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(54) **Natural composition based on Chilean monofloral honey extract from native vegetable species for bacterial infection control in vegetables and flowers**

Natürliche Zusammensetzung auf Grundlage von chilenischem monofloralem Honigextrakt aus einer nativen Pflanzenart zur Kontrolle bakterieller Infektionen in Gemüse und Blumen

Composition naturelle basée sur un extrait de miel monofloral Chilien à partir d'espèces végétales natives pour le contrôle des infections bactériennes dans la culture des légumes et des fleurs

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Description

[0001] The present application addresses a natural composition based on Chilean monofloral honey extract from native vegetable species for bacterial infection control in vegetables at all. By way of illustration, the composition of the present invention showed to be especially useful for the control of soft rot (*Erwinia spp*) in potatoes, vegetables and flowers in general.

Previous design

[0002] Bacterial diseases affect vegetable crops causing losses for agriculture. One example for this kind of diseases is the soft rot in vegetables caused by bacteria of the gender *Erwinia.* Within this bacterial gender, the species *Erwinia carotovora* pv. *carotovora* affects various crops like potatoes, artichokes, celery, sunflowers, asparagus, Brussels sprouts, cabbage, cauliflower, lettuce, aubergines, radish, turnip cabbage, melons, onions, paprika, rhubarb, tomatoes and beets. It also affects some fruit, ornamental plants like hyacinth bulbs and callas and native plants. In general, the susceptibility of plants being infected is due to the presence of an organ with abundant parenchymatic tissue, cells in which bacteria can develop due to the presence of a huge vacuole with water and other nutritive elements.

[0003] The putrescence consists in maceration and final rupture of the parenchymatic tissue of all plant organs, which finally results in her death causing the ruining of the production. This is due to the action of enzymes that dissolve the middle sheet, destroying the cellular wall and impeding plasmodesmic connection, leading to an interruption in translocation of indispensable solutes for organic or plant life. On these cells starts the developing.of a mucilaginous bacteria mass, of unbearable smell, typical of bacterial putrescence, hence the name of the disease.

[0004] Global losses of vegetables and flowers ascribed to soft rot add up to US$ 100,000,000 per year. (Ciampi et al., 1997). The disease can occur on fields, in gardens, in greenhouses during crop time or in the post crop phase. It can also happen during transportation and on selling places. In potato tubers (*Solanum tuberosum* L.), when conditions are favourable for the development of the disease, the total loss can add up to 75% of production, being normal ranges between 2 and 5%. It is estimated that on a field level a total of 5 % is lost, whereas in stored tubers this cipher can add up to 15 % (Ciampi *et al.,* 1997) what equals to an estimated loss in Chile of 167,250 tons of potatoes, taking as a base a national production of 1,115,000 tons (source ODEPA (bureau for agrarian studies and politics) 2005).

[0005] Calla industry in New Zeeland losses approximately NZ$ 2,000,000 because of this disease, what equals to 1,360,000 USD, affecting between 15-20% of stored tubers. If conditions are favourable for the disease, losses in field conditions can be total (Vanneste, 1997) for this reason currently only the *in vitro* propagation is used on a commercial level; this makes crop production more expensive. (personal communication, PIGA Seed S.A. Manager, 2005).

[0006] Currently there are serious problems for controlling these pathogens due to the lack of commercial bactericides; farmers just throw away vegetable products with putrescence.

[0007] Control measures are preventive, like: the use of healthy tubers, certified seeds, resistant cultures, perform rotation with non lodging crops, throw way sick plants, use soils with good draining and manage the relative humidity in storage Eradication of the pathogen is difficult and in experimental field conditions no good control levels have been shown. Literature mentions the use of formaldehyde, sodium hypochlorite, citric acid and some bactericide formulations like Strepto Plus (gentamicyne sulphate with oxytetracycline chlorohydrate) utilizing a dose between 60-120 g/HL. Another not so often used product because of dyeing the eatable organ blue is copper oxychloride.

[0008] On the other hand, controlling these bacteria becomes complicated as the different bacterial subspecies of the complex *Erwinia carotovera* can cause soft rot and black leg depending on the conditions of the environment where the plants are cropped. Furthermore, the latent infection risk in tuber-seeds allows the appreciation of disease symptoms only once the crop has been established. Another related actor is that pathogens frequently are protected within the lenticels or vascular system and therefore are not affected by the usually used commercial disinfectants.

[0009] Within the area of control with organic based products we can mention the possibility of using a product coming from citric fruit extracts (CITRUPAR 80) acting as a "natural antibiotic" for the control of these pathogens.

[0010] It is important to mention that the existence of extracts from vegetable origin is appreciated; as for example the one disclosed in the Chilean invention patent applications 2886-2001 and 2377-2004, insecticides as well as acaricides, and agents from the active substances Hederacocido and Alfa-Hederina. Also observed is the patent application 67-1999 that comprises honey combined with other ingredients, useful as cosmetics and for sanitary disinfections.

[0011] On the other hand, the Russian patent application RU2236248 dated 27^{m} December 2001 and published on 20th September 2004, discloses an immunotropic 5 preparation for medical and pharmaceutical use where a preparation based on honey obtained from bees fed with a composition that comprises honey obtained from one sole flower extracted from the plant family Compositae and that comprehends specific flavonoids to include the synthesis of interferona Alfa and Beta, presenting high effectiveness in the treatment of viral and bacterial prophylaxis. Similar compositions 10 can be observed in the Russian applications RU2236247 and RU2236244.

