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# *Panax ginseng* has anti-infective activity against opportunistic pathogen *Pseudomonas aeruginosa* by inhibiting quorum sensing, a bacterial communication process critical for establishing infection

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## Abstract

Virulent factors produced by pathogens play an important role in the infectious process, which is regulated by a cell-to-cell communication mechanism called quorum sensing (QS). *Pseudomonas aeruginosa* is an important opportunistic human pathogen, which causes infections in patients with compromised immune systems and cystic fibrosis. The QS systems of *P. aeruginosa* use N-acylated homoserine lactone (AHL) as signal molecules. Previously we have demonstrated that *Panax ginseng* treatment allowed the animals with *P. aeruginosa* pneumonia to effectively clear the bacterial infection. We postulated that the ability to impact the outcome of infections is partly due to ginseng having direct effect on the production of *P. aeruginosa* virulence factors. The study explores the effect of ginseng on alginate, protease and AHL production. The effect of ginseng extracts on growth and expression of quorum-sensing (QS)-controlled virulence factors on the prototypic *P. aeruginosa* PAO1 and its isogenic mucoid variant (PAO*mucA22* or PDO300) was determined. Ginseng did not inhibit the growth of the bacteria, enhanced the extracellular protein production and stimulated the production of alginate. However, ginseng suppressed the production of LasA and LasB and down-regulated the synthesis of the AHL molecules. Ginseng has a negative effect on the QS system of *P. aeruginosa*, which might be part of the mechanisms that ginseng helped the bacterial clearance from the animal lungs *in vivo* in our previous animal study. It is possible that enhancing and repressing activities of ginseng are mutually exclusive as it is a complex mixture, as shown with the HPLC anaylsis of the hot water extract of ginseng that was performed in this study. Though

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ginseng is a promising natural synergetic remedy, it is important to isolate and evaluate the ginseng compounds associated with the anti-QS activity.

### Keywords

Ginseng; *Pseudomonas aeruginosa*; anti-quorum sensing; LasA; LasB; Alginate

## Introduction

The leading pathogen responsible for morbidity and mortality among patients with cystic fibrosis (CF) is *Pseudomonas aeruginosa* that grows in a biofilm-mode in the airways. The initial and intermittent colonization of CF lungs by nonmucoid *P. aeruginosa* can be eradicated by early aggressive antibiotic therapy but such treatment usually fails if the colony morphology of bacteria isolated from sputum samples is mucoid owing to overproduction of a capsule-like polysaccharide called alginate. Extensive efforts have been carried out to elucidate the molecular mechanisms responsible for overproduction of alginate in planktonic cells. However, despite our knowledge on the regulation of alginate genes in *P. aeruginosa* (Mathee *et al.*, 2002), the successful control, if not complete elimination, still remains a challenge in the treatment of *P. aeruginosa* infection in CF patients.

The infection of CF patients with *P. aeruginosa* strains, especially after their conversion to alginate-producing mucoid form, is rarely eradicated by antibiotics (Høiby, 1974; Pedersen *et al.*, 1992). In the hope of finding new and efficacious measures for the treatment of *P. aeruginosa* pneumonia in CF patients, alternative treatments have also been explored. Immunization with *P. aeruginosa* vaccines and adjuvants, or the treatment with IFN-γ or Chinese herbal medicine, *Daphne giraldii Nitsche*, could decrease the inflammatory response and enhance bacterial clearance in a rat model (Johansen *et al.*, 1994; Johansen *et al.*, 1995; Johansen *et al.*, 1996; Song *et al.*, 1996). The possibility of using the even more popular Chinese herbal supplement, ginseng has also been studied.

Ginseng is one of the best-known Chinese medicinal herbs and it has been widely used in China for thousands of years (Huang, 1993; O'Hara *et al.*, 1998). The toxicity of ginseng is quite low, the LD50 for intravenous injection in mice is 16.5 mg/kg (Bensky, Gamble, 1993). The herb is reported to influence the cardiovascular, nervous, endocrine and immune systems; in addition, it affects metabolism, possesses some anti-cancer, anti-inflammation and anti-aging effects (Huang, 1993). As a modulator of the endocrine system and immune system, ginseng has been shown to have roles in modulating the production of specific antibodies and the functions of phagocytes and natural killer (NK) cells (Huang, 1993; Yang *et al.*, 1983; Yang *et al.*, 1987).

