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Antioxidant and Antibacterial Activities against Food Pathogenic and Spoilage Bacteria by *Hibicus Sabdariffa* L. (Roselle) Extract

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Abstract Recently, natural ingredients from herb or herbal extract have received increasing attention as sources of natural antioxidants and food nutrients and additives. In this study, the antioxidant activities of the ethanol extract of *Hibicus sabdariffa* L. (roselle)'s calyx was screened for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, reducing power, anti-lipid peroxidation ability, and total phenolic content. The results showed that the extracts' half maximal inhibitory concentration (IC_{50}) were 932.25 mg/L for DPPH radical-scavenging activitiy and 217.07 mg/L for anti-lipid peroxidation. Moreover, the extract's reducing power at half maximal effective concentration (EC_{50}) value was 937.20 mg/L. The total extract phenolic content expressed as gallic acid equivalents was 6.76 µg/g dry weight. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the roselle extract against food pathogenic and spoilage bacteria were also studied. Among all of the tested bacterial strains, *Salmonella typhimurium* TISTR 292 was found to be the most sensitive showing an MIC value of 12.5mg/ml., followed by *Staphylococcus aureus* TISTR 118, *Escherichia coli* TISTR 780, *Listeria innocua* ATCC 33090^T, *Pseudomonas fluorescens* TISTR 358, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T, and *Lactobacillus sakei* subsp. *sakei* JCM 1157^T (25 mg/ml). An MBC value of 25 mg/ml was found against *S. aureus* TISTR 118 and *L. innocua* ATCC 33090^T, while MBC values for the rest of the bacteria were found to be 50 mg/ml. The bactericidal effects of roselle that was studied by the viable count technique at different exposure times, demonstrated that *S. typhimurium* tested with 50 mg/ml extract was completely killed at 1 min and at 14 min. for *S. aureus* tested with 25 mg/ml extract. The results of the present study revealed that the roselle calyx ethanol extract presented as a source of natural antioxidant that demonstrated free radical scavenging activity, reducing power, and inhibition of lipid peroxidation, in addition to inhibit some food pathogenic and spoilage bacteria. With promising antioxidant and antibacterial properties, dried roselle calyx has potential to be developed for a natural food preservative.

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Keywords: roselle extract, pathogenic bacteria, spoilage bacteria, antioxidant activity, phenolic content

Introduction

Herbs, which are widely used as food ingredients or medicinal herbs, have received much attention in recent years as sources of natural antioxidants and are recognized as food antioxidants and nutrients in addition to health-promoting products. Plant-derived phytochemicals or antioxidants can function in binding of transition metal ion catalysts, in a reductive capacity, or in scavenging free radicals. They are of great importance in terms of protection from and therapeutics for diseases involving free radicals because they maintain a balance between free radicals and the antioxidant defenses of the body (Mc Cord, 2000; Lobo *et al*., 2010). The human body requires antioxidants from external sources, which are obtained by consuming fruits and vegetables.

Foodborne pathogens are the major cause of disease and human mortality and have become a major public health concern (Leroy *et al*., 2006; Fullerton *et al*., 2011; Khalaphallah and Soliman, 2014). Various foods and beverages can serve as source of foodborne pathogens such as *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* (Nørrung and Buncic, 2008; Verkade and Kluytmans, 2014). Natural substances such as plant extracts have been investigated to replace chemical preservatives and extend shelf-life and safety. Many antioxidative substances, including herbs for controlling pathogens or toxin-producing microorganisms in food, have been isolated from plant materials. Bajpai *et al.* (2007) reported that essential oils, methanol extracts, and various organic sub-fractions of methanol extracts of *Metasequoia glyptostroboides* exhibited great potential for antibacterial activity against *Bacillus subtilis*, *Listeria monocytogenes*, *S. aureus*, and *Pseudomonas aeruginosa*. The *Eupatorium lindleyanum* DC obtained by water decoction exhibited the maximum inhibitory effect against *S. aureus*, *B. subtilis*, *B. cereus*, *E. coli*, *Enterococcus faecium*, and *S.* Typhimurium. When the *E. lindleyanum* DC was used at a concentration of 0.4 mg/ml in commercial orange juice, an antimicrobial effect similar to potassium sorbate at the level of 0.2 mg/ml was observed (Ji *et al*., 2008). Lipid oxidation, which occurs during the storage of raw materials, processing, and heat treatment, is one of the main causes of rancidity in food products (Lizcano *et al*., 2012). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG), have been commonly added to food products to retard lipid oxidation. Even though the chemical treatment proved to be effect method to extend shelf life, consumers have become particularly aware of health concerns (Turantaş *et al*., 2015). The benefits of antioxidants from plant materials in food storage have been studied by many