[0012] However, none of the publications of the previous design resolve the problem of bacterial infections suffered

by vegetables as described previously. As described, one of the most affected crops by these bacteria is the potato. *{Solarium tuberosum* L). Therefore, the use of a toxic compound is not feasible, as it wouldn't allow consumption of the tuber. In spite of the good results obtained *in vitro* using chemicals for controlling *Erwinla* spp. it hasn't been effective as a method for disinfecting tubers.

[0013] In the specific case of controlling soft rot in potato tubers, products like streptoplus (sulphate) have been used, sodium benzoate and chlorine dioxide, o-phenylphenate, experimental bactericides like CGA 78039, sodium hypochlorite and citric acid in 1%, formaldehyde, followed by drying with blast.

[0014] As most of the horticultural products affected with putrescence by *Erwinia* are consumed as food, for the control of this rot no synthetic products derived from antibiotics that can have toxic effects on the human species can be used. Furthermore, globalization nowadays doesn't allow us the use of antibiotics, as the major part of the international market corresponds to countries with barriers regarding products derived from antibiotics for controlling this type of bacterial diseases.

[0015] Document from Database WPI Week 200470, Derwent Publications Ltd., London GB., AN 2004-716837, XP-002446355 relates the immunotropic activity of a honey obtained from an Asteraceae (Compositae) family plants, propolis and flower and leaves extract, not separating the specific effect of each component as immunotropic.

[0016] Patent JP-03240451A concerns to a mixture of water and honey coming from the vegetable species *Tithymalus helioscopius (Syn Euphorbia heiloscopia),* and therefore neither being Ulmo honey *{Eucryphia cordifoila)* nor existing phylogenetic relation between both vegetable species. Moreover, JP-03240451A states that the obtained mixture is useful to keep the food fresh, but, unlike the present invention, it does not point out that this mixture is able to avoid the "soft rot" disease in vegetables caused by bacteria of the gender *Erwinia carotovora* pv. *carotovora* (Syn *Pectobacterium carotovorom* subp. *carotovorum).*

[0017] Patent WO-2005/120250A discloses the classification of the manuka honey, *(Leptospermum scoparium),* vegetable species from New Zealand, and its antibacterial 5 activity known as unique manuka factor (UMF), valid classification for manuka honeys.

[0018] Document of Molan P.C. "The antibacterial activity of honey 1. The nature of the antibacterial activity" BEE WORLD, BEE RESEARCH ASSOCIATION, GERRARDS CROSS, GB, VOL 1-2, 1992, PAGES 5-29 XP-002802515, issn: 0005-772X points out the antibacterial properties inherent to bee honey from any botanical origin, not disclosing in the list the susceptibility of the *Erwinia carotovora pv. Carotovora* bacteria.

[0019] The articles "Pollen analysis of honeys from the Los Lagos region of southern 10 Chile," HELMUT HORN and MARIA JESUS AIRA, 1997 Scandinavian University Press and "Estudio palinologico en 10 muestras de miel de ulmo (Eucryphia cordifolia CAV.)", Miguel Angel Triveili, Alimentos, Vol. 12 - N° 3, 1987 relate to a study of the Ulmo honey from the south of Chile based on the pollen types on these honeys, but do not mention its bacterial properties.

[0020] The solution of the present invention solves the previously described problemsby a product based on monofloral honeys corresponding to an innocuous organic compound whose "raw material is unique to the world" as the botanic origin of the honey to be used indicate that it only can be produced in Chile. This allows the antibiotic soft rot controlling product to be produced in Chile and to benefit the control of bacteria in potatoes, vegetables and fruit by means of a natural product that doesn't harm the qualities of the species applied on and fulfilling all international norms with regards to this matter. On the other hand, it offers a big opportunity for the national apicultural sector in general.

[0021] To sum up, the advantages or benefits of the invention's object are as follows:

- 100% natural product Non-toxic to humans and animals
- Wide spectrum germicide, of rapid action and very effective against Erwina carotovora pv. Carotovora
- It doesn't irritate skin or eyes of humans or animals and it is inoffensive to the mucous membrane.
- It has conservation properties, that is, bacteriostatics.
- Optimal stability in ph 2 to 12 and temperatures up to 130°C.
- It isn't volatile.
- It is "selective".
- It is no antibiotic, but it works in a similar way.
- It is compatible with antibiotics, sulphates etc.

Brief description of the Figures

[0022]

Figure 1: Corresponds to a graphic showing the incidence of soft rot in potato tubers of the variety Desireé treated with monofloral honey in post crop conditions.

Figure 2: Corresponds to a graphic showing the botanical origin of one of the tried honeys, M182, to exemplify the invention.

Figure 3: Corresponds to a graphic showing the botanical origin of one of the tried honeys, M84, to exemplify the invention. This honey represents a monofloral Ulmo honey, as the frequency of the Ulmo pollen grains found was over 50%.

Figure 4: Corresponds to a graphic showing the botanical origin of one of the tried honeys, M335, to exemplify the invention.

