Bioactivity of *Rosmarinus officinalis* extracts in combination with *Camelus dromedarius* whey proteins

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Abstract: The present study was performed to evaluate the antibacterial potential of camel wheyand *Rosmarinus officinalis* aqueous and ethanol extracts as well as their effect on glutathione-S- transferases (GSTs) enzymes activity. The antibacterial activity of all samples and combinations was tested against *Staphylococcus aureus* and *Escherichia coli* using micro-broth dilution method. In addition to that, the effect of camel whey and plant extracts on GSTs was studied by spectrophotometric method using 1-chloro-2,4-dinitro-benzene (CDNB) as substrate. The obtained results showed that ethanol extract was the most effective when compared to the aqueous extract and whey proteins. Synergistic antibacterial activity was observed for whey and ethanol extract against *E. coli* with MBC value equal to 0.39 mg/ml. In contrast, whey combination to aqueous extract showed antagonistic bacteriostatic and bactericidal effect against both bacteria. Moreover, results revealed that all samples with 1 mg/ml concentration increased the activity of GSTs *in vitro*. The recorded GSTs activity was higher after combination of whey to both extracts. The best results obtained after combining the ethanol extract to whey proteins. The obtained results provided the necessity for further bioactivity studies between active ingredients of both camel whey and plant extracts.

Keywords: *Rosmarinus officinalis*; Plant Extracts; Whey; Antibacterial; Glutathione-s-transferase.

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Introduction:

Medicinal plants considered as one of the rich sources of antimicrobial agents. These agents are secondary metabolites produced by plants as a defence mechanism against pathogens (Obeidat et *al.,* 2012). One of these medicinal plants is *Rosmarinus officinalis*, L. (Rosmary) from the Lamiaceae family (Cuvelier et *al.,* 1996). Rosemary extracts have been used in the treatment of different diseases (Raškovic et *al.,* 2014; Habtemariam et *al.,* 2016). Phytochemical analysis of *R .officinalis* showed the presence of several phenolic compounds, including rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid and the antioxidants carnosic and carnoso (Aliyu, 2006). In this aspect, there are several studies that outlined the antioxidant and antimicrobial activities of *R .officinalis* (Lo et *al.,* 2002; Moreno et *al.,* 2006). This bioactivity may be attributed to the presence of phenolic compounds that exhibited high antimicrobial activity against both Gram-positive and Gram-negative bacteria (Moreno et *al.,* 2006).

In addition to plants, animals contain many medicinal ingredients that are wildly used. Functional foods are defined as food substances that demonstrated physiological benefits and are designed to lower the risk or delay the onset of certain diseases (Al-Alawi and Laleye, 2008). Camel milk is one of these foods that have many properties which make it a very useful choice to cure certain diseases (Attia et *al.,* 2001). Studies showed that camel milk has bactericidal activity due to higher content of antimicrobial factors like lysozyme, lactoferrin and immunoglobulins, which give camel milk high biological value (Elagamy et *al.,* 2000; El-Hatmi et *al.,* 2007). Moreover, camel milk contains unique types of IgG antibodies as they lack the light chains and so called heavy chain antibodies. Scientists found that these antibodies acquired unique characteristics. Their high stability allows them to participate in natural preservation of camel milk in deserts without using refrigerators (El-Hatmi et *al.,* 2007).

From this point of view, this study was conducted to evaluate the antibacterial potential of aqueous and ethanol extracts from *Rosmarinus officinalis* in combination with camel whey proteins. All combinations were tested against two bacterial isolates which are *Staphylococcus aureus* and *Escherichia coli*. The Gram-positive *Staphylococcus aureus* is one of the leading causes of human infections of the skin, soft tissues, bones and joints, abscesses, as well as normal heart valves (Karlowsky et *al.,* 2003). The other studied bacterium is the Gram-negative *Escherichia coli* of the Enterobacteriaceae family that considered as one of the

most important bacterial pathogens that is associated with gastrointestinal tract infections and extrain testinal infections (Beutin, 1999).

In addition to that, the *in vitro* effect of camel whey, plant extracts and their combinations on sheep liver glutathione-S-transferases activity was also studied in the current research. Glutathione-S-transferase isoenzymes (GSTs) are widely distributed in nature and present in different organisms such as microbes, plant, fish, insect, birds and mammals (Hayes and Pulford, 1995).

Glutathione-S-transferases contains two super families of enzymes that possess transferase activity (Hayes and Strange, 2000). The first enzyme super family type is cytosolic with eight families in mammals and four families in bacteria, insect and plants (Mannervik, 1985; Board et *al.,* 1997). The second enzyme family type is membrane-associated protein in eicosanoid and glutathione metabolism (MAPEG) (Hayes and MCLellan, 1999). Cytosolic GSTs are mostly involved in the metabolism of foreign chemicals, such as carcinogens, environmental pollutants and cancer chemotherapeutic drugs as well as the detoxification of potentially harmful endogenously derived reactive compounds (Hayes and Pulford, 1995). By contrast, MAPEG members are not principally involved in detoxification reaction, but instead involved in the biosynthesis of leukotrienes and other things (Hayes and MCLellan, 1999). Never the less, their activity is motivated by the action of the cytochrome P450 (CYP) enzymes. As they catalyze the introduction of a functional group into inactive xenobiotic. The electrophilic center of the functional group attacked by the reduced glutathione (GSH), which is catalyzed by GSTs through the conjugation reaction (Mannervik, 1985). The addition of GSH to the toxic molecule gives it a molecular flag, which allow the xenobiotic-conjugate to be removed from the cell (Board et *al.,* 1997).

In this regards, several studies showed that *in vivo* induction of GSTs is potentially beneficial in protecting the cells from several compounds that cause diseases such as cancer and neurodegenerative diseases (van Haaften et *al.,* 2003).

Materials and methods:

Bacterial Isolates

The *in vitro* antibacterial activities of plant extracts and camel whey were evaluated against two reference bacterial isolates which were obtained from the American Type Culture Collection (ATCC). The studied isolates were *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922).

Plant Extracts Preparation

Rosmarinus officinalis was collected from Nablus, West Bank, Palestine. The studied plant species was identified by Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University, Palestine. The plant leaves were washed, air dried, ground into powder using a grinder and stored at room temperature until they were used. For aqueous extraction, five grams of dried plant leaves powder were soaked in 100 ml warm distilled water, for three days with continuous shaking at room temperature. Then the mixture was centrifuged for 5 min at 4000 rpm and the supernatant was evaporated by freeze-dryer. For ethanol extraction the same procedure was applied using 70% ethanol as a solvent and evaporation done by rotary evaporator (Omar et *al.,* 2013). To adjust the final concentration at 1mg/ml for enzyme assay and 100 mg/ml for antibacterial assay (Abdallah et *al*., 2017). The extracted powder from the examined plant species was dissolved in distilled water for aqueous powders, while ethanol powders were dissolved in 5% dimethyl sulfoxide (DSMO) (Figure 1).

Whey Preparation

Milk sample was collected from one female camel (Jenin, West Bank) by a veterinary specialist. For whey protein preparation, the casein was precipitated from the skimmed milk sample by the addition of commercially available rennin. The coagulated milk was heated to 56°C for 10 minutes. Casein separation from lactoserum was carried out by filtration. For final clarification, the lactoserum was again centrifuged at 10,000 rpm for 30 min at 4°C. The obtained supernatant was filtered and subjected to freeze-drying to produce whey powder (Brüssow et *al.,* 1987). The prepared whey powder was dissolved in distilled water to a final concentration equal to 1mg/ml for enzyme assay and 100 mg/ml for antibacterial assay and sterilized by microfiltration (Abdallah et *al*., 2017; Abdallah et *al*., 2019) (Figure 2). The total protein content of camel whey sample was determined by Biuret method (Gornall et *al.,* 1949).

Glutathione-S-Transferase Preparation

Fresh sheep liver was homogenized at 4°C with 50 mM phosphate buffer containing 1 mM EDTA pH 7. The homogenate was centrifuged at 10,000 rpm for 25 min. The obtained supernatant was centrifuged at 20,000 rpm for 120 min and the supernatant that contains cytosolic glutathione-s-transferases was precipitated by ammonium sulfate at (30-70) %. The obtained solution was purified by gel filtration column chromatography using (Sephadex G-100 column, Sigma) with 0.2 M phosphate buffer pH 7. The protein level for all fractions was

determined using (Warburg and Christian, 1942) method and GSTs activity was determined by (Habig et *al.,* 1974) method. The fractions with GSTs activities were pooled and applied to affinity column (GSH-agarose, Sigma). The column was equilibrated and washed with 0.2 M phosphate buffer pH 7. After elution, all fractions were determined for protein level and GST activity (Warburg and Christian, 1942; Habig et *al.,* 1974). The fractions with GSTs activities were pooled, lyophilized and used for the activity studies (Figure 3).