researchers. Incorporation of dried holy basil powder to cooked ground pork was effective in retarding lipid oxidation (Juntachote *et al*., 2007). Broccoli powder extract applied at 2% was found to be efficient in inhibiting lipid oxidation in nuggets, which was similar to results result obtained from the product with 100 ppm BHT (Banerjee *et al*., 2012).

Among natural antioxidants, *Hibicus asbdariffa* L. (roselle) is used for flavoring, cooking, food coloring, and folk medicine. The edible part of roselle is the calyx, the red, fleshy cover enclosing the flower's seed pod, which is dried and widely used as a nutritional soft drink with significantly high amounts of ascorbic acid and minerals such as calcium, magnesium, and iron. It has been reported that that both ethanol and water roselle extracts displayed antimicrobial activity against many microorganisms such as *B. subtilis, E. coli* O157:H7, *P. aeruginosa, S. aureus*, *Micrococcus leuteus, Serratia marcessens, Clostridium sporagens,* and *Streptococcus* spp. (Tolulope, 2007; Fullerton *et al*., 2011; Al-Hashimi, 2012; Khalaphallah and Soliman., 2014). Therefore, the demand for natural antioxidants has attracted considerable attention because of consumers' positive perception about their safety.

Objectives: Determination of roselle extract efficacy on antioxidant activities and investigation of the *in vitro* antibacterial activity of roselle extracts against pathogenic and spoilage bacteria.

Materials and methods

Preparation of calyxes of roselle extracts

Dried red roselle calyxes were purchased from a local market in Bangkok, Thailand. They were ground to a fine powder (100 mesh) in an electrical blender. One kilogram of powder was extracted with 95% ethanol at room temperature for 72 h. The extract was filtered through four layers of cheesecloth to remove any fiber debris and filtered with Whatman No. 1 filter paper to obtain the extract for antioxidant experiments. Subsequently, the extract was evaporated in the rotary evaporator at 45 °C to give a crude extract. This crude extract was stored at 5°C for antimicrobial experiments.

Determination of antioxidant activities of roselle extracts

DPPH radical scavenging activity

The antioxidant activity of the ethanol roselle calyx extract was measured using the stable DPPH radical (Miliauskas *et al*., 2004). Two milliliters of different ethanol extract concentrations (100–5000 mg/L) were mixed with 100 μM of an ethanol solution of the 2,2-diphenyl 2-picrylhydrazyl (DPPH radical).

The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min. Absorbance of the solution was then measured at 517 nm with a Spectronic^{TM} GENESYS spectrophotometer (Thermo Electron Corporation, USA). Ascorbic acid and BHT were used as standard controls. The inhibition of DPPH radicals by the samples was calculated according to the following equation:

$$\text{Inhibition of DPPH radicals (\%)} = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

A_{blank} and A_{sample} are the absorbance of the control and test samples, respectively. Sample concentrations providing 50% inhibition (IC_{50}) were calculated from the graph plotted for inhibition percentage against sample concentrations.

Reducing power assay

The reducing power of the roselle calyx ethanol extract was determined according to a previous method described by Oyaizu *et al*., 1986. One ml of various concentrations of ethanol extract was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50ºC for 20 min. Afterwards, 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min at 25 ºC. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as a standard. The value of half maximal effective concentration (EC_{50}) is the effective concentration at which absorbance was, an optical density of 0.5 for reducing power, and obtained by interpolation from a linear regression analysis.

Antioxidant activity against lipid peroxidation in egg yolk homogenates

Inhibition of lipid peroxidation by the roselle extract was determined by quantification of the lipid peroxide decomposition product (malondialdehyde) based on the reaction with thiobarbituric acid using egg yolk as oxidizable substrate (Wanasundara and Shahidi, 1998). Egg yolk (2.5% w/v) was homogenized in 100 mM phosphate buffer saline (pH 7.4) for 30 min using a magnetic stirrer. The mixture containing 1 ml of egg yolk homogenates, 0.1 ml of 1 mM ferrous sulfate, and 0.1 ml of either PBS or one of the extracts was incubated at 37°C for 60 min. After incubation, the mixture was tested for formation of thiobarbituric acid-reactive substances (TBARS). The mixture was added to 0.5 ml of the 15% (w/v) trichloroacetic acid and 1 ml 1% (w/v) of the

thiobarbituric acid and immediately vortexed. The mixture was boiled for 10 min, cooled on ice, and centrifuged at 1710×g for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. Synthetic antioxidant of BHT was used as standard controls. The percentage of lipid peroxidation inhibition by the control and test samples was calculated. The concentration in the samples that provided 50% inhibition (IC_{50}) was calculated from the graph plotted for percentage inhibition against sample concentrations. Results were expressed as a value of IC_{50} as mg/ml.

Total phenolic content

Total phenolic content of roselle extract was determined using the Folin–Ciocalteu assay (Kahkonen *et al*., 1999). The extract was diluted in ethanol to 2500, 5000, and 10,000 mg/L was then mixed with 0.5 ml of Folin–Ciocalteau reagent (previously diluted 10-fold with distilled water), and this mixture was allowed to stand for 5 min before the addition of sodium carbonate (4 ml; 7.5% w/v). After 30 min at room temperature, absorbance was read at 765 nm using a spectrophotometer. Results were expressed as µg gallic acid equivalents in 1 g of dried sample (µg GAE/g dry weight).

Determination of Antibacterial activity

Bacterial preparation

The tested bacteria included both pathogenic and spoilage bacteria. Lactic acid bacteria were propagated in MRS (de Man, Rogosa and Sharpe: Merck, Germany). *S.* Typhimurium, *S. aureus*, *E. coli*, and *P. fluorescence* were cultured in tryptic soy broth (Merck, Germany). The initial cell culture was used at 5×10^5 cfu/ ml.

Determination of minimum inhibitory concentration (MIC)

The MIC was considered the lowest concentration of extract which prevented growth and reduced the inoculum by ≥99.9% within 24 h. The MIC was achieved by broth microdilution method [Clinical Laboratory Standard Institute (CLSI), 2002]. Two fold serial dilutions of plant extract concentrations from 50 to 12.5 mg/ml were mixed with tryptic soy broth in sterile 96 well microliter plates. Ethanol at 10% was used as a negative control. Twenty microliters of each tested bacterial suspension were added to each well. The microplates were then incubated at 35 ^oC for 18 h. The lowest concentration without visible growth was defined as the MIC. The experiments were done in triplicate.

Determination of minimum bactericidal concentration (MBC)

The MBC was examined by comparing the number of remaining viable bacteria with the internal number of bacteria. All wells from the MIC experiments that showed no visible turbidity were serially diluted and spreaded on tryptic soy agar plates for viable cell counts. The plates were incubated at the appropriate temperature for each of the tested bacteria (Table 1) for 24 h and then recorded as log cfu/ml. MBC was then recorded as the lowest concentration that killed at least 99.9% of the initial number of bacteria. The experiments were evaluated in triplicate (CLSI, 2002).

Determination of bacteria killing time

To determine the exposure time of roselle extract required to inhibit and kill pathogenic bacteria as determined by the bacterial MBC values, 50 mg/ml for *S.* Typhimurium and 25 mg/ml for *S. aureus* were used. Total bacterial cells were counted for different contact times and recorded as log cfu/ml. The total number of *S.* Typhimurium and *S. aureus* bacterial cells were investigated by plating on tryptic soy agar.

Statistical analysis

All experiments were done using the completely randomized design (CRD). Significant differences between means were separated using Duncan's new multiple range test (DMRT).