Figure 5 - corresponds to example 3 where visual differences of soft rot in lettuce type Great Lakes can be appreciated. From left to right you can see not infected lettuce, infected lettuce, infected lettuce treated with M349 extract and not infected lettuce treated with M349 extract.

Figure 6 - ELISA plate where you can observe the *in vitro* growth inhibition of *Erwinia carotovora pv. carotovora* with M349 honey extract.

Figure 7 - ELISA plate with P. *syringae pv. Syringae.* The first three lines correspond to in vitro growth of the bacteria in environments with M349 extract and soy medium. The bacteria growth can be appreciated from the fourth column on.

Figure 8 - feasibility of rat spermatozoids exposed to different concentrations of M349 honey extract expressed in percentage of living rat spermatozoids (n=100) and observation time.

Detailed description of the invention

[0023] The invention corresponds essentially to a composition obtained from extract of flavonoids and/or phenols of monofloral honey, preferably Chilean honey derived from different species and who act independently as controllers for different bacterial infections in various vegetable species.

[0024] The botanic origin of each honey is determined through a separation process of the pollen grains by centrifugation and its posterior staining and observation under a light microscope. Data was quantified statistically. With this method honey is considered monofloral when more than 50% of the pollen present in the honey correspond to one sole vegetable species.

[0025] To perform honey extraction, and only for illustrative means as an experienced medium-level technician could clearly extrapolate the following mentioned quantities, a process is carried out that contains the following phases:

1. 50 gr honey is weighed
2. They are dissolved with 100 ml distilled water acidified with HCI (pH=2).
3. The solution is put into a volumetric flask of 250 ml and it is filled to said volume with acid water.
4. The solution is filtered with cotton and passed through a column of Amberlite XAD-2 resin (250 mm height by 20 mm de diameter), at a dropping speed of 2 ml/min. Phenolic compounds will be retained in the column.
5. The column is cleaned with 100 ml acid water. The liquid is thrown away.
6. It is washed a second time with 200 ml neutral distilled water. The liquid is thrown away.
7. It is washed a third time with 300 ml pure methanol. Methanol will separate the phenolic compounds of the column. Methanol is recollected in a clean glass or flake and it is passed to a balloon for 500 ml rotoevaporator.
8. The methanolic solution concentrates until dryness in the rotoevaporator at 45°C (approximated time: 12 hrs at high rotation speed).
9. The residue is re-suspended in 5 ml distilled water.
10. The suspension is put in a funnel for decantation, and 5 ml diethylic ether. The ethereal phase is collected (lower coloured phase), and it is extracted again twice with 5 ml ether.
11. The ethereal solution concentrates until dryness in the rotoevaporator at 45°C (approximated time: 1 hrs at high rotation speed).
12. The residue is re-suspended in 2 ml sterilized distilled water with autoclave at 15 pound pressure (125°C) for 15 minutes, afterwards the extract is filtered through syringe filters of 0,45 um (pore size) and it is stored at -20°C.

[0026] The before described process made it possible to obtain monofloral honey extracts with what different tests were performed for the control of bacterial soft rot in potato tubers in general.

[0027] A chromatographic analysis to analyze the phenolic and flavonoid compounds present in the honey allowed to determine that the present compounds in the extract correspond to galic acid, cumaric acid, pherulic acid, salicylic acid, naringenine and kaempherol.

[0028] In spite of the procedure described above it is also possible to obtain ethanolic honey extracts. To this end, in point 4 of the process, part of the diluted honey is collected and filtered with 0,45 um filters and stored at -20°C. In point 9 of the process the residue is re-suspended in 85° ethylic alcohol then it is filtered with 0,45 um filters and stored at -20°C. In point 12 of the process the residue is re-suspended in 85° ethylic alcohol then it is filtered with 0,45 um filters and stored at -20°C.

[0029] This procedure allowed obtaining an innocuous and organic honey product of simple elaboration and compatible

with integrated plague and disease management. As it is an innocuous product it can be used in conventional and organic agriculture. Afterwards the extract can be formulated in different ways, like for example and not limiting to, dissolution in distilled water that can be presented in a spray product (atomization) or as a bactericide solution.

[0030] When applying the product, its action mode is due to compounds with floral origin that are not peroxidic, acting by inhibiting the bacterial development with a bacteriostatic action on the pathogen. That is, it is a product for contact.

[0031] Application method for its use in bacterial development inhibition in vegetables is given by solutions that contemplate a dose higher than 10% of the extract. Notwithstanding, studies have been performed with other formulations and results have been also successful.

[0032] The following examples show the efficiency in disease control and development inhibition of *Erwinia carotovora pv. Carotovora,* by extending the post-crop time for vegetable products and lowering the associated losses.

Example 1: *In situ* and *in vitro* growth inhibition of the bacteria *Erwinia carotovora pv. carotovora,* a bacteria that causes black leg and soft rot of potato tubers (*Solanum tuberosum* L.)

[0033] In the experimental study performed to show the efficiency of the product presented in this patent application, the *in vitro* and *in situ* inhibition of growth of the *Erwinia carotovora pv. carotovora* bacteria, that causes black leg and humid rot of potatoes (*Solanum tuberosum*) was observed, through the effect of solutions obtained out of 3 monofloral Ulmo honeys (*Eucryphya cordifolia*), dominant native species of the Bosque Templado in southern Chile.

[0034] The culture medium used for bacteria growth was B de King (KB). The inoculum used was bacterial isolation coming from commercial potato tubers from the region of San Fernando. Afterwards a biochemical battery was used to determine the bacteria and the stump plus the gram- staining, resulting in the stump *Erwinia carotovora pv. carotovora* (Ecc). The in vitro bacterial growth inhibition analysis was based on the quantification of bacterial growth inhibition halo measuring the inhibition area with a planimeter, the MIC (minimum inhibition concentration) of each honey was also measured and the technique of agar diffusion was used (well diffusion agar) adding honey into the culture medium to different volumes observing the bacterial growth. Concentrations between 10^5 to 10^7 UFC (colony forming units/ml) were used. Statistic analysis was based on a completely random design with ANDEVA and TURKEY-Kramer statistic analysis to 95-5 confidence each. On the other hand, honeys were analyzed with a pH 4 adjusted sugared solution that contained in an average proportion the most important sugars present in honeys.

[0035] Afterwards an analysis of the *in situ* activity of monofloral Ulmo honeys was performed. The inhibition of bacterial growth in potato tubers of the variety Desireé (principal potato in the Chilean market) was analyzed in two ways: 1) Inoculating through a 0,5 ml syringe with bacterial solution and distilled water with a concentration of 10^7 UFC and putting on the inside of the tuber honey solutions in different concentrations with an other syringe. 2) Inoculating through a 0,5 ml syringe with bacterial solution and distilled water with a concentration of 10^7 UFC and brushing the outside of the tuber with different honey solutions.

[0036] The honeys that showed highest bactericide activity against Ecc were the honeys coded as M182, M84 and M335 whose botanic origins can be observed in the figures that complete the present description.

Result of the effect of honey M182 and M84 on bacteria Ecc colonies

[0037] The organic and innocuous honeys were prepared in different aqueous solutions that corresponded to: 1:10; 1:4; 1:1 (v/v). All solutions showed inhibition, however the concentration to 50% had the highest data. Results showed the inhibition of the bacterial growth in a range of from 40 to 80% in dose 1:10 v/v of honey and distilled water in potato tubers with a bacterial dose of (3,7 x 10^7 UFC). The number of experimented samples showed significant differences of inhibition in relation to control.

[0038] Figure 1 shows the high impact the application of the product based on monofloral honeys had on the infected sample. Said graphic shows that a 50% of concentration v/v had a high control percentage in the incidence of soft rot.

[0039] As to honey M335, whose botanic origin can be observed in figure 4, it is possible to deduce significant results in its application. This honey was tested in dissolutions of 10% v/v. It showed a 60% *in vitro* bacterial growth inhibition comparing to sugar and ph 4 adjusted solutions, present in average in honeys (fructose 38,5% (w/w), glucose 31%, 7,2% maltose, 1,5% sucrose) (D'Arcy, 2001. Data USDA), solution that didn't give or showed to be less effective for inhibition in vitro of *Erwinia carotovora pv. Carotovora.*

[0040] Honey M335 corresponded to an extract of flavonoids derived from organic monofloral ulmo honey that controls bacterial soft rot of potato tubers post crop. Its composition is as follows:

Compound	Concentration (ug/mL)
Galic acid	0,236 ± 0,005 ug/ml
Cumaric acid	0.221 ± 0,005 ug/ml

(continued)

Compound	Concentration (ug/mL)
Pherulic acid	0.235 ± 0,005 ug/ml
Salicylic acid	0.435 ± 0,005 ug/ml
Naringenine	0.456 ± 0,005 ug/ml
Kaempherol	0.174 ± 0,005 ug/ml

pH : 4,9 a 5,5

Example 2: In vitro and in situ growth inhibition of bacteria *Erwinia carotovora pv. carotovora,* in seed tubers of five potato varieties (Desiree, Cardinal, Atlantic, Karu-INIA and Pukara-INIA) in storage conditions.

[0041] For evaluating the effectiveness of the described invention on bacterial soft rot the following study was performed using seed tubers of five different potato varieties (Desiree, Cardinal, Atlantic, Karu-INIA and Pukara-INIA), putting them under storage conditions. The following treatments were used: 1 Healthy sample 2. Inoculated with *Erwinia carotovora pv. carotovora,* 3. Inoculated with *Erwinia carotovora* pv. *carotovora* plus application of honey M349 extract, 4. Inoculated with *Erwinia carotovora* pv. *Carotovora,* plus application of the product Citrupar-80.

[0042] The procedure or preparing the honey extract as well as the preparation of the inoculums and the inoculation of the five potato varieties corresponded to the same protocol described in example 1.

[0043] Afterwards the tubers were distributed in plastic boxes with a completely random factorial design to be put in storage, in darkness and covered with Rachel mesh, and they were analyzed after 50 days.

[0044] Severity was evaluated cutting the core of each tuber in length, using the Horsfall-Barrat (1945) scale that considers area percentage of the tuber with putrescence according to the following scale:

Grade	Percentage
1	0%
2	1-3%
3	3-6%
4	6-12%
5	12-25%
6	25-50%
7	50-75%
8	75-87%
9	87-93%
10	93-96%
11	96-99%
12	100%

[0045] Evaluation data was submitted to statistic analysis (Andeva), separation occurred according to the DMS test ($p<0,05$).

[0046] Prior to Andeva the data of the variable incidence transformed to square root of % plus 0.5

Chart 1.- Soft rot severity comparison between different treatments with diverse potato varieties in storage conditions, La Platina, 2006. Averages with the same letter, in the same column, do not differ among each other, according to test DMS ($p<0,05$)

[0047]

Treatments	VARIETIES				
	Atlantic	Cardinal	Desiree	Karu-INIA	Pukara-INIA
Control (+): sample without inoculation	1,76 b[1]	1,06 d	1,06 b	1,15 d	1,03 c
Control (-): sample inoculated with Ecc	2,41 a	1.7 a	1.18 b	1.46 a	1.26 b
Inoculated plus "organic product"	1.31 c	1.38 c	1.85 a	1.5 a	1.65 a
Inoculated plus Citrupar-80	2.38 a	1.56 b	1.00 b	1.3 b	1.18 b

[1] different letters correspond to different statistics among varieties, the average values of severity are expressed according to Horsfall-Barrat scale

[0048] The results obtained in this essay allowed concluding the evaluation of severity in which interaction was found among the varieties through the described statistic parameters. The product had a good effect in reducing soft rot damage in the varieties Atlantic and Cardinal. (Chart 1)

Example 3: *In vitro* and *in situ* growth inhibition of the bacteria *Erwinia carotovora pv. carotovora,* in lettuce type Great Lakes with honey M349 extract.

[0049] In order to evaluate growth of the bacteria *Erwinia carotovora pv. carotovora* lettuce type Great Lakes (n=18) was used distributed in 4 treatments: healthy sample, infected sample, lettuce treated with M349 extract and infected lettuce treated with M349 extract in a solution of 10% v/v. Artificial infection of the lettuce was carried out with a dripping atomizer covering the total surface of each infected lettuce. Afterwards the treatments were sprinkled. Each lettuce was packed in transparent bags and put under greenhouse conditions at 30°C for seven days.

[0050] Severity was evaluated cutting the core of each tuber in length, using the Horsfall-Barrat (1945) scale that considers area percentage of the tuber with putrescence according to the following scale:

Grade	Percentage
1	0%
2	1-3%
3	3-6%
4	6-12%
5	12-25%
6	25-50%
7	50-75%
8	75-87%
9	87-93%
10	93-96%
11	96-99%
12	100%

[0051] Evaluation data was submitted to statistic analysis (Andeva), separation occurred according to the DMS test ($p<0,05$).

[0052] Prior to Andeva the data of the variable incidence transformed to square root of % plus 0.5

Chart 2.- Soft rot severity comparison between different treatments in lettuce variety Great Lakes (n=20) in storage conditions, PUC, 2007.

[0053]

Treatment	Putrescence severity expressed in Horsfall-Barrat scale
Healthy sample	2,0±2,0 a[1]
Not infected lettuce treated with M349 extract	2,6±2,3[a]
Infected lettuce treated with M349 extract	3,2±2,2[a]
Infected sample	7,4 ±1,3[b]
[1] Averages with the same letter do not differ among each other, according to test DMS (p<0,05)	

[0054] 7 days later the infected lettuce treated with M349 extract didn't show any significant changes as to the presence of the disease compared to healthy samples and lettuce treated with M349 extract, obtaining soft rot levels in those treatments between 1 and 6% of affected area with disease, on the other hand the statistically different lettuce group corresponded to treatment of infected sample where the reached putrescence levels are close to 80% of putrescence.

[0055] The treatment in lettuce infected and treated with M349 extract controlled the disease in this evaluation period that was observed with putrescence corresponded to sick sample. (Image 5)

Example 4: Evaluation of the bacteriostatic activity of the aqueous honey extract M349 stored in different storage conditions.

[0056] The bacteriostatic activity of the aqueous honey extract M349 stored for 30 days at room temperature and at -20°C, was evaluated about the growth control in vitro of *Erwinia carotovora pv. carotovora,* determining the minimum concentration of the extract able to inhibit the bacterial growth (image 6).

[0057] From the obtained results we can observe that the M349 extract stored at 25°C inhibited the *in vitro* growth of *Erwinia carotovora pv. carotovora* at concentrations higher than 0,188 μl of extract/? μl of solution. On the other hand the M349 extract stored at -20°C inhibited the *in vitro* growth of *Erwinia carotovora pv. carotovora* at concentrations higher than 0,188 μl of extract/? μl of solution.

[0058] The results show that the activity of the honey extract presented in the patent application is able to go on inhibiting the growth of the bacteria causing Soft Rot even under storage conditions in shelves at room temperature (25°C) and storage at low temperatures.

[0059] No significant differences of the bacteriostatic activity were found between these two extracts stored under different storage conditions, the temperature didn't affect the bacteriostatic activity of the extract.

Example 5: Evaluation of the bacteriostatic activity of the aqueous honey extract M349 on *Pseudomonas syringae pv. syringae,* agent that causes bacterial cancer of cores (apricots, plums, cherries, almonds, peaches)

[0060] *Pseudomonas syringae pv. syringae* is a Gram negative bacteria, that causes the disease called bacterial cancer, that attacks fruit trees like: mazzard, almond tree, cherry-tree, apricot tree, peach tree, pear tree and some crops like rice. It affects young and adult trees unleashing a series of symptoms (syndrome). Among the most important damages we find the not uniform and retarded sprout, acid sap, and exudation of gum. However, an uncontrolled or bigger attack is able to generate death of mother branches, buds, flowers and even the entire tree (Figure 7). *Ps. syringae pv syringae* on his part is able to affect the fruits, provoking a brown leisure with an aqueous margin that impedes its commercialization. All these damages are because of the bacteria producing syringomicine fitotoxin, which consist in a greasy acid of variable length linked to a cyclic peptide.

[0061] The ways of dissemination of the bacteria are various, being the main one eluviation by rain and penetration by prune wounds.

[0062] Disease control is allotted to cultural measures, like for example, not to prune with wet wood, or to disinfect the scissors and saws, and the elimination of affected branches (who, as last solution, are burnt): For this reason the search for efficient products that ensure a good control of the disease and are also environmental friendly (from its production to its discard) is preferential for national agro industry. On account of this, there is currently a huge interest in studying antimicrobial properties of plant extracts and other products that can ensure a good control of this and other diseases with similar characteristics.

[0063] The activity of the honey extract M349 was evaluated through determining the minimum concentration of the

extract able to inhibit bacterial growth in ELISA plates (Figure 7).

[0064] The extract M349, inhibited in vitro growth of *Pseudomonas syringae pv. syringae* in concentrations higher than 0,188 ul of extract/ μl DE ?ul of solution.

[0065] The extract M349 *in vitro,* shows a promising activity on the agent causing bacterial cancer in cores, with a potential use as biocontroler of the disease.

Example 6: Evaluation of bacteriostatic activity *in vitro* of extract M349 on human pathogens.

[0066] The analyzed bacteria species and their stump belonged to:

1. *Enterobacter aerogenes* Hormaeche and Edwards ATCC 13048, enteric bacteria causing intrahospitalarian gastrointestinal disorders.
2. *Escherichia coli* (Migula) ATCC 25922, bacteria of enteroagregative type (ECEAgg) able to survive long time in human intestine, producing through toxins diarrhea in a severe infection.
3. *Pseudomonas aeruginosa* (Schroeter) Migula ATCC 27853. Causing skin infections associated mainly to its presence in nosocomial infections (intrahospitalarian)
4. *Salmonella typhi* STH 2370 Causing typhoid fever, like other enteric pathogens, infection by *S. typhi* are transmitted by food and water contaminated by human feces with acute infection, persistent excretors or asymptomatic chronically carriers. Humans are the only host of *S. typhi;* there are no environmental reservoirs.
5. *Vibrio cholerae* ISP, causing cholera, acute associated gastrointestinal disease.
6. *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC 25923, bacteria found on skin and nasal cavities of healthy people, causing huge variety of diseases, from minor infections to the skin (furuncles, blister, bladders) and cutaneous abscesses up to diseases that can endanger life like pneumonia, meningitis, endocarditis, toxic shock syndrome (TSS) and sepsis.
7. *Streptococcus pneumoniae* type B common agent of low and high respiratory diseases, like pneumonia and medium acute earache (infections to the middle ear), and meningitis, that affect children and adults globally.

[0067] The activity of the honey extract M349 was evaluated determining the minimum concentration of the extract capable to inhibit bacterial growth on ELISA plates with microbiology technique of microdilution in elisa plates with 96 wells (Broth microdilution methods) (Chart 3).

Chart 3.- Minimum concentration of *in vitro* inhibition of bacterial growth of human pathogens of extract M 349.

[0068]

Bacteria	MIC (μl of extract/μl of solution
Enterobacter aerogenes	0,188
Escherichia coli	0,188
Pseudomonas aeruginosa	0,188
Salmonella typhi	0,188
Vibrio cholerae	0,047
Staphylococcus aureus	0,188
Streptococcus pneumoniae type B	0,047

[0069] The extract percentage column was eliminated as it is explained in the MIC column.

[0070] The honey extract M349 inhibited the *in vitro* growth of all bacteria, distinguishing two groups according to the minimum concentration of inhibition (MIC) of the extract.

[0071] For bacteria: *Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus,* the obtained MIC was 0,188 μl of extract/μl of solution.

[0072] Extract M349, inhibited *in vitro* growth to a minor concentration of the bacteria *Vibrio cholerae and Streptococcus pneumoniae* type B with an obtained MIC of 0,047 μl of extract / μl of solution.

Example 7: Cellular Toxicity evaluations of extract M349

[0073] Rat spermatozoids were used (Balb-c), these cells are considered the most sensitive for this kind of evaluation. Spermatozoids were cultured in a medium completing with different concentrations of the honey extract M349 (1:10, 1:

100, 1:1000, 1:10000 v/v).

[0074] The cellular viability of the spermatozoids was evaluated at different time intervals (t=0 hr, t=1 hr, t=2 hrs, t=4 hrs), approx 100 spermatozoids were observed in each interval and the percentage of spermatozoids presenting motility, parameter for cellular viability, was obtained. (Figure 8). Figure 8 shows that besides the concentration 1:10 (high dose to determine toxicity), about 50 % of the spermatozoids are observed active after 4 hours of observation. Furthermore, after 4 hours, the number of observed living spermatozoids is equal or superior to the one observed in the middle of the control to the concentrations C4 (1:10000), C3 (1:1000) y C2 (1:100), this result is interpreted as a very low or almost non toxicity of the extract on these cells.

Claims

1. Natural composition for controlling bacterial infections in potatoes, lettuce, vegetables, cores and flowers in general, **characterised in that** it is elaborated based on extracts from organic monofloral honey flavonoids and/or phenols that act independently as a controller for various bacterial infections, said organic monofloral honey being an Ulmo honey, composed in such a way that at least 50% of the total pollen grains present in them correspond to one sole vegetal species and with the following composition:

Compound	Concentration (ug/mL)
Gallic acid	0.236 + 0,005 ug/ml
Cumaric acid	0.221 + 0,005 ug/ml
Pherulic acid	0.235 + 0,005 ug/ml
Salicylic acid	0.435 + 0,005 ug/ml
Naringenine	0.456 + 0,005 ug/ml
Kaempherol	0.174 + 0,005 ug/ml

with a PH: 4,9 to 5,5.

2. Procedure for obtaining a organic monofloral honey extract useful in controlling bacterial infections in potatoes, lettuce, vegetables, cores and flowers in general, said organic monofloral honey being an Ulmo honey and composed in such a way that at least 50% of the total pollen grains present in them correspond to one sole vegetal species **characterised in that** it comprises the following phases:

   a) weighing the quantity of honey to use;
   b) diluting said honey in distilled water acidified with HCl (pH=2);
   c) putting the solution of the prior phase in a volumetric flake and filling it up with acid water;
   d) filtering said solution and passing it through a column of cationic exchange at a constant dropping speed for the phenolic compounds to be retained in said column;
   e) cleaning said column with acid water and throwing away the remaining liquid,
   f) cleaning said column a second time with neutral distilled water and throwing away the remaining liquid;
   g) cleaning for the third time with pure methanol to elude the phenolic compounds of the column, then gathering said extract and giving it to a balloon to rotoevaporate until vacuum dryness with a temperature of 45°C, in order to eliminate the solvent and concentrate the obtained phenolic compounds
   h) re-suspending the residue in distilled water;
   i) decanting the suspension and adding diethylic ether, in order to collect an ethereal phase and extract again with ether twice;
   j) concentrating said ethereal solution in dryness in rotoevaporator; and
   k) re-suspending the residue in sterilized distilled water to subsequently filtering the extract with nitrocellulose syringe filters of 20 um and storing it at -20°C.

3. Procedure according to claim 2, **characterised in that** in phase d) the filtering process is done with hydrophobe cotton or filter paper type Watman No. 2, and the used column can be selected as cationic exchange column with resin Amberlite XAD-2, and the dropping speed can be in the range of 2±0,2ml per minute in a constant way.

4. Procedure according to claim 2, **characterised in that** alternatively it is possible to obtain ethanolic honey extracts, by collecting in said phase d), part of the diluted and filtered honey and filter it in order to store it at -20°C; in said

phase I) re-suspending the residue in ethylic alcohol of 85°, filter and storing it at -20°C; and in said phase k) re-suspending the residue in ethylic alcohol of 85°, filtering and storing it at -20°C.

5. Use of a composition elaborated on the basis of extracts from flavonoids and/or phenols from organic monofloral honeys, said monofloral honey being an Ulmo honey and composed in such a way that at least 50% of the total pollen grains present in them correspond to one sole vegetal species, **characterised in that** it serves for controlling bacterial infections in potatoes, lettuce, vegetables, cores and flowers in general.

6. Use of a composition according to claim 5, **characterised in that** it serves for controlling the growth of bacteria *Erwinia carotovora pv. Carotovora,* which causes black leg and soft rot in potato.

7. Natural composition for controlling bacterial infections comprising the composition of claim 1, **characterised in that** it is formulated based on dissolution in water that allows presenting it as a spray product (atomization) or as a bactericide solution.

8. Natural composition for controlling bacterial infections comprising the composition of claim 1, **characterised in that** said composition comprises at least 10% v/v of monofloral honey based on extracts from monofloral honey.

Patentansprüche

1. Natürliche Zusammensetzung zur Kontrolle von bakteriellen Infektionen bei Kartoffeln, Blattsalat, Gemüsen, Kerngehäusen und Blumen im Allgemeinen, **dadurch GEKENNZEICHNET, dass** diese aus Extrakten von Flavonoiden bzw. Phenolen von organischem sortenreinem Honig hergestellt wird, die unabhängig zur Kontrolle von unterschiedlichen bakteriellen Infektionen wirken, wobei es sich bei dem sortenreinen Honig um Honig der chilenischen Scheinulme (*Eucryphia cordifolia*) handelt, wobei dieser Honig eine solche Zusammensetzung hat, dass mindestens 50% der gesamten Pollenkörner einer einzigen Pflanzenart angehören:

Bestandteile	Konzentration (ug/mL)
Gallussäure	0,236 ± 0,005 ug/ml
Kumarinsäure	0,221 ± 0,005 ug/ml
Ferulasäure	0,235 ± 0,005 ug/ml
Salicylsäure	0,435 ± 0,005 ug/ml
Naringenin	0,456 ± 0,005 ug/ml
Kaempherol	0,174 ± 0,005 ug/ml

mit einem pH-Wert der zwischen 4,9 und 5,5 liegt.

2. Verfahren zur Gewinnung des Extrakts aus organischem sortenreinem Honig zur Kontrolle von bakteriellen Infektionen bei Kartoffeln, Blattsalat, Gemüsen, Kerngehäusen und Blumen im Allgemeinen, wobei es sich bei dem sortenreinen Honig um Honig der chilenischen Scheinulme *(Eucryphia cordifolia*) handelt, der sich so zusammensetzt, dass mindestens 50% der gesamten Pollenkörner einer einzigen Pflanzenart angehören, **dadurch GEKENNZEICHNET, dass** dieses Verfahren die folgende Verfahrensschritte umfasst:

a) die Honigmenge abwiegen, die verwendet werden soll;
b) den Honig in destilliertem Wasser, das mit HCl (pH=2) angesäuert wird, auflösen;
c) die im vorangegangenen Schritt gewonnene Lösung in einen Messbecher geben und mit saurem Wasser auffüllen;
d) die Lösung filtern und durch eine Kationentauschersäule geben, dies in einem konstanten Tröpfchenrhythmus, damit die phenolischen Bestandteile in der Säule zurückbleiben;
e) die Säule mit saurem Wasser säubern und den Rest der Flüssigkeit entsorgen;
f) die Säule ein zweites Mal säubern, dieses Mal mit neutralem destilliertem Wasser und den Rest der Flüssigkeit entsorgen;
g) die Säule ein drittes Mal säubern, jetzt mit reinem Methanol, um Phenolverbindungen zu eluieren, anschließend das Extrakt aufnehmen und in einen Kolben geben, um mittels Rotoevaporation bei einer Temperatur von 45°C eine Vakuum-Trockenheit zu erreichen und so das Lösungsmittel zu eliminieren und die erhaltene phe-

nolische Verbindung zu konzentrieren;
h) erneut den Rückstand in destilliertem Wasser suspendieren;
i) die Suspension dekantieren und Diethylether hinzufügen, um eine Etherphase aufzunehmen und erneut zweimal mit Ether extrahieren;
j) die Etherlösung in einem Rotoevaporator bis zur Trockenheit konzentrieren; und
k) erneut den Rückstand in sterilisiertem destillierten Wasser suspendieren, um das Extrakt anschließend mit einem Cellulosenitratfilter von 20 um zu filtern und anschliessend bei -20°C zu lagern.

3. Verfahren nach Patentanspruch 2, **dadurch GEKENNZEICHNET, dass** in der Phase d) der Filtervorgang mit hydrophober Watte oder einem Papierfilter vom Typ Watman Nr. 2 vorgenommen wird und die verwendete Säule als Kationentauschersäule mit Amberlit XAD-2 Harz eingesetzt werden kann, wobei die Tropfgeschwindigkeit einen konstanten Wert in einem Bereich von $2 \pm 0{,}2$ ml pro Minute umfasst.

4. Verfahren nach Patentanspruch 2, **dadurch GEKENNZEICHNET, dass** es alternativ möglich ist, in der Phase d) einen Teil des aufgelösten und gefilterten Honigs aufzunehmen und so ethanolische Honigextrakte zu gewinnen, wobei diese gefiltert und bei -20°C gelagert werden, wobei in der Phase i) der Rückstand erneut in Ethylalkohol von 85° suspendiert wird, gefiltert und bei -20°C gelagert wird, und in der Phase k) der Rückstand noch einmal in Ethylalkohol von 85° suspendiert, gefiltert und anschliessend bei -20°C gelagert wird.

5. Verwendung einer Zusammensetzung aus Flavonoiden- bzw. Phenolextrakten aus organischem sortenreinem Honig, wobei es sich bei dem sortenreinen Honig um Honig der chilenischen Scheinulme *(Eucryphia cordifolia*) handelt, der sich so zusammensetzt, dass mindestens 50% der gesamten Pollenkörner einer einzigen Pflanzenart angehören, **dadurch GEKENNZEICHNET, dass** diese Zusammensetzung zur Kontrolle von bakteriellen Infektionen an Kartoffeln, Blattsalat, Gemüsen, Kerngehäusen und Blumen im Allgemeinen dient.

6. Verwendung einer Zusammensetzung nach Patentanspruch 5, **dadurch GEKENNZEICHNET, dass** sie dazu dient, das Wachsen der Bakterie *Erwinia carotovora pv. Carotovora* zu kontrollieren, die bei der Kartoffel Schwarzbeinigkeit und Knollennassfäule hervorruft.

7. Natürliche Zusammensetzung zur Kontrolle von bakteriellen Infektionen, die die Zusammensetzung nach Anspruch 1 umfasst, **dadurch GEKENNZEICHNET, dass** sie in einer Wasserlösung zubereitet wird und so als Spray (Versprühen) bzw. bakterizