

(12) **UK Patent Application** (19) **GB** (11) **2578146** (13) **A**

(43) Date of A Publication **22.04.2020**

(21) Application No: **1817003.5**

(22) Date of Filing: **18.10.2018**

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(51) INT CL:  
**A01N 43/16** (2006.01) **A01N 37/46** (2006.01)  
**A01P 1/00** (2006.01) **A23L 3/3463** (2006.01)  
**A23L 3/3508** (2006.01) **A23L 3/3562** (2006.01)  
**A61K 8/49** (2006.01) **A61K 8/88** (2006.01)  
**A61K 31/7048** (2006.01) **A61Q 19/00** (2006.01)  
**C02F 1/50** (2006.01)

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**KR 20160007839**  
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(58) Field of Search:  
 INT CL **A01N, A01P, A23L, A61K, A61P, C02F**  
 Other: **EPODOC, WPI, Patent Fulltext, BIOSIS,**  
**MEDLINE, CAS Online**

(54) Title of the Invention: **Bioflavonoid compositions and their use for water purification and food preservation**  
 Abstract Title: **Bioflavonoid compositions and their use in water purification, food preservation and surface disinfection**

(57) Pharmaceutical compositions and their use for preventing, eradicating or ameliorating infections. More particularly, the compositions comprise a bioflavonoid mixture and caprylic acid and/or polylysine. The flavonoid mixture may include naringin and neohesperidine. The exemplified bioflavonoid mixture is Citrox HXT powder (RTM). The composition may also comprise willow bark extract which contains salicylic acid. Such compositions can, for example, be employed for reducing or eliminating bacteria, fungi and other parasites from water, such as water intended for drinking or from food. The composition may be in the form of a gel, liquid soap or hand foam suitable for use as a surgical sanitizer. The hand foam composition may be used in a commercial misting device to produce a mist for disinfection of surfaces, such as those in an ambulance.

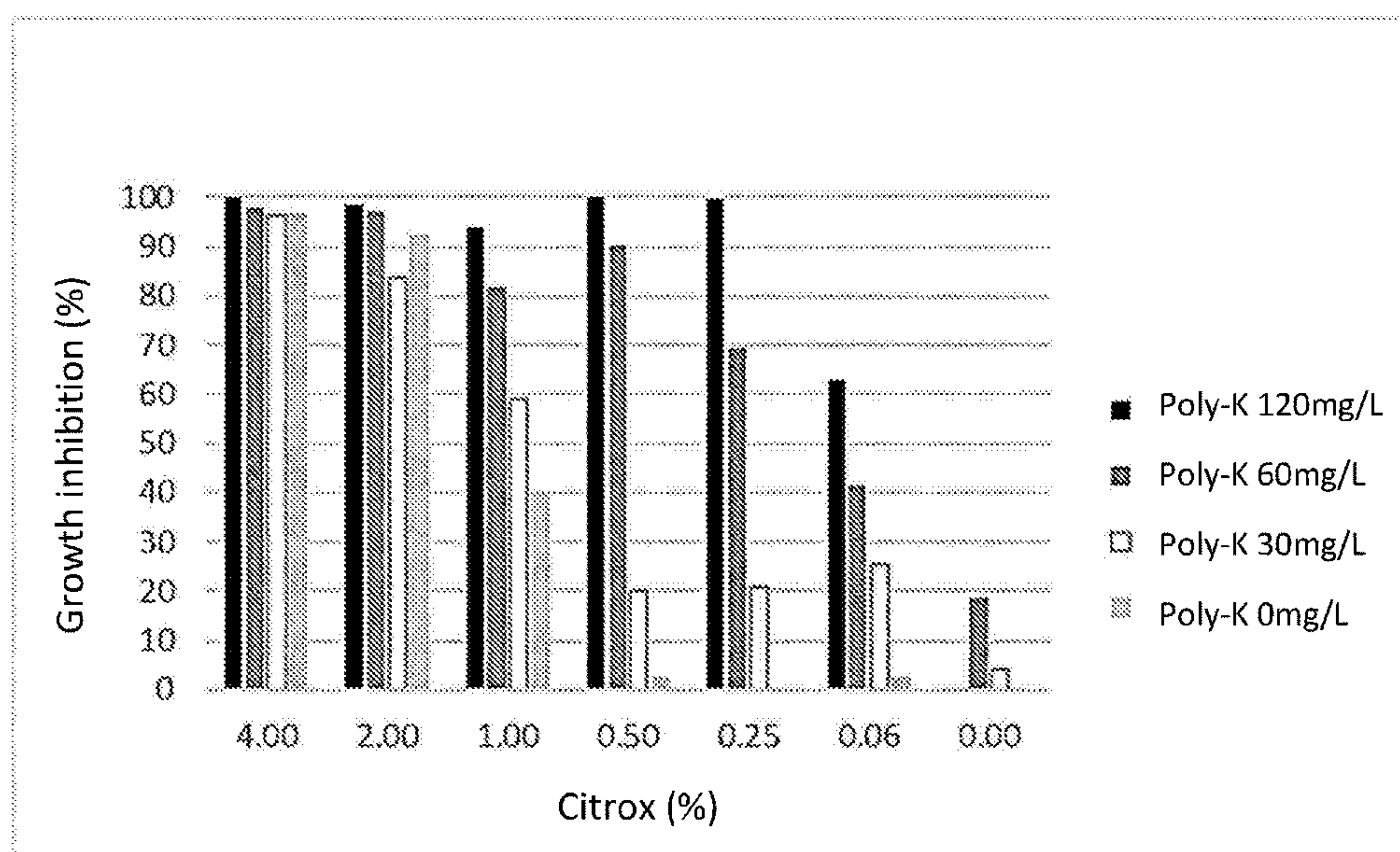


Figure 1.

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# DRAWINGS

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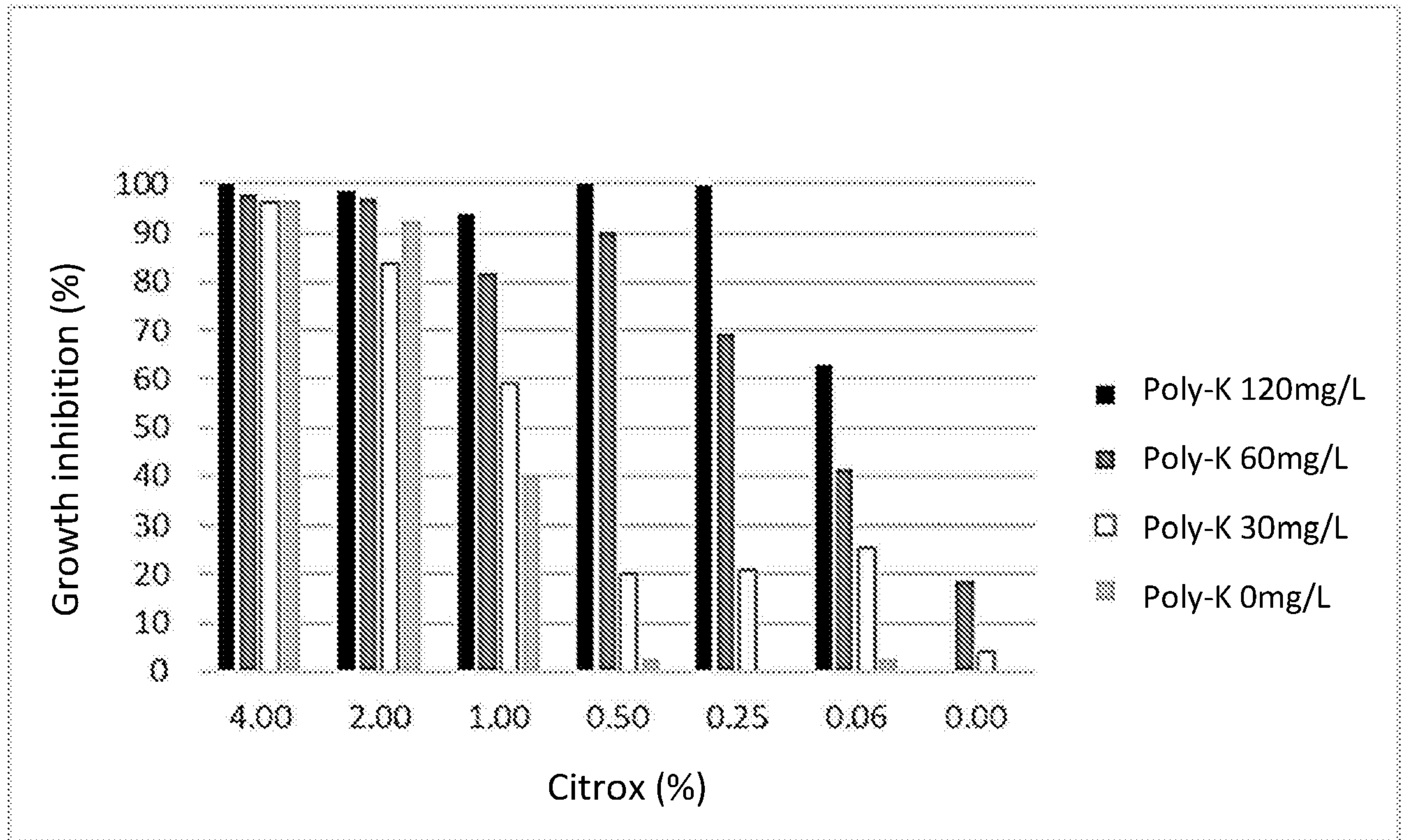


Figure 1.

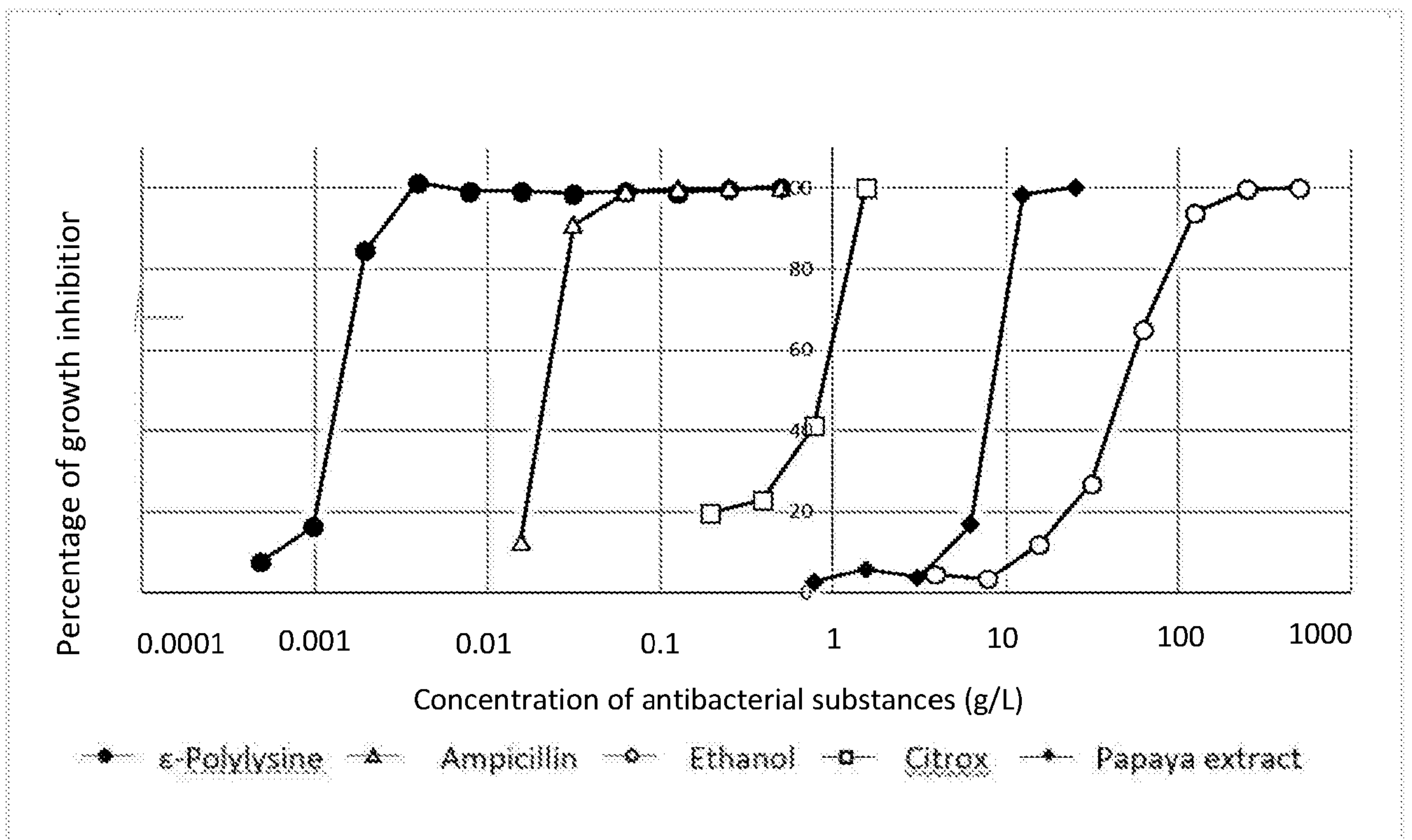


Figure 2.



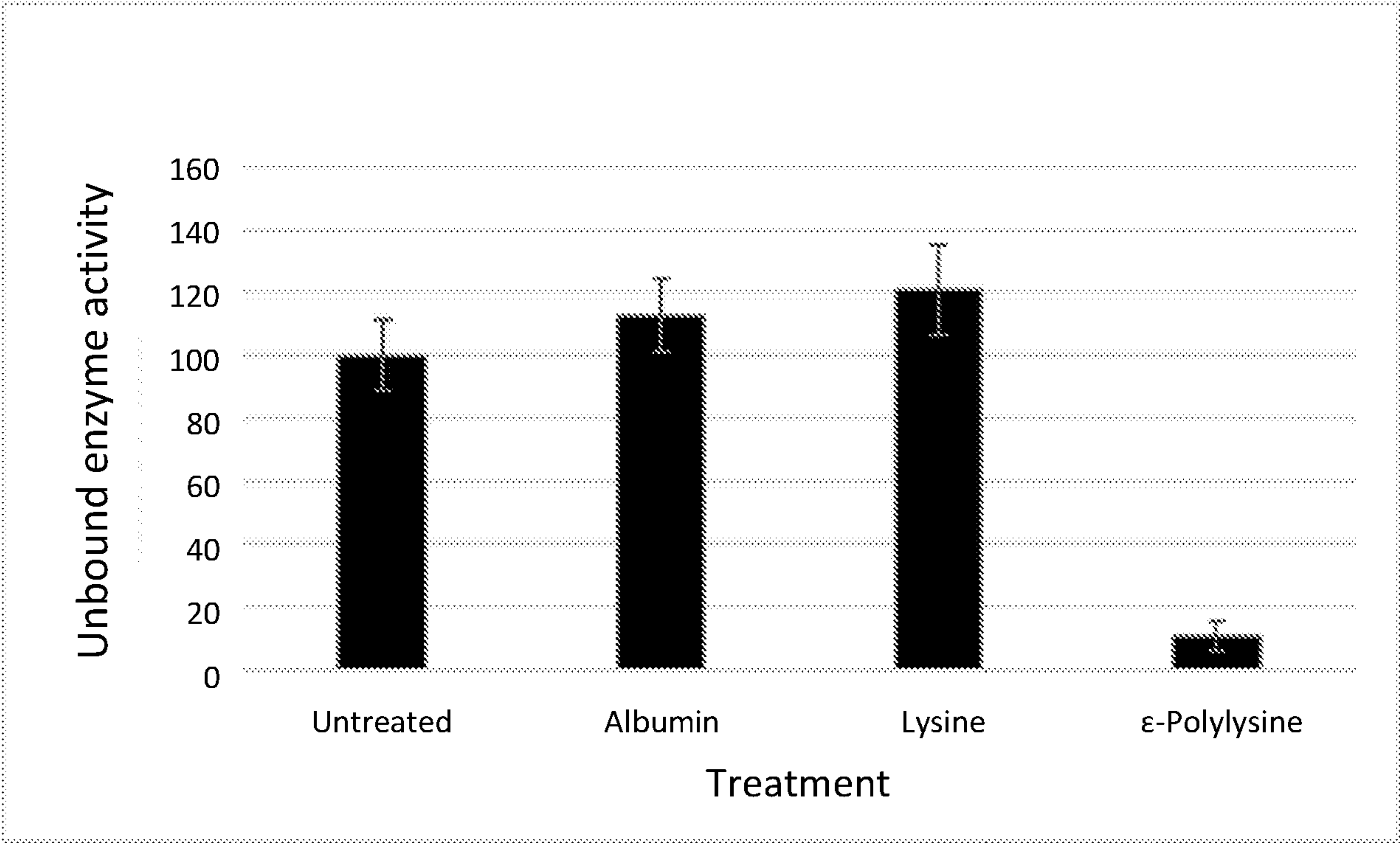


Figure 3.

**BIOFLAVONOID COMPOSITIONS AND THEIR USE FOR WATER PURIFICATION  
AND FOOD PRESERVATION**

5 The present invention provides compositions suitable for preventing, eradicating or ameliorating infective organisms. More particularly the present invention relates to compositions which contain bioflavonoids and polylysine and/or caprylic acid for use topically or by systemic administration.

**Background of the invention**

10

Various flavonoids have been suggested to possess antibacterial properties. PCT/GB2007/002756, PCT/GB2007/002758 and EP2198862 describe particularly effective compositions containing flavonoids (and which are incorporated herein by cross-reference).

15

One family of commercial products is available under the trade mark Citrox which have proved particularly advantageous in respect of antibacterial properties.

20

Nevertheless, known bioflavonoid anti-infective agents are not always as effective as could be wished when treating certain organisms in all circumstances. Thus, for example an enhancement in effectiveness is often desirable in the case of particularly difficult problems such as preventing, eradicating or ameliorating infections resulting from the presence of clostridium difficile spores and methicillin resistant staphylococcus aureus, or certain biofilm forming bacteria.

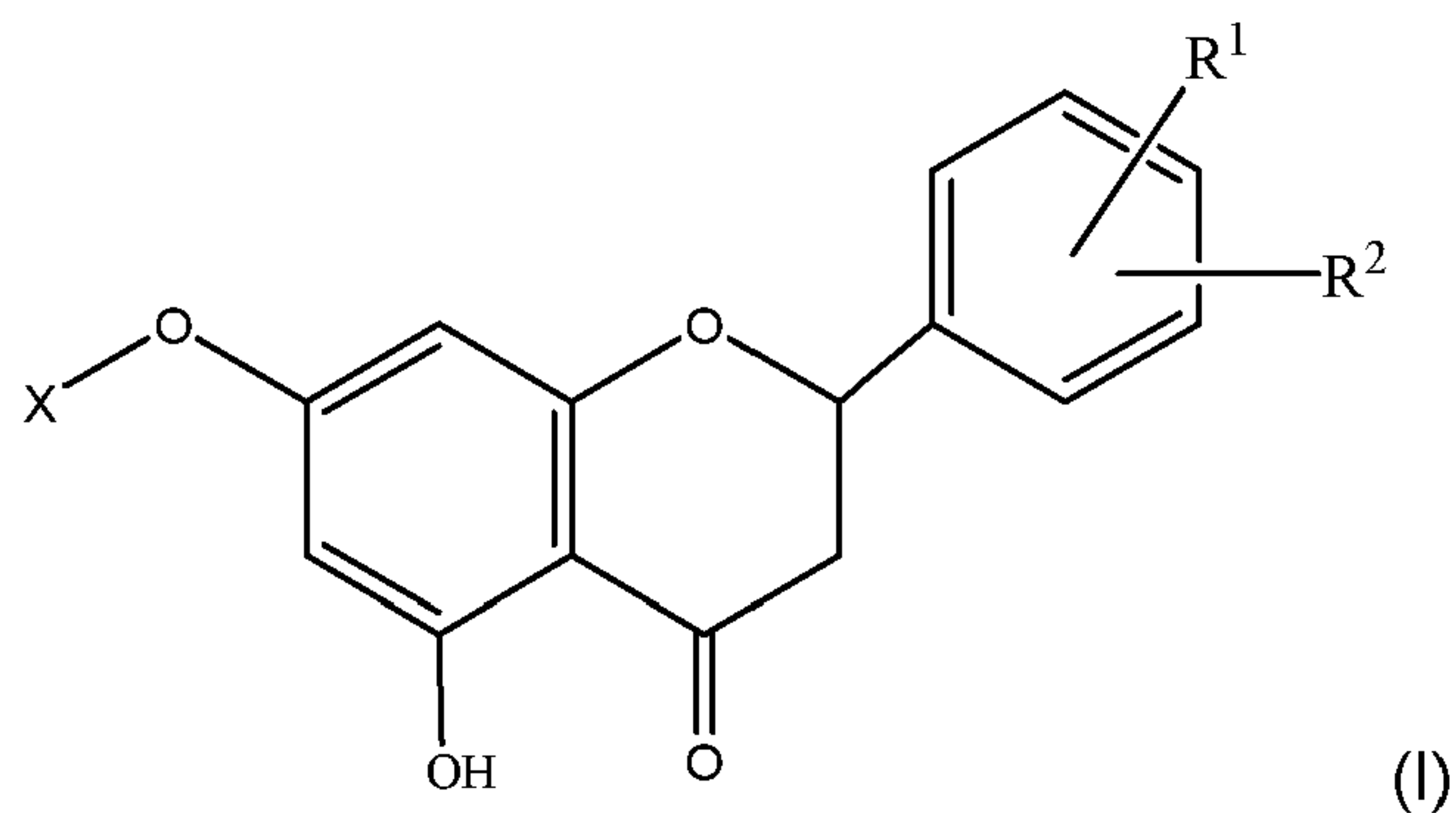
25

The present invention addresses such problems by providing compositions which comprise a bioflavonoid component and polylysine and/or caprylic acid component.

**Description**

30

The present invention provides a composition which comprises polylysine and/or caprylic acid and one or more flavonoids of Formula (I)



wherein wherein  $R^1$  is hydroxyl or methoxyl and  $R^2$  is hydrogen, hydroxyl or methoxyl and X is hydrogen or a saccharide.

5

Aptly in the first component  $R^2$  is hydrogen and  $R^1$  is in the 3- or 4- position. Alternatively, aptly in the first component  $R^2$  is 3-hydroxy and  $R^1$  is 4-methoxyl.

Suitably X in a compound of the Formula (I) is H.

10

Suitably X in a compound of Formula (I) is a saccharide.

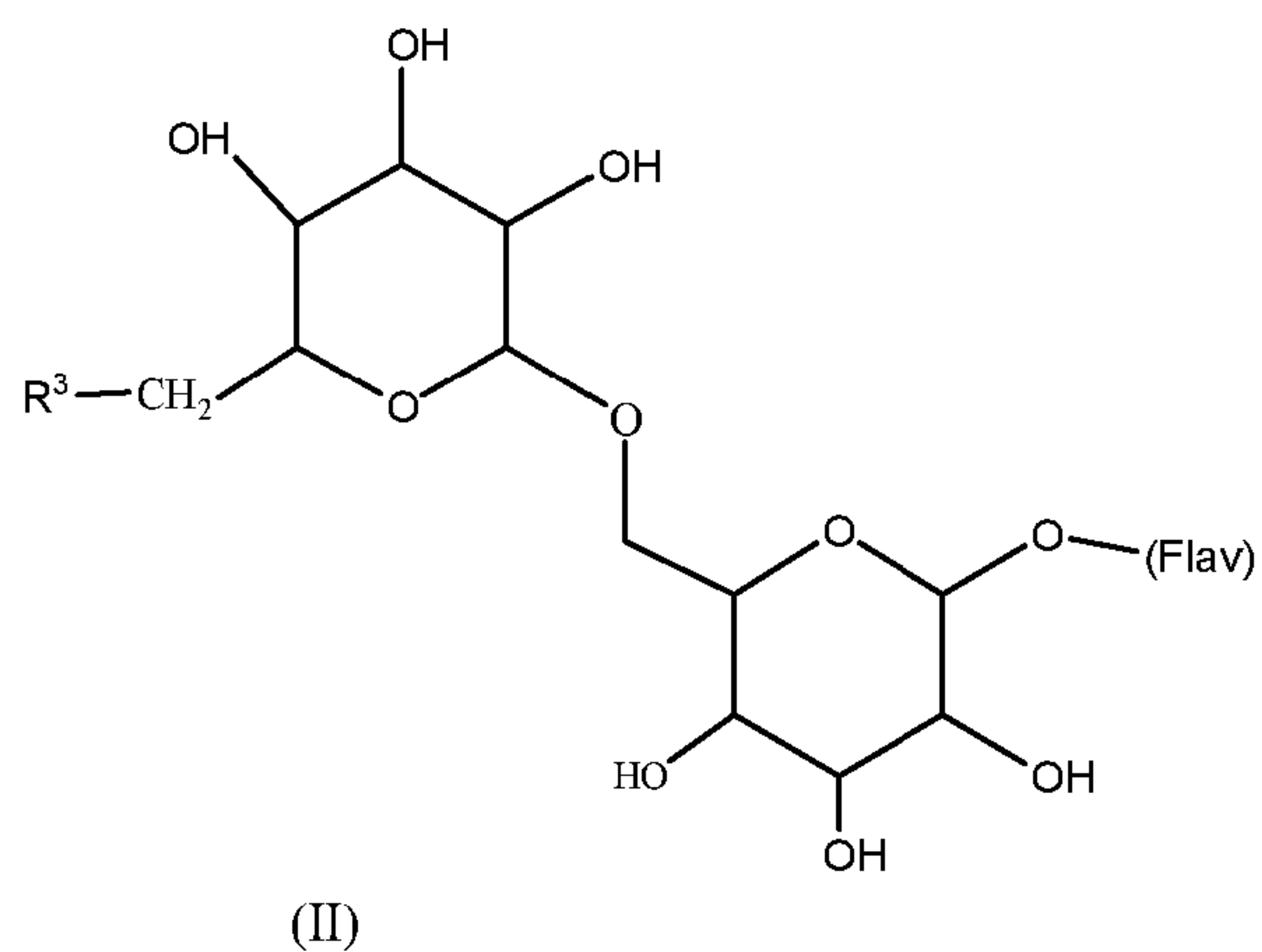
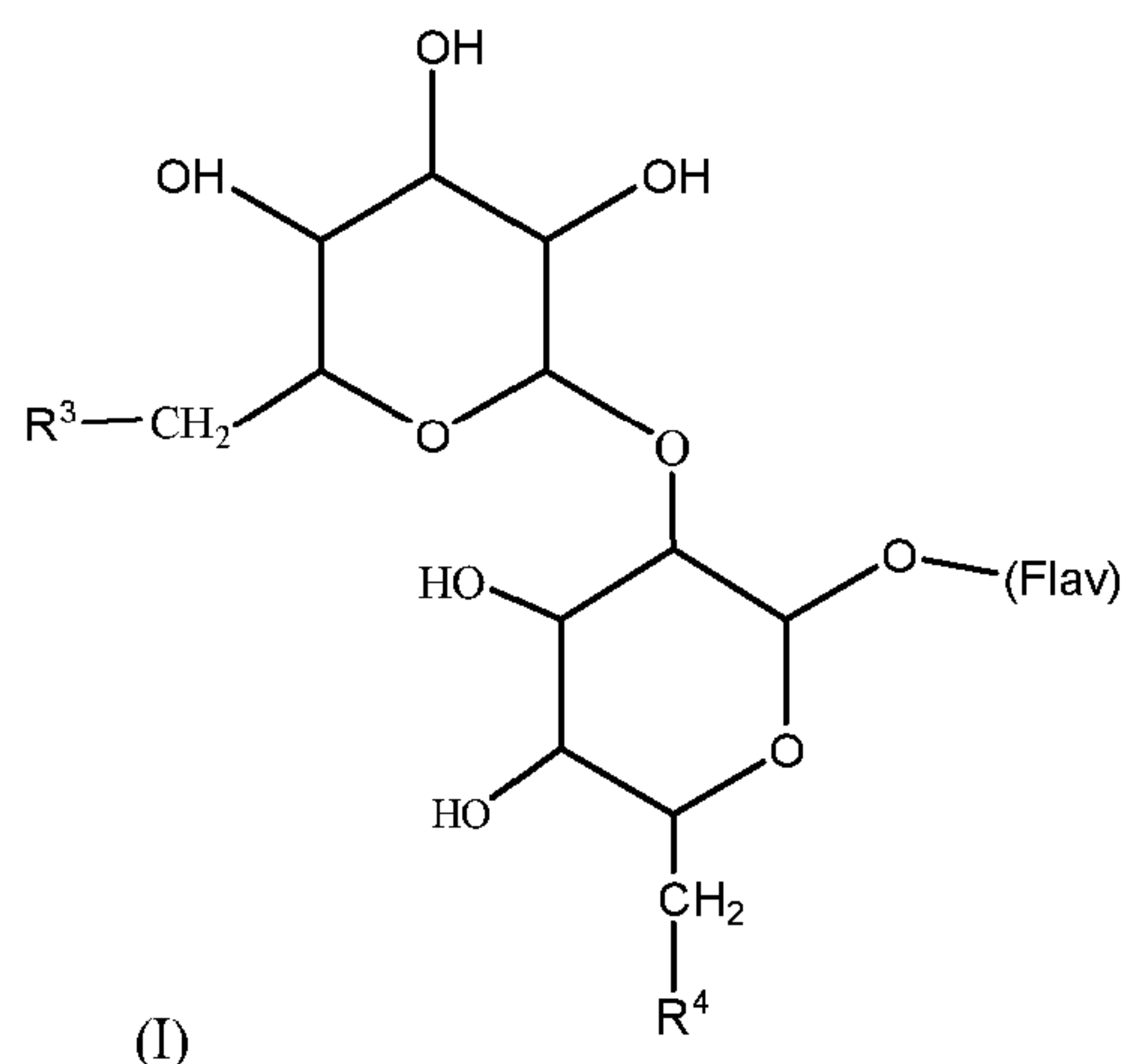
Favourably X is a disaccharide.

15

Suitable disaccharides include combinations of two monosaccharide, suitably pyranoses, linked by a glycosidic bond, for example rhamnose and glucose, for example L-rhamnose and D-glucose.

Suitable disaccharides can have the structure:

20



wherein one of R<sup>3</sup> and R<sup>4</sup> is H and the other OH or both are H or both are OH. Aptly R<sup>3</sup> is H and R<sup>4</sup> is OH so that the disaccharide is rutinose.

5 Favoured glycosyl groups of flavonoids for use in this invention are the disaccharides 6-O-(alpha-L-rhamnopyranosyl)-beta-D-glucopyranose, also known as rutinose, and 2-O-(alpha-L-rhamnopyra-nosyl)-beta-D-glucopyranose.

10 It is presently believed that the flavonoid of Formula I very suitably comprises naringin or neohesperidin or mixtures thereof. Mixtures of one or both of naringin and neohesperidin with for example, one, two or three other flavanoids of the Formula I are presently believed particularly favoured for use in this invention. Such mixtures can be obtained from extraction from bitter oranges.

15 Suitable compounds of Formula (I) include Neoeriocitrin, Isonaringin, Naringin, Hesperidin, Neohesperidin, Neodiosmin, Naringenin, Poncirin and Rhiofolin.

Favoured compositions for use include those which comprise either of naringin and neohesperidin or preferably both.

20 Particularly aptly the invention will contain naringin and neohesperidin and other flavonoids of the Formula (I).

25 The mixture of flavonoids may aptly contain neohesperidin and naringin, and one or more of isocriocrin, isonaringin, naringin, hesperidine, neohesperidin, neocliomin, naringenin, poncrin and rhiofolin. Such a mixture of flavonoids can be obtained from bitter oranges. Suitable mixtures can include 2, 3, 4, 5, 6, 7, 8, 9 or more compounds of Formula (I). Thus, a mixture comprising 2, 3, 5, 6, 7, 8 or 9 of the above named flavonoids is aptly, for example containing 3, or containing 4, or containing 5, or containing 6, or containing 7, or containing 8 or containing 9 of said flavonoids.

30

It is presently believed that mixtures of such flavonoids have advantages over the use of a single flavonoid. It is particularly advantageous that extract of bitter oranges may be employed without the need for isolating individual flavonoids if desired. The use of the composition generally comprising biomass that enhances solubility of the flavanoids. Generally, the flavonoids are present in mixtures with biomass by about 35 10% to 75%, more aptly 30% to 60%, for example 40% to 50%, preferably about



45%. The biomass comprises pectins and other sugar derived materials. Typically, about 40% of low molecular weight pectins are present in such biomass.

5 If it is desired to avoid biomass, other solubilising agents such as dextrans, for example cyclodextrin, may be employed if desired, but this is not presently envisaged as generally advantageous.

10 Aptly the mixture of flavonoids will comprise at least 25%, more suitably at least 40% and preferably at least 50% of naringin. More aptly the mixture will contain from 40% to 65% of naringin (wt/wt of flavonoids present).

15 Aptly the mixture of flavonoids will comprise at least 15%, more suitably at least 20% and preferably at least 25% of neohesperidin. More aptly the mixture will contain 20% to 35% of neohesperidin (wt/wt of flavonoid present).

In a favoured form the mixture of flavonoids will contain at least 75% of neohesperidin and naringin (wt/wt).

The composition will also comprise polylysine and/or caprylic acid.

20 Favourably, the amino acids in polylysine should be capable of forming charged polymers, for instance  $\epsilon$ -polylysine is preferred to  $\alpha$ -polylysine because the free amino acid groups may have a positive charge in non-basic media. Polylysine consists of 20-50, favourably 25-40 L-lysine residues with a molecular weight of 2400  
25 Da to 6000 Da, favourably 3000 Da to 5000 Da.

The concentration range of polylysine, particularly  $\epsilon$ -polylysine employed is aptly 0.01 to 1.00 wt-%, for example 0.1 to 0.75 wt-%, such as 0.25 to 0.5 wt-%.

30 The concentration range of the bioflavonoids employed is aptly 0.05 to 6 wt-%, for example 0.1 to 4 wt-% such as 0.2 to 3 wt-%.

35 A particular advantage of many compositions of the invention is that they may employ compounds of natural origin. Thus, for example, it is preferred to employ bioflavonoids which obtained from bitter oranges. However, synthetically or semi-synthetically obtained compounds may be employed if desired instead of the ones directly extracted from natural sources although this tends to be less favourable in

view of cost and less acceptable to those who prefer agents which are naturally derived from renewable resources.

5 The compositions of this invention show synergistic anti-microbial effectiveness between the bioflavonoids and the polylysine and/or caprylic acid.

It is presently believed that the preferred range is between 30mg/L and 240mg/L of polylysine and 0.06% to 4% bioflavonoids as best synergy is believed to then occur.

10 Aptly the composition comprises flavonoids of Formula I and polylysine.

Aptly the composition comprises flavonoids of Formula I and caprylic acid.

15 Aptly the composition comprises flavonoids of Formula I and polylysine and caprylic acid.

Such compositions may desirably contain a mixture of for example 65 % to 75% of naringin and neohesperidin together with polylysine and/or caprylic acid (wt/wt).

20 It has been found that compositions of this invention are particularly effective in the presence of an additional component, which is one or more organic acids.

25 A surprisingly effective acid for use include citric acid or salicylic or lactic acid or pharmaceutically acceptable salt thereof, optionally together with a further organic acid or pharmaceutically acceptable salt.

30 Favourably an organic acid may be present, such as acids of up to 8 carbon atoms which are monobasic (i.e. 1 CO<sub>2</sub>H group), di-basic or tri-basic acid which optionally contain 1, 2 or 3 hydroxyl groups. Such organic acid may be one or more of citric acid, malic acid, lactic acid, tartaric acid, fumaric acid and the like.

Such compositions can provide an approximately neutral or acid pH, when used, for example pH of from 3-8, more aptly 3.5-7, for example 4-5.

35 In compositions containing a flavonoid of Formula I, polylysine and/or caprylic acid (and optionally a further organic acid) the weight/weight ratio of the compound(s) of Formula (I) to polylysine and/or caprylic acid or pharmaceutically acceptable salt



thereof is 1300:1 to 1:10, more aptly 100:1 to 1:2, favourably 50:1 to 1:3 and preferably is 25:1 to 3:1, for example 20:1.

5 Such compositions may include a solubilising agent, for example a dextrin such as cyclodextrin, although use of biomass extracted from bitter oranges can avoid the need for this if required.

10 Compositions of the invention may be adapted for application to external surfaces including external surfaces of rooms, ambulances, hospital areas, plants or animals, or for internal administration to an animal and preferably a human.

15 The compositions of the invention show activity against a wide range of organisms including gram positive bacteria, gram negative bacteria, fungi, virus, protozoans and insect parasites. Particularly surprising the compositions may be employed against difficult bacteria such as methicillin resistant staphylococcus aureus (MRSA), clostridium difficile (C.diff) helicobacter pylori (H.py), and vancomycin resistant enterobacteria. The compositions of this invention may also be used against norovirus and other pathogens whereby transmission is by contact or air.

20 It is a particular advantage that the compositions are effective against spores of clostridium difficile which can otherwise be particularly intransigent, and also against film forming bacteria.

25 The compositions may be administered systemically or locally if an animal is to be treated. Suitable animals include humans and food and companion animals such as cows, pigs, horses, chickens, sheep, goats, dogs and cats. Hence the compositions may be formulated for oral administration, topical administration, injection or the like as required by the medical practitioner. Particularly suitably such compositions are suitable for use to treat humans, especially by oral or topical administration.

30 If the composition is intended for food preservation, topical administration is apt, for example by washing, spraying or the like.

35 The compositions of the invention are also useful for use in conjunction with a further antibacterial agent.

Since the compositions of the invention are particularly effective for sterilizing surfaces, they are very suitably formulated in a composition useful for external use.

5 These may be in the form of solutions, gels, soaps, body wash, shampoo, dusting powders and aerially dispersible powder and liquids and the like.

Such compositions may be used to reduce the bacterial count on body surfaces, clothing and in the general environment particularly in hospitals, ambulances, nursing homes especially for the elderly or the like where it is particularly desirable to reduce  
10 the presence of bacteria such as C. diff or film forming bacteria.

Compositions suitable for washing the hands are particularly useful, for example in a hospital environment, for medical staff, patients and visitors.

15 Such compositions may also be employed to wash stethoscopes or other medical equipment.

The substantivity of the compositions (as opposed to rapid diminution of effectiveness of ethanol) is a considerable advantage.

20 Use against organisms showing resistance to the sterilizing effects of ethanol is proposed.

If external surfaces of enclosed spaces, such as ambulances, operating theatres, wards, kitchens (and even mortuaries) and so on are to be treated, it is particularly  
25 suitable to do so by "misting". In this a fine aerial dispersion of powder or microdroplets of composition are dispersed within the enclosed space. This can then offer a non-toxic alternative to the presently employed methods which often employ noxious gases. Since the compositions of the invention have such low toxicity they  
30 may be employed on patients and their visitors and associated clothing, linen and the like by "misting". Such "misting" is of use in vehicles such as ambulances which are required to be free of pathogens but likewise free of residual odors that are typically left following the use of noxious gases. This equally applies to other areas requiring treatment. Compositions used in this way may be in the form of a dispersible liquid,  
35 for example akin to the soap or shampoo or skin foaming compositions described hereinafter. These can also be used to whet the surfaces to similar ends.



A particular use includes antibacterial soaps, gels, detergents, lotions and the like for treating inter alia human skin and hair in order to reduce or eliminate undesired organisms.

5 Thus, it is possible by using compositions of this invention presented in such forms, to treat hands, the face and skin generally and the hair, both on the head and elsewhere. This can be employed to reduce bacterial count and so help to reduce the spread of methicillin resistant staphylococcus aureus, clostridium difficile and its spores and other bacteria. Similarly, the composition may be used to reduce  
10 microorganisms associated with acne, body odor or the like. A further benefit is that such compositions may be used to reduce viral transmission, for example for influenza virus, which can occur by hand contamination. Other virus that may be on the skin or membranes include HIV, herpes and the like which are also minimized by use of the compositions of the invention adapted for administration to the skin or  
15 membranes.

Parasite infestation may be treated with compositions of the invention. Such parasites include internal parasites such as protozoa which can lead to diseases of humans such as malaria, leishmaniasis and trypanosomiasis and various diarrhoeas.  
20 Other internal parasites that may be treated include flukes. External parasites that may be treated include lice, especially head lice, and scabies and fleas. Soaps and shampoos are favoured for such external application although solutions, lotions and gels are also particularly suitable. Composition may be analogous to those described in EP2198862, with the addition of the lysine and/or caprylic acid components.

25 Fungi for treatment include those responsible for dandruff, thrush, athlete's foot and the like, for example, candida albicans. Conditions such as dandruff may be treated with soaps and shampoo but other formulation types named herein may also be used. Athlete's foot may also be treated with dusting powder. Candida albicans or  
30 other infecting agents in the vagina may be put up in the form of a pessary.

The compositions of the invention may also be adapted for use in the eye, for example for prophylaxis or treatment of conjunctivitis (red eye). Such compositions are aptly not at a pH below 5, for example may be buffered to pH 5 to 6. The  
35 composition of the invention may also be used for sterilising contact lenses or other material that comes into contact with the eye.



The compositions of the invention may also be used for the prophylaxis or treatment of dandruff or the like.

5 Compositions of the invention may therefore also suitably contain a pharmaceutically acceptable salt of choline such as choline chloride. This can further enhance effectiveness further against organisms such as clostridium difficile and spores or clostridium difficile.

10 Formulations may be composed of conventional carriers, as long as they are compatible with the active component of the compositions herein.

15 Thus soaps, shampoos, gels and the like may aptly contain surfactants. Many conventional surfactants may be employed but it appears certain effective formulations will employ non-ionic surfactants. Particularly effective non-ionic surfactants include alkyl polycyclosides and/or alkenyl polyglycosides (APGs) such as those containing up to 10 sugar residues coupled to a hydrocarbon chain. Oligomerisation of up to about 4 sugar residues can be desirable. Such surfactants are available under the trade name "Plantacare" for example from Henkel as "Plantacare 2000".

20 In some compositions minor amounts of typical anionic surfactants may be employed together with the non-ionic surfactant. Amphoteric surfactants may also be present, for example and preferably, with the non-ionic surfactants, for example those having secondary or tertiary amino and water solubilising anionic groups, such as sulphate, phosphate, phosphonate or carboxylate groups. Such amphoteric surfactants include those available under trade names such as Miranol (of Rhone-Poulenc) and Betaine, such as Dehyton from Henkel.

30 The compositions of the invention may optionally comprise thickening agents. Suitable thickening agents include polysaccharide thickeners such as xanthan gums, gellan gums, pectins, carageenans and the like. An apt thickening agent is xanthan gum such as Keltrol CG which is a high molecular weight polysaccharide produced by microbial fermentation. Viscosity may also be selected by use of an amphoteric surfactant such as a cocamido-propyl betaine or Tego Betaine F50 as a thickening agent as well as surfactant agent.

Due to growing fears that the use of synthetic chemical preservatives in the food industry can cause health hazards, there has been an increased demand for safe, natural food preservatives. Both Citrox (which includes the aforementioned bioflavonoids) and polylysine are natural, organic Gras-approved preservatives with activity against a range of organisms that could contaminate food and reduce shelf-life due to spoilage.

The compositions of the invention may be employed for the treatment of food stuffs to reduce or eliminate unwanted pathogens or organisms leading to reduction in storage life of food stuffs. Thus vegetable, fruits and meat may be treated, for example lettuce, tomatoes, cucumbers, peppers, cereals such as wheat and maize, fruit such as apples, grapes, pears and figs, and meats such as beef, pork, lamb, bacon and the like. Methods of treatment include washing, spraying, misting and the like.

The said combination may be included in chewing gums for treatment of bad breath or mouth ulcers and in deodorants to aid in their effectiveness.

The compositions of this invention may also be employed in order to purify water where local supplies are deemed of inadequate purity. This offers, for example, users in remote areas the possibility of improved drinking or washing water at acceptable cost without resorting to synthetic and possibly environmentally damaging antibacterial anti-infective agents.

The compositions of this invention may be used in the form of a solution or powder or gel enclosed in a dissolvable bag, pod or other dissolvable container.

WHO reports that over 2.6 billion people lack access to clean water, which is responsible for about 2.2 million deaths annually, of which 1.4 million are in children. Improving water quality can reduce the global disease burden by approximately 4%.

Pathogen contamination in freshwater environment (ground water, rivers and lakes and reservoirs) includes *Salmonella*, *E.coli*, *S.faecalis*, enteroviruses, shigellas, *Bacillus megaterium*, *Staphylococcus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, fecal coliform, streptococci, *Salmonella newport* and fecal bacteria.



When used to prevent, eradicate or ameliorate infective organisms such as bacteria, fungi, protozoa, e.g. plasmodia, and other parasitic organisms, the compositions of this invention may be administered topically to an extended surface of the body, such as the skin, or may be administered directly into the blood, for example by injection or  
5 by absorption from the gastrointestinal tract having been administered thereto, for example by swallowing. Particular plasmodia infections which may be treated or addressed by prophylaxis include those causing malaria or leishmania. The compositions may also be employed where the causative organism is a trypanosomal parasite such as *Trypanosoma brucei*. Particular plasmodia which  
10 may be countered include *Plasmodium falciparum* and *Plasmodium chabaudi*, especially chloroquine-resistant strains.

When used ex-vivo, compositions of the invention were found to reduce malaria parasite levels by 50%, even at a dilution of 1:6000. Several strains of parasite are  
15 killed, including *P. falciparum*, which is often fatal, including strains resistant to chloroquine.

When using the compositions continuously for 76 hours, no schizonts are formed and no ring stages form new cycles of infection. The combination of the Citrox and  
20 polylysine and/or octanoic acid halts the life-cycle of the parasite. The use of the combination therefore disrupts the malaria parasite inside the human red blood cell.

Withdrawal of the test compounds in vitro did not result in resurgence of parasitaemia, indicating that the result is not due only to immune-protection. At 48  
25 hours post-withdrawal, untreated controls increased six-fold, whereas treated samples showed no significant levels of parasitaemia.

When treating chloroquine-sensitive *Plasmodium chabaudi* infection in mice at 20 to 40 days post-treatment was not different from mice treated with chloroquine from  
30 those treated with a composition of the invention (approx. zero infection).

When treating mice, no reoccurrence of infection was seen 30 days post-treatment, indicating the malaria parasite had been killed and not merely suppressed. This also  
35 occurred if treatment was withdrawn before peak parasitaemia.

When mice were infected with a very virulent strain of plasmodium which kills within 10 days, all treated mice were still alive 16 days post-infection.



Preliminary data indicates it is possible that the compositions of the invention also enhance activation of the immune system.

5 *Ex-vivo*, the composition of the invention killed four strains of trypanosomes, including *T. brucei* and a strain thereof resistant to chloroquine. In a mouse model, infection was greatly reduced, with several mice completely cleared of parasites.

10 *Ex-vivo*, the compositions of the invention were found to kill leishmania parasites even at a five-fold log dilution.

No significant toxicities were observed in any *in-vivo* test.

### **Brief description of the figures**

15

Figure 1 shows the inhibitory effect on *Escherichia coli* growth of pretreatment at different concentrations of Citrox and/or  $\epsilon$ -polylysine in a high binding microtiter plate.

20 Figure 2 shows the inhibitory effect on bacterial growth (*E. coli*) of direct exposure to Citrox or  $\epsilon$ -polylysine compared to other antibacterial substances.

Figure 3 shows that  $\epsilon$ -polylysine promotes immobilization of Glucose oxidase onto silicate as assessed by measurement of residual activity in solution.

25

### **Examples**

#### **Example 1**

30 A Gel Suitable as a Surgical Sanitizer

Water (481.5g; 96.3%) was added to a beaker and stirring commenced. Keltrol CG-SFT (9.0g; 1.8%) was added and stirring continued until dissolved. Citrox HXT powder (2.5g; 0.5%) was added and stirring continued until dissolved. White willow bark extract (2.0g; 0.4%) was added and stirring continued until dissolved. Glycerol (5.0g; 1.0%) was added and stirring continued until dissolved.

The resulting viscous gel was de-aerated. The pH was 4-5. The viscosity 7000-10000 cp at 20°C (spindle 4/0 rpm). The pH may be adjusted with citric acid if required to bring it within the stated range.

5

The Willow Bark extract contains 90% of salicylic acid.

The Citrox HXT powder (Citrox Biosciences) comprises 7.5% of residues of extraction from bitter oranges together with the following mixture of bioflavonoids:

10

<b>Bioflavonoid mixture</b>	<b>% bioflavonoid (component in biomass)</b>
Neohesperidin	1.1
Isonaringin	1.2
Naringin	23.4
Hesperidin	1.4
Neohesperidin	12.5
Neodiosmin	1.4
Naringenin	1.5
Poncirin	2.0
Other (Rhiofolin)	0.5
<b>Total</b>	<b>45%</b>

15 **Example 2**

Hand Foam Composition

This may be prepared by mixing ingredients as described in Example 1.

20

Salicylic acid	0.25%
Citric acid	0.15%
Bioflavonoid mixture	0.0375%

(Example 1)	
$\epsilon$ -Polylysine or caprylic acid	0.015%
Betaine BP20	1.0%
Glycerine	0.5%
Dermosoft GMCY	1.0%
Water	97.0%

$\epsilon$ -Polylysine is from Everguard PL, Impag AG.

When tested against spores of Clostridium difficile (NCTC 11209) according to BS EN 13704, satisfactory sporicidal activity was found with a 15 minutes contact time at 20°C.

### **Example 3**

#### 10 Sanitizing Gel

This maybe prepared by mixing as described in Example 1.

Keltrol CG-SFT	1.7%
Bioflavonoid mixture (Example 1)	0.0375%
$\epsilon$ -Polylysine or caprylic acid	0.015%
Citric acid	0.15%
Salicylic acid	0.25%
Dermosoft GMCY	1.0%
Glycerine	1.0%
Water	95.8%

15 Such a gel provides satisfactory sporicidal activity against the spores of C. difficile.

### **Example 4**

#### Liquid Soap

20



Keltrol CG-SFT	1.8%
Plantacare 2000	13.56%
Tego Betain F50	9.48%
Glycerine	1.0%
Bioflavonoid mixture (Example 1)	0.0375%
$\epsilon$ -Polylysine or caprylic acid	0.015%
Citric acid	0.15%
Salicylic acid	0.25%
Dermosoft GMCY	1.0%
Water	72.66%

### **Example 5**

#### 5 **Aerially Dispersible Form**

The hand foam composition of Example 2 is used in a commercial misting device to produce a mist for disinfection of surfaces.

#### 10 **Example 6**

A commercial hand held misting device is used to direct mist at the surfaces in an ambulance and to the air space. The misting is continued until the operative is satisfied surfaces have been thoroughly treated.

15

The ambulance may be occupied twenty minutes after the completion of the misting.

### **Example 7**

20

#### **Immobilisation and release of antibacterial activity of Citrox in presence of $\epsilon$ -Polylysine**

25

High Binding plates were pre-treated with 0.1 mL of pure Citrox/ $\epsilon$ -Polylysine solutions incubated 60' at room temperature. The concentrations of Citrox range from 0.06% to 4%. The  $\epsilon$ -Polylysine concentrations range from 30 mg/L to 120 mg/L. The wells have been emptied and rinsed with 0.2 mL bi-distilled water. 0.2 mL

of a diluted *Escherichia coli* culture (turbidity: 5 mOD at 600 nm) were given to the pre-treated wells and incubated for 3 hours at 37°C. Turbidity were measured at 600 nm and percentage of growth inhibition in comparison to untreated well calculated (Figure 1).

5

### **Example 8**

#### **Antibacterial activity of Citrox and $\epsilon$ -Polylysine against *Escherichia coli***

10

The antibacterial activity of Citrox and  $\epsilon$ -Polylysine has been tested by incubating 0.2 mL mini cultures of *Escherichia coli* (0.1 OD<sub>600nm</sub>) with varying concentrations of antibacterial substances. Growth inhibition was monitored by measuring turbidity of mini cultures after 5 hours of aerobe incubation at 37°C under agitation.

15

Solutions were diluted to the half in cascade. The lowest concentration of the substance yielding less than 2% (6% in case of ethanol) of growth in comparison to an untreated control is considered the minimal inhibitory concentration (MIC).

20

#### **Short-term antibacterial activity of Citrox toward *S. mutans* (Bactericidal effect)**

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<u>Minimal inhibitory concentration (MIC)</u>	
$\epsilon$ -Polylysine	4 mg/L
Ampicillin	60 mg/L
Ethanol	12.5 %
Citrox	0.16 %
Papaya extract	1.25 %

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Table 1. Summary of MIC (*E. coli*) extrapolated from Figure 2 (1% corresponds to 10 g/L).

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The concomitant pretreatment with Citrox and  $\epsilon$ -polylysine emphasizes the effectivity of Citrox pre-treatment. The apparent MIC of pretreating Citrox (0.25% pretreating Citrox with  $\epsilon$ -polylysine 120 mg/L) approaches MIC of Citrox in directed exposure (0.16%).

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With  $\epsilon$ -polylysine 60 mg/L, 0.5% pretreating Citrox is required to reach inhibition, which is still significantly lower than the 2% required in absence  $\epsilon$ -polylysine.

30 mg/L  $\epsilon$ -polylysine corresponds to the highest concentration that does not affect significantly the antibacterial effect of Citrox pretreatment. Therefore, the preferred range of synergy is between 30 mg/L and 240 mg/L of  $\epsilon$ -polylysine, and 0.06% to 4% of Citrox.

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### **Example 9**

#### **Immobilisation of enzymatic activity in presence of $\epsilon$ -Polylysine**

0.5 mg/mL glucose oxidase enzyme was incubated for 15 minutes at room temperature in an artificial saliva buffer composition including:

	Na <sub>2</sub> HPO <sub>4</sub>	G/L 0.26
15	NaCL	6.70
	KSCN	0.33
	KH <sub>2</sub> PO <sub>4</sub>	0.20
	KCL	1.20
	NaHCO <sub>3</sub>	1.50,

20 with 2 mg/mL silicate and 0.25 mg/mL of an amino acid based compound, bovine serum albumin (BSA), lysine or  $\epsilon$ -polylysine. The mixture was centrifuged in order to pellet the silicate and the supernatant tested for its glucose oxidase activity in presence of glucose, lactoperoxidase and a chromogen substrate (ABTS). The resulting activities were compared with the enzyme incubated with silicate in  
25 absence of an amino acid compound (Figure 3).

### **Example 10**

#### **Antibacterial activity of $\epsilon$ -polylysine against *S. mutans***

The antibacterial activity of  $\epsilon$ -polylysine was semi-quantitatively assessed in a inhibition zone assay. 0.1 mL of a confluent *Streptococcus mutans* culture was spread over a Brain Heart Infusion (BHI) agar plate. Filter platelets (5 mm  
35 diameter) soaked with 0.01 mL substance were applied on plate. Plates were incubated 48h at 37°C under anaerobe conditions. The diameter of outer limit of the inhibition zones were measured, the diameter of the platelet deduced and the half result considered the radius of inhibition rings. Concentrations were tested in triplicate and their results averaged.

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Antibacterial substance	Radius
$\epsilon$ -Polylysine 1000 mg/L	6.5 mm
$\epsilon$ -Polylysine 100 mg/L	2.5 mm
$\epsilon$ -Polylysine 10 mg/L	0.5 mm
Ethanol	5 mm
Water peroxide 0.1%	1.5 mm
Water peroxide 1 %	5 mm

Table 2. Radius of inhibition rings around platelets diffusing the corresponding antibacterial substance across a *Streptococcus mutans* culture on agar plate.

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### **Example 11**

#### **Short-term antibacterial activity of Citrox against *S. mutans* (Bactericidal effect)**

The short-term antibacterial activity of Citrox was semi-quantitatively assessed in a short exposure assay. A *S. mutans* culture, diluted in order to contain 5000 Colony Forming Units, was incubated for 10 minutes in presence of the antibacterial substance and extemporarily plated onto a BHI agar plate. Plates were incubated at 37°C under anaerobe conditions and colony counted. Citrox at final concentrations of 5% resulted in complete absence of colony formation, as did water peroxide 0.05% under the same conditions. Citrox 0.5% reduced the count of CFU to the half (<3000 CFU).

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### **Example 12**

#### **Assay format circumvents intrinsic antibacterial effect of $\epsilon$ -Polylysine**

The experimental results show the remarkable antibacterial activities in the case of a direct exposure (in solution in a low binding plate) of  $\epsilon$ -polylysine with a minimal inhibitory concentration (MIC) of 4 mg/L (Figure 2 and Table 1). The pretreatment of a high binding plate with  $\epsilon$ -polylysine does not yield any inhibitory effect despite of titers overpassing largely the MIC of direct exposure assay. This fulfils the purpose of this work that aims to circumvent the strong intrinsic antibacterial effect of  $\epsilon$ -polylysine in order to prevent it masking the anti- bacterial effect of Citrox.

35 A possible explanation of the successful quenching of antibacterial effect of  $\epsilon$ -polylysine in this format is that  $\epsilon$ -polylysine irreversibly binds to the plate, which is

a known property of the  $\epsilon$ - polylysine. Increasing the concentration of  $\epsilon$ -polylysine will accordingly not result in antibacterial activity, the surface being likely to be already saturated at the tested concentrations.

5 Since  $\epsilon$ -polylysine strongly adsorbs onto solid surface and is not significantly released in solution, it does not affect the growth of bacteria in suspension. Although it is very likely that it prevents the surface from being colonized by bacteria, this effect concerns only the close proximity of the surface, thus a limited volume, so that it remains undetected.

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**Example 13**

Antibacterial effect upon delayed release of Citrox is enhanced by  $\epsilon$ -Polylysine

15 The tests demonstrate that the antibacterial activity of Citrox is retained by the high binding plates but also allows its release in solution, thus allowing to affect the growth of the bacteria in suspension. Quantitatively, pretreating a high binding plate with Citrox 2% reaches the same inhibitory effect of a direct exposure with Citrox 0.16 % (MIC Citrox).

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**Example 14**

Tested concentrations of Citrox and  $\epsilon$ -Polylysine employed are as follows:

25	Sample No.	Citrox wt-%	Polylysine wt-%
	1	4	0.012
	2	4	0.06
	3	4	0.03
	4	4	0
30	5	2	0.012
	6	2	0.06
	7	2	0.03
	8	2	0
	9	1	0.012
35	10	1	0.06
	11	1	0.03
	12	1	0
	13	0.5	0.012
	14	0.5	0.06
40	15	0.5	0.03
	16	0.5	0
	17	0.25	0.012
	18	0.25	0.06
	19	0.25	0.03
45	20	0.25	0
	21	0.06	0.012
	22	0.06	0.06
	23	0.06	0.03

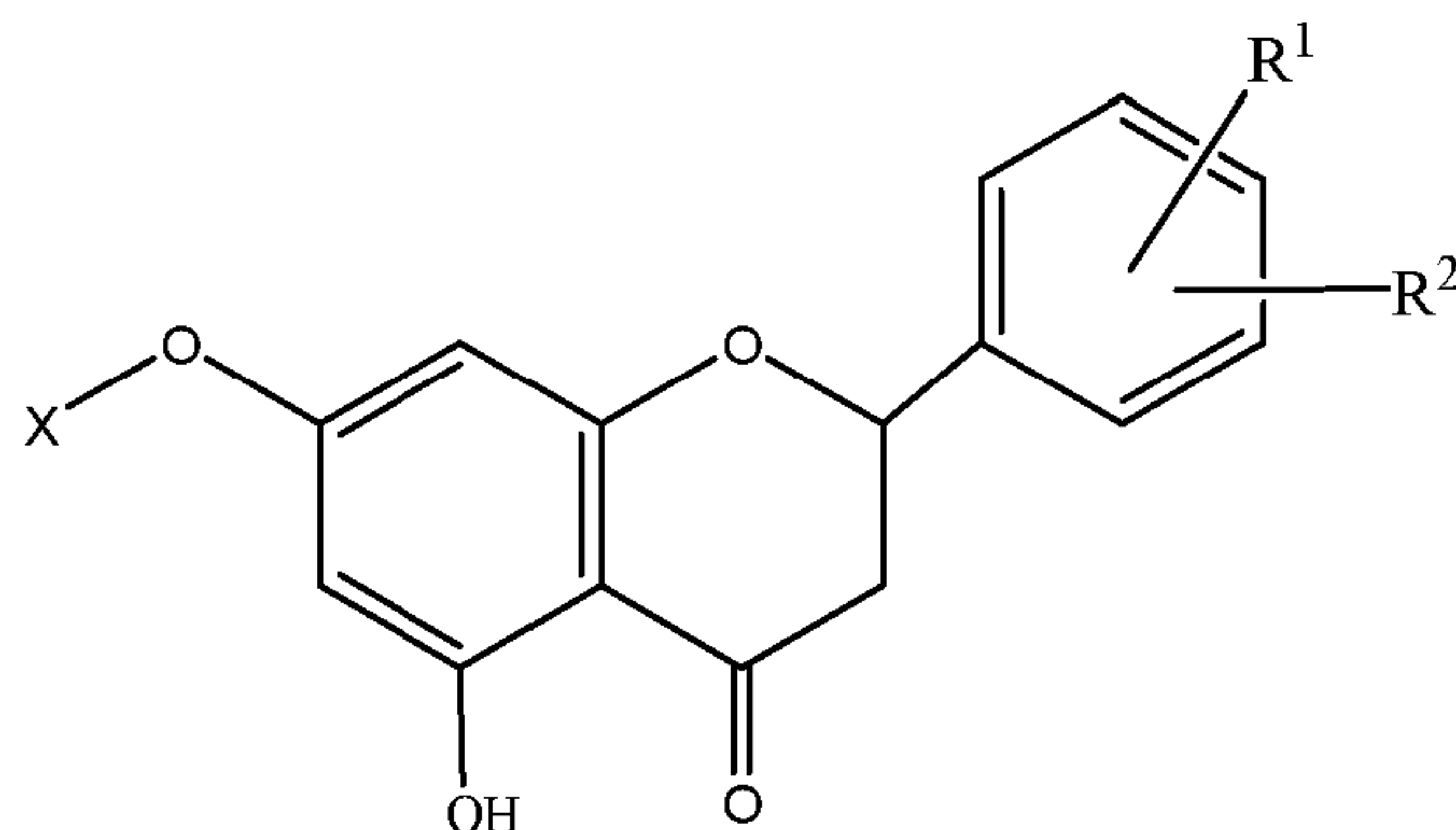
	24	0.06	0
	25	0	0.012
	26	0	0.06
	27	0	0.0
5	28	0.06	0

These demonstrated that using both agents proved anti-bacterially more effective than the control tests employing singular composition comprising either Citrox or  $\epsilon$ -Polylysine (sample No 4, 8, 12, 20, 24-28).



## Claims

1. A composition which comprises polylysine and/or caprylic acid and one or more flavonoids of Formula (I)



wherein R<sub>1</sub> is hydroxyl or methoxyl and R<sub>2</sub> is hydrogen, hydroxyl, methoxyl and X is hydrogen or a saccharide.

2. A composition as claimed in claim 1 wherein the composition includes a mixture of flavonoids comprising naringin and neohesperidin and other flavonoids of Formula I.
3. A composition as claimed in claim 1 or claim 2 which comprises a mixture of flavonoids comprising 40% to 65% of naringin (wt/wt of flavonoids) and 20% to 35% of neohesperidin (wt/wt of flavonoids present).
4. A composition as claimed in any one of claims 1 to 3 which comprises  $\epsilon$ -polylysine of molecular weight of 3000 Da to 5000 Da.
5. A composition as claimed in any one of claims 1 to 4 which comprises bioflavonoids and  $\epsilon$ -polylysine in a ratio (wt/wt) of 50:1 to 1:20, for example 25:1 to 3:1.
6. A composition as claimed in any one of claims 1 to 5 wherein the composition comprises 0.06% to 4% of bioflavonoids and 30 mg/L to 240 mg/L of  $\epsilon$ -polylysine.
7. A composition as claimed in any of claims 1 to 6 for use in reducing bacterial numbers on a surface.
8. A composition as claimed in any one of claims 1 to 6 for use in purifying water.
9. A composition as claimed in claim 8 for use in reducing or eliminating bacteria, viruses, fungi and other parasites from water, such as water intended for drinking, or a source of water that could be contaminated.
10. A composition as claimed in any one of claims 1 to 6 for use in food preservation.
11. A composition for use as claimed in claim 7 in the form of a solution, gel, soap, body wash, shampoo, dusting powder, or aeriably dispersible powder or liquid.

12. A composition for use as claimed in claim 7 for reducing bacterial numbers in a hospital environment or external surfaces of enclosed spaces.
- 5 13. A composition as claimed in any of claims 1 to 6, for use in reducing methicillin resistant staphylococcus aureus, clostridium difficile and its spores, and/or bacteria causing acne or body odour, or viruses such as influenza or HIV.
- 10 14. A sanitizing gel which comprises a composition as set forth in any one of claims 1 to 6, for example for use in reducing spread of infection, for example in hospitals or as a hand gel, for example for use in reducing spread of infection by, for example, protective hand gloves, respiratory masks and packaging of non-sterile products.
- 15 15. A composition as claimed in any of claims 1 to 6, for use in the treatment or prophylaxis of an internal parasite or an external parasite.
- 20 16. A composition as claimed in any of claims 1 to 6, for use in the treatment of prophylaxis of a protozoan infection, particularly *Plasmodium falciparum* or *Plasmodium chabaudi*.
17. A composition for use as claimed in any of claims 7 to 10 and 12 to 16 in the form of a solution or powder or gel enclosed in a dissolvable bag, pod or other dissolvable container.



**Application No:** GB1817003.5

**Examiner:** Mr Chris Archer

**Claims searched:** 1-17 (in part)

**Date of search:** 18 April 2019

**Patents Act 1977: Search Report under Section 17**

**Documents considered to be relevant:**

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-14, 17	KR 20050063182 A (NONG SIM) see WPI AN: 2006-566032 attached.
X	1-14, 17	JP 2005152784 A (KIKUSUI KAGAKU KOGYO) see English language abstracts attached and paragraph [0026].
X	1-14, 17	KR 20190011844 A (HOSEO UNIV) see English abstracts attached and paragraph [0089].
X	1, 10	CN 106261893 A (HUIZHOU YINGDIL) see English abstracts attached.
X	1, 10	KR 20160007839 A (CHOI) see English abstracts attached and paragraph [0055].
X	1, 7	CN 107468584 A (JINGXIAN COUNTY MAILAN NETWORK TECH) see English abstracts attached.

**Categories:**

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

**Field of Search:**

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC<sup>X</sup> :

Worldwide search of patent documents classified in the following areas of the IPC

A01N; A01P; A23L; A61K; A61P; C02F

The following online and other databases have been used in the preparation of this search report

EPODOC, WPI, Patent Fulltext, BIOSIS, MEDLINE, CAS Online





**International Classification:**

<b>Subclass</b>	<b>Subgroup</b>	<b>Valid From</b>
A01N	0043/16	01/01/2006
A01N	0037/46	01/01/2006
A01P	0001/00	01/01/2006
A23L	0003/3463	01/01/2006
A23L	0003/3508	01/01/2006
A23L	0003/3562	01/01/2006
A61K	0008/49	01/01/2006
A61K	0008/88	01/01/2006
A61K	0031/7048	01/01/2006
A61Q	0019/00	01/01/2006
C02F	0001/50	01/01/2006