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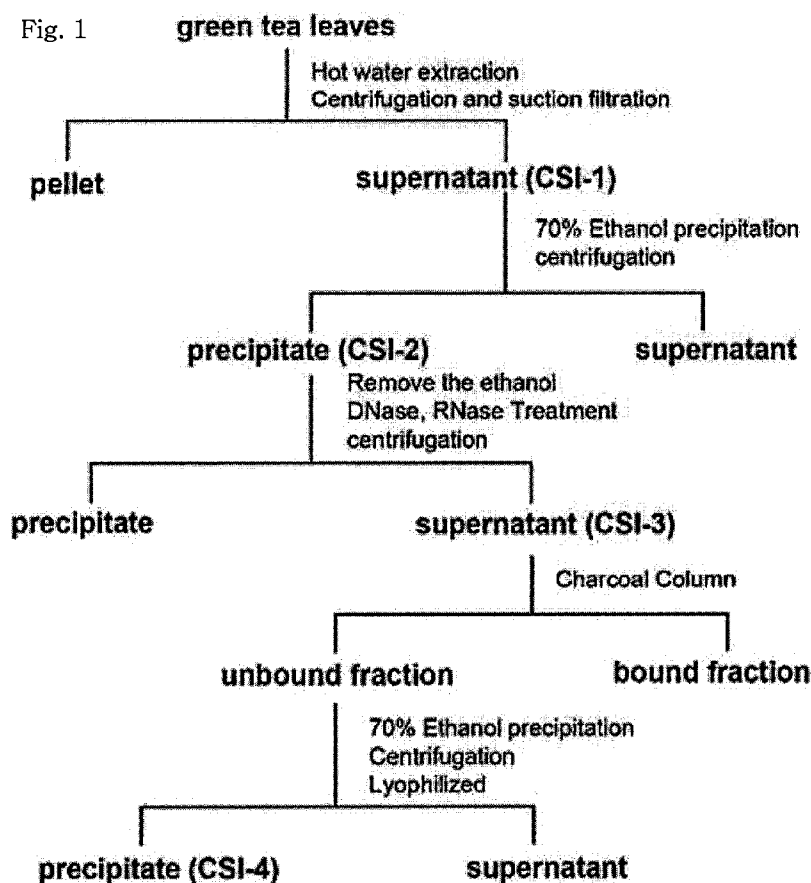
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(54) Title: PRODUCTION METHODS OF GREEN TEA EXTRACTS FOR PREVENTING ADHESION OF PATHOGENIC BACTERIA TO HUMAN CELL

Fig. 1



(57) Abstract: The present invention relates to a method for preparing green tea extracts having inhibitory activity for adhesion of pathogenic bacteria to human cells, which comprises the following steps: hot-water extracting dried green tea leaves, precipitating the extract using ethanol, and then filtering the ethanol precipitate with charcoal column. According to the present invention, we can prepare the green tea extract, having inhibitory activity for adhesion of pathogenic bacteria to human cells, by using charcoal column filtration in much higher yield than that of the conventional chromatography. Moreover, the present method can be used as a novel technology suitable for large-scale production due to simple procedure. The green tea extracts prepared by the present method have inhibitory activity for adhesion of Periodontitis-inducing bacteria, Acne vulgaris-inducing bacteria, Atopic dermatitis-inducing bacteria and Gastric disease-inducing bacteria to human cells. Therefore, they can be used as drugs for preventing and curing Gastric disease, Periodontitis, especially, Acne vulgaris and Atopic dermatitis.



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【DESCRIPTION】**【Invention Title】**

Production methods of green tea extracts for preventing adhesion of pathogenic bacteria to human cell

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【Technical Field】

The present invention relates to a method for preparing green tea extracts having inhibitory activity for adhesion of pathogenic bacteria to human cells, which comprises the following steps: hot-water extracting dried green tea leaves, precipitating the extract using ethanol, and then filtering the ethanol precipitate with charcoal column.

10

【Background Art】

Helicobacter pylori exclusively colonizes humans and primates and has been identified as a principal etiological agent of chronic active or type B gastritis, duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tumors (Buck, 1990). *Propionibacterium acnes* is a primary inhabitant of adult human skin (McGinley et al., 1978). This bacterium has been implicated primarily in acne, the most frequently encountered disease of the skin (Borjar and Holland, 2004). *Staphylococcus aureus* has been recognized as largely responsible for the known complication of atopic dermatitis (Henocq et al., 1982) and also as the primary causative agent of nosocomial infections (Projan and Novick, 1997). Adhesion of commensal or pathogenic bacteria to host cell surface is a crucial event in colonization and infection (Gibbons, R.J., 1989; Hultgren et al., 1993). Commensal bacterial species protect against pathogens by competing with pathogens for host binding sites (Tannock, 1995), and specificities of bacteria-host interactions have been the subject of many recent studies. It is now well established that cell surface carbohydrates mediate the pathogen-host cell recognition through single or multiple interactions.

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Green tea made from *Camellia sinensis* (Theaceae family) is a widely consumed beverage which provides a dietary source of biologically active

compounds considered to be beneficial to human health. The health benefits associated with tea consumption have resulted in the inclusion of green tea extracts as common ingredients in dietary supplements, nutraceuticals, and cosmetics. Green tea extract contains polyphenol, tannin and caffeine, and catechins have been most widely studied to prove the pharmacological action of green tea (Roberts and Wood, 1951). They have demonstrated significant antioxidant, anticarcinogenic, antiinflammatory, thermogenic, probiotic, and antimicrobial properties in numerous studies (Dongfeng et al., 2001). Given that *C. sinensis* is consumed mainly as a hot water infusion of fresh green shoots, other water soluble components of *C. sinensis* have been relatively less investigated.

Water soluble polysaccharides from green tea have been found to possess immunological, anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV, antioxidant, and hypoglycemic qualities (Roedig-Penman and Gordon, 1997; Bao et al., 2002). An acidic polysaccharide CSF2 isolated from *C. sinensis* leaves have shown remarkable inhibitory effects against the adherence of *H. pylori* to human gastric epithelial cells and *P. acnes* and *S. aureus* to NIH3T3 cells including erythrocytes (Lee et al., 2006). However, the purification yield of CS-F2 was very low, less than 0.02% on average. Water soluble green tea infusions (CSI) were instead prepared from *C. sinensis*, by hot water extraction, ethanol precipitation, enzyme treatment, and charcoal filtration. The objective of the present study was to evaluate the anti-adhesive role of the tea infusions against the pathogens as well as beneficial and commensal bacteria.

Periodontitis is a wide gingival disease in which tissues attached on teeth and alveolar bones are destroyed by infection of bacteria. It is known that periodontitis is an adult disease of which incidence increases as getting old, especially, periodontitis is a chronic disease from 40s up to old age, and a very important factor in health of old age. Finally, periodontitis makes teeth loose and has an great effect on health. The *Porphyromonas gingivalis* (hereinafter, referred to as "*P. gingivalis*"), one of bacteria inducing the periodontitis, is known as gram negative bacteria and the most important pathogenic bacteria inducing the chronic serious periodontitis in human body.

Acne vulgaris is one of common skin diseases from an adolescent period up to a middle age. Acne vulgaris is an inflammatory lesion of hair follicles and sebaceous glands within a skin, occurred in many sebaceous glands more than 3 millions in the skin, and an inflammatory disease induced mainly in a face, back and breast. Acne vulgaris occurs as a result of stimulation in a follicle epithelium wall by *Propionibacterium acnes* living in sebaceous gland due to increasement of sebum, which thickens keratins inside of the epithelium. In this period, a obstruction of the follicle caused by parakeratosis occurs and an inflow of oxygen within the follicle is decreased. These events become a very good condition for growth of anaerobic *P. acnes* which is bacteria inducing the acne vulgaris, and the proliferation of the bacteria is promoted in the acne lesions. Generally, bacteria such as *Propionibacterium acnes* and *Staphylococcus aureus* are existing on the healthy skin. In accordance with individuals, the number of the *Propionibacterium acnes* existing on 1 cm² of the skin reaches several tens to hundreds of thousands.

