via plasmid transmission has been reported amongst certain bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter* spp. [6]. These plasmid-mediated multidrug resistance phenotypes are well known for affecting the treatment of infectious human diseases [7]. Unfortunately, synthetic plasmid-curing agents such as acridine orange, ethidium bromide and sodium dodecylsulfate are unsuitable for therapeutic application owing to their toxic nature. There is therefore a need to develop new antibiotics with alternative mechanisms to overcome bacterial resistance, particularly as resistance modifying efflux inhibitors or plasmid curing compounds [8, 9]. In this context, plant-derived products have been proposed as a new source of drug leads to combat bacterial resistance mechanisms [10] through the reversal of MDR phenotypes facilitating the re-establishment of use of existing antibiotics.

Rosa canina L (Rosaceae; RC) known as Rose Hip or Dog Rose is mostly used traditionally as a dietary supplement and a herbal remedy for the prevention and treatment of diverse human diseases such as diabetes, kidney disorders, inflammation, common cold, ulcer and cancer, but only a few of these have any *in vitro* scientific data to support these indications [11]. The strong anti-microbial potential of *Rosa canina* against many micro-organisms has been documented [12]. The folkloric medicinal provides a significant source of ascorbic acid, tocopherol, tannins, pectin, minerals, fatty acids, carotenoids and phenolic compounds [13, 14]. It has previously been reported that tellimagrandin I isolated from RC reduced the MIC of oxacillin and tetracycline against four MRSA strains [15]. Presently, there is no scientific

data on the antibacterial properties of RC against strains with characterised efflux-mediated mechanisms of resistance or the capacity to inhibit plasmid transfer. This study therefore aimed to investigate the antibacterial, plasmid conjugation inhibition and antibiotic modulation potential of the methanol extract of *Rosa canina* fruit against selected MDR *S. aureus* strains. In addition, their differential cytotoxicity against mammal cells expressing efflux pumps was also determined using the Sulphorhodamine B (SRB) assay with human liver carcinoma cells (HepG2).

2. Results and discussion

2.1 Total phenolic content (TPC)

Due to the lack of an appropriate standard, the TPC of the RC extract was expressed as tannic acid equivalents (TAE). The TPC value was found to be 60.86±0.04 mgTAE/g for RC, extrapolated from the standard tannic acid curve: Y= 0.1216x; R^2= 0.93651. The data recorded for RC fruits was contrary to that of Montazeri et al [16] who reported 424.6±1.8 mg/gallic acid equivalent (GAE)/g but corroborated with the findings (59.69±0.89 mg GAE/g dry matter) of IIbay et al. [17]. The observed inconsistency could be attributed to different climatic conditions, the standard equivalent used, solvent system and/or processing methods. Moreover, Folin Ciocalteu reagent (FCR) has been used to measure other compounds and hence may not give a concise amount of the phenolic compounds present. However, a HPLC/DAD chromatogram with detection at 360 nm revealed the presence of seven major peaks of phenolic compounds for the RC extract (data not shown) between a retention time of 2 and 38 min.

2.2 Antibacterial activity of RC extract

The methanol extract showed a mild to poor antibacterial activity with minimum inhibitory concentration (MIC) values ranging between 256 and >512 mg/L whereas the standard antibiotics used as controls ranged from ≤0.03 to 0.5 mg/L as summarized in Table 1. According to Rios and Recio [18] extracts can be classified as significant (MIC < 100 mg/L), moderate (100 < MIC = 512 mg/L) or weak (MIC > 512 mg/L) depending upon their respective activities against the corresponding pathogens. Utilising this scheme, the RC extract demonstrated moderate antibacterial activity against EMRSA-15 and EMRSA-16 [3, 4], the major epidemic methicillin-resistant *S. aureus* strains occurring in UK hospital, as well as *Proteus vulgaris* 10330 and *Pseudomonas aeruginosa* 10662 with MIC values

ranging between 256 and 512 mg/L, but was less active towards other MDR organisms (MIC >512 mg/L). The poor antibacterial activity of RC fruit is consistent with the report of Yilmaz and Ercisli [19] against certain bacteria. Although the MIC values obtained from extracts against the test organisms were much higher compared to the antimicrobial agents this is likely to be due to the complex mixture of the phytochemicals in the sample.