Several studies using animal models have shown that ginseng might play a role in enhancing immune response and bacterial clearance (Song *et al.*, 1997a; Song *et al.*, 1997b; Song *et al.*, 1998; Song *et al.*, 2002). Ginseng treatment appears to down-regulate the antibody response or shift the immune reaction from a Th2 type to a Th1 type and improve phagocytosis by PMNs, and perhaps downregulate inflammation (Song *et al.*, 1997b). Furthermore, ginseng treatment significantly decreases the lung pathology and enhances bacterial clearance from the lung in the animal models of chronic *P. aeruginosa* lung infection (Song *et al.*, 1997a; Song *et al.*, 2002). It also down-regulates the serum IgG response against *P. aeruginosa* antigens, improves the blood PMN oxidative burst, and activates the phagocytosis of *P. aeruginosa* by blood PMNs and alveolar macrophages (Song *et al.*, 1998). In addition, serum IgG1 decreases, IgG2a increases and mast cell numbers in the lung foci decrease. Furthermore, production of IFN-γ in the lung cell culture in the ginseng-treated group increased and IL-4 production decreased.

These suggest that the therapeutic effect of ginseng is associated with its Th1-like modulating effect. The down-regulation of serum IgG leads to less formation of immune complexes, which in turn reduces the infiltration of PMNs and the damage of lung tissues (Song *et al.*, 1997a; Song *et al.*, 1997b).

All the studies described above have addressed the *in vivo* efficacy of ginseng against *P. aeruginosa* infection and the potential mechanisms involved using an animal model system. In this study we determined if ginseng directly affected the bacterial expression of virulence factors using a set of appropriate isogenic strains containing a well-characterized mutation. PAO*mucA22* (PDO300) carries a mutation in the *mucA* gene that encodes the anti-sigma factor MucA, resulting in a constitutive mucoid phenotype (Mathee *et al.*, 1999a; Mathee *et al.*, 1999b) Since the efficacy of antibiotics and antibacterials against the mucoid and non-mucoid forms of *P. aeruginosa* differs significantly, we chose to determine the effect of ginseng extracts on the growth and expression of virulence factors under the control of quorum sensing (QS), in the wild type PAO1 (Holloway, Matsumoto, 1984) and the mucoid PDO300 (Mathee *et al.*, 1999b) strains of *P. aeruginosa.* Our results suggest that although ginseng inhibited the expression of virulence factors such as LasA protease and LasB elastase in the non-mucoid strain, PAO1, addition of a lower concentration of ginseng (< 2.5 μg) stimulated growth and alginate production in the mucoid strain, PDO300. Therefore, for ginseng to be effectively used as a therapeutic agent, it becomes imperative to consider several factors including the concentration of ginseng extract, as well as the mucoid or non-mucoid state of the *P. aeruginosa* infection.

## Materials and methods

### Bacterial strains

Non-mucoid wild-type *P. aeruginosa* PAO1 (Holloway, Matsumoto, 1984) and its isogenic mucoid variant $Alg^+$ PAO*mucA22* (PDO300) (Mathee *et al.*, 1999a; Mathee *et al.*, 1999b) were used. LB media containing various concentrations of ginseng were used for bacterial growth under rapid shaking at 37°C. For growth curve experiments, culture samples were removed every hour for measurement of $OD_{600}$ for 20 hours.

### Ginseng preparation

Six-year old ginseng roots from Jilin, People's Republic of China were dried and ground into powder. Luria-Bertani (LB) broth extracts of different concentrations of ginseng were prepared as described previously (Song *et al.*, 1997b). In brief, different amounts of ginseng powder were mixed with LB medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride), heated to 90°C for 30 minutes, filtered and then used for culturing bacteria.

### Determination of acylhomoserine lactones (AHL)

AHLs were extracted from ABT culture supernatants with acidified ethyl acetate, dried under nitrogen, and quantified by electrospray mass spectroscopy (ESMS) after the methods of Makemson *et al* (Makemson *et al.*, 2006). Peak intensities for BHL (m/z = 172) and OdDHL (m/z = 298), and their sodium adducts (m/z=194 and 230 respectively) were combined and converted to concentration using a standard curve generated from the pure compounds. Background readings from samples extracted with alkaline ethyl acetate were subtracted from those of the acid-extracted bacterial cultures before conversion. Acetic acid preserves the AHL while NaOH opens up the charged lactone ring, resulting in the loss of AHL during the extraction with organic solvent, ethyl acetate.

### LasA staphylolytic assay

LasA protease activity was determined by measuring the ability of culture supernatants to lyse boiled *S. aureus* cells (Kong *et al.*, 2005). Supernatants from 18-hour culture at 37°C under shaking condition were taken for protease activity evaluation. A 100-μl aliquot of *P. aeruginosa* LB culture supernatant with or without plant extracts was added to 900 μl of boiled *S. aureus* suspension. $OD_{600}$ was determined after 0, 5, 10, 20, 30, 45, and 60 minutes. Activity was expressed as $\Delta OD_{600}$/hour per μg protein.

### Elastase activity

LasB protease (elastase) activity was measured in a spectrophotometric assay using elastin Congo red (ECR; Sigma, St. Louis, MO) as a substrate as reported previously (Ohman *et al.*, 1980). A 100-μl aliquot was added to 900 μl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg ECR. This was then incubated with shaking for 3 hours at 37°C. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. Cell-free AB medium, alone, and with ginseng were used as negative controls. Activity was expressed as $\Delta OD_{495}$ per μg protein.

### Quantification of alginate

The content of uronic acid polymers (the component of alginate) in the supernatants of overnight culture was analyzed by the carbazole-borate assay with D-mannuronic acid lactone (Sigma) (Knutson, Jeanes, 1968).

### Quantification of proteins

Bio-Rad dye-reagent concentrate was diluted to 25% with distilled water. 20 μl of the sample was mixed with 20 μl of Triton X-100 or NaOH solutions in varying concentrations at 20°C and incubated for 1 minute prior to the addition of 1.0 ml of the diluted dye reagent. The optical density was read at 595 nm in the interval of 5 to 60 minutes after addition of the dye reagent.

### High performance liquid chromatography (HPLC) analysis

The hot water extract was analyzed using an HPLC Spectra system P4000 (Thermo Finnigan, Waltham, MA) with a spherical reverse phase column (5 μm, 4.6 mm × 150 mm) (Waters Spherisorb® ODS2, Milford, MA). In addition, six ginsenoside standards, $Rb_1$, $Rb_2$, Rc, Rd, Re, and $Rg_1$, were purchased (ChromaDex™, Irvine, CA), that were used as controls to confirm the presence of these ginsenosides in the extract. A mixture containing all six ginsenosides at a concentration of 30 mg/ml dissolved in water was made and analyzed on HPLC. To spike the water extract with the standards, 20 μl of the ginsenoside standards mixture (30 mg/ml) was added to 300 μl of the water extract. Samples were introduced by autoinjector, with a 10 μl or 20 μl injection volume. Using a slightly modified protocol that was previously described (Li *et al.*, 1996), mobile phases were (A) water and (B) acetonitrile (HPLC grade; Fisher Chemical, Pittsburgh, PA), with a flow rate of 1 ml/min and the following gradient for B: 0–22 min, 20–22%; 22–40 min, 22–50%; 40–50 min, 50–55%; 52–54 min, 65–20%; and 54–70 min, 20%. The peaks were detected by UV spectrophotometry at 205 nm.

## Results

### Effect of ginseng on bacterial growth

To determine if ginseng has any effect on *P. aeruginosa* growth, PAO1 and PAO*mucA22* (PDO300) were grown in the presence of 1.25, 2.5 and 5% ginseng (Fig. 1). In general, the growth of these two strains was not significantly inhibited by the three different concentrations of ginseng in LB medium. In fact, 1.25% of ginseng seemed enhanced growth of the bacteria; the growth of the bacteria was exactly the same in 2.5% of ginseng as in the LB control, whereas

the growth of *P. aeruginosa* in 5% of ginseng showed a relatively slower growth from hour 6 to 12 in PAO1 and hour 6 to 15 in PAO*mucA22*.

### Effects of ginseng on the synthesis of alginate

The prototypic PAO1 does not produce alginate unlike its isogenic variant PAO*muc22*. The effect of ginseng on alginate production was compared between the strains with 2.5% and 5% ginseng (Fig. 2). Both concentrations did not stimulate any alginate production in PAO1. However, both 2.5% and 5% ginseng stimulated the production of alginate from the mucoid strain in a non-dosage-dependent way after over-night culture in LB medium.

### Effect of ginseng on *P. aeruginosa* protease activities

In a previous paper, we have shown that *P. aeruginosa* PAO1 and PAO*mucA22* expressed similar levels of protease activities (Mathee *et al.*, 1999a; Mathee *et al.*, 1999b). The effect of ginseng on the production of LasA and LasB proteases was determined in *P. aeruginosa* PAO1 (Fig. 3).

LasA staphylolytic protease is a 20-kDa zinc metalloendopeptidase belonging to the β-lytic endopeptidase family of proteases (Kessler, 1995). LasA production was inhibited only slightly by 2.5% ginseng, and completely with 5% of ginseng (Fig. 3A).

LasB elastase is a zinc metalloprotease capable of destroying or inactivating a wide range of biological tissues and immunological agents (Bever, Iglewski, 1988). There was a significant suppression of elastase activity with 5% ginseng, whereas 2.5% of ginseng slightly enhanced the production (Fig. 3B).

### Effect of ginseng on total extracellular protein concentrations in culture supernatants

Total extracellular protein concentration was measured to determine if suppression of the protease activity is correlated with decrease in protein production. The synthesis of extracellular proteins from *P. aeruginosa* PAO1 was slightly downregulated in the two media containing different concentrations of ginseng on hour 4 of the culture. However, 5% ginseng greatly upregulated the synthesis of extracellular protein from the bacteria on hours 7 and 10, whereas 2.5% ginseng did not affect the synthesis of extracellular protein (Fig. 4).

### Effect of ginseng on the production of AHLs

To determine if ginseng interfered with the production of AHL molecules, the amount of BHL and OdDHL produced in the supernatant was quantitated using mass spectroscopy in the absence and presence of ginseng. Ginseng inhibited the production of both BHL and OdDHL in the supernatants of over-night culture of *P. aeruginosa* PAO1 in a concentration-dependent manner (Fig. 5).

### HPLC analysis of the ginseng extract

In order to provide a general compositional view of the extract as well as confirmation of the presence of ginsenosides, HPLC of the aqueous extract was done and compared using the elution profile of ginsenoside standards (Figs. 6A and 6B). Initially, retention times were determined for each ginsenoside standard individually. Subsequently, the six ginsenoside standards were combined and the HPLC retention times were determined. The ginsenoside standards produced six peaks with retention times of 26.8 min, 28.0 min, 37.0 min, 37.6 min, 38.3 min, and 39.6 min corresponding to $Rg_1$, Re, $Rb_1$, Rc, $Rb_2$, and Rd, respectively (Fig. 6A). The water extract of ginseng contained six peaks with similar retention times, indicating the presence of $Rb_1$, $Rb_2$, Rc, Rd, Re, and $Rg_1$ (Fig. 6B). In addition, the hot water extract was spiked with standards to provide further confirmation of the presence of the ginsenosides (Fig.

6C). The peaks corresponding to the ginsenoside standards retention time were increased in size, thus verifying the presence of the six ginsenosides (Fig. 6C).

## Discussion

*P. aeruginosa* is the major pathogen responsible for the secondary infections in patients with burn trauma, diffused pan-bronchitis, chronic obstructive pulmonary disease, CF and with immune defects (Driscoll *et al.*, 2007). *P. aeruginosa* remains one of the major causes of nosocomial infections (Rosenthal *et al.*, 2008). The pathogenesis of *P. aeruginosa* is associated closely with the production of a myriad of extracellular virulence factors and the formation of biofilm, both of which are regulated by QS systems in the bacterium (Davies *et al.*, 1998; Kharazmi, 1989; Meyer *et al.*, 1996; Morihara, Homma, 1985). There is increasing evidence indicating that QS might be a novel target for developing new antimicrobial agents (Kaufmann *et al.*, 2008).

Previously, we found that ginseng treatment helped to speed up the clearance of *P. aeruginosa* from animal lungs with *P. aeruginosa* pneumonia, to decrease the severity of lung pathology by changing the type of immune response from a TH2 to a TH1 response and to stimulate the phagocytosis by neutrophils and monocytes (Song *et al.*, 1997a; Song *et al.*, 1997b; Song *et al.*, 1998; Song *et al.*, 2002; Song *et al.*, 1999). It has also been shown that *P. aeruginosa* virulence can be reduced using anti-QS inhibitors (Hentzer *et al.*, 2003). Thus, we postulated that protective effect of ginseng could be partially due to its ability to reduce the efficacy of virulence factor production that is often under QS-control.

### Ginseng exhibits no toxicity against *P. aeruginosa*

The toxicity of ginseng in mice is quite low, the LD50 for intravenous injection is 16.5 mg/kg (Bensky, Gamble, 1993). In the present study, ginseng did not inhibit the growth of neither mucoid nor non-mucoid *P. aeruginosa* (Fig. 1). Instead, lower concentrations of ginseng seemed to stimulate growth. Thus, it is likely that the effect of ginseng seen in *in vivo* studies is not due to bacterial killing. One should also be mindful not to prescribe a lower dose of ginseng that might enhance its growth properties and consequently have a negative outcome in patients.