Results and discussion

Antioxidant activity was assayed using various methods such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging (Diplock, 1997). Use of at least two methods with different approaches and mechanisms is recommended for precise characterization (Sakanaka and Ishihara, 2008). The roselle extract used in this experiment showed antioxidant activity in three different *in vitro* assays. Antioxidant activities of roselle extract were determined via DPPH scavenging ability. In this assay, antioxidant activity was expressed as a value of IC_{50}, where a low value indicates high antioxidant activity (Table 1). The IC_{50} value for DPPH radical-scavenging activity of the roselle calyx ethanol extract was 932.25 mg/L. The DPPH scavenging ability of the roselle extract was lower than that of the two reference antioxidants, BHT (IC_{50} = 21.82 mg/L) and ascorbic acid (IC_{50} = 3.16 mg/L). In this study, the test samples' DPPH radical scavenging activity increased with increasing concentrations. Antioxidant

activity of dried roselle calyx extract at 2500 mg/L was similar to BHT at 100 mg/L and ascorbic acid at 7.5 mg/L. The radical scavenging capacity of the plant extract was tested using the stable free radical, DPPH, and measured in terms of hydrogen-donating or radical scavenging abilities (Prior *et al*., 2005). DPPH is a free radical compound that has been widely used to test free radical scavenging ability of various samples (Chan *et al*., 2007; Boligon *et al*., 2009; Wijekoon *et al*., 2011).

The sample reducing power was reported as EC_{50}. Increasing absorbance at 700 nm indicates an increase in reducting ability. Table 2 shows dose-response curves for the reducing powers of the extract and ascorbic acid. It was found that the reducing power of the roselle ethanol extract increased with an increase in its concentrations. The antioxidant power of dried roselle calyx extract, with an optical density of 0.578 at 2500 mg/L, was similar to ascorbic acid, which had an optical density of 0.529 at 50 mg/L. The reducing power essay measures the ability of antioxidants to reduce ferric ions to the ferrous form in the presence of antioxidants. Antioxidant agents may serve as electron donors and can reduce the oxidized intermediates of the lipid peroxidation process (Yen and Chen, 1995). The presence of electron donors in roselle extracts reduced the Fe^{3+} ion/ferricyanide complex used in this study to ferrous form.

Anti-lipid peroxidation was measured by monitoring TBARS. The anti-lipid peroxidation in dried roselle calyces was compared with that of BHT in Table 3. In 2.5% egg yolk homogenized with 100 mM phosphate buffer saline at pH 7.4, the roselle extract effectively inhibited the formation of TBARS in a dose-dependent manner. The value of IC_{50} for inhibition of lipid peroxidation by the roselle extract was 217.07 mg/L. The anti-lipid peroxidation by dried roselle calyx extract (78.92 % at 250 mg/L) was similar to BHT (81.58 % at 5000 mg/L). The synthetic BHT antioxidant has been widely used to prevent food from undergoing peroxidative damage (Wanasundara and Shahidi, 1998; Pokorńy, 2007). Lipid peroxidation is one of the main causes of food spoilage during storage or processing because it produces rancid and unpleasant flavors and decreases safety and nutritional value (Lin and Liang, 2002; Wang *et al*., 2002). The ability of anti-lipid peroxidation by the roselle calyx ethanol extract was about three-fold more than that of BHT (Table 1). This result shows that antioxidants obtained from an ethanol extract of roselle has potential for prevention of food spoilage.

In the current study, the dried roselle calyx ethanol extract was found to have phenolic compounds (6.76 µg gallic acid equivalents/g dry weight) with a dose-dependent increase (Fig. 1). Plant phenolic compounds have been shown to contain high levels of antioxidant capabilities. Previously, results of the

DPPH free radical scavenging and ferric-reducing antioxidant power assay in leaves of *Etlingera* species (Zingiberaceae) showed trends identical to that of total phenol content (Chan *et al*., 2007).

Table 1. The percentage inhibition (%) of free-radical DPPH and IC_{50} of roselle calyx in ethanol extract, ascorbic acid and BHT.