Antibacterial Activity Assay:

The antibacterial activity of both *R. officinalis* extracts and whey proteins were determined by micro-broth dilution method (NCCLS, 2000) (Figure 4). The prepared samples were serially diluted two fold in Mueller-Hinton broth medium. Duplicates of each dilution (50.0, 25.0, 12.5, 6.25, 3.125, 1.563, 0.781 0.391, 0.195 and 0.98 mg/ml) were inoculated with 1 µl of $1*10^5$ CFU/ml. The last two duplicate wells were not inoculated and considered as negative controls. After that, the inoculated microtiter plates were incubated at 37°C for 18 h. The lowest extract concentration that inhibited the growth of tested microorganisms was considered as MIC. For the determination of minimum bactericidal concentrations (MBC), the contents of the wells resulting from MIC were streaked on agar plates free of antibacterial agents and incubated at 37ºC for 18 hours. The lowest concentration of the extract which showed no bacterial growth was considered as MBC. The same procedure was performed for camel whey and the prepared combinations.

Glutathione-S-Transferase Assay

Glutathione-S-transferase activity using 1-chloro- 2, 4- dinitrobenzene (CDNB) as substrate was assayed spectrophotometrically as described by (Habig et *al.,* 1974) with modification (Abdallah et *al*., 2019). The cuvettes (final volume of 1.0 ml) contained 0.1 M phosphate buffer (pH 7), 1.5 mM GSH and 1.5 mM of CDNB and 50 µl of diluted enzyme (10 µg/ml). Change in absorbance at 340 nm was followed against a blank containing all reactants except CDNB activity was expressed as µmol conjugate formed/ min/ml using a molar extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$. To determine the effect of *R. officinalis* extracts, whey proteins and their combinations on the activity of GST enzyme, 1 mg/ml from each of the previous samples was added to the enzyme reaction mixture. Then the effect of each sample on the activity of GSTs was measured by Seacomam spectrophotometer at 340nm and expressed as activation percentage.

$$\text{Activation \%} = (\text{GSTs activity under treatment} / \text{GSTs activity without treatment} \times 100\ \%.$$

Results and discussion

Results

The first part of this study was conducted to evaluate the antibacterial potential of camel whey and *Rosmarinus officinalis* aqueous and ethanol extracts. The antibacterial activity of all samples and combinations was quantitatively tested against *S. aureus* and *E. coli* using micro-broth dilution method. The obtained results showed that the ethanol extract was the most effective when compared to the aqueous one and whey proteins (Figure 5 and 6). Synergistic antibacterial activity was observed for whey and ethanol extract against *E. coli* with MBC value equal to 0.39 mg/ml. Additionally, the same observation was noticed for the combined effect between ethanol extract and whey proteins against *S. aureus*. In the contrary, no synergies was noticed for whey combination to aqueous extract against both studied bacterial isolates.

Moreover, results of the second parts of the current research revealed that all samples with 1 mg/ml concentration increased the activity of GSTs *in vitro* (Figure 7). This was clearly noticed as aqueous extract, ethanol extract and whey proteins increased GSTs activity up to 155.6 %, 277.7 % and 153.7 % respectively compared to the control (Figure 8). The recorded GSTs activity was higher after combination of whey proteins to both extracts. The best activation results obtained after combining the ethanol extract to whey proteins as it reached 428.9 % activation when compared to the used control.

Discussion

Camel milk as well as *Rosmarinus officinalis* considered as rich sources of many potent and powerful antimicrobial agents. Therefore, the current study was carried out to estimate the antibacterial activity of aqueous and ethanol extracts from *R. officinalis* in combination with camel whey proteins against *Staphylococcus aureus* and *Escherichia coli*. Furthermore, the effect of camel whey, plant extracts and their combinations on the activity of sheep liver glutathione-S-transferases was also explored in this research. The results in the current research showed that rosemary aqueous and ethanol extracts had a powerful bacteriostatic and bactericidal activity against both examined bacterial isolates. In this regard, the obtained results are compatible with several studies that focus on the antibacterial potential of *R. officinalis* against *E. coli*. Among these studies, Abdel-Massih et *al*., (2010) demonstrated that