### Ginseng stimulates alginate production

Recovering alginate-producing *P. aeruginosa* patients spells poor prognosis for CF patients (Høiby, 1974). Early aggressive antibiotic therapy is able to eradicate initial and intermittent colonization of the CF lungs by *P. aeruginosa* (Frederiksen *et al.*, 1997). However, when the colony morphology of bacteria isolated from sputum samples is observed to convert to the $Alg^+$ form, the organisms can no longer be eliminated from the lungs despite aggressive antibiotic therapy (Frederiksen *et al.*, 1997). The selection pressure for mucoid conversion common to *P. aeruginosa* strains that thrive in the complex CF respiratory environment is not well understood. However, it has been established that repeated exposure of a *P. aeruginosa* biofilm *in vitro* to activated polymorphonuclear leukocytes (PMNs), or to low-levels of hydrogen peroxide, can give rise to mucoid variants with defects in *mucA* gene, mimicking that seen *in vivo* (Mathee *et al.*, 1999b). Thus, any antibacterial or anti-QS agents used have to suppress alginate production for effective eradication of *P. aeruginosa* infection. In this study, ginseng stimulated alginate production (Fig. 2). The animal studies to date point to ginseng as a promising natural medicine for stimulation of the immune system in CF patients with *P. aeruginosa* lung infections. However, these studies were done largely with nonmucoid strains. The results from this study suggest that ginseng might not be appropriate for patients with chronic infections of mucoid *P. aeruginosa*. Moreover, similar results were obtained in mice infected with PAO1 and PAO*mucA22* (Song *et al*, unpublished observations). However,

the caveat is that the effect of ginseng on alginate production *in vivo* is not known. We believe that it can certainly be used for early infection where the immune response has to be stimulated as seen in an activation of innate immunity after ginseng treatment (Song *et al.*, 1997a). Alternatively the ginseng components with anti-QS activity should be separated from the alginate-enhancing components.

### Ginseng exhibits anti-QS activity

It has been demonstrated that the success of *P. aeruginosa* as an opportunistic pathogen is due to the battery of QS-regulated arsenals that it produces to combat host-associated aggression (antibiotics and immune response) (Davies *et al.*, 1998; Kharazmi *et al.*, 1989; Meyer *et al.*, 1996; Morihara, Homma, 1985). As such, targeting bacterial QS as potential anti-infective therapy has been of interest (Kaufmann *et al.*, 2008; Fast, 2003). Synthesizing chemical analogs has yielded compounds with anti-QS activity, but they are yet to make their way to pharmaceutical use (Rice *et al.*, 2005; Janssens *et al.*, 2007). However, the promise is in the naturally occurring compounds that can be potentially non- or mildly toxic. Anti-QS activity has been demonstrated in algae and terrestrial plants (Manefield *et al.*, 1999; Adonizio *et al.*, 2006; Adonizio *et al.*, 2008a; Adonizio *et al.*, 2008b; Singh *et al.*, 2009). This study clearly demonstrates that 5 % ginseng has the ability to counter the QS system. Ginseng was able to suppress the protease activities (Fig. 3). This suppression is not due to a decrease in cellular growth or protein synthesis, as 5% ginseng stimulated the synthesis of extracellular proteins in the supernatants, but not the virulence factors (Fig. 4). It appears that the reduction of protease activity may be due to the decrease in the production of BHL and OdDHL (Fig. 3), critical components that stimulate the production of virulence factors. It is of concern that 2.5% ginseng stimulated the synthesis of elastase from *P. aeruginosa*. Thus, it is important to determine the appropriate dose for ginseng to be administered as an effective natural remedy to combat *P. aeruginosa* infection.

Ginseng has been used widely as dietary supplement in the orient. In recent years, efforts have been made to validate its use. This study suggests that ginseng is a potentially promising remedy to treat *P. aeruginosa* infections in future. Ginseng extract is a complex mixture containing ginsenosides saponins and other root components such as polysaccharides, sterols, organic acids, phenolic acids, flavonoids, essential oils, vitamins and trace elements. The aqueous extract used in this study contains ginsenosides as well as other unidentified compounds (Fig. 6B). It is known that traditional Chinese medicine is characterized by multiple compounds working synergistically and isolating these activities are difficult (Lee, 2000). A number of these compounds could be responsible for the anti-QS activity. An acidic polysaccharide isolated from *P. ginseng* displayed anti-adhesive activity against pathogenic bacteria (Lee *et al.*, 2006). The activity of other ginseng-derived compounds has not been researched as extensively as ginsenosides. While ginsenosides have been shown to have a beneficial effect on a number of mammalian systems, the effects of ginsenosides on bacteria have not been widely examined (Liu, 1992). It is very likely the components that enhance growth, alginate production are different from those that exhibit anti-QS activity. It is important that these components are separated and tested before ginseng can be advocated for use against *P. aeruginosa* infections.

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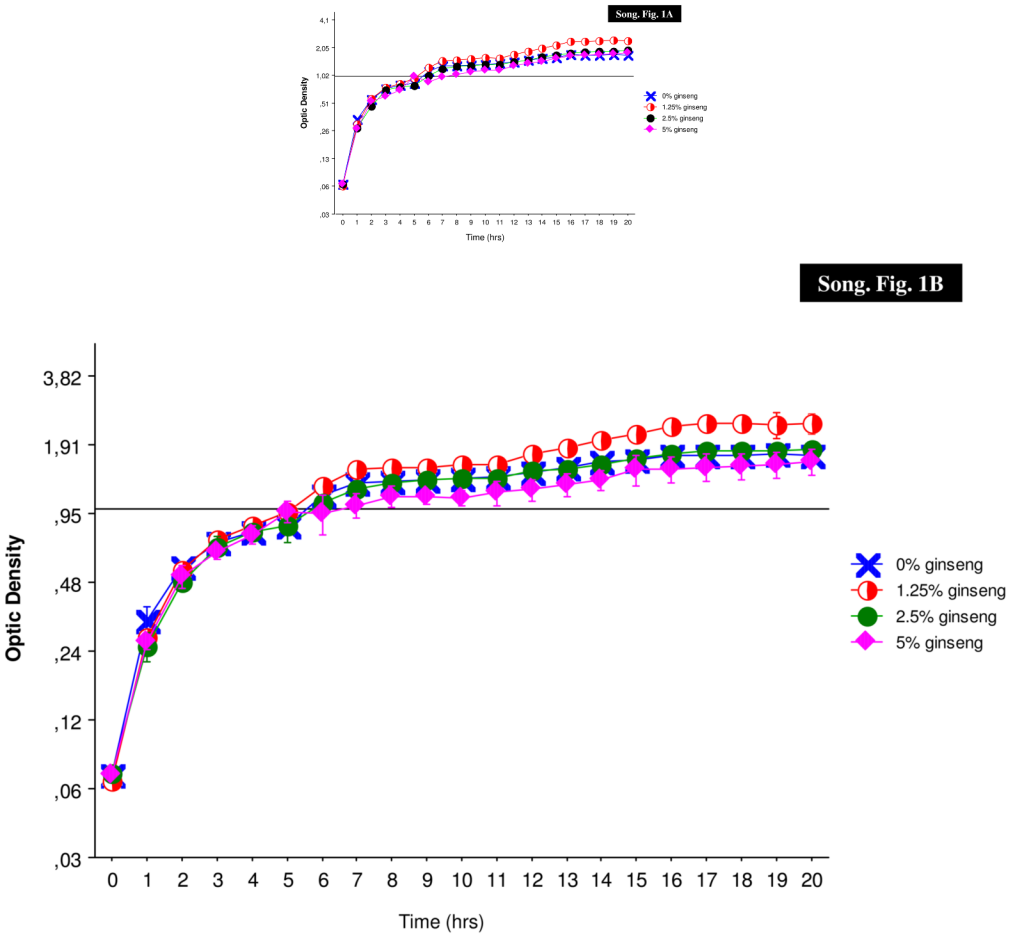



**Fig. 1.**

Growth curves of *P. aeruginosa.* The prototypic PAO1 (A) and PDO300 (B) were grown in the absence (X) and presence of 1.25% (open circle), 2.5% (filled circle) and 5% (diamonds) of ginseng.

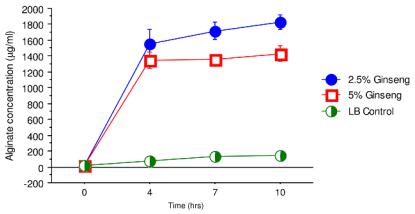



**Fig. 2.**

Alginate production in *P. aeruginosa*. The amount of alginate produced in the absence (X) and presence of 2.5% (filled circle) and 5% (diamonds) of ginseng were quantified.

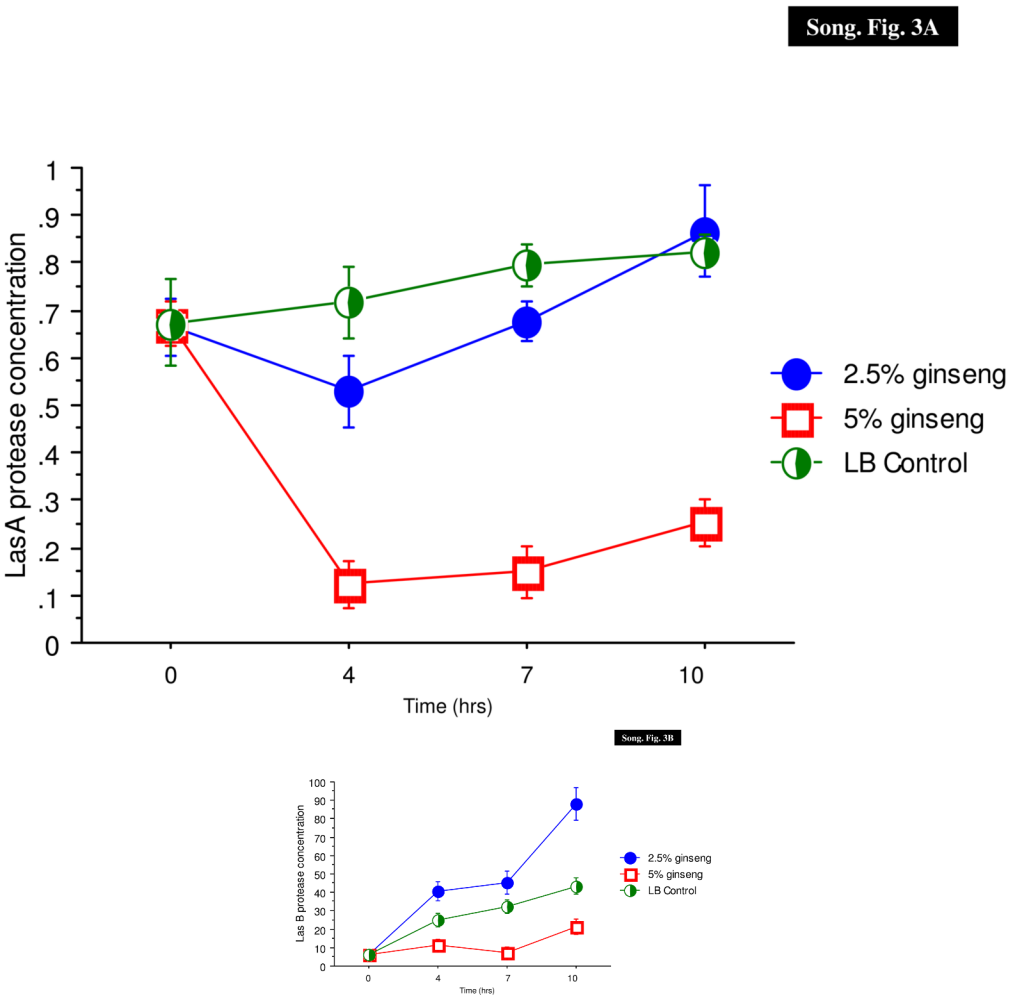



**Fig. 3.**

Effect of ginseng on *P. aeruginosa* PAO1 protease activities. The staphylolytic LasA (A) and elastolytic LasB (B) activities were monitored in the absence (X) and presence of 2.5% (filled circle) and 5% (diamonds) of ginseng.

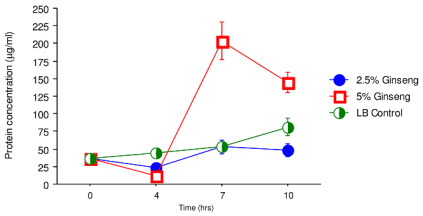
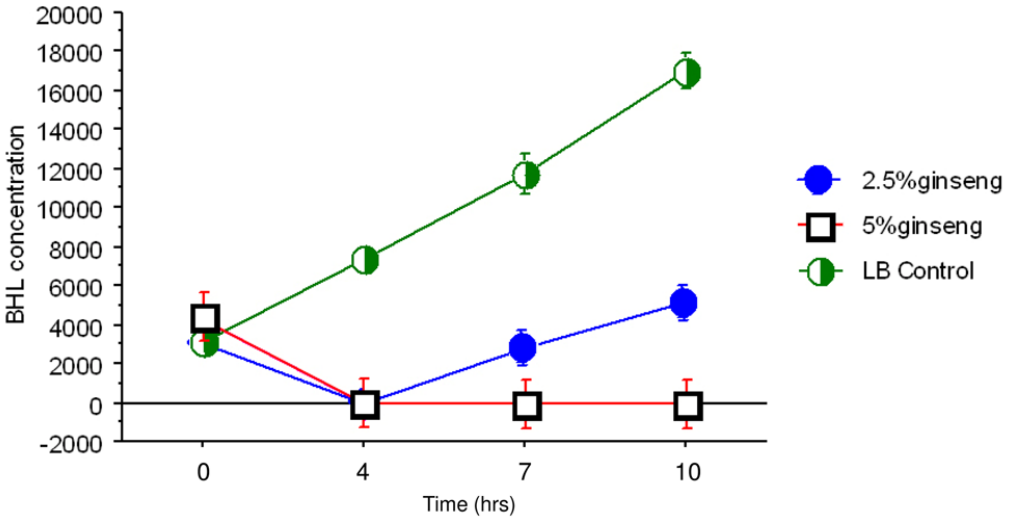



**Fig. 4.**

Extracellular protein levels in *P. aeruginosa* PAO1. The protein levels in the supernatant were quantified in the absence (X) and presence of 2.5% (filled circle) and 5% (diamonds) of ginseng.

Song. Fig. 5A




Song. Fig. 5B

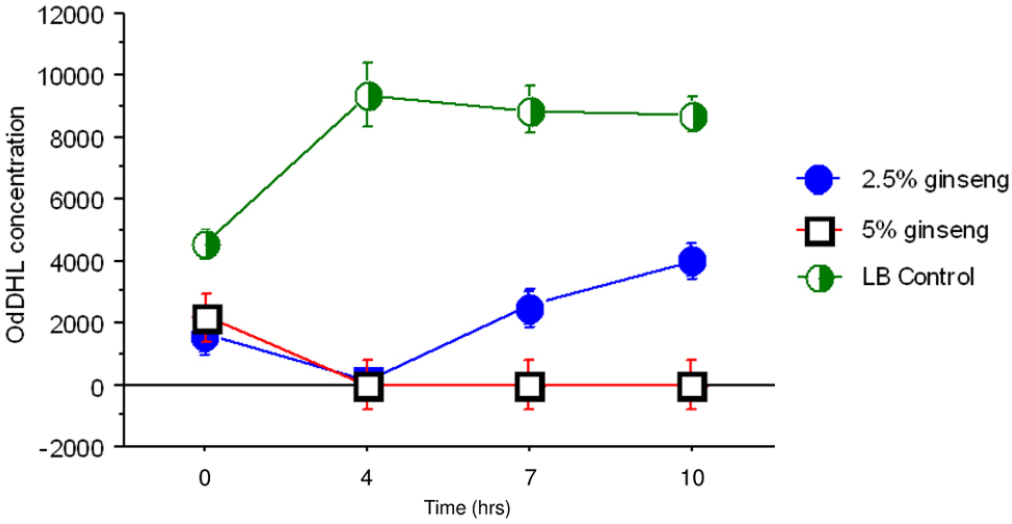



**Fig. 5.**

AHL production in *P. aeruginosa* PAO1. The BHL (A) and OdDHL (B) levels in the supernatant were quantified in the absence (X) and presence of 2.5% (filled circle) and 5% (diamonds) of ginseng.

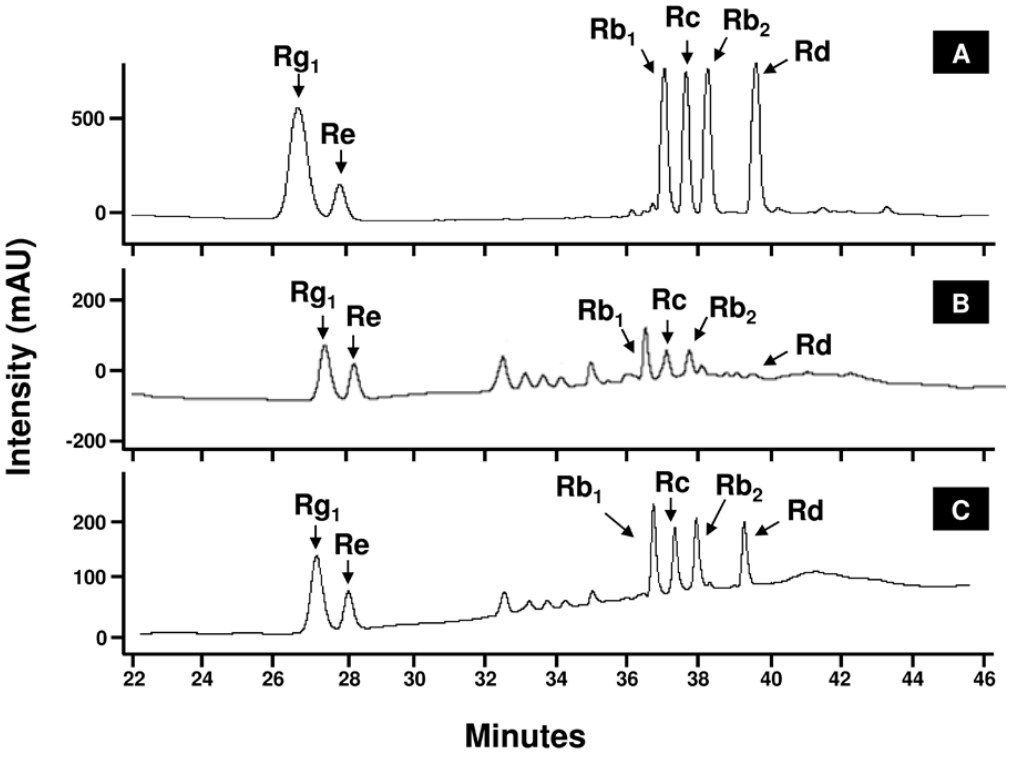



**Fig. 6.**

HPLC - UV analysis of ginseng extract. The chromatogram show combined ginsenoside standards (A) and aqueous ginseng extracts (B). Peaks are a function of intensity measured in milliabsorption units over time in minutes. Peaks corresponding to the six ginsensosides used as standards are labeled.