2.3 Antibiotic resistance modifying activity

The bacterial resistance-modifying activity of the RC extract in the presence of either norfloxacin or tetracycline against four bacterial strains (SA1199B, XU212, ATCC 25923 and EMRSA-16) is depicted in Table 2. The combination of RC extract with norfloxacin had no effect on the activities towards EMRSA-16 and ATCC 25923. Interestingly, an increase in the MIC of norfloxacin was recorded against SA1199B, suggesting antagonism. Moreover, when tested against the tetracycline-resistant strain (XU212) expressing the TetK efflux transporter, a sixty-four-fold reduction in the MIC was noted at a sub-inhibitory concentration (SIC). When reserpine, a known efflux inhibitor, was combined with norfloxacin or tetracycline a four-fold potentiation was observed towards EMRSA-16, ATCC 25923 and XU212 while eight fold reductions in the MIC of norfloxacin was displayed against SA1199B. The development of efflux pump inhibitors has been proposed to be used in conjunction with existing antibiotics to improve therapeutic efficacy and suppress the emergence of resistant variants that may arise during treatment [20]. Although, the plant extract showed promising resistance modifying properties against XU212 and appeared to overcome the efflux mechanisms present, the isolation of their active principles could provide useful drug leads to reverse antibiotic resistance mechanisms in clinically-relevant pathogens.

2.4 Plasmid conjugal inhibition activity

The search for inhibitors of plasmid-mediated resistance is currently gaining new ground, with only a small number of synthetic compounds identified [21], which are unsuitable for clinical application due to their neurotoxicity. Consequently, recent studies have concentrated on natural products in order to identify natural inhibitors of bacterial conjugation [22]. Here, the RC extract was evaluated to assess its capacity to reduce the transfer frequency of the plasmids TP114 and PKM10. TP114 is a self-transmissible plasmid belonging to $Incl_2$ compatibility group isolated from *E. coli* [23] and encodes a kanamycin resistance determinant. PKM 101 belongs to the IncN compatibility group, is a construct from the parent plasmid R46 [24], and confers ampicillin resistance via β-lactamase production.

Moderate inhibitory activity against the transfer of TP114 and PKM101 was demonstrated by RC despite a weak antibacterial and resistance modifying effect, signifying a different mechanism (Figure 1). RC is notable for its phenolic constituents and this class of compound are typified by bharangin isolated from *Pygmacopremna herbacea* (Roxb), together with gossypetin and gossypin, known to cause the loss of the TP181 plasmid as well as a penicillinase-conferring plasmid in *E. coli* 46R41 [25]. Novobiocin was used as the reference control at 10 mg/L, having 110% and 83% plasmid conjugal transfer frequency for PKM 101 and TP114, respectively. Synergy in whole plant medicines or crude extract treatments is very commonly responsible in bioactive extracts, especially given that many folklore medicines are co-administered with one or more preparations [10]. In RC, the combinatorial effect of the crude extract may possibly facilitate transport across the bacterial cell wall, which could aid maximum absorption of the drug to assist inhibition of the conjugation process. However, the exact mechanism of action of the crude extract is not known but further bioassay-guided isolation of the active extract is currently being examined.

2.5 SRB cytotoxicity potential of the extracts

Numerous assays such as MTT (3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3–bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt) and SRB (sulphorhodamine B) have been employed to evaluate toxic effects of chemicals on the membrane integrity, mitochondrial function or protein synthesis in mammalian cells [26]. The sulphorhodamine B assay is considered the preferable approach based on the ability to bind to protein basic amino acid residues of trichloroacetic acid (TCA) fixed cells [27]. It is sensitive, simple, reproducible and more rapid than the formazan-based assays with better linearity without a time sensitive measurement as regarding the MTT or XTT assays [28, 29]. The effect of the RC (IC_{50}>500mg/L) extract on HepG2 cell viability was assessed using a colorimetric assay that evaluates cell number indirectly by staining total cellular protein with the SRB dye [28] as presented in Figure 2. The RC extracts did not impair lysosomes as compared with the standard doxorubicin (0.5mg/L: 52.45%) a chemotherapeutic agent used for the treatment of many different cancer types. A similar observation was reported by Davis et al. [30] and thus supports the safe use of this botanical as complementary and alternative therapy in the prevention or treatment of certain health problems. The weak toxic effect could be linked to its hydrophilic nature inhibiting the interaction with the cell membranes where signal transduction pathways occur [31].

3. Materials and Methods

3.1 Plant extracts preparation

Rosa canina L (RC) fruits were obtained in fully dried and powdered form from Herbs in a Bottle Ltd UK. Fifty grams (50g) of the dried powdered materials were extracted in 300 mL of methanol, at room temperature by constant mechanized agitation in an ultrasonic bath, for 45 min. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The volume was concentrated under vacuum at 40°C to recover the methanol, air-dried, weighed and stored in a refrigerator at 6^{o}C for future use.

3.2 Bacterial strains

The antibacterial assay was performed against strains of *Staphylococcus aureus*: SA1199B and XU212 are MDR strains that overexpress the NorA and TetK efflux transporter proteins with a high level of resistance to norfloxacin and tetracycline, respectively. EMRSA-15 and EMRSA-16 are the major epidemic methicillin-resistant *S. aureus* strains in UK hospitals [3, 4]. *S. aureus* ATCC25923, *E. coli* NCTC 10418 and *P. aeruginosa* NCTC 10662 are standard susceptibility testing control strains. *S. aureus* RN-4220 is a macrolide-resistant strain, while *K. pneumoniae* 342 and *P. vulgaris* 10330 are MDR clinical isolates. The plasmid conjugal inhibitory potential of RC extract was tested on a conjugal transfer system of *E. coli* bearing the TP114 plasmid, which encodes for kanamycin resistance and *E. coli* harbouring the PKM 101, which codes for ampicillin resistance. The recipient did not harbour plasmids but had chromosomally encoded resistance to streptomycin. All the bacteria and plasmids tested were obtained from Dr. Paul Stapleton UCL School of Pharmacy.

3.3 Cell culture and maintenance

The HepG2 cell lines (American Type Culture Collection) were obtained from Prof. Andreas Kortenkamp formerly at the UCL School of Pharmacy. HepG2 cells were replenished with new growth medium every 2-3 days and sub-cultured every 3-4 days when they were 80% confluent. Cells were maintained in DMEM (Invitrogen, UK) containing 10% foetal bovine serum (GIBCO 10010) and antibiotics [penicillin (100 IU/mL) and streptomycin (100 mg/L)], incubated at 37^{o}C in a 5% CO_2 humidified atmosphere and split when confluent. The cell density was adjusted to7.5 x 10^4 cells/mL before exposure to different concentrations of plant extracts prepared in 1% w/v DMSO.

3.4 Total phenolic content (TPC)

The total phenolic content of the methanol extract from RC was determined with Folin Ciocalteu reagent using the method of Lister and Wilson [32] with slight modification. A volume of 0.5 mL of the plant extract (1000 mg/L) was added to 2.5 mL of 10% v/v Folin-Ciocalteu reagent (FCR) and 2 mL of 20,000 mg/L Na_2CO_3. The resulting mixture was incubated at 45ºC with shaking for 15 min, followed by measuring the absorbance at 765 nm. The calibration curve of the standard tannic acid (1 mL; 25-400 mg/L) was prepared following the same method. The experiment was conducted in triplicate, and the results were reported as mean ± SD values. Tannic acid equivalents (TAE) in the plant extract was calculated by the following formula: T = C.V/M. Where T was the TPC (mg/g) of the extract in TAE; C was the concentration of tannic acid established from the calibration curve, V was the volume of the extract (mL) and where M was the weight of the extract (g).

3.5 Minimum Inhibitory Concentration (MIC) assay

The minimum inhibitory concentrations (MICs) of the antibiotics and plant extract were determined against resistant bacteria using the method described by Shiu and Gibbons [33]. The bacterial strains and isolates were grown at 37°C overnight on nutrient agar and inocula of the test organisms were prepared in normal saline (9 g/L) and compared with a 0.5 McFarland standard and diluted to achieve 5×10^5 CFU/mL. A volume of 100 µL of Mueller Hinton broth (MHB) was dispensed into 96 wells of microtitre plate. A stock solution of antibiotics or drug extracts was prepared in dimethyl sulfoxide (DMSO) (Sigma) and further diluted in MHB (1.5 mL) to reduce DMSO concentration to 1% or below. The stock solution (100 µL) was serially diluted into each well, mixed with 100 µL of standardized bacterial inoculum to give a final concentration that ranged between 512 and 1 mg/L for the extract and 128 to 0.03 mg/L for the antibiotics. All procedures were performed in duplicate; the plates were incubated at 37°C for 18 h. A volume of 20 µL of a 5 g/L methanol solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well and incubated for 30 min. A blue coloration indicated bacterial growth while the MIC was recorded as the lowest concentration at which no visible growth was observed.

3.6 Bacterial resistance modulation assay

For evaluation of extracts as antibiotic resistance modulators, MICs of norfloxacin and tetracycline were determined in the presence or absence of sub-inhibitory concentrations

(SIC) of the extract (64 - 512 mg/L) against susceptibility testing control strain *S. aureus* ATCC 25693 and three resistant *S. aureus* isolates: XU 212, SA1199B and EMRSA-16 following the procedure described by Oluwatuyi et al. [34] with slight modification. A volume of 100 µL of MHB was dispensed in each well except wells in column 1 that contained 100 µL of the extract. The samples at the stock concentrations (256 - 2048 mg/L) were introduced into wells 2-10 while 100µL of the appropriate antibiotic (MICs) was added into well 1 and serially diluted (512 to 1 mg/L) across the plate leaving well 11 empty for final growth control and well 12 as sterility controls. To the wells in rows four to six were added 100 µL of 20 mg/L reserpine (a modulatory agent). All strains were cultured on nutrient agar slope before MIC determination. Overnight cultures of each strain were prepared in 9 g/L saline to an inoculums density of 5×10^5 CFU/mL. To the wells were added 100 µL of a standardised inoculum, but wells in rows seven and eight were maintained free from the extracts and reserpine. All experiments were performed in triplicate under aseptic conditions. The MIC was determined as mentioned above.

3.7 Plasmid conjugation inhibition assay

The plasmid conjugal transfer inhibition assay was performed by the broth mating method described by Rice and Bonomo [35] with some modifications. Mating between the plasmid-containing donors strains *E. coli* K12 J53 and the recipients *E. coli* ER1793 and JM 109 were performed in Luria-Bertani (LB) broth. The independent overnight cultures of the plasmids were inoculated into 5 mL of fresh LB broth and incubated overnight with shaking at 37^{o}C. Donor and recipient cultures were mixed 1:1 in 100 µL of LB with 100 mg/L of each of the extracts or 10 mg/L of standard novobiocin to a final volume of 200 µL and incubated overnight at 37^{o}C. For time course experiments, overnight cultures were mixed and mated in the presence of the drug and incubated for the time periods stated. Only the bacteria that had successfully taken up the resistance gene and become resistant will grow indicating plasmid conjugation.

3.8 Sulphorhodamine B (SRB) assay

The method described by Houghton et al. [29] was adopted to test for the toxicity of the plant extract using the HepG2 cell line. A volume of 100 µL cell suspension of optimum density was introduced into each well of a 96-well plate. Plant extract concentrations ranged between 15.625 and 500 µg/mL and were prepared in the culture medium and 100µL of each extract concentration was added to the cell monolayer and control well. Monolayer cells were

incubated with the drug extracts or standard doxorubicin (positive control) for 48 h, and cells were fixed with ice-cold TCA for 1 h at 4^{o}C. After incubation, the plates were washed five times in sterilized distilled water and then air dried. A volume of 50 µL of sulphorhodamine B (0.4%w/v in 1%v/v acetic acid) solution was added to each well of the dry 96-well plates and allowed to stain at room temperature for 30 min. The SRB unbound dye solution was removed after staining by washing the plates five times with 1% v/v acetic acid. The bound SRB dye was solubilised by adding 100 µL of 10 mM non-buffered Tris Base (pH 10.5) to each well, shaking for 5 min. The plates were read in a 96-well plate reader at 492 nm. The mean background absorbance was subtracted and the mean values of each drug concentration were calculated. The IC_{50} values of tested extract was the calculated.

3.9 Statistics

Data analysis was done with Microsoft Excel to obtain descriptive statistics. Means were separated by the Duncan multiple test using SAS. The different levels of significance within the separated groups were analyzed using one way analysis of variance (ANOVA). Values were considered significant at $P<0.05$.

4. Conclusion

We have shown that while the RC extract had mild to poor antibacterial activity, the sample was able to potentiate tetracycline activity against tetracycline-resistant strain XU212 possessing a multidrug efflux mechanism and had a moderate inhibitory effect on plasmid conjugation. Encouragingly, the RC extract did not exhibit any significant toxic effect to mammalian cells suggesting the inhibitory was specific in nature.

Acknowledgement

The authors thank National Research Foundation of South Africa and doctoral research supported by commonwealth scholarship commission, UK (NGCA-2010-59) for their financial support.

Author Contributions

S.O.O and B.O.O designed the experiments; S.O.O and B.O.O performed the experiment; S.O.O and B.O.O wrote the paper; S.G, P.S, J.M.P edit the paper; P.S, J.M.P and S.G provided the lab reagents; R.M.C provided the funding; S.O.O, and B.O.O; analyzed the data.

Conflicts of Interest

The authors declare no conflict of interest

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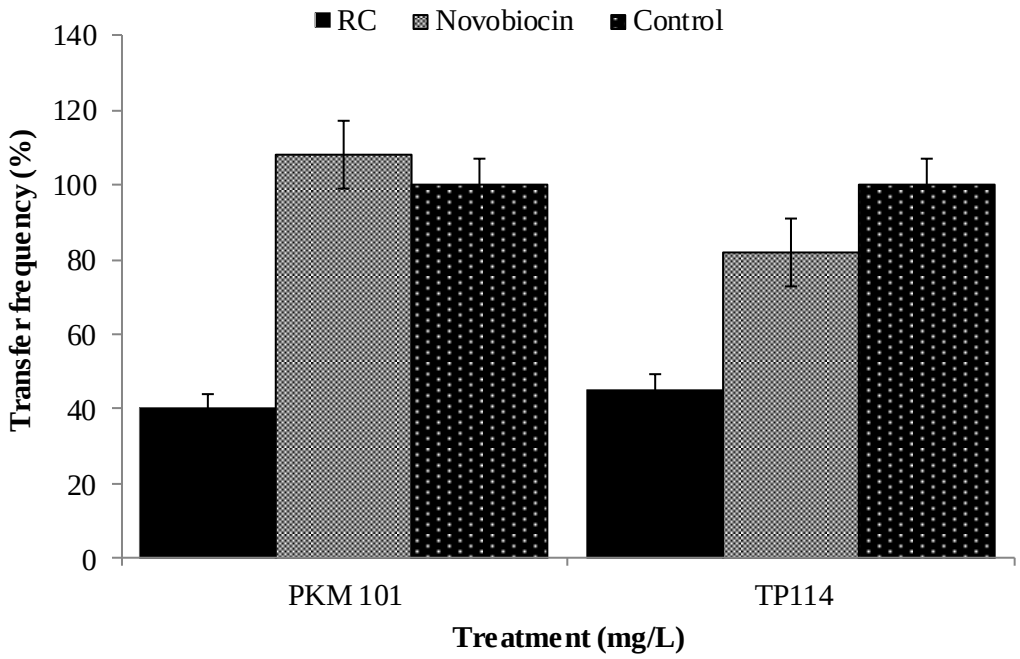

Figure 1: Percentage (%) plasmid transfer frequency of PKM 101 and TP114 in the presence and absence of *Rosa canina* extracts (100 mg/L). **P<0.05 compared to novobiocin (10 mg/L) treatment.

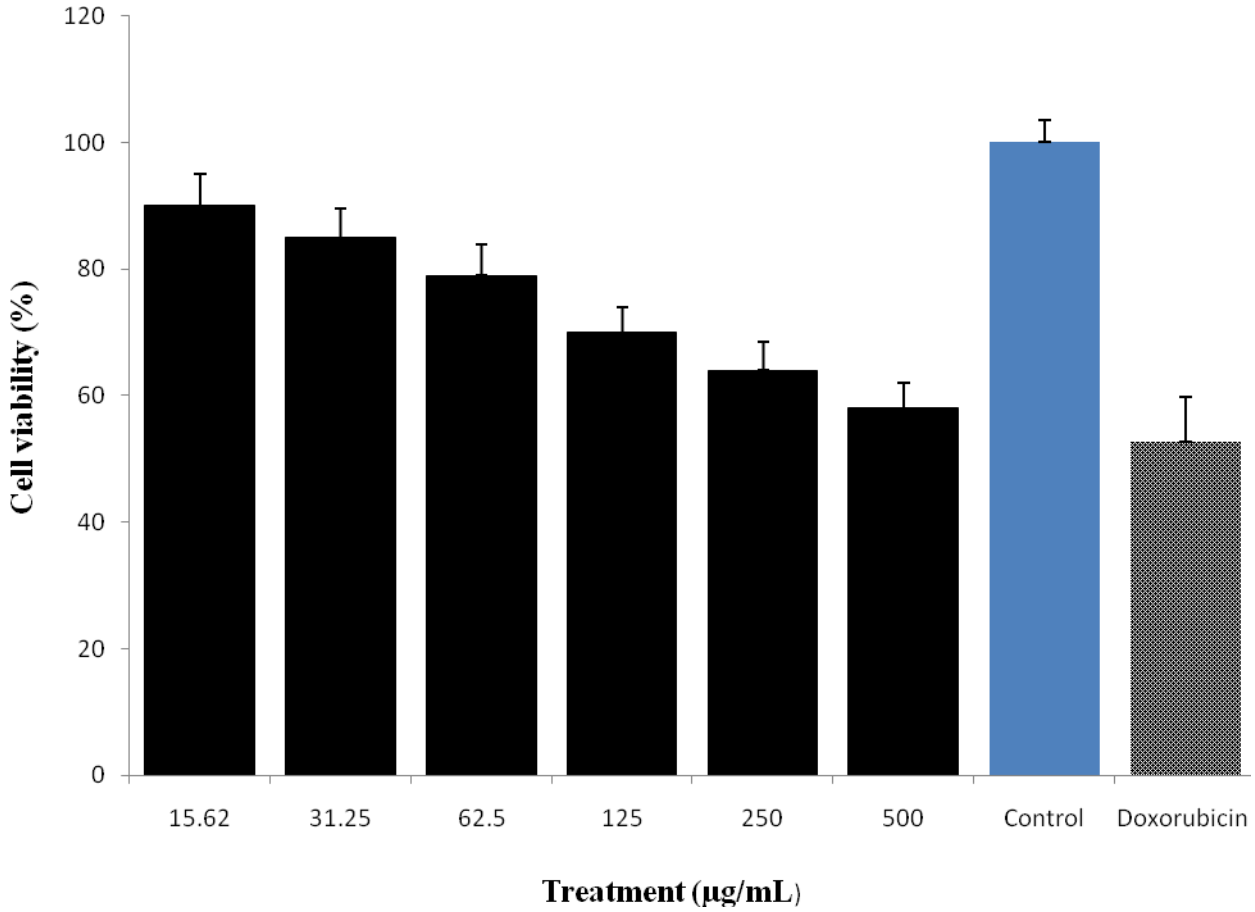

Figure 2: Sulphorhodamine B viability assay of HepG2 cells treated with methanol extracts from *Rosa canina* (RC) fruits. Confluent cultures were treated for 48 h prior to SRB assay. Data are expressed as % of control ± SD (n=6). Doxorubicin (0.5mg/L) was used as postive control.

Table 1: Antibacterial activities of methanol crude extracts from *Rosa canina* fruits against MRSA and MDR bacterial strains

	MIC of plant extracts/ antimicrobial agents (mg/L)				
Bacteria	RC	Ciprox	Tet	Norx	Ery
Proteus vulgaris (10330)	512	32			
Klebsiella pneumonia (342)	>512	≤0.03			
Pseudomonas aeruginosa (10662)	256	≤0.03			
Escherichia coli (NCTC 10418)	>512	≤0.06			
EMRSA-15	256		0.25		
EMRSA-16	512		0.25		
¶XU212	>512		16		
¶SA1199B	>512			32	
*ATCC25923	>512			0.5	
¶RN4220	>512				32

Norx stands for Norfloxacin; Tet: Tetracycline; Ciprox; Ciprofloxacin and Ery; Erythromycin. RC; *Rosa canina*.¶ denotes bacteria with efflux protein transporter, SA-1199B possesses the NorA efflux transport protein, RN4220 (MsrA) XU212 (TetK), EMRSA-15 and -16 (mecA) and*ATCC25923 a standard laboratory strain.

Table 2: Minimal inhibitory concentrations (mg/L) of selected antimicrobial agents in the presence and absence of plant extracts (MIC/4) and reserpine (20mg/L)

Treatment	EMRSA-16 (mecA)	SA1199B (norA)	ATCC25923	XU212 (TetK)
Norfloxacin	128	32	0.5	-
Tetracycline	-	-	-	128
RC + Norfloxacin	128(0)[§]	64	0.5(0)[§]	-
Reserpine + Norfloxacin	128(4)[§]	4(8)[§]	0.125(4)[§]	-
RC + Tetracycline	-	-	-	2(64)[§]
Reserpine + Tetracycline	-	-	-	32(4)[§]

RC, *Rosa canina*; Norfloxacin; Tetracycline and Norfloxacin were used as positive control. [§] denotes potentiation values