Roselle extract conc. (mg/L)	**Inhibition (%)**	**Ascorbic acid conc.**	**Inhibition (%)**	**BHT conc.**	**Inhibition (%)**
100	8.23	0.5	8.29	6.25	21.95
250	13.11	1	14.11	12.5	35.71
500	26.99	2.5	35.44	25	53.18
750	32.19	5	63.54	50	70.43
1000	52.18	7.5	83.42	100	84.41
2500	76.50	10	91.53	200	90.27
5000	100.00				
IC_{50} (mg/L)	932.25	IC_{50} (mg/L)	3.16	IC_{50} (mg/L)	21.82

Table 2. Reducing antioxidant power and EC_{50} of dried roselle calyx in ethanol extract and ascorbic acid.

Roselle extract conc. (mg/L)	**AU**	**Ascorbic acid conc.**	**AU**
100	0.218	30	0.315
250	0.273	40	0.426
500	0.369	50	0.529
750	0.447	60	0.629
1000	0.516	70	0.725
2500	0.578		
5000	0.852		
EC_{50} (mg/L)	937.20	EC_{50} (mg/L)	3.16

Table 3. Anti-lipid peroxidation and IC_{50} of dried roselle calyx in ethanol extract and BHT.

Roselle extract conc. (mg/L)	**Inhibition (%)**	**BHT conc.**	**Inhibition (%)**
100	11.98	500	45.80
250	59.97	1000	52.78
500	78.92	2500	70.52
750	87.88	5000	81.58
1000	89.16	10000	84.85
2500	91.23		
5000	94.24		
IC_{50} (mg/L)	217.07	IC_{50} (mg/L)	683.50

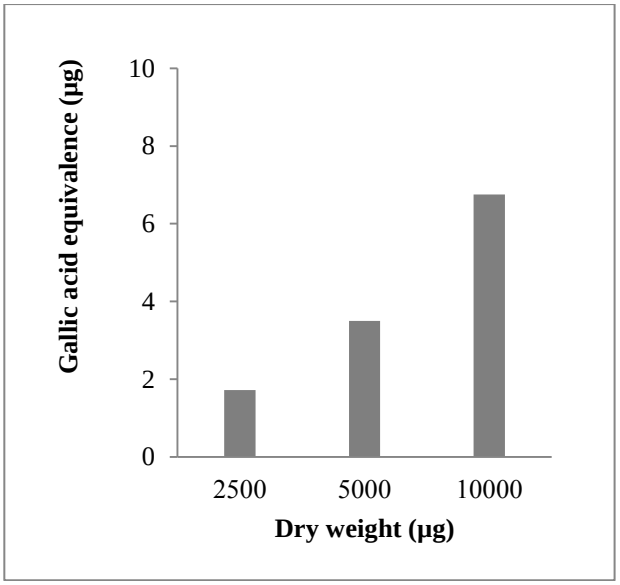

Figure 1. Total phenolic content of different concentrations of ethanolic extract of roselle calyx. Mean ± SD of three tests, expressed as gallic acid equivalents.

MICs and MBCs of roselle extract against pathogenic bacteria (*S.* Typhimurium TISTR 292, *S. aureus* TISTR 118, *E. coli* TISTR 780, and *L. innocua* ATCC 33090^T) and spoilage bacteria (such as *P. fluorescens* TISTR 358, *L. sakei* subsp. *sakei* JCM1157^T, and *Leu. mesenteroides* subsp.*mesenteroides* JCM 6124^T) are shown in Table 4. In general, the MICs and MBCs of the roselle extract against seven bacterial strains ranged from 12.5 mg/ml to 50 mg/ml. The MIC values for the extract showed greater