Atopic dermatitis is one of the most common skin disease and the chronic inflammatory skin disease. Atopic dermatitis usually occurs from a three-month-old baby in neonate, has prevalence rate in more than 10% of infants and more than 30% of children in school, and shows high increase as other allergic ailment. Causes of atopic dermatitis include aromatics, antiseptics, disinfectants, solvents, as well as indoor dusts, house dust mites, molds, foods, pollens, and pet dogs. The antigens penetrate protective barrier of the skin injured by atopic dermatitis and become the cause of contact dermatitis. It is known through many studies that *Staphylococcus aureus* propagates in the skin of 90% of atopic patients, and the epithelium of atopic patients is infected by *Staphylococcus aureus*. *S. aureus* is a second cause of atopic dermatitis as well as a primary cause of atopic dermatitis, which occurs as a skin follicle is infected, a sweat gland is infected in a stuffy state, or a wound is penetrated by *S. aureus*. *S. aureus* have been found in 90% of atopy patients but within 10% of the normal. An enterotoxin released by *S. aureus* worsen the atopic dermatitis seriously. *S. aureus* also participate in occurring a toxic shock syndrome induced by superantigen in a blood vessel. More important thing is that an increasement of antibiotic resistant bacteria, such as MRSA

(methicillin-resistant *Staphylococcus aureus*) which is the mutant of *S. aureus*, brings about some problems. In order to cure the atopic dermatitis, therefore, removal of *S. aureus* as well as treatment of allergy should be carried out. Moreover, the treatment is needed which can be used in combination with antibiotics or as substitute antibiotics.

Generally, normal cutaneous flora that play the role of mixing a sweat and sebum are good for the skin as beneficial normal flora. On the other hand, excessive increasement of harmful normal flora such as *P. acnes* and *S. aureus* is causative of breaking the balance of normal cutaneous flora. *Staphylococcus epidermidis* as normal cutaneous flora is gram positive, non-pathogenic, and coagulase-negative bacteria. But *Staphylococcus epidermidis* may be often pathogenic if they infect an operation-regions of patients, unlike the normal skin, after a surgical or internal operation. *Lactobacillus acidophilus* is important for maintenance of intestinal bacterial flora. As existing within intestines, *Lactobacillus acidophilus* plays a beneficial role such as promoting growth of host and protecting it from the pathogenic bacteria. *Escherichia coli* is a facultative anaerobe living in gastrointestinal tract of mammals. Although the bacteria such as *E. coli* O-157 bring about human diseases, most of *E. coli* strains except for *E. coli* O-157 assist food digestion in digestive organs and produce vitamin B and K. Accordingly, these bacteria existing in the human body are divided into beneficial and harmful bacteria. It is known that some bacteria can be changed into pathogenic bacteria causing diseases in accordance with a circumstance or a region of the body, even if the bacteria is beneficial.

Helicobacter pylori is known to cause a chronic and acute gastritis, a gastric ulcer, a stomach cancer, etc. as gram-negative bacteria. It is reported that more than half of 60 years old are being infected in the industrial society, and most of people are being infected from their childhood in the developing country. In case of children from 2 to 8 years, approximately 10% of them are infected every year, so most of them come to show a tendency to infection when being teenagers. Recently it is a general opinion that the inflammation is occurred at the time of infection by *Helicobacter*. When the inflammation spreads over submucous layer

within the stomach, it comes to develop into the gastric ulcer. Although it does not progress directly to stomach cancer, it is known that a possibility to progress to stomach cancer may get higher more than 3-5 times when infected by *Helicobacter*.

5 *Helicobacter* binds to the mucous epithelium within the stomach, the binding mechanism has been reported to depend mainly on the interaction between carbohydrates and bacterial surface proteins recognizing it. Especially, the recognition carbohydrate are reported to be a sulfated carbohydrate of mucin glycoprotein, a sialyllactose, a fucosylated Lewis b antigen, etc.. Recently,
10 researchers are trying to inhibit the adhesion of bacteria to human cells by using glycosphingolipids or mucin proteins in saliva.

The antibiotics are mainly used for curing and protecting the infectious disease, particularly acne and atopy. However, many pathogenic bacteria have a resistance against the antibiotics because of misuse and abuse of the antibiotics.
15 The life of antibiotics also gets shorter, so the use of them are increasing.

Carbohydrates have several functions in vivo and carbohydrates such as glycoprotein and glycolipids on the cell surface have a function of ligand recognizing proteins, so the carbohydrates provide attachment sites of extraneous pathogenic bacteria, virus, toxic materials and hormone as well as cell-to-cell
20 interactions. As the pathogenic bacteria penetrate into human cell, the bacteria induce inflammations and diseases, and so blocking up completely adhesion of the bacteria to human cells in an early stage can play a role of preventing diseases. The early infection of the pathogenic bacteria can be blocked if anti-adhesion drugs are developed by using the carbohydrates of human cell surface playing a
25 important role in early stage of pathogenic bacterial infection.

Accordingly, as the anti-adhesion treatment removes and prevents adhesion of infective mediators, specifically decreases the contact between human tissue and pathogenic bacteria, and specifically permits the adhesion of beneficial bacteria, a development of bio-friendly treatment maintaining a balance of normal
30 cutaneous flora is possible. Consequently, studies for the interaction and binding between the carbohydrates and bacterial surface proteins recognizing it will

contribute to understand the adhesion mechanism of these bacteria to human skin cells. The bacteria cells usually show hemaagglutination, which is a reaction occurred as a result that adhesins on the bacteria recognize carbohydrates or proteins on the surface of human cells such as red blood cells. The hemagglutination is extensively applied to cell-to-cell recognition reaction because the surface of red blood cells have the same or similar carbohydrate-composition as that of human epithelial cells. It is possible to study the recognition between the bacterial surface proteins and human cells through hemagglutination. The important thing is that inhibiting the adhesion by the interaction can be used for curing and preventing diseases induced by pathogenic bacteria, because the carbohydrate-compositions of skin cells and red blood cells is the same or similar.

In order to overcome above-mentioned problems of conventional methods, the present inventors have tried to develop novel methods for inhibiting the adhesion of pathogenic bacteria. As a result, we have identified that we can produce and purify the green tea extracts inhibiting remarkably human cell-adhesion of pathogenic bacteria, for example, *Porphyromonas gingivalis*, *Propionibacterium acnes*, *Staphylococcus aureus* and *Helicobacter pylori*, without inhibitory effects on other normal flora, simply in high yield by extracting green tea leaves with hot-water, precipitating the extracts with ethanol and filtrating the precipitates using charcoal column, and completed the present invention successfully.

【Disclosure】

【Technical Problem】

Accordingly, it is an main object of the present invention to provide a method for producing green tea extracts inhibiting remarkably human cell-adhesion of pathogenic bacteria, for example, *Porphyromonas gingivalis*, *Propionibacterium acnes*, *Staphylococcus aureus* and *Helicobacter pylori*, without inhibitory effects on other normal flora, simply in high yield.

【Technical Solution】

In one aspect of the present invention, there is provided a method for preparing green tea extracts having inhibitory activity for adhesion of pathogenic bacteria to human cells, which comprises the following steps:

a) extracting dried green tea leaves with hot water;

5 b) centrifuging the hot-water extracts, and precipitating the supernatant using ethanol;

c) filtering the ethanol precipitates with charcoal column; and

d) freeze-drying the filtrates.

The present invention relates to a new method for producing green tea
10 extracts(aqueous infusions of green tea leaves) which comprise acidic polysaccharides extracted from the green tea(*Camellia sinensis*) leaves and are known to be effective in the present inventor's Registered Patent (Korean Patent Registration No. 10-0700912) more simply and in higher yield. In conventional methods, the yield of extraction of the acidic polysaccharides was very low,
15 because they had been prepared using Gel-Filtration Chromatography after collecting only an active fraction among fractions obtained by Ion-Exchange Chromatography. However, in the method of the present invention, green tea extracts were prepared by filtrating the hot-water extracts using open column filled with activated charcoal that is easy to purchase commercially, therefore, the
20 method of the present invention may be comparatively simple procedure, suitable for large-scale procedure due to high economical efficiency, and easy to apply industrially. As seen in Example 3 of the present invention, the green tea extracts could be obtained in the higher yield of more than 300 times and, none the less, the green tea extract represented inhibitory activity for the adhesion of infective
25 bacteria to human cells, which was equal to that of the green tea extract prepared by collecting only active fractions using the chromatography according to the conventional method.

In the method of present invention, the green tea extracts are prepared by extracting the green tea with a hot distilled water according to usual intake and a
30 extraction method of green tea, which is considered to be human body-friendly in contrast to the conventional extraction method using organic solvents that is hard

to apply to cosmetics and drugs. The green tea extracts have a high uronic acid content and are greatly specific to inhibiting the attachment of *P. gingivalis*, *P. acnes*, *S. aureus* and *H. pylori* on human skin cells.

The conventional extraction methods of green tea leaves depend on organic solvents, which are in contraposition to ingesting green tea boiled in water. In present invention, green tea extracts extracted by hot distilled water(90℃) have 20-30% of uronic acid contents, the Minimum inhibitory concentration(MIC) inhibiting hemagglutination and adhesion to fibroblast by *P. gingivalis*, *P. acnes*, *S. aureus* and *H. pylori* shows an effective inhibitory activity at 0.05-0.5 mg/ml, which is a low concentration when based on high molecular weight.

In the present invention, preferably, the preparing method may further comprise a step, dissolving the ethanol-precipitates in phosphate buffer solution and removing nucleic acids, after the step b). To remove the nucleic acids, the ethanol-precipitates are treated with RNase and DNase. This may improve viscosity and physical property of green tea extracts and increase inhibitory activity for adhesion to cells.

In the preparing method of the present invention, the charcoal column in the step c) removes catechins using granular activated carbons. Although the charcoal may be powder type of activated carbons(charcoal), it is preferably a granular type of activated carbons in order to be filled in column and used as Examples of the present method. The catechins are well known as a main active component of the green tea and have several useful activities such as anti-oxidant activity. However, green tea extracts of the present invention contain uronic acids, not catechins, as active components by removing catechins from the extracts. In addition, the present invention demonstrates indirectly that this green tea extracts have inhibitory activity for the adhesion of a pathogenic bacteria to human cells.

In another aspect of the present invention, there is provided a composition for inhibiting adhesion of pathogenic bacteria to human cells, which comprises green tea extracts prepared by the above present methods.

In the present invention, preferably, the pathogenic bacteria are selected from a group consisting of periodontitis-inducing bacteria, acne vulgaris-inducing

bacteria, atopic dermatitis-inducing bacteria, or gastric disease-inducing bacteria.

In the present invention, it was found that green tea extracts of the present invention suppressed hemaagglutination by *Porphyromonas gingivalis* (periodontitis-inducing bacteria), *Propionibacterium acnes* (acne vulgaris-inducing
5 bacteria), *Staphylococcus aureus* (atopic dermatitis-inducing bacteria) or *Helicobacter pylori* (gastric disease-inducing bacteria), and was great effective for inhibiting adhesion of them to fibroblast in skin (Fig 3 and 4).

The green tea extracts of the present invention prepared by the above method may be used as drugs for preventing and curing Gastric disease,
10 Periodontitis, especially Acne vulgaris and Atopic dermatitis, because they have strong inhibitory activities against hemaagglutination mediated by *Porphyromonas gingivalis* as Periodontitis-inducing bacteria, *Propionibacterium acnes* as Acne vulgaris-inducing bacteria and *Staphylococcus aureus* as Atopic dermatitis-inducing bacteria, and have also the inhibitory activity for *Helicobacter pylori* as
15 Gastric disease-inducing bacteria.

The present invention has many advantage in the industrial application aspect as compared with the conventional methods for preparing green tea extracts. The present invention is superior in time and economical aspects to ion-exchange and Gel-filtration Chromatography because the purification of green tea
20 extracts is more simple, and it is appropriate for large scale of production. In addition, unlike the conventional methods, the preparing method of the present invention using the activated carbons permits green tea pigments to be removed conveniently and active extracts with lots of the uronic acid to be gained.

In order to be used as a pharmaceutical composition for preventing and
25 curing the above described diseases, the green tea extracts according to the present invention may be prepared by conventional methods known in the pharmaceutical field. And the composition may be prepared in several formulations including, but not limited to, powder, granules, tablets, capsules or injectable agents by mixing with pharmaceutically acceptable carriers, excipients
30 and diluents. Further, the composition can be administered orally or parenterally.

The dosage of the green tea extracts according to the present invention may

vary depending on factors such as patient's age, sex, and physical condition, and severity of disease or symptom. Preferably, the green tea extracts or its powder can be administered to an adult patient in the range of 1-500 mg per day.

We previously reported strong inhibitory effects of acidic polysaccharide (CS-F2) from *Camellia sinensis* against the adhesion of *Helicobacter pylori*, *Propionibacterium acnes* and *Staphylococcus aureus* to host cell lines (Korean Patent No. 10-0700912). However, the purification yield of CS-F2 was very low (0.012%) from the green tea leaves. In our manuscript, we described the preparation of water infusions, to show a green tea infusion (CSI-4) with the maximum yield of 4% and with strong inhibitory activity on the adhesion of pathogens to host cell lines. The effects were comparable to those given by CS-F2, and were the highest against *P. acnes* that causes acne. More importantly, CSI-4 exhibited no inhibitory effects against *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Escherichia coli*, or *Staphylococcus epidermidis*, suggesting that it may exert a selective anti-adhesive effect against certain pathogenic bacteria with no adverse effects against beneficial bacteria.

Water extracts containing the polysaccharide from green tea (*Camellia sinensis*) leaves were prepared by hot water extraction, ethanol precipitation, enzyme treatment, and charcoal filtration. This process resulted in the isolation of a green tea infusion (CSI-4) with the maximum yield of 4%, which contains a major proportion of carbohydrates and lack of catechins. CSI-4 showed strong inhibitory activities against hemagglutination mediated by pathogens *Helicobacter pylori*, *Propionibacterium acnes* and *Staphylococcus aureus* with the minimum inhibitory concentrations of 0.01-0.5 mg/mL, and on their adhesion to host cell lines with the IC₅₀ values of 0.14-2.3 mg/mL. It exhibited the highest activity against *P. acnes* that causes acne. Importantly, no inhibitory effects were observed against *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Escherichia coli*, or *Staphylococcus epidermidis*. Our results suggest that CSI-4 may exert a selective anti-adhesive effect against certain pathogenic bacteria with no adverse effects against beneficial bacteria.

An aqueous infusion of green tea leaves has been a popular beverage in

Asia for centuries and provides a dietary source of biologically active compounds considered to be beneficial to human health (Lee et al., 2006). It has a naturally high content of polyphenols including catechins that make up approximately 30-45% of the solid green tea leaves (Lee et al., 2004). In a case-control study, the infection rate of *H. pylori* in the stomach was low in the group that consumed a large quantity of green tea (Graham et al., 1992). CS-F2 from green tea leaves was previously reported to have high inhibitory properties against the adherence of *H. pylori* to AGS gastric cells and of *P. acnes*, and *S. aureus* to NIH 3T3 fibroblast cells (Lee et al., 2006). Due to the extremely low yield of CS-F2 (0.012%) from green tea leaves, an aqueous infusion CSI-4 was instead prepared by hot water extraction, ethanol precipitation, enzyme treatment, and charcoal filtration. The purification yield of CSI-4 (5%) containing a major portion of carbohydrates without catechins was increased more than 300 times that of CS-F2. Green tea pigments abundant in CSI-3 could be removed most effectively by the use of activated charcoal filtration, which was turned out to be successful to exclude the polyphenol compounds (Table 1).

CSI-3 has potent anti-adhesive activities, similar to that of CSI-4, but showed the inhibitory activity against a commensal bacterium *E. coli*. CSI-1 and CSI-2 showed antiadhesive activities against more commensal or beneficial bacteria. Thus, the anti-adhesive activities of the tea infusions become more pronounced against the pathogens and less effective against good bacteria, as the purification proceeds, simultaneously with the increase in the proportion of acidic carbohydrates. Citrus pectin, a high uronic acid polysaccharide, exerts a strong anti-adhesive effect against *P. gingivalis*, *P. acnes*, and *S. aureus* without affecting commensal or beneficial bacteria (Lee et al., 2004). Negatively charged polysaccharides, such as dextran sulfate, also inhibit the adhesion of respiratory pathogens, including *P. aeruginosa*, *Burkholderia cenocepacia*, *Burkholderia pseudomallei*, *L. pneumophila*, *Bacillus anthracis*, and *Y. pestis* (Thomas and Brooks, 2004). Our results are strongly suggestive that the infusion CSI-4 shows the excellent selective anti-adhesive activity against pathogens without adverse effect on beneficial bacteria and that the selectivity of CSI-4 against pathogens

may arise from the acidic polysaccharide CS-F2.

The attachment of pathogenic bacteria to human cells is a pivotal step in the initiation of infections, which lead to the development of a variety of diseases (Hultgren, 1993, Sharon and Ofek, 2000). Pathogens have a broad spectrum of different specificities in adhesion to host cells, and a number of cell surface carbohydrates that mediate pathogen-cell interactions have been previously discovered (Ascencio et al., 1993, Veerman et al., 1997). In particular, the Lewis b blood group carbohydrate antigen has been demonstrated to mediate the adherence of *H. pylori* to human gastric mucosa, and the formation of biofilm from the fibrin fibers and glycocalyx is an important first step in the adhesion of *S. aureus* to the skin (Ilver et al., 1998, Krautgartner et al., 2005). The acidic polysaccharide CS-F2 inhibits the *H. pylori*-mediated hemagglutination (0.12 μ M), higher than that of *P. ginseng* or *A. capillaris*, (1-15 μ M) and much higher than sialyllactose (ca. 1.1 mM) (Lee et al., 2004). The tea infusion CSI-4 was proved to inhibit the adhesion of *H. pylori*, *P. acnes*, and *S. aureus* to host cell lines as highly as CS-F2. Furthermore, CSI-4 showed additional anti-adhesive activity against *P. gingivalis*, a major etiological pathogen in advanced adult periodontitis (Haffajee and Socransky, 2000), which was not observed in CS-F2. The basis for the difference in specificity to this oral pathogen is yet to be determined. Notably, CSI-4 exhibited dramatically reduced adhesion of *P. acnes* to NIH3T3 fibroblast cells, reaching approximately 80-90% inhibition at concentrations of 0.25 mg/mL or above, indicating that the anti-adhesive activity of CSI-4 was the highest against *P. acnes* that causes inflammatory acne.

Bacteria that are attached to host cells are very difficult to dislodge. CSI-4 demonstrated to be able to detach *H. pylori* that had adhered to AGS human gastric epithelial cells in a concentration-dependent manner. In this context, CSI-4 could play a role as an analog of glycoconjugates on the cell surface and further dislodge the attached bacteria from the host cell. It was also shown that sialyllactose not only inhibited *H. pylori* binding to duodenum-derived human cells, but also can detach bacteria that were already bound to cells via their surface lectin. Soluble carbohydrates can block adhesion of the bacteria to a variety of

host cells, and ligand-coated bacteria would decrease the host cell adhesion. Several inhibitory oligosaccharides which are common constituents of human milk play a protective role against different infectious agents in breast-fed babies (Sharon and Ofek, 2000). Importantly, CSI-4 can be nontoxic substance without
5 detrimental effects on cell viability of host cells.

In the present invention, the green tea extracts may be referred to green tea aqueous infusion throughout the Examples.

【Description of the Drawings】

10 Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Figure 1 shows extraction scheme of green tea infusions. The steam and dried green tea leaves were homogenized and extracted in hot distilled water,
15 followed by 70% ethanol precipitation, DNase and RNase treatment, and filtration using activated charcoal.

Figure 2 shows micrograph images of hemagglutination inhibition by CSI-4 and catechin (magnification x 100). A positive control (PC) for each bacterium is shown in left column. The anti-adhesive activities of CSI-4 and catechin are shown
20 for each bacterium at their minimum inhibitory concentrations.

Figure 3 shows anti-adhesive activity of CSI-4 on the adhesion of *H. pylori* to AGS cells. The activity was determined by urea phenol red method. The positive control without inhibitor was used to establish 100% attachment. The results are the means $\pm$ SE for three independent experiments. Asterisk (*) indicates significant
25 difference from control ($p < 0.05$ by paired t-test). (A) Anti-adhesive activities, after preincubation of CSI-4 and *H. pylori* followed by addition to AGS cells. (B) anti-adhesive activities, after preincubation of AGS and *H. pylori* followed by the addition of CSI-4.

Figure 4 shows anti-adhesive activity of CSI-4 on the adhesion of (A) *P. gingivalis* to KB cells, (B) *P. acnes* and (C) *S. aureus* to NIH 3T3 cells. The activity
30 was determined by colony counting method. The results were expressed as the

relative percentage of attached bacteria, compared with the positive control as 100%. The results are the means $\pm$ SE for three independent experiments. Asterisk (*) indicates significant difference from control ($p < 0.05$ by paired t -test).

Figure 5 shows an effect of CSI-4 on host cell viability. AGS, KB, and NIH3T3 cells were treated with various concentrations of CSI-4 and examined by MTT assay. The results are means $\pm$ SD of three independent experiments in triplicate. Asterisk (*) indicates significant difference from control ($p < 0.05$ by paired t -test).

10 【Best Mode】

Now, the present invention is described in detail based on the following Examples. The Examples are only for illustrative purpose and the present invention is not limited thereto.

15 **Example 1: Bacterial cell growth**

H. pylori (ATCC 43504 and 49503) cells were grown in Brucella broth (Difco) containing 10% (v/v) fetal bovine serum (FBS), 0.2% (w/v) 2,6-di-o-methyl- β -cyclodextrin (CD). The cells were incubated in a 10% CO₂ atmosphere at 37°C for 2 days. The cultured bacteria were identified by urease and catalase reactions, and confirmed by polymerase chain reaction based on *VagA*, *Cag1*, *IceA1*, *IceA2*, *BabA*, and *UreA* primers (Lee et al., 2004). *P. gingivalis* (ATCC 33277) and *A. actinomycetemcomitans* (ATCC 29522) were grown for 2 days in trypticase soy broth (Difco) supplemented with 0.6% yeast extract at 37°C under a condition of 80% N₂, 10% H₂ and 10% CO₂. In case of *P. gingivalis*, hemin (10 mL/L) and vitamin K3 (0.2 mL/L) were included. *P. acnes* (ATCC 6919) was grown for 2 days in brain-heart infusion (Difco) at 37°C in an atmosphere of 80% N₂, 10% H₂ and 10% CO₂. *S. aureus* (ATCC 27217) and *Staphylococcus epidermidis* (ATCC 12228) were grown in brain-heart infusion (Difco) at 37°C. *Escherichia coli* strains, BL21(DE3) and BL21(DE3)pLysS from Novagen (Madison, U.S.A.), were cultured on Luria-Bertani (LB) agar plates. All bacterial cell stocks were maintained with 20% (w/w) glycerol in a liquid nitrogen tank until required. *Lactobacillus*

acidophilus (ATCC 4356) and *Bifidobacterium bifidum* (ATCC 29521) were directly used from the stocks obtained from the Korean Culture Center of Microorganisms (Seoul, Korea), without further growth.

5 **Example 2: Mammalian cell growth**

AGS (ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line), KB (ATCC CRL 17, a human adenocarcinoma oral cell line) and NIH 3T3 cells (ATCC CRL-1658, a mouse embryo fibroblast epithelial cell line) were grown to confluence in tissue culture flasks (100 mm²) in RPMI 1640 or DMEM medium
10 supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotics in a 5% CO₂ incubator at 37 °C for 2-3 days.

Example 3: Preparation of green tea extracts (infusions)

The tea leaves (50 g) were homogenized and extracted with hot distilled
15 water (500 mL, 90 °C) for 3 h. After high speed centrifugation and filtration for the removal of any insoluble materials, the supernatant was precipitated using ethanol (final 70% concentration) at 4 °C. The precipitate was dried and dissolved in PBS (100 mM sodium phosphate, pH 7.4, and 150 mM NaCl) containing 10 mM MgCl₂ and 1 mM CaCl₂, and then incubated for 3 h with RNase and DNase at 37 °C. The
20 enzyme reactions were stopped by heating at 100 °C for 10 min. After centrifugation, supernatant was concentrated by using rotary evaporator, and passed through the activated charcoal-packed column (Duksan company, KR) to remove the color substance of the extract. The pass-through eluent was precipitated by cold ethanol (final 70% concentration) and lyophilized.

25

Extraction and characterization of green tea infusions

The preparation of green tea infusions from *C. sinensis* followed a different procedure from the one reported previously (Fig. 1) (Lee et al., 2006, Korean Patent No. 10-0700912). The tea leaves (50 g) were dissolved in hot distilled
30 water (500 ml). After centrifugation to remove insoluble materials, the supernatant (CSI-1) was precipitated using ethanol. The ethanol precipitate (CSI-2) was

dissolved in PBS and treated with RNase and DNase. After centrifugation, the supernatant (CSI-3) was passed through a charcoal filter column. The filtrate was precipitated using 70% ethanol and lyophilized (CSI-4).

Our new procedure provided green tea infusions from CSI-1 to CSI-4 with significantly high yields (Table 1). The recovery of CSI-4 from the tea leaves was found to be 4% (2 g) which is approximately 300-fold increase over that of CS-F2 (0.012%) (Korean Patent No. 10-0700912). Only a trace amount of proteins was found and catechin was absent in CSI-4, suggesting that both catechins and green tea pigments could be effectively removed by activated charcoal. Neither protein nor catechin was detected in the acidic polysaccharide CS-F2 (Lee et al., 2006). As the purification proceeds, the proportion of uronic acids in the infusions increased.

[Table 1] General characteristics of CSI from *C. sinensis*

	Purification Yield Gram (%) ^a	Total sugars (μg/mg)	Uronic acids (μg/mg)	Catechins (μg/ml)	Protein (μg/mg)
CSI-1	13.35 (26.7)	569.59	223.49	51.33	46.88
CSI-2	3.15 (6.3)	359.02	251.69	27.79	87.74
CSI-3	3 (6.0)	196.42	221.44	16.33	52.44
CSI-4	2 (4.0)	204.15	373.23	0	9.72
CS-F2	0.006 (0.012)	177.13	457.13	0	0

^aThe purification yield was calculated from 50 g of green tea leaves.

Example 4: Hemagglutination assays

Human erythrocytes suspended in 10 mL PBS were incubated with trypsin (2 mg) at 37 °C for 3 h, washed three times, and suspended at 2% (w/v) in PBS. Equal volumes of two-fold dilutions of the bacterial suspensions with isolated infusions were mixed and incubated at room temperature for 30 min. Each mixture was then added to O-type blood samples in Ushaped 96 microwell plates. The extent of the inhibition of hemagglutination was visualized by microscopic

inspection. Aspartic and glutamic acids were tested as non-specific reactions, and exhibited no inhibitory activities. Erythrocytes in the bacterial suspension in the absence of anti-adhesive agents were used as a positive control and erythrocytes without bacterial suspension were employed as a negative control (Molin, 2001).

5

Green tea infusions inhibit pathogen-mediated hemagglutination

CSI-4 exhibited strong inhibitory activity on hemagglutination mediated by *H. pylori*, *P. gingivalis*, *P. acnes*, and *S. aureus*, with a minimum inhibitory concentration (MIC) of 0.01-0.5 mg/mL (Table 2), but no inhibitory activity against
10 *A. actinomycetemcomitans*. It was previously reported that the polysaccharide CS-F2 inhibited the attachment of *H. pylori*, *P. acnes*, and *S. aureus* to erythrocytes, at concentrations of 0.01-0.1 mg/mL (Lee et al., 2006). Our data suggest that CSI-4 demonstrates strong anti-adhesive activities against these pathogens comparable to those of CS-F2, in addition to *P. gingivalis*. Catechin was examined to show
15 weak activity against the skin pathogens with MIC values of 1.0 mg/mL, and little activity against other pathogens (Table 2).

CSI-3 was found to have potent anti-adhesive activities, similar to those of CSI-4, but it showed the activity against *E. coli* with an MIC of 0.5 mg/mL. CSI-1 and CSI-2 did not exhibit any activities against the pathogens even at
20 concentrations of 2.0 mg/mL, and they rather showed anti-adhesive activities against commensal bacteria *E. coli*, and *S. epidermidis*. In case of CSI-1, *E. coli* was blocked at a low concentration of 0.05 mg/mL (Table 2).

[Table 2] Inhibition of bacteria-induced hemagglutination and adhesion to host
25 cells by green tea infusions

Inhibitors (Mw Da)		Minimum inhibitory concentration (mg/mL) ^a							
Red Blood Cells	<i>H. p</i> ^b	<i>P. g</i>	<i>A. a</i>	<i>P. a</i>	<i>S. a</i>	<i>S. e</i>	<i>E. c</i>	<i>L. a</i>	<i>B. b</i>
CSI-1	-	-		-		0.1	0.05		-
CSI-2	-	-		-		0.1	1.0		-
CSI-3	0.05	-		0.01	0.5	-	0.5		-
CSI-4	0.01	0.05	-	0.05	0.5	-	-		-
CS-F2 (80,000)	0.01	-	-	0.05	0.1	-	-	-	-
Catechin (442.4)	-	-	-	1.0	1.0	-	-	-	-

Inhibitors		IC ₅₀ values (50% inhibition of adhesion)				
Mammalian cells*	AGS	KB	KB	NIH3T3	NIH3T3	NIH3T3
CSI-4	1.26	2.3	-	0.14	0.83	-
CS-F2	1.10	-	-	1.35	1.71	-

^aThe minimum inhibitory concentrations (MIC) in mg/mL were used in hemagglutination assays, while IC₅₀ values at which 50% inhibition of adhesion occurs were used in host cell adhesion assays. All the values correspond to the average in triplicates. '-' represents no inhibition at high concentrations above 2.0 mg/mL. Aspartic and glutamic acids were used as non-carbohydrate acidic compounds, which did not cause non-specific reaction (data not shown). The green tea infusions have no influence on cell viability of erythrocytes and host cell lines below 2.0 mg/mL.

^b*H. p*: *Helicobacter pylori*, *P. g*: *Porphyromonas gingivalis*, *A. a*: *Actinobacillus actinomycetemcomitans*, *P. a*: *Propionibacterium acnes*, *S. a*: *Staphylococcus aureus*, *S. e*: *Staphylococcus epidermidis*, *E. c*: *Escherichia coli*, *L. a*: *Lactobacillus acidophilus*, *B. b*: *Bifidobacteria bifidum*, Asterisk * represents the result by using urea phenol red or visible colony counting method.

Example 5: Anti-adhesion assay by urea phenol red method

AGS cells (100 μ L) were seeded in 96-microwell plates (Corning, USA) at a density of 5×10^4 cells/mL (Simon, 1997). After achieving confluence in 1.5-2 days,

the confluent monolayers on the plates were washed 3 times with PBS. Non-specific binding was blocked by incubation with 0.5% BSA, prior to two rinses in PBS. 50 μ L of *H. pylori* suspensions ($OD_{600nm}=1.0$; 1×10^8 bacterial cells/mL) were incubated with inhibitors at various concentrations for 30 min. The bacteria-carbohydrate mixture (100 μ L) was added to the AGS cells in each well of the 96-microwell plates and incubated for 1 h. 0.03% phenol red in 2% urea solution (pH 5.2) was added and absorbance was measured at 560 nm with a microplate reader. The percentage of attached *H. pylori* cells was calculated as follows: attached % = $100 - [(OD_{\text{experimental}} - OD_{\text{negative}}) / (OD_{\text{positive}} - OD_{\text{negative}}) \times 100]$. The negative control contained only the AGS cells and the positive control contained the epithelial cells and bacteria, which were used to establish 100% attachment. For non-specific binding, FITC-labeled *E. coli* and AGS cells were incubated in the dark for 1 h. After washing three times with carbonate buffer (pH 6.0), adhesion was examined fluorometrically by monitoring the emission spectrum of FITC-labeled *E. coli* bound to AGS at 520 nm with excitation at 480 nm.

CSI-4 Inhibits Pathogenic Adhesion to Host Cells

With regard to its ability to inhibit the adhesion of *H. pylori* to human AGS gastric epithelial cells, a significant inhibition by CSI-4 was observed with a 20-80% reduction of *H. pylori* attachment at concentrations between 0.25 and 2.0 mg/mL (Fig. 3A). Moreover, when the AGS cells were pre-incubated with *H. pylori* followed by the addition of the inhibitor CSI-4, it also revealed an inhibitory activity, albeit reaching a 10% reduction of attachment at 2 mg/mL. Although it was less effective to detach the bacterial cells which had already attached to the host cell, CSI-4 could detach *H. pylori* from AGS cells in a concentration-dependent manner at concentrations from 0.125 to 2 mg/mL (Fig. 3B).

Example 6: Anti-adhesion assay by colony counting method

KB and NIH3T3 cells were seeded at 1×10^4 cells/mL in DMEM medium containing 10% fetal bovine serum and 1% antibiotics in 96-microwell plates. After achieving confluence in 1.5-2 days, the confluent monolayers on the plates were

washed 3 times with PBS. Various concentrations of green tea infusions were incubated with bacteria (1×10^7 cfu/mL) for 30 min. A 100 μ l aliquot of each mixture was added to the host cells, incubated at 37°C for 30-60 min. After treatment of the cells with PBS containing 0.1% Triton X-100, the recovered cell extracts were serially diluted with PBS and plated on 5% sheep blood agar or trypticase soy agar. After incubation for several days, colonies were counted visibly or by using stereomicroscope. The activity to inhibit bacteria from binding to KB or NIH3T3 cells was estimated as follows: attached (%) = (cfu of bound bacteria in the presence of polysaccharide/cfu of bound bacteria in the absence of polysaccharide) x 100 (Lee et al., 2006).

CSI-4 Inhibits Pathogenic Adhesion to Host Cells

CSI-4 was found to reduce the attachment of *P. gingivalis* to human KB cells by ca. 20% at 1.0 mg/mL, confirming the result from the hemagglutination assay (Fig. 4A). However, CSF2 was previously reported to show no activity against *P. gingivalis* (Lee et al., 2006). Both CSI-4 and CS-F2 did not show any inhibition against *A. actinomycetemcomitans* (data not shown).

50% inhibition by CSI-4 against the adhesion of *P. acnes* to NIH 3T3 fibroblast cells was observed at a concentration of 0.14 mg/mL (Table 2). The IC50 value against *P. acnes* was the lowest among the pathogens examined by CSI-4, which reduced the adhesion of the pathogen by ca. 80% at 0.25 mg/mL (Fig. 4B). The attachment of *S. aureus* to NIH3T3 cells was significantly reduced to ca. 40% at 1.0 mg/mL (Fig. 4C). Our results suggest that CSI-4 has very strong anti-adhesive activity on the adhesion of skin pathogens to host cells.

Example 7: Hemolysis and cytotoxicity assays

Samples of blood suspension were treated with the selected infusion concentrations in 96-well microtiter plates and centrifuged at 1000 x g for 5 min. Hemoglobin release was monitored spectrophotometrically at 414 nm. Percent hemolysis was calculated as follow: % hemolysis = $(\text{Abs}_{\text{Inhibitor}} - \text{Abs}_{\text{PBS}}) / (\text{Abs}_{\text{TritonX-100}} - \text{Abs}_{\text{PBS}}) \times 100$, where 0% and 100% hemolysis were determined in PBS and

0.1% Triton X100, respectively. Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). AGS, KB, and NIH 3T3 cells were treated with the infusion inhibitors for 24 h and MTT was added to each solution (final 0.5 mg/mL). After incubation for 2 h, the supernatant was removed and a solubilization solution was added. The absorbance was measured at 595 nm and control cells were treated in the same way without the infusion (Maher and McClean, 2006).

Effects of CSI-4 on Erythrocytes and Host Cells

CSI-1 induced the hemolysis of 20-45% at 1-2 mg/mL, but CSI-4 didn't induce the hemolysis in concentrations of 0.125 to 2 mg/mL (data not shown). CSI-4 did not significantly influence on cell viability of AGS, KB, and NIH3T3 cells in concentrations between 0.125 and 2 mg/mL (Fig. 5).

【Industrial Applicability】

As described hereinabove, according to the present invention, we can prepare the green tea extract, having inhibitory activity for adhesion of pathogenic bacteria to human cells, by using charcoal column filtration, in much higher yield than that of the conventional chromatography. Moreover, the present method can be used as a novel technology suitable for large-scale production due to simple procedure. The green tea extracts of the present invention prepared by the above method may be use as drugs for preventing and curing Gastric disease, Periodontitis, especially Acne vulgaris and Atopic dermatitis, because they have strong inhivitory activities against hemaagglutination mediated by *Porphyromonas gingivalis* of Periodontitis-inducing bacteria, *Propionibacterium acnes* of Acne vulgaris-inducing bacteria and *Staphylococcus aureus* of Atopic dermatitis-inducing bacteria, and have also the inhibitory activity for *Helicobacter pylori* of Gastric disease-inducing bacteria.

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【CLAIMS】**【Claim 1】**

A method for preparing a green tea extract having inhibitory activity for adhesion of pathogenic bacteria to human cells, which comprises the following steps:

- a) extracting dried green tea leaves with hot water;
- b) centrifuging the hot-water extract and precipitating the supernatant using ethanol;
- c) filtering the ethanol precipitate with charcoal column; and
- d) freeze-drying the filtrate.

【Claim 2】

The method according to claim 1, which further comprises a step, dissolving the ethanol-precipitates in phosphate buffer solution and removing nucleic acids, after the step b).

【Claim 3】

The method according to claim 1, wherein the charcoal column in the step d) removes catechins using granular activated carbons.

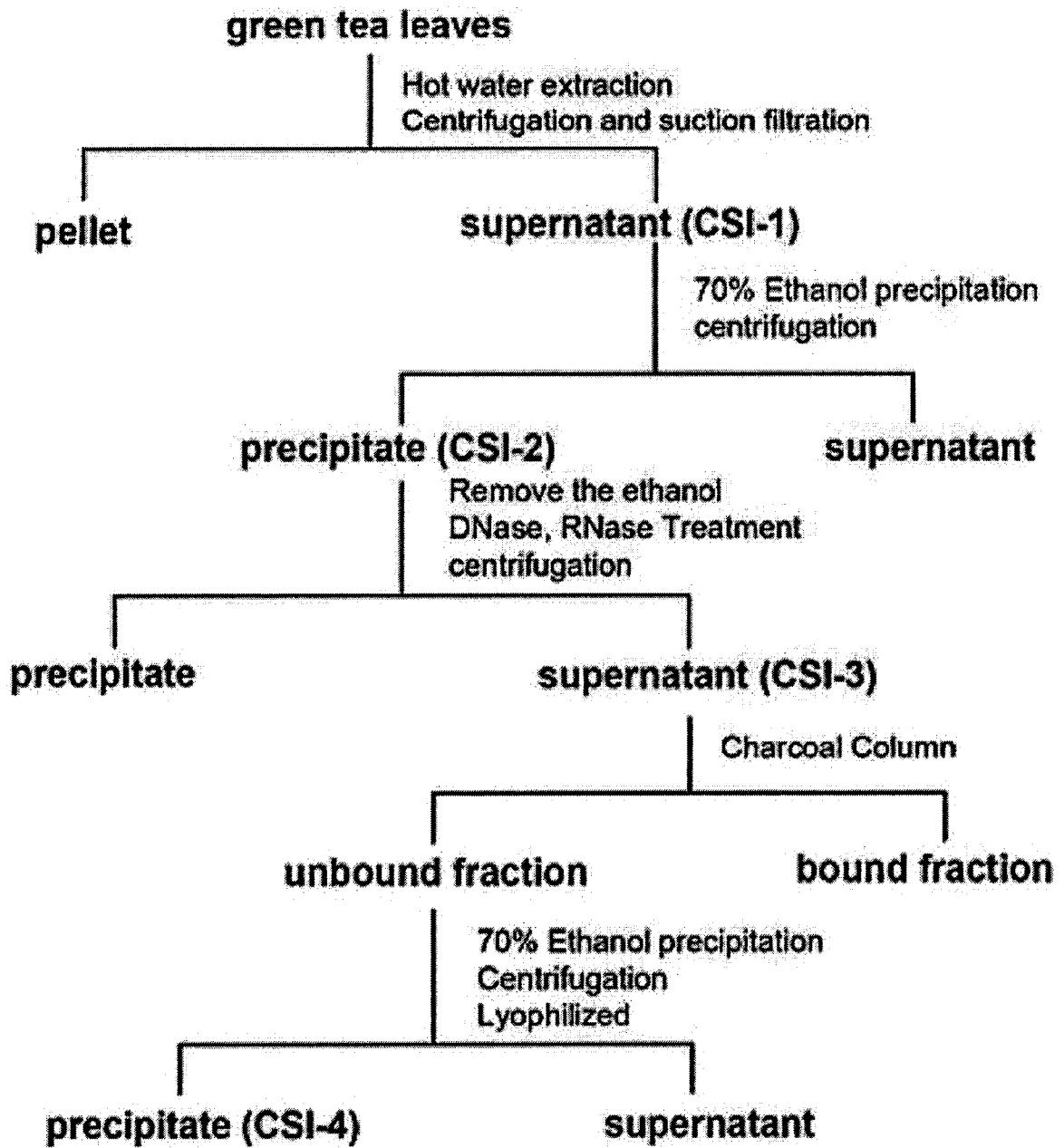
【Claim 4】

A composition for inhibiting adhesion of pathogenic bacteria to human cells, which comprises green tea extracts prepared by the method of any one of Claim 1 to 3.

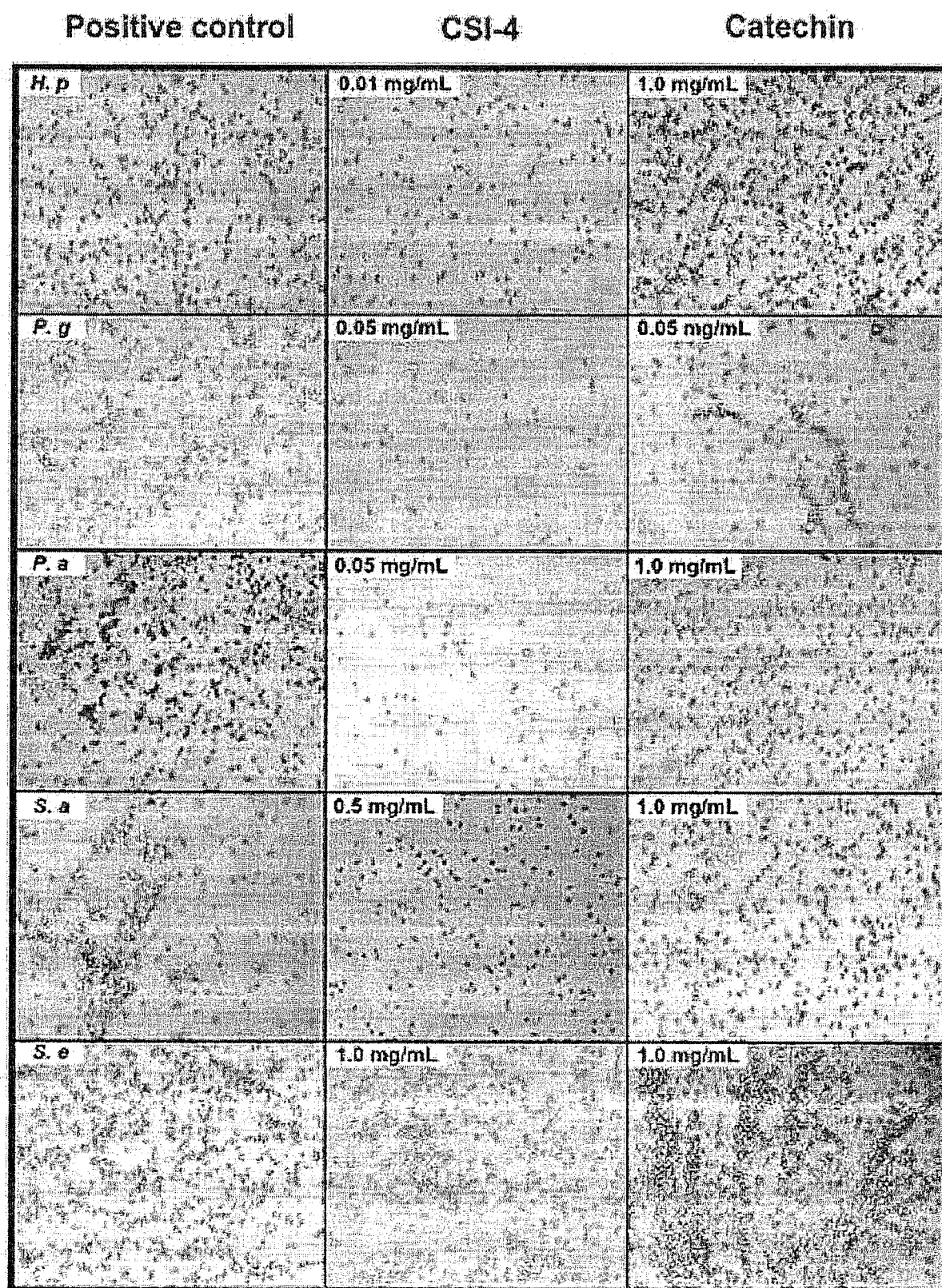
【Claim 5】

The composition according to claim 4, wherein the pathogenic bacteria are Periodontitis-inducing bacteria, Acne vulgaris-inducing bacteria, Atopic dermatitis-inducing bacteria, or Gastric disease-inducing bacteria.

[Fig. 1]

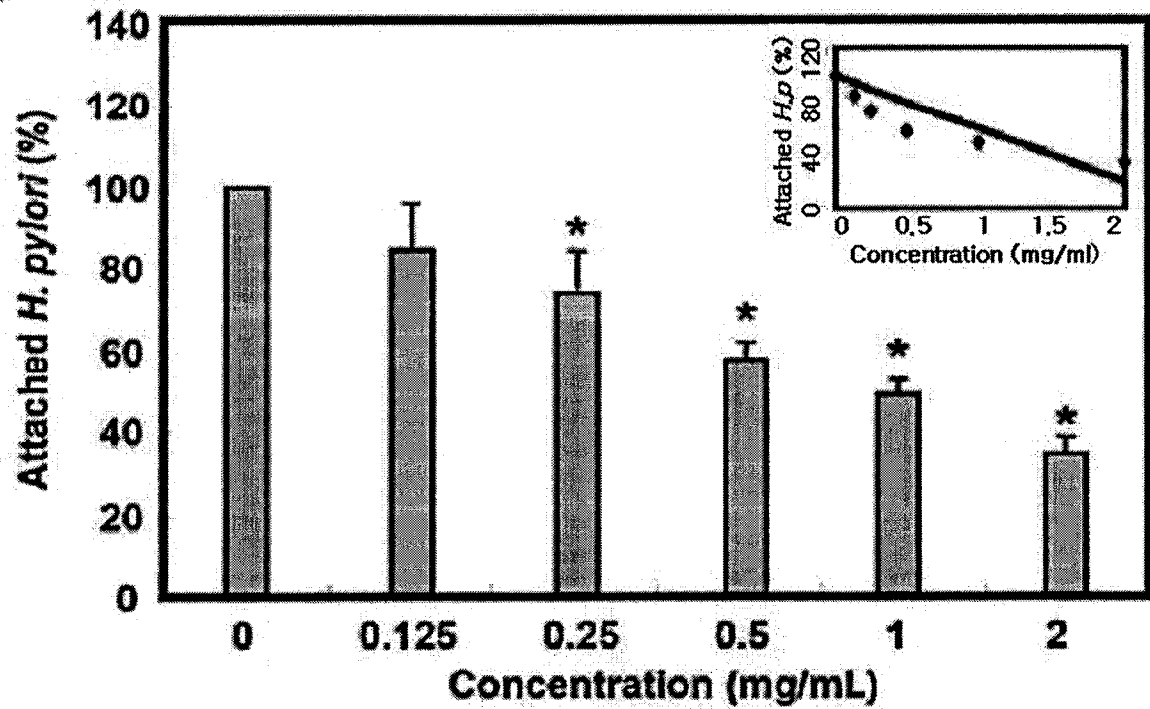


[Fig. 2]

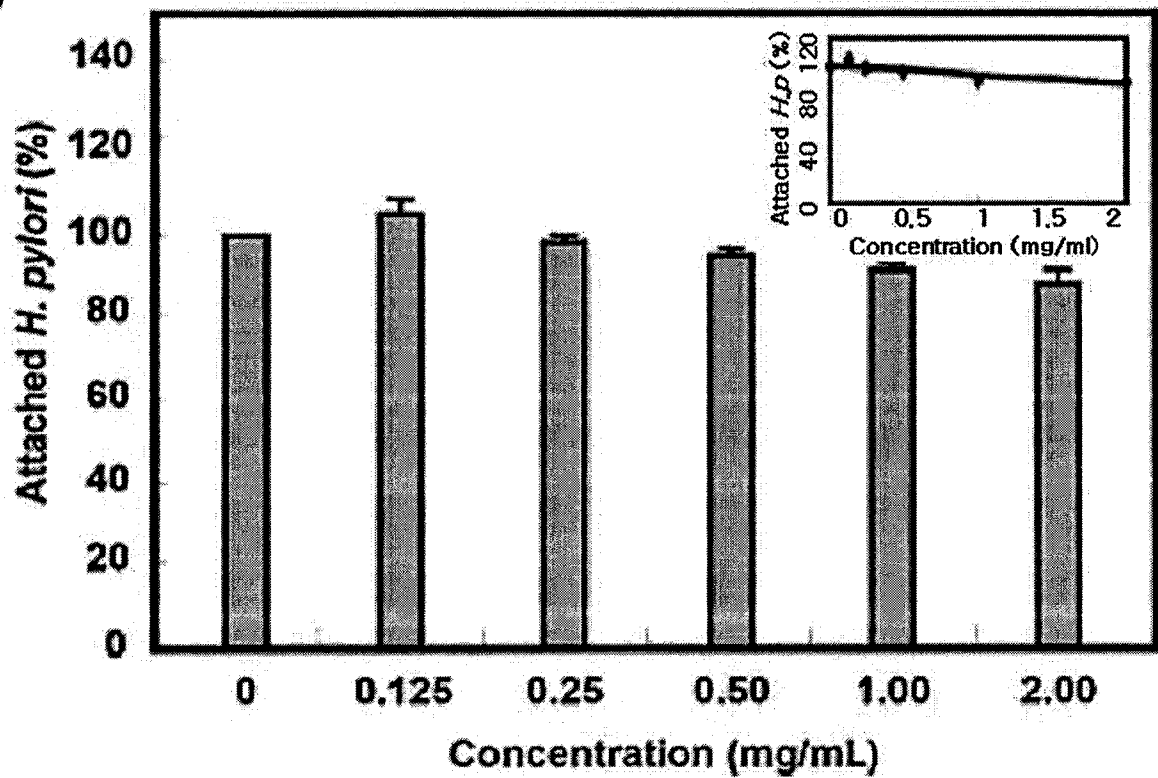


[Fig. 3]

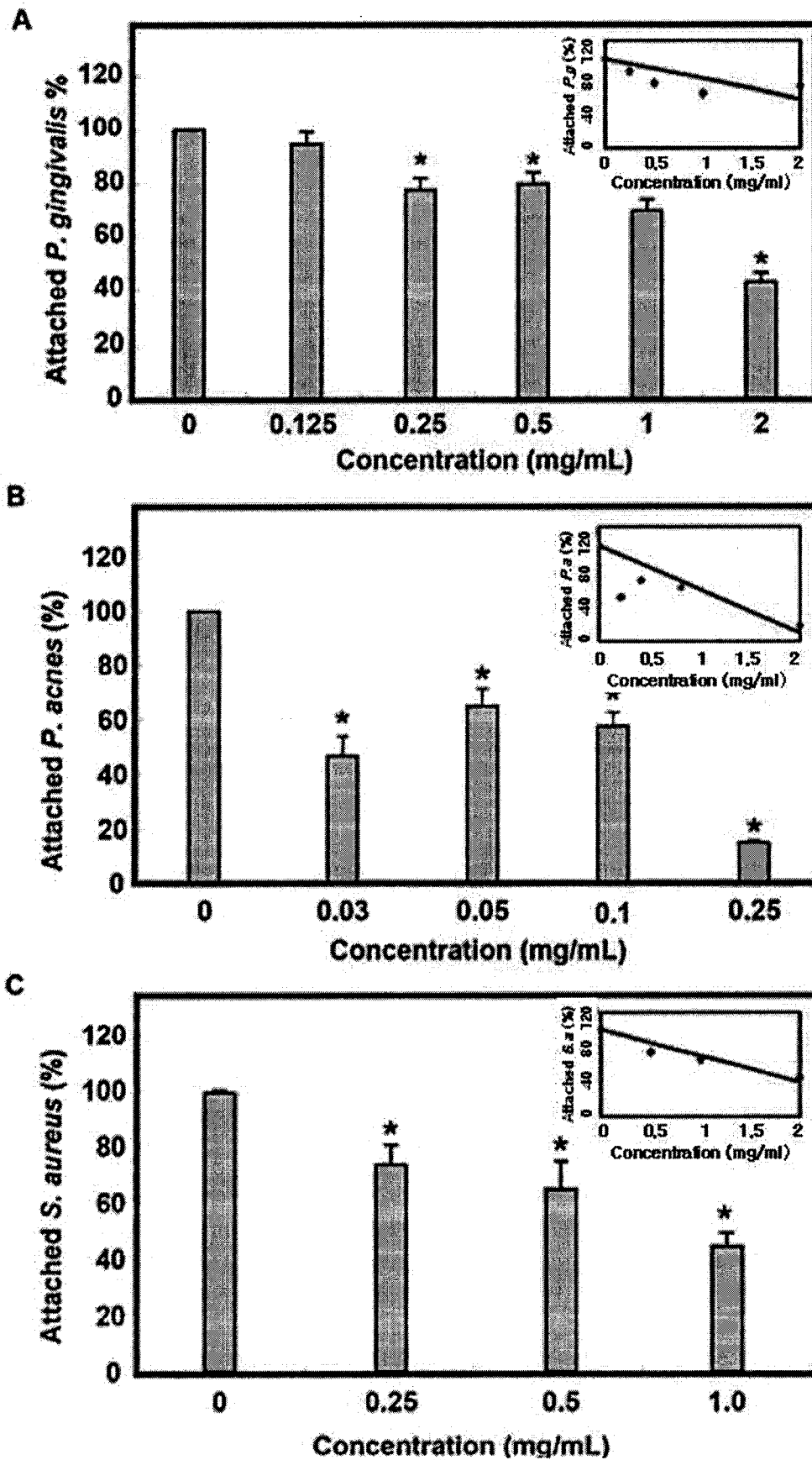
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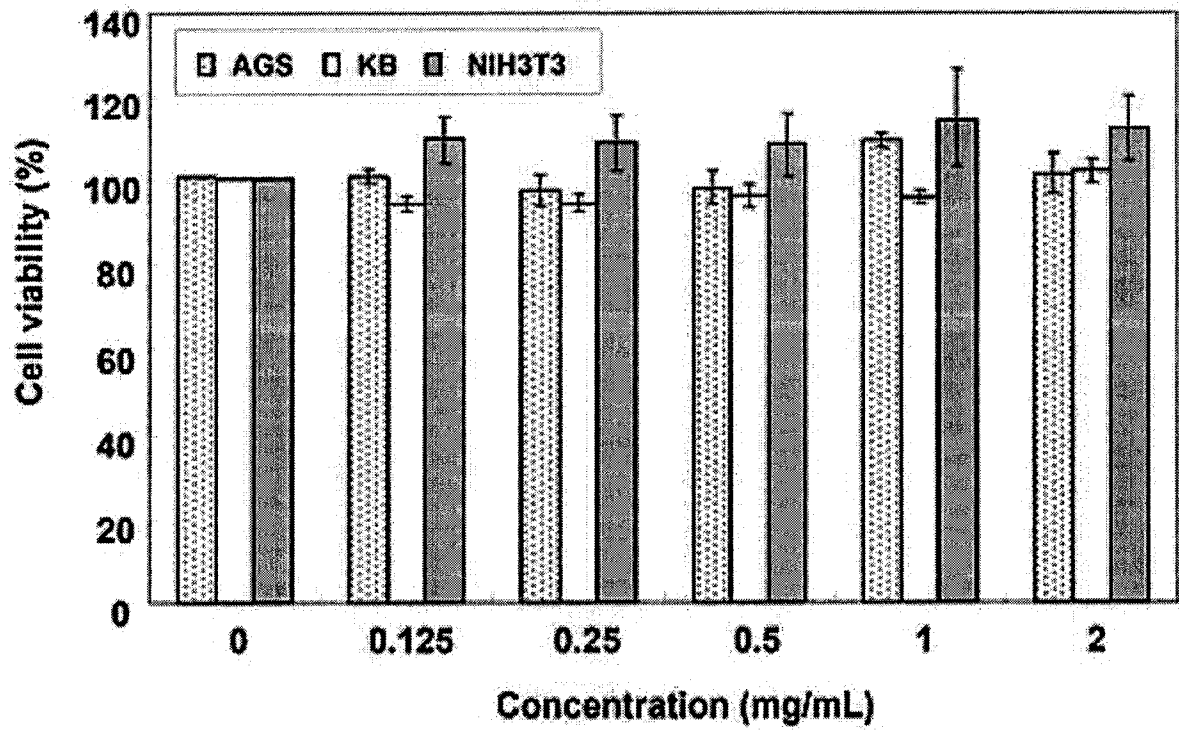
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[Fig. 4]



[Fig. 5]



A. CLASSIFICATION OF SUBJECT MATTER*A61K 36/82(2006.01)i, A61P 31/04(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models since 1975Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS(KIPO internal), PubMed on-line**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KR 10-0700912 B1 (KOREA UNIVERSITY INDUSTRY AND ACADEMY COOPERATION FOUNDATION) 28 March 2007 See abstract; claims 1-5, figure 1.	1-5
A	LEE, J.H. et al. Inhibition of pathgenic bacterial adhesion by acidic polysaccharide from green tea (Camellia sinensis). J. Agric. Food Chem. 2006, Vol. 54, No. 23, pages 8717-23. See abstract; page 8718, left column, line 55 - right column, line 8.	1-5
A	KR 10-0602841 B1 (KOREA UNIVERSITY INDUSTRY AND ACADEMY COOPERATION FOUNDATION) 19 July 2006 See abstract; claims 1-7; figure 1.	1-5
A	JP 10-101573 A (MORINAGA & CO., LTD.) 21 April 1998 See abstract; paragraph [006]; table 2.	1-5
A	JP 05-255101 A (SUNTORY LTD.) 05 October 1993 See abstract; paragraphs [0016], [0039]-[0041]; tables 1-2.	1-5



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/004139

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 10-0700912 B1	28.03.2007	NONE	
KR 10-0602841 B1	19.07.2006	NONE	
JP 10-101573 A	21.04.1998	NONE	
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