R. officinalis crude extract displayed antibacterial activity against the clinical strains from *E. coli*. Moreover, other studies showed that ethanol extract of rosemary leaves exhibited antibacterial activity against pathogenic bacteria including *S. aureus* and *E. coli* (Gazwi et *al.,* 2020). Additionally, Kloy et *al.,* (2020) summarized the highest antibacterial effect of rosemary in several studies and it is clearly noticed that the most effect was against *E.coli* and *S. aureus*. The main component of *R. officinalis* extract that cause the inhibitory effect against bacteria is carnosic acid (Moreno et *al.,* 2006). According to the previous report, carnosic acid is more efficient against gram-positive bacteria than rosmarinic acid (Klancnik et *al.,* 2009). The antibacterial behavior of these constituents may be due to their ability to interact with the cell membrane proteins that produced the loss of membrane functionality and its structure (Fung et *al.,* 1977). In addition to that, some constituents disrupt the bacterial membrane, and therefore promote bacterial lysis which leads to the leakage of cellular components (Bajpai et *al.,* 2012).

Besides plants, animals have a numerous antimicrobial system that often evolved as part of their defense mechanisms. Antibacterial results in the running study revealed that camel whey inhibited and killed the examined bacterial isolates at 12.5 mg/ml and 25 mg/ml respectively. In literature, the inhibitory effect of camel milk against different bacterial isolates has been investigated (Duhaiman, 1988; Benkerroum et *al.,* 2004). Otherwise, Gakkhar et *al.,* (2015) showed that camel milk produced no antibacterial activity against *E. coli* and *S. aureus*. The antibacterial ability of camel whey may be due to the fact that it contains several antibacterial factors, including lactoglobulins, lactoferrin, albumin and immunoglobulins (Eigel et *al.,* 1984; Elagamy, 2000). Studies confirmed that lactoferrin has bacteristatic and bactericidal activity because of its ability to bind iron, which considered as vital ion for bacterial growth and virulence (Dionysius et *al.,* 1993). Likewise, El Sayed et *al.,* (1992) proved that camel immunoglobulins had little effect against *E. coli* and *S. aureus*. In the present study, the ethanol extract of *R. officinalis* exhibited a remarkable synergistic activity after combination to whey proteins. This was clearly reflected by the marked changes in the MIC and MBC values to both examined bacterial isolates. The observed synergism coincides with another study that carried out between camel whey and other plant extracts including *B. undulata* and *R. chalepensis* ethanol extracts (Abdallah et *al.,* 2017).

Despite that, the synergic mechanism of action between plant extracts and camel whey is still unknown. The possible explanation for such synergism may attribute to the presence of biologically active components that disturb cell membrane permeability and thereby facilitate

the influx of other ones with intracellular activity (Zhao et *al.*, 2000). The acquired synergism is similar to the results obtained from the combination between *R. officinalis* extract and antibiotic ceproflaxine against MRSA (Jarrar et *al.*, 2010).

The other aim of this study is to find out the effect camel whey, plant extracts and their combinations on the activity of sheep liver glutathione-S-transferases. As the induction of phase II drug metabolizing enzymes is an important aspect in drug discovery, the ability to induce GST activity is a property of many chemopreventive agents (Gweshelo et *al.,* 2016). One of those chemopreventive agents is natural compounds that derived from plants like flavonone and flavones (Ogbonnia et *al.,* 2009). In addition to that, plants contain antioxidant metabolites which also coordinately elevate GSTs through a common transcriptional activation pathway (Vargas et *al.,* 1998). Rosemary herb is also known to exhibit antioxidant activity which mainly attributed to its major constituents diterpene, carnosic acid and essential oils (Frankel et *al.,* 1996). Additionally, *R. officinalis* act as an antioxidant agent and hence improve GST-dependent detoxification systems *in vivo* (Sotelo-Felix et *al.,* 2002). The obtained results in the current research matched with the previous findings as both aqueous and ethanol extracts of this plant had the ability to induce GSTs activity *in vitro*.

Similar to plants, dietary constituents like camel milk have a valuable role in different biological activities including chemoprevention (Yagil et *al.,* 1982). It was recorded that camel milk increased the expression of chemo-protective genes which in turn increased the levels of several antioxidant enzymes. These enzymes prevent the formation of highly reactive oxygen species and so protect DNA and cell damage (Habib et *al.,* 2013). The running research showed that camel whey proteins induced the activity of GSTs *in vitro*. These obtained results can be supported by an *in vivo* experiment relaying on camel milk (Lebda et *al.,* 2012). In their *in vivo* study, rats treated with camel milk showed an increase in the activity and affinity of GST enzyme.

Conclusions:

In conclusion, the results presented in this research highlight the potential of rosemary extract as well as camel whey proteins as a source of several bioactive compounds that had antibacterial activity and an inductive potential for phase II drug metabolizing enzymes GSTs. This bioactivity showed a synergy between rosemary extracts and whey proteins. So, further work is required to characterize the action mechanisms of these interesting compounds responsible for the synergistic bioactivity.

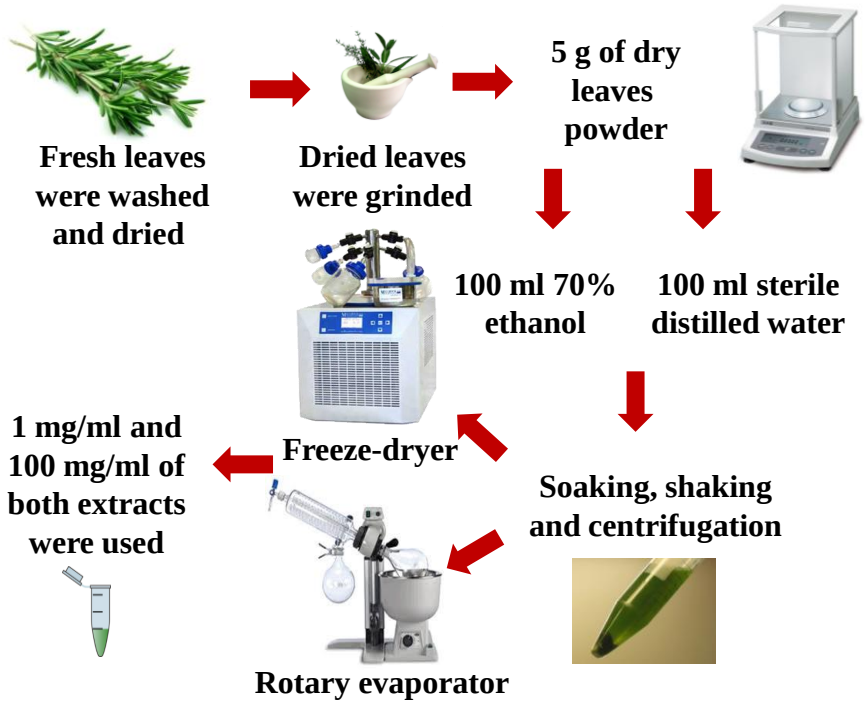

Figure 1. *Rosmarinus officinalis* extract preparation

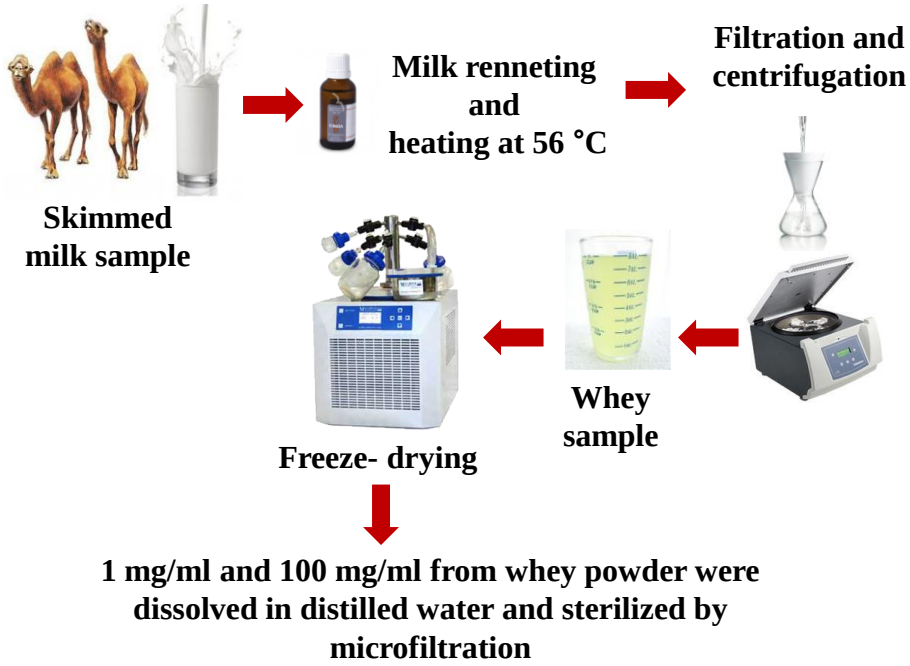

Figure 2. Whey Proteins preparation

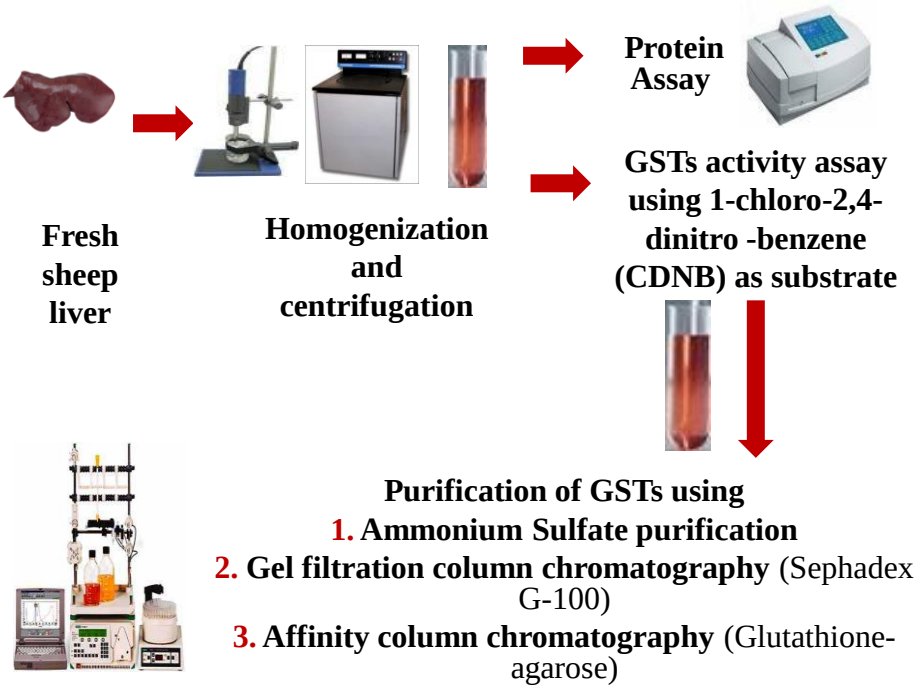

Figure 3. Glutathione-S-transferase preparation

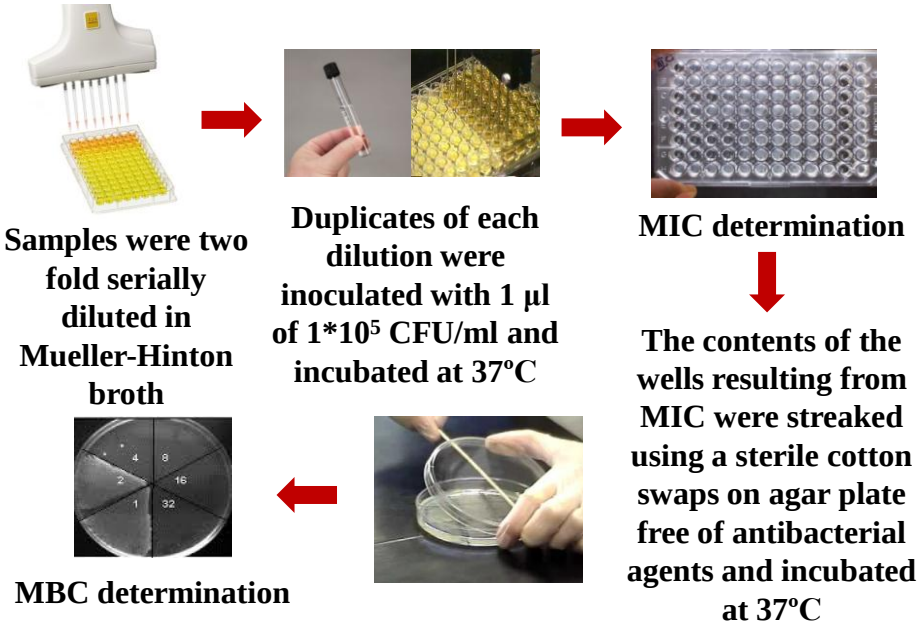

Figure 4. Micro-broth dilution method for antibacterial activity

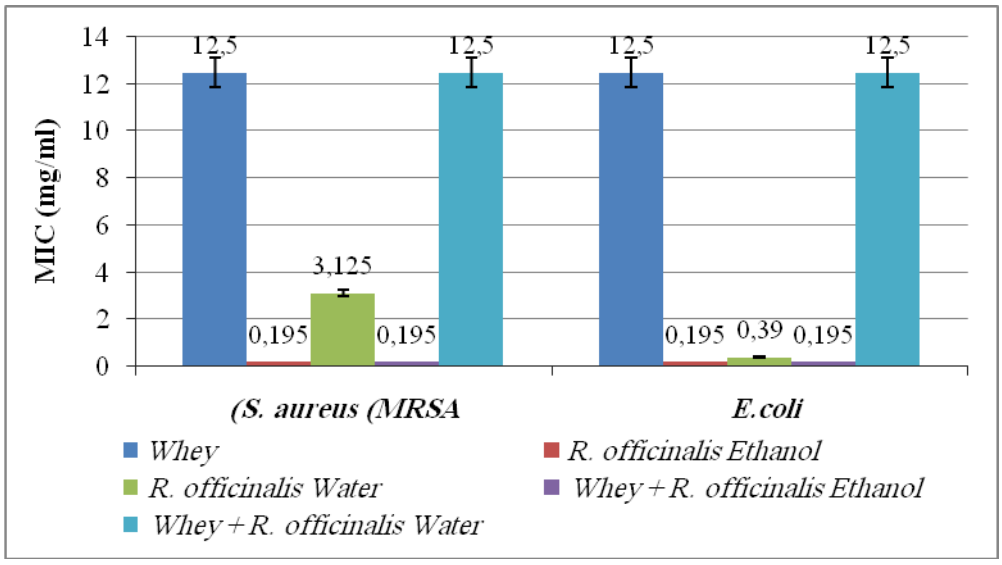

Figure 5. Antibacterial activity of *R. officinalis* aqueous and ethanol extracts in addition to whey proteins and their combinations against two bacterial isolates using micro-broth dilution method; (MIC) minimum inhibitory concentration (mg/ml).

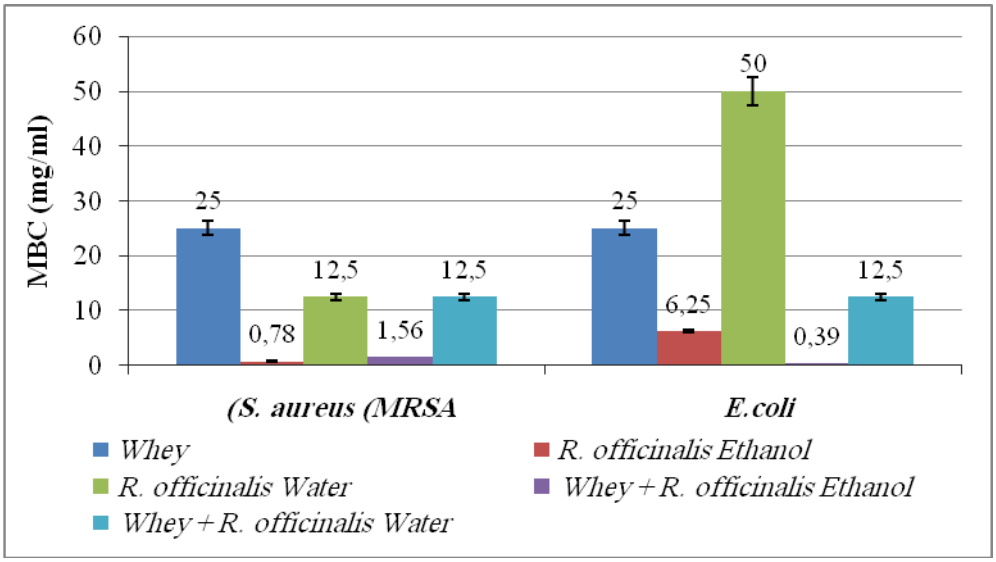

Figure 6. Antibacterial activity of *R. officinalis* aqueous and ethanol extracts in addition to whey proteins and their combinations against two bacterial isolates using micro-broth dilution method; (MBC) minimum bactericidal concentration (mg/ml).

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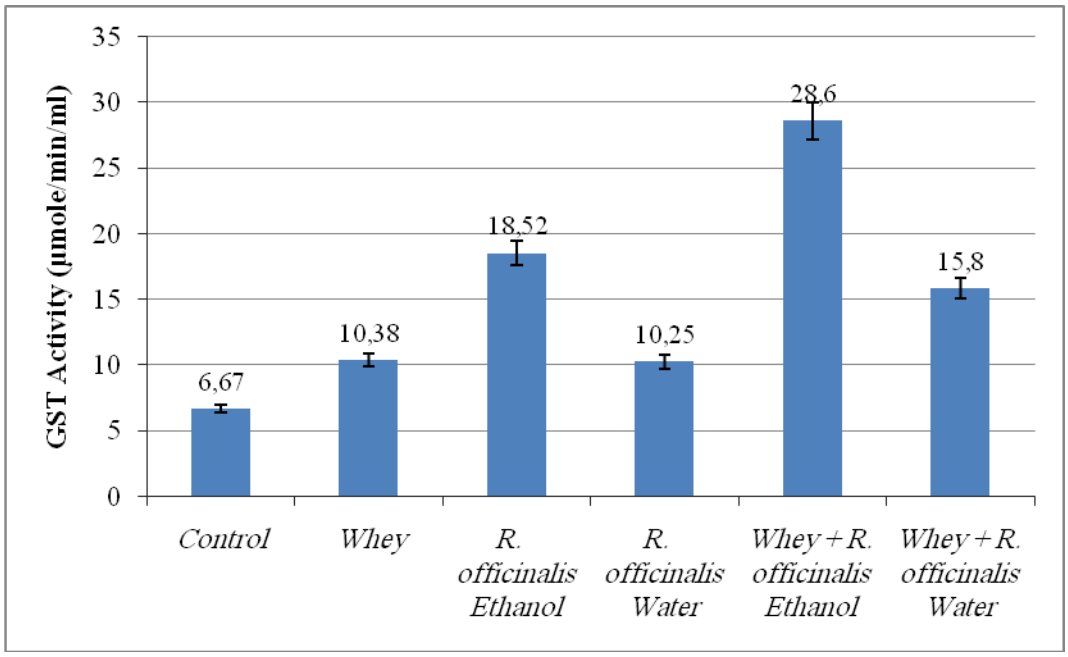

Figure 7. Effect of *R. officinalis* aqueous and ethanol extracts in addition to whey proteins and their combinations on the activity of sheep liver Glutathione-S transferases.

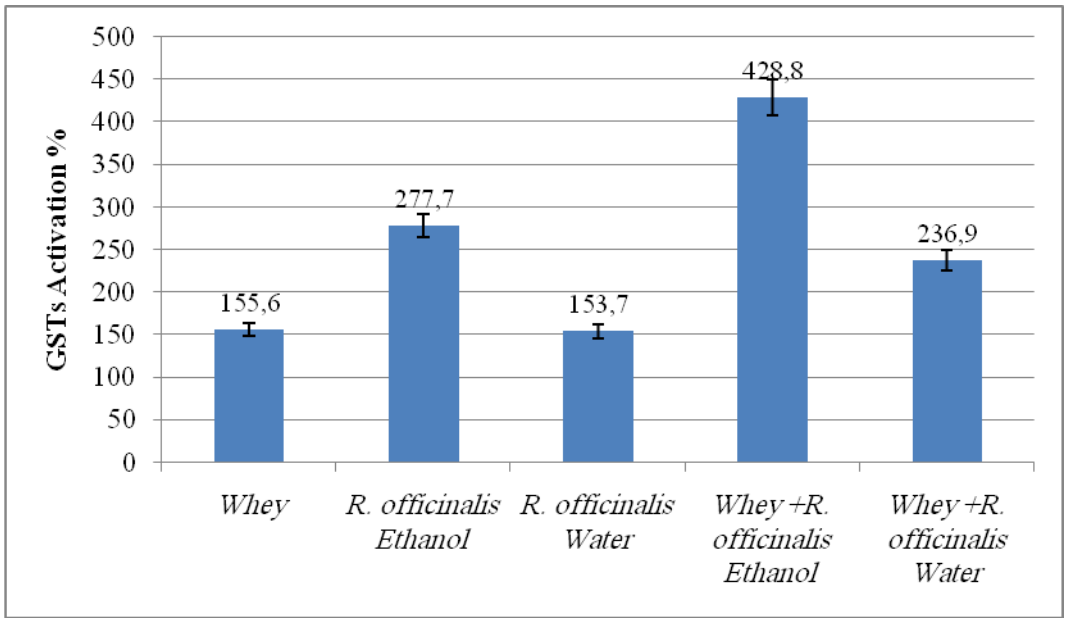

Figure 8. GSTs activation percentage (%) by *R. officinalis* aqueous and ethanol extracts in addition to whey proteins and their combinations on the activity of sheep liver Glutathione-S-transferases.

Competing interests
The authors declare that they have no competing interests.

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