susceptibility of *S*. Typhimurium TISTR 292 (12.5 mg/ml) than *E. coli* TISTR 780, *L. innocua* ATCC 33090^{T}, *P. fluorescens* TISTR 358, *L. sakei* subsp. *sakei* JCM1157^{T}, or *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^{T} (25 mg/ml). An MBC value of 50 mg/ml was observed against *S.* Typhimurium TISTR 292, *E. coli* TISTR 780, *P. fluorescens* TISTR 358, *L. sakei* subsp. *sakei* JCM 1157^{T}, and *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^{T}, whereas an MBC value at 25 mg/ml was found against *S. aureus* TISTR 118 and *L. innocua* ATCC 33090^{T}. MIC is defined as the highest dilution or smallest concentration of extract that inhibits organism growth. Determination of MIC is important in diagnostic laboratories because it helps to confirm resistance of microorganisms to an antimicrobial agent, and it monitors the activity of new antimicrobial agents. The MBC was considered the concentration of a plant extract that completely killed the bacteria. The MBC value is almost two fold higher than the MIC value (Sen and Batra, 2012). The roselle extract completely killed *S.* Typhimurium at 50 mg/ml after 1 min and *S. aureus* at 25 mg/ml after 14 min (<10 cfu/ml) (Fig. 2).

The results from this study agree with several reports in which antimicrobial activity of roselle extract on both foodborne and food spoilage microorganisms was studied. Al-Hashimi (2012) reported that aqueous and alcoholic roselle extracts showed antibacterial activity against *E. coli, S. aureus, S. mutans, and P. aeruginosa.* Khalaphallah and Soliman (2014), who reported that the ethanolic roselle extract had antibacterial activity against *B. subtilis*, *P. aeruginosa*, and *E. coli.* Some studies have claimed that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Jalosińska and Wilczak, 2009). Nychas (1995) proposed that phenolic extracts could react with the phospholipid cell membrane components of *P. aeruginosa, P. flagi, and P. fluorescence*; thereby an increase in cell membrane permeability could cause significant changes in fatty acid composition and phospholipid content of these organisms. In addition, Puupponen-Pimia *et al*. (2001) and Vattem *et al*. (2004) reported that plant phenolic compounds can cause damage to bacterial cell membranes because they act as strong oxidizers.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of roselle extracts against some pathogenic and spoilage bacteria.

Bacterial strains	**Roselle concentration (mg/ml)**	
	MIC	**MBC**
Salmonella Typhimurium TISTR 292	12.5	50
Staphylococcus aureus TISTR 118	25	25
Escherichia coli TISTR 780	25	50
Listeria innocua ATCC 33090^{T}	25	25
Pseudomonas fluorescens TISTR 358	25	50
Lactobacillus sakei subsp. *sakei* JCM1157^{T}	25	50
Leuconostoc mesenteroides subsp.*mesenteroides* JCM 6124^{T}	25	50

ATCC = American Type Culture Collection, Rockville, Md
JCM = Japanese Culture of Microorganism, Wako, Japan
TISTR = Thailand Institute of Science and Technological Research, Thailand

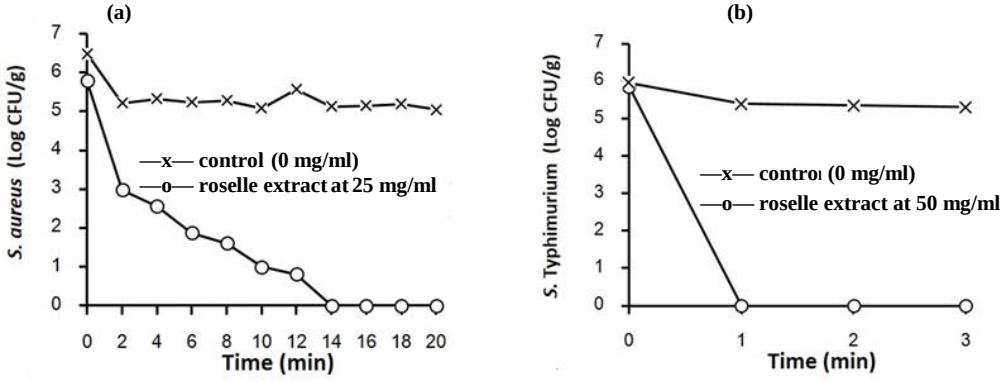

Figure 2. Exposing time of *S. aureus* TISTR 118 (a) and *S*. Typhimurium (b) in tryptic soy broth adding 25 and 50 mg/ml of roselle extract on survival of cells.

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