Effects of selected Taiwanese endemic plants on anti-lipid peroxidation and antibacterial activities against clinically isolated extended-spectrum-β-lactamase producing Klebsiella pneumoniae and Escherichia coli

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Research Article

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Abstract

It has been a challenge for many clinicians to treat a complicated extended-spectrum-β-lactamase (ESBL) producing *Klebsiella Pneumoniae* (*Kp*) and *Escherichia coli (E. coli)* infection due to widespread antibiotic abuse with renal damage as one of its common side effects. Therefore, this study aimed to assess the antibacterial activity of extracts from several Taiwanese folk medicinal plants against ESBL- *Kp* and *E. coli*. with renal protecting ability against lipid peroxidation (LPO) on mice kidney mitochondria. Preliminary antibacterial activities of ethanol extracts from twenty (20) Taiwanese folk medicinal plants were measured by agar-dilution method against standard *ESBL strains of E. coli* (ATCC 25922, ATCC 35218) and *Kp* (ATCC 23856, ATCC 700603). *Rhus semialata* var. *roxburghiana* DC. (RSR) exerted the most inhibitory effect and then further extracted with *n*-hexane, ethyl acetate, acetone, ethanol, and water, respectively. Each extract also evaluated against the four standard ATCC microorganisms. Their MIC_{50}, $MIC_{90,}$ and time kill assay were adapted with detecting the maximum inhibitory activities and the antibacterial spectrum range of each extract was measured against twenty-four (24) kinds of microbes. Which were used including gram-positive, gram-negative bacteria and fungus by agar dilution method. Finally, renal protective ability was detected inhibitory effect of ferrous induced lipid peroxidation on mice mitochondria. Among 20 Taiwanese folk medicinal plants tested, *Rhus semialata* var. *roxburghiana* DC. (RSR) exhibited maximum inhibition against clinical ESBL-producing *Kp* and *E. coli* strains with acetone extracts showing MIC_{50}/MIC_{90} values at 1000 µg/mL, the course of antimicrobial action was bacteriostatic and with inhibitions to all 24 kinds of microbial including Gram positive and negative bacteria and fungi. Furthermore, result of thiobarbituric acid reactive substances (TBARS) assay from this extract showed high lipid peroxidative (LPO) protective capability on mice kidney mitochondria (IC_{50}: 29.29 ± 0.35µg/mL). RSR acetone extract, with its maximum activity against clinical isolated ESBL-producing *Kp* and *E. coli*, antimicrobial effect against other wide spectral range bacteria and relatively high LPO protective ability on mice kidney mitochondria, is a potential source, albeit further studies have yet to be conducted, to develop an antimicrobial drug against ESBL-*Kp* and *E. coli*.

Introduction

Medicinal plants have been the major source of new medicines and offer an alternative to usual drugs (Tepe at al., 2004). As the primary source for human treatment, World Health Organization (WHO) reported that approximately 80% of the world population relies on plants or derived products (WHO, 1993). In case of infectious diseases, one of the reasons of unsuccessful treatment could be attributed to increasing number of drug-resistant bacteria (Al-Mariri and Safi, 2014). This indeed is associated with the potencies of existing antibiotics are decreasing steadily (Shahghasi et al., 2004). Since multidrug resistance of microorganisms is a major medical concern, screening of natural products in search for new antimicrobial agents is the need of the hour (Zgoda and Porter, 2001).

Treatment of complicated extended-spectrum-β-lactamase (ESBL) *Klebsiella Pneumoniae* (*Kp*) and *Escherichia coli* infection is a challenging for clinicians due to the antibiotic abuse (Hoban et al., 2001)

(Sah et al., 2019)(Tan et al., 2020). ESBL-producing bacteria is able to hydrolyze third-generation cephalosporin, aminoglycoside and fluoroquinolone (Harish et al., 2007). *Kp* and *E. coli* are normal flora in human intestine. *Kp* is a frequent agent causing nosocomial or community-acquired bacteraemia as well as pneumonia, pyogenic brain abscess, meningitis, and pyogenic liver abscesses (Tsai et al., 2010) (Hui et al., 2007). ESBL- *E. coli* urinary tract infection is increasing, and in more serious cases, the bacteria enter the bloodstream and cause toxemia. (Rodríguez-Baño et al., 2004). Several studies have been reported that utilizes antibacterial properties of plant extracts against *Kp* and *E. coli* (Sharmeen et al., 2012)(Shaik et al., 2014)(Haroun and Al-Kayali, 2016). Moreover, renal damage is one of the side effects of antibiotics therapy with increasing possibility leading to acute renal failure in newborn (Fanos and Cataldi, 1999). In Taiwan, the risk of end-stage renal disease (ESRD) increased among those that reported regular use of antibiotics and has become another public health problem shouldered by the health insurances on medicinal care (Tsai et al., 2009).

Twenty (20) plant extracts, some are endemic in Taiwan, were collected with extracts have been traditionally employed in Taiwan as detoxicant. This study aimed to investigate these Taiwanese folk medicinal plants for their antimicrobial activities that can be utilized for the development of potential antimicrobial agent against the antibiotic-resistant bacteria -ESBL-producing *Kp* and *E. coli* with kidney protective activity.

Material And Method

Herbal Materials

Twenty (20) kinds of dried Taiwanese folk medicinal plants with antitoxic ability were purchased from the crude drug market in Taipei, Taiwan: *Amaranthus viridis* Linn., *Angelica dahurica* var. *formosana* Yen, *Bidens pilosa* L. var. *minor* (blume) Sherff, *Broussonetia papyrifera* (Linn) L'Herit. Ex Vent., *Centella asiatica* L., *Equisetum ramosissimum* subsp..*debile* (Roxb.) Hauke., *Euphorbia hirta* L., *Euphorbia thymifolia* L., *Eupatorium formosanum* Hay., *Kyllingia brevifolia* Rottb., *Litsea cubeba* (Lour.) Pers., *Persoon Actinodaphne* citrata (Blume) Hayata, *Lygodium japonicum* (Thunb.) Sw., *Pinellia ternate* (Thunb.) Breit., *Plantago asiatica* L., *Polygonum perfoliuatum* Linn., *Portulaca oleracea* var. *sativa* DC., *Pteris multifida* Poir., *Rhus semialata var. roxburghiana* DC., *Serissa japonica* (Thunb.) Thunb., *Urena lobata* L.. All were identified by Prof. Ling-Ling Yang and the specimens were stored at the Department of Pharmacognosy, Taipei Medical University.

Microorganisms

Twenty-eight (28) kinds of standard microorganism were purchased from Food Industry Research and Development Institute (FIRDI) in Taiwan. Standard strains include *Klebsiella Pneumoniae* ATCC 23856, (ESBL) ATCC 700603, *Escherichia coli* ATCC 25922, ATCC 35218, *Staphylococcus. aureus* (MSSA) ATCC 29213, (MRSA) 85/2082, (hVISA) Mu3, (VISA) Mu50, *Staphylococcus epidermidis* ATCC 12228, Group B *streptococcus* ATCC 12401, *Enterococcus faecalis* ATCC 29212, ATCC 29212, *Pseudomonas aeruginosa* ATCC 14207, ATCC 27853, *Samonella enteric Typhimurium* ATCC 13076, *Samonella multivorum* ATCC

35656, *Samonella typhimurium* ATCC 13311, *Samonella paratyphi* (A) ATCC 9150, *Samonella anatum* (E) ATCC 9270, *Samonella choleraesusis* ATCC 10744, ATCC 115462, *Shigella boydii* ATCC 9207, *Shigella dysenteriae* ATCC 13983, *Shigella flexneri* ATCC 10772, *Shigella sonnei* ATCC 15965, ATCC 25931, *Candida albicans* ATCC 90018, *Candida parapsilosis* ATCC 22019. While the thirty-three (33) clinical strains were isolated and collected from Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan, which labeled as *Klebsiella Pneumoniae* (ESBL) 1520914, 1527226, 1527920, 1520160-1, 1521656, 1522657, 1523941, 1524581, 1526783, 1543407, 1541487, 1542299, 1545756, 80, 81, 91, 98, and *Escherichia coli* (ESBL) 1526829, 1522652, 1530404, 1521949, 1521624, 1521569, 1530090, 1520331, 1520641, 1522991, 1522953, 1519678, 1520264, 1527958, 1528051, 1527082).

Chemicals and reagents

All chemicals and reagents were analytical grade including dimethyl sulfoxide (DMSO), gallic acid, Folin-Ciocalteu's reagent, rutin, aluminum chloride, epicatechin, ferrous chloride ($FeCl_2$), trolox, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), bovine serum albumin, phosphoric acid, methanol, sodium hydroxide (NaOH) and 4-dimethylaminocinnamaldehyde (DMACA) were purchased from Sigma Chemical (St. Louis, MO).

Animals

Imprinting Control Region (ICR) mice were purchased from BioLASCO Taiwan Co., Ltd., housed in plastic cages in a temperature and humidity-controlled environment and bred at the Experimental Animal Center of Taipei Medical University. All experiments were approved and performed in accordance with the guidelines for Experiments Animal Center of Taipei Medical University and the Laboratory Animal Ethics Committee of Taipei Medical University with the guiding principles for the care and use of laboratory animals approved by the Chinese Society of Laboratory Animal Sciences, Taiwan (No. LAC980119). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Extraction and sample solution preparation

Dried plants were washed continuously with tap water to remove impurities, cut into small pieces and then dried in the air circulating oven at 60℃ for 48 hr. Each 100 g of dried material was extracted twice with 1000 mL of ethanol, filtered, and combined the filtrates, then concentration and removed the solvent *in vacuo* by vacuum rotary evaporator (Eyela CCA-1111) to 50mL. The concentrated extract solutions were dried by lyophilizer (Eyela FDU-1200) to obtain the extract. The preliminary antimicrobial activity to standard strains of *Kp* and *E. coli* ATCC bacteria of each extract was identified to find RSR the most potent extract from the 20 Taiwanese endemic plants. Therefore, RSR was further extracted by *n*-hexane, ethyl acetate, acetone, ethanol and water, respectively. Each crude extract (20 mg) was dissolved in 1 mL DMSO as a stock solution and serial dilution with PBS for the following assays. (DMSO <0.05%).

Protocol of an antimicrobial agent development from natural plants source procedure

Five (5) steps were carried out to determine the most potent extract with antibacterial capability against *Kp* and *E. coli* and renal damage protective effect (Figure 1). Agar-dilution method was used to preliminary screening assess the antibacterial activity of crude extracts from 20 Taiwanese plants against standard strains of *Kp* and *E. coli* ATCC bacteria (*E. coli* ATCC 25922, ATCC 35218 and *Kp* ATCC 23856, ATCC 700603). RSR crude extract exhibited the maximum inhibitory effects and then subjected to further extract by *n*-hexane, ethyl acetate, acetone, ethanol and water, respectively. Each of these extracts was prepared as mentioned previously prior to antimicrobial assay against the same 4 bacteria by agar-dilution method.

The most potential extract of RSR (acetone extract) was chosen to evaluate the MIC_{50} and MIC_{90} against clinical *Kp* and *E. coli.* Time kill assay against *Kp* (ATCC) 23856, (ATCC) 700603, *E. coli* (ATCC) 25922**,** (ATCC) 35218, and clinical ESBL-*Kp* 1520914 and ESBL- *E. coli* 1526829 were also conducted.

To assess the antibacterial spectral range of the extract, agar-dilution method was performed against 24 kinds of microbes including Gram positive, gram negative bacteria and fungi. Finally, the renal protective capability was measured by ferrous ion-induced lipid peroxidation (LPO) on mice mitochondria.

Agar-dilution method

Agar-dilution method described by Nauman and Arshad (2011) was used with some modification. Briefly, extracts were added in appropriate amounts of 20 mL Mueller-Hinton (MH) agar to yield a two-fold serial dilution. The bacteria were plated onto the MH-agar surface (10^4 CFU per 0.3 µL per spot) by an A400 Multipoint Inoculators (Jencons Co. UK) and incubated at 37°C for 24 hours. MIC value was evaluated as the lowest concentration (µg/mL) of antimicrobial agent with no visible growth. MIC_{50} and MIC_{90} were detected with the concentration of antibacterial agent preventing 50% and 90% of colony formation of the strains tested.

Time-kill assay

Method as described by Darah et al. (2013) was used with some modification. Briefly, exponential-phase ESBL-producing *Kp* (ATCC) 23856, (ATCC) 700603, *E. coli* (ATCC) 25922, (ATCC) 35218, and clinical ESBL-*Kp* 1520914 as well as ESBL- *E. coli* 1526829 were diluted to 4×10^4 to 1×10^5 CUF/ mL and then exposed to RSR acetone extract (1, 2 and 4 x MIC), 1 mL DMSO, polymyxin B sulfate salt (PB) (0.5 and 1 x MIC) and 1 x MIC RSR acetone extract combined with 0.5 x MIC PB, respectively, incubated for 48 hours period in a total volume of 2 mL of MH media. Viable cell count was determined by agar plating (onto MH agar). Bactericidal activity was defined as > 3 log decrease in cell counts. For all strains in the present report, time kill assays were performed at least twice independently with similar results.

Inhibitory capability of LPO on mice kidney mitochondria

Preparation of mice kidney mitochondria and protein content quantitation

Method described by Lin et al. (2013) was used with some modification. Male ICR mice (4~6 weeks) were sacrificed by carbon dioxide and the kidneys were removed as soon as possible and perfuse with ice-cold PBS (0.1 M, pH 7.4) prior to homogenizing in a Potter Elvehjem homogenizer. The homogenate was suspended in PBS and centrifuged at 2000 rpm for 10 min at 4°C to separate the nuclear debris. The clear suspensions were re-centrifuged at 13000 rpm for 10 min at 4°C to obtain mitochondrial fraction and then suspended in PBS. Different concentrations of mitochondrial suspended solution were pipetted into 1.5 mL eppendorf tubes, and total volume was adjusted to 50 µL with PBS. Protein reagent was added and mixed with a vortex-mixer before measurement of absorbance at 595 nm by ELISA spectrophotometer (Synergy H4 Hybrid Reader). Quantitative analysis of the protein content was determined from bovine serum albumin standard curve.

Inhibitory capability of RSR (*Rhus semialata* var. *roxburghiana*) acetone extract on ferrous chloride induced LPO production in mice kidney mitochondria

The LPO inhibitory activity was determined by thiobarbituric acid reactive substances (TBARS) assay by quantitatively measure the $MDA(TBA)_2$ products using ELISA reader (Synergy H4 Hybrid Reader). The reaction mixture solution (total volume of 500 µL) containing 100 µL of kidney mitochondria, 200 µL of PBS buffer, 100 µL of an $FeCl_2$ solution (4 mM), and 100 µL of RSR acetone extract or positive control (trolox or gallic acid) was incubated at 37 °C for 1 hour, then centrifuged at 4000 rpm for 10 min. A 375 µL of H_3PO_4, 200 µL of distilled water, and 125 µL of TBA were added and the mixture was further incubated at 90 °C for 66 min prior to place in an ice bath to stop the reaction. Then, 350 µL of methanol-NaOH (9.1: 0.9 v/v) was added, mixed and absorbance of $MDA(TBA)_2$ product was measured at 532 nm. The data was recorded and the inhibition of LPO calculated according to the following formula:

Inhibition percentage = $[(A_{\text{control (532 nm)}} - A_{\text{sample (532 nm)}}) / (A_{\text{control (532 nm)}} - A_{\text{blank (532 nm)}})] \times 100\%$ while IC_{50} was calculated by a linear regression analysis, and results were expressed as mean ± SD.

Results And Discussion

Infectious diseases caused serious threats to the existence, health and survival of humans (Yamac and Bilgili, 2006). With the growing demands of search and development of new antibacterial agent against the rapid rise of antibiotic-resistant strain worldwide, studies are currently focusing on natural sources due to its efficacy, safety and cheaper nature. Medicinal plants have long been used as a source of antibacterial compounds due to its vast number and diversity of secondary metabolites. In this study, 20 Taiwanese folk medicinal plants traditionally used as detoxicant and some are endemic, were screened for antimicrobial properties against the antibiotic-resistant bacteria -ESBL-producing *Kp* and *E. coli* with kidney protective activity.

Preliminary screening of the ethanol extracts in DMSO from 20 Taiwanese plants collected against two *Kp* and *E. coli* bacteria strains showed that only *Rhus semialata* var. *roxburghiana* DC. (RSR) gave a positive inhibition against *Kp* (ATCC 23856) strain but negative to other *Kp* (ATCC 700603) strain as well as *E. coli* (ATCC 25922 and ATCC 35218) in the preliminary screening whereas the rest of the studied extracts were all have negative inhibitions (Table 1). These results were in contrast to some previous reports from other plants screened having antimicrobial properties against other microorganisms (Baruah et al., 1994)(Williamson, 2002)(Hellión-Ibarrola et al., 2012)(Shu et al., 2012)(Narasimhulu And Mohamed, 2014)(Babu et al., 2015)(Li, 2006)(Zhao et al., 2016)(Subba et al., 2016)(Syed et al., 2016). *Klebsiella spp* are known opportunistic pathogens naturally present in soil, water and vegetables that causes serious infections in human especially since this specie is becoming increasingly antibiotic-resistant, so that many are now labeled as "Multidrug-resistant (MDR) *Klebshiella spp*. (Yan et al., 2001)(Ktari et al., 2006) (Wei et al., 2008).

More recent studies focused on extraction of phytochemical constituents using different solvents of variable polarities to analyze their pharmacological properties. Hence, extracts from RSR where further partitioned in different solvents to obtain the crude extracts with *n*-hexane, ethyl acetate, acetone, ethanol and water, respectively, and the same antibacterial tests were applied using the same four microorganisms from preliminary screening. Results showed that all RSR extracts partitioned in solvents except in ethanol, showed inhibitions against the two *Kp* (ATCC 23856 and 700603) strains with high MIC value observed in the acetone extracts (500 µg/mL) against *Kp* (ATCC) 23856 (Table 2). Similarly, EA and acetone extracts gave positive inhibitions against *E. coli* (ATCC 25922 and ATCC 35218) except *n*-hexane extract, which gave negative inhibitions. In addition, both ethanol and water extracts exhibited negative inhibitions against all *E. coli* strain used. These maybe associated with the polar constituents present in the RSR acetone extracts such as phenolics that are often related to antioxidant properties. Anokwuru et al. (2013) reported that acetone is highly effective at extracting biomolecules that are antioxidant. Although there is no direct correlations between antimicrobial and antioxidant activities, many studies on plant extracts reported that biomolecules are antioxidant and antimicrobial too but further studies have yet to be conducted on these extract to validate this relationship.

Since acetone extract from RSR gave a good inhibition against the four *Kp* and *E. coli* strains, the same extract was used to evaluate its property against seventeen (17) and sixteen (16) clinically isolated *Kp* and *E. coli* strain, respectively. Results showed that acetone extract of RSR exhibited positive inhibitions against all thirty-three (33) clinically isolated *Kp* and *E. coli* strains with MIC values ranging from 500 to 1000 µg/mL (Table 3). No inhibitions were observed in DMSO against all the strains used.

Time-kill assay was used to assess the mode of action of RSR against the tested *Kp* and *E. coli* strains. Although time consuming, the time-kill assay provides a dynamic picture of antibiotic action over time (Perez et al., 2016). In this study, the time-kill kinetics of RSR acetone extract against *Kp* (ATCC) 23856 and 700603 showed a gradual increased in the number of viable cells in a dose-dependent manner, as well as when combined with polymyxin B sulfate salt (PB) (0.5X MIC) at 1X MIC. However, against clinical ESBL-*Kp* 1520914, almost same number of viable cells was observed at 4X MIC over the

first 6 to 12 hours followed by a gradual rise up to 24 hour (Figure 2). Similar trends have been observed in the time-kill kinetics of RSR acetone extract against *E. coli* (ATCC) 25922 as well as when combined with polymyxin B sulfate salt (PB) (0.5X MIC) at 1X MIC. However, against (ATCC) 35218 and clinical ESBL-*E. coli* 1526829, almost the same number of viable cells was observed at 4X MIC over the first 6 hours followed by a gradual rise up at 12 hours (Figure 3). Overall, time-kill assay suggest that RSR acetone extract demonstrated a slight bacteriostatic action with strain-selectivity at relatively high MIC (4X MIC).

Disrupted balance between essentiality and toxicity of iron as an important trace element in the body is known to induce oxidative stress (OS) via Fenton reaction (Fischer et al., 2002)(Jomova and Valko, 2011) (Pari et al., 2015). Measurement of lipid peroxidase product, MDA, as the end product of poly-unsaturated fatty acid peroxidation and a marker of free radical mediated LPO injury have been applied to detect renal injury and its levels were found to significantly increase in kidney after treatment of ferrous ion indicating an increased LPO activity. In this study, LPO induced by ferrous ion on mice kidney mitochondria was significantly affected by the addition of RSR acetone extract, gallic acid and trolox in a dose-dependent manner (Table 4). However, IC_{50} value of the extract (29.29**±**0.35 µg/mL) is only comparable to gallic acid (27.07±6.61 µg/mL) than to trolox (4.44**±**0.23 µg/mL).

Antimicrobial properties of RSR acetone extract were assessed over a wide range of bacterial spectrum and fungi. Results showed that RSR acetone extract has inhibitions to all the microorganisms used (Table 5). However, positive inhibition was also observed from DMSO against *Samonella multivorum, Candida albicans* and *Candida parapsilosis* suggesting that the inhibitions observed from RSR acetone extract against these microorganisms were not purely attributed from the extract alone.

Conclusions

Out of 20 Taiwanese folk medicinal plants tested, only RSR exhibited a positive inhibition in the preliminary antimicrobial screening. RSR acetone extract, with its maximum activity against clinical isolated ESBL-producing *Kp* and *E. coli*, antimicrobial effect against other wide spectral range bacteria and relatively high LPO protective ability on mice kidney mitochondria, is a potential source, albeit further studies have yet to be conducted to isolate and purify the agents or molecules from this extract which is responsible for the antimicrobial properties and may serve as potential antibiotics against ESBL-*Kp* and *E. coli*.

Abbreviations

Extended-spectrum-β-lactamase (ESBL)

Klebsiella pneumoniae (*Kp*)

Minimum inhibitory concentration (MIC)

American type culture collection (ATCC)

Methicillin-sensitive *Staphylococcus aureus* (*MSSA)*

Heterogeneously vancomycin-intermediate *Staphylococcus aureus* (*hVISA)*

Methicillin-resistant Staphylococcus aureus (*MRSA*)

Vancomycin intermediate *Staphylococcus aureus* (VISA)

Vancomycin-resistant *enterococci* (VRE)

Mueller-Hinton agar (MH)

Mycorrhiza helper bacteria (MHB)

Lipid peroxidation (LPO)

Malondialdehyde (MDA)

Thiobarbituric acid (TBA)

Thiobarbituric acid reactive substances (TBARS)

Rhus semialata var. *roxburghiana* (RSR)

Declarations

Conflicts of Interest

All the authors report no conflicts of interest.

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Tables

Table 1. Antibacterial effect of extracts from 20 Taiwanese plants

Plant Extracts / Control (+ and -)	MIC (μg/ml)			
	***Kp* (ATCC) 23856**	***Kp* (ATCC) 700603**	***E. coli* (ATCC) 25922**	***E. coli* (ATCC) 35218**
Polymyxin B sulfate salt	≦1	≦1	≦1	≦1
Gentamycin	≦4	≦4	≦4	16
0.5% DMSO in PBS	-	-	-	-
Amaranthus viridis Linn.	-	-	-	-
Angelica dahurica var. *formosana* Yen	-	-	-	-
Bidens pilosa L. var. *minor* (blume) Sherff	-	-	-	-
Broussonetia papyrifera (Linn) L'Herit. Ex Vent.	-	-	-	-
Centella asiatica L.	-	-	-	-
Equisetum ramosissimum subsp..*debile* (Roxb.) Hauke.	-	-	-	-
Euphorbia hirta L.	-	-	-	-
Euphorbia thymifolia L.	-	-	-	-
Eupatorium formosanum Hay.	-	-	-	-
Kyllingia brevifolia Rottb.	-	-	-	-
Litsea cubeba (Lour.Pers. *Persoon Actinodaphne* citrata (Blume) Hayata	-	-	-	-
Lygodium japonicum (Thunb.) Sw.	-	-	-	-
Pinellia ternate (Thunb.) Breit.	-	-	-	-
Plantago asiatica L.	-	-	-	-
Polygonum perfoliuatum Linn.	-	-	-	-
Portulaca oleracea var. *sativa* DC.	-	-	-	-
Pteris multifida Poir.	-	-	-	-
Rhus semialata var. roxburghiana DC.	+	-	-	-
Serissa japonica (Thunb.) Thunb.	-	-	-	-
Urena lobata L.	-	-	-	-

+: inhibition to bacteria growth; **-** : no inhibition to bacteria growth

Table 2. Minimum Inhibitory Concentrations (MICs) of each RSR extracts (μg/mL)

Extract	*n*-hexane	EA	Acetone	Ethanol	H_2O	Acetone	EA
						MIC (μg/ml)	
Kp (ATCC) 23856	+	+	+	+	+	500	250
Kp (ATCC) 700603	+	+	+	-	+	1000	1000
E. coli (ATCC) 25922,	+	+	+	-	-	1000	1000
E. coli (ATCC) 35218	-	+	+	-	-	1000	>1000

+: inhibition to bacteria growth; **-** : no inhibition to bacteria growth

Table 3. Minimum inhibitory concentration (**MIC,** μg/ml) of RSR acetone extract and positive antibiotis against standard strains and clinically isolated *Kp* and *E.coli strains*

	Control			Acetone Extract	
	DMSO	polymyxin B sulfate salt	Vancomycin	Gentamycin	
Kp		**MIC** (μg/ml)			**MIC** (μg/ml)
*ATCC 700603	-	1	>16	>16	+	1000
*ATCC 23856	-	0.25	>16	≦0.5	+	500
1520914	-	0.5	>16	64	+	1000
1527226	-	1	>16	128	+	750
1527920	-	0.5	>16	64	+	1000
1520160-1	-	0.5	>16	128	+	1000
1521656	-	0.5	>16	1	+	750
1522657	-	0.5	>16	32	+	1000
1523941	-	0.5	>16	128	+	1000
1524581	-	0.5	>16	>128	+	1000
1526783	-	0.5	>16	64	+	1000
1543407	-	0.5	>16	64	+	1000
1541487	-	>16	>16	>128	+	>1000
1542299	-	0.5	>16	≦0.5	+	>1000
1545756	-	0.5	>16	≦0.5	+	>1000
80	-	0.5	>16	>128	+	750
81	-	0.25	>16	>128	+	1000
91	-	0.5	>16	64	+	750
98	-	0.5	>16	16	+	500
E. coli						
*ATCC 25922	-	0.5	>16	1	+	1000
*ATCC 35218	-	0.25	>16	1	+	1000
1526829	-	0.25	>16	>128	+	1000
1522652	-	0.25	>16	2	+	750
1530404	-	0.25	>16	>128	+	750
1521949	-	0.25	>16	>128	+	750
1521624	-	≦0.125	>16	>128	+	1000
1521569	-	0.25	>16	128	+	1000
1530090	-	0.25	>16	1	+	1000
1520331	-	0.25	>16	64	+	750
1520641	-	0.25	>16	2	+	750
1522991	-	0.25	>16	128	+	1000
1522953	-	0.25	>16	1	+	500
1519678	-	0.25	>16	1	+	750
1520264	-	0.25	>16	64	+	500
1527958	-	0.25	>16	1	+	1000
1528051	-	0.25	>16	1	+	500
1527082	-	0.25	>16	1	+	1000

*** Antibacterial activity to standard strain**

***Kp*- MIC_{50}: 1000μg/ml ; MIC_{90}: >1000μg/ml; *E.coli*- MIC_{50}: 750μg/ml; MIC_{90}: 1000μg/ml**

+: inhibition to bacteria growth; -: no inhibition to bacteria growth

Table 4. Inhibitory effect IC_{50} (µg/mL) of RSR acetone extract to ferrous ion-induced LPO on mice kidney mitochondria

	IC_{50} (µg/ml)
RSR acetone extract	29.29±0.35
Gallic acid	27.07±6.61
Trolox	4.44±0.23

Positive control agents are Gallic acid and Trolox

Table 5- Antibacterial spectrum range of RSR acetone extract against other 24 kinds of microbial strains

Figures

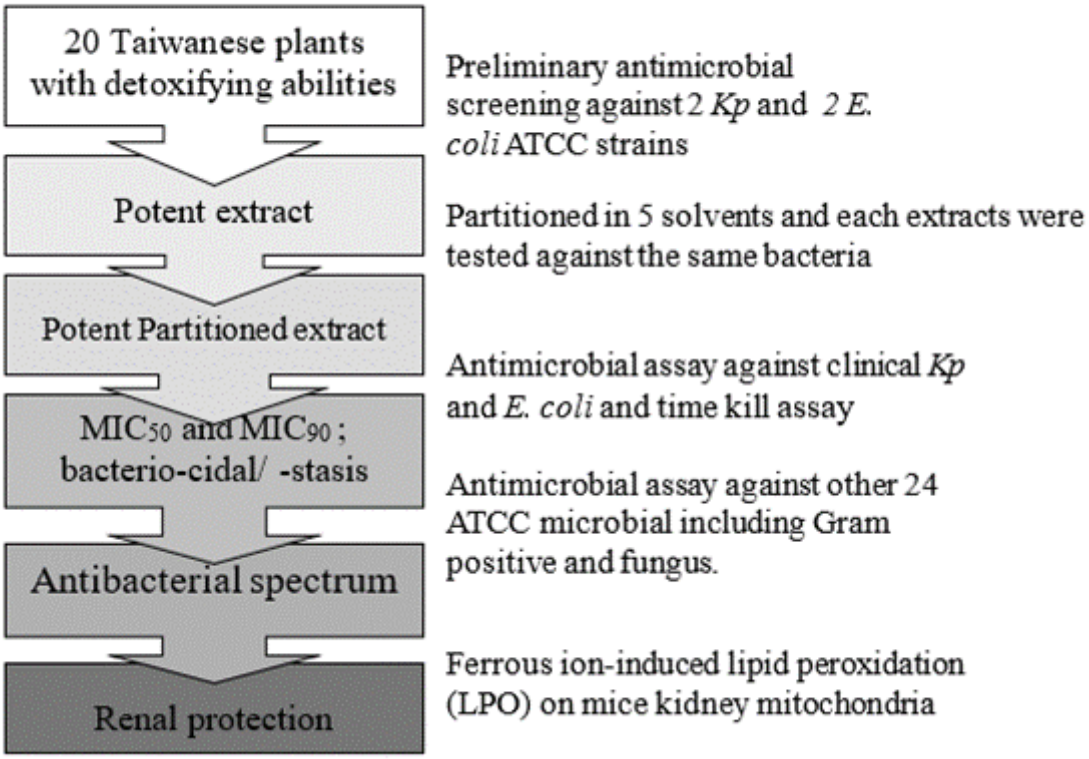

Figure 1

The flow chart of antibacterial capability against Kp and E. coli detection procedure and renal damage protective effect.

Bacteria	Strain	+/- Bacterium or fungus	DMSO	polymyxin B sulfate salt	Gentamycin	RSR Acetone extract
				MIC (μg/ml)		
MSSA	ATCC 29213	(+)	-	>16	≦4	+
MRSA	85/2082	(+)	-	>16	≦4	+
hVISA	Mu3	(+)	-	>16	>128	+
VISA	Mu50	(+)	-	>16	>128	+
Staphylococcus epidermidis	ATCC 12228	(+)	-	8	≦4	+
Group B *streptococcus*	ATCC 12401	(+)	-	>16	128	+
Enterococcus faecalis	ATCC 29212	(+)	-	>16	16	+
Enterococcus faecalis	ATCC 51299	(+)	-	>16	>128	+
Pseudomonas aeruginosa	ATCC 14207	(-)	-	≦1	≦4	+
P. aeruginosa	ATCC 27853	(-)	-	2	≦4	+
Samonella enteric Typhimurium	ATCC 13076	(-)	-	≦1	≦4	+
Samonella multivorum	ATCC 35656	(-)	+	>16	64	+
Samonella typhimurium	ATCC 13311	(-)	-	≦1	≦4	+
Samonella paratyphi (A)	ATCC 9150	(-)	-	≦1	≦4	+
Samonella anatum (E)	ATCC 9270	(-)	-	≦1	≦4	+
Samonella choleraesusis	ATCC 10744	(-)	-	≦1	≦4	+
Samonella choleraesusis	ATCC 115462	(-)	-	≦1	≦4	+
Shigella boydii	ATCC 9207	(-)	-	>16	>128	+
Shigella dysenteriae	ATCC 13983	(-)	-	≦1	≦4	+
Shigella flexneri	ATCC 10772	(-)	-	≦1	≦4	+
Shigella sonnei	ATCC 15965	(-)	-	≦1	≦4	+
Shigella sonnei	ATCC 25931	(-)	-	≦1	≦4	+
Candida albicans	ATCC 90018	fungus	+	>16	128	+
Candida parapsilosis	ATCC 22019	fungus	+	>16	>128	+

+: inhibition to bacteria growth; **-** : no inhibition to bacteria growth

, Control:0.5%DMSO and Positive control agents :polymyxin B sulfate salt, gentamycin

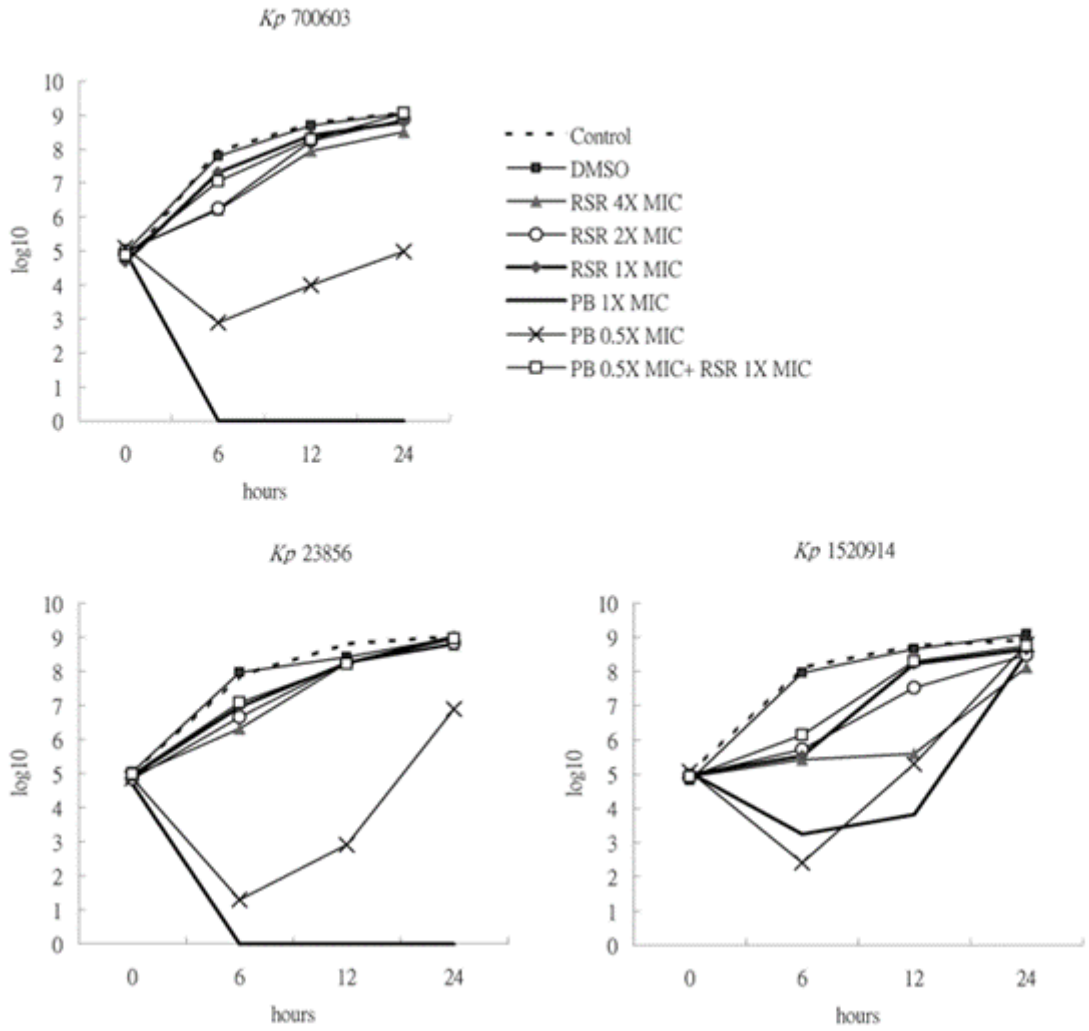

Figure 2

Time-kill kinetics of RSR acetone extract, 1 mL DMSO (- control) and polymyxin B sulfate salt (PB) (+ control) against Kp (ATCC) 700603, (ATCC) 23856 and clinical ESBL-Kp 1520914

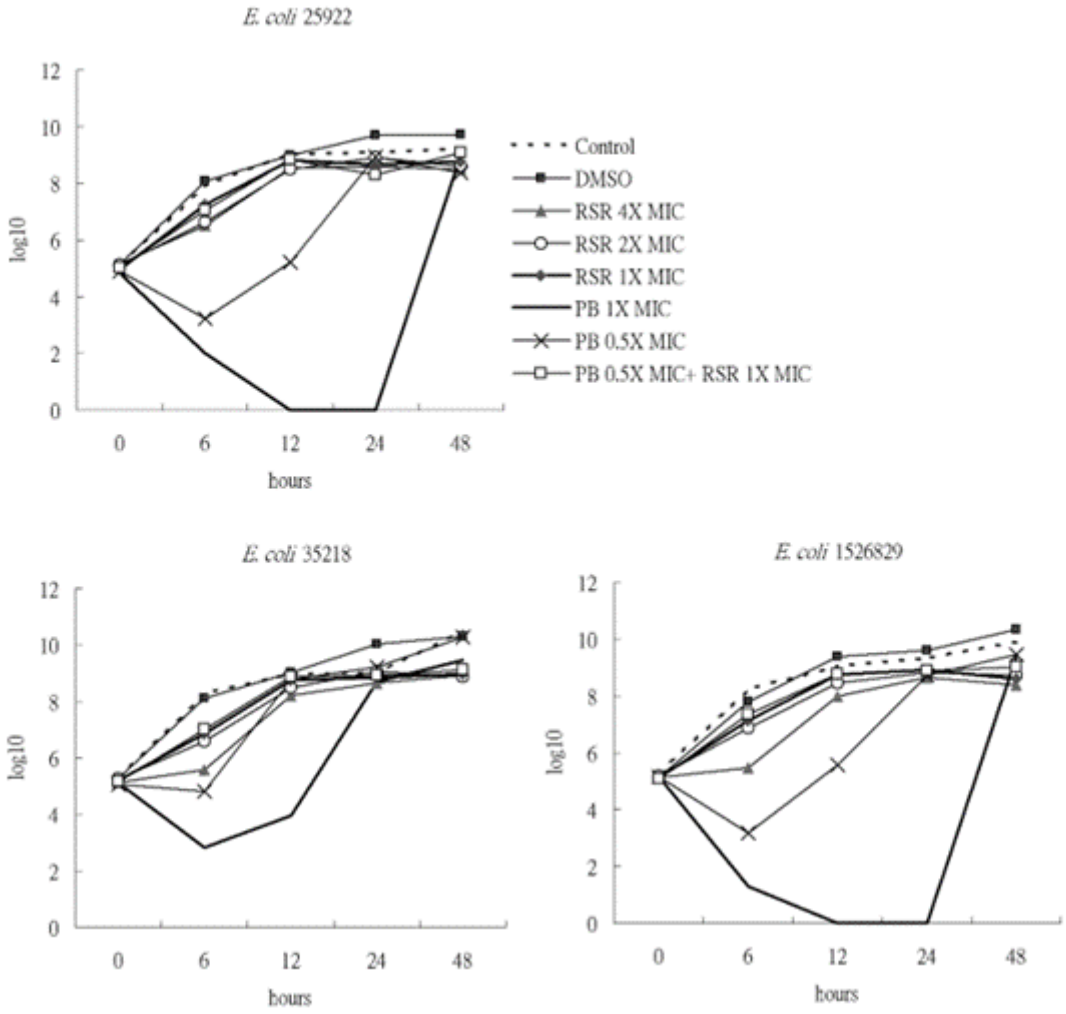

Figure 3

Time-kill kinetics of RSR acetone extract, 1 mL DMSO (control) and polymyxin B sulfate salt (PB) against E. coli (ATCC) 25922, (ATCC) 35218, and clinical ESBL- E. coli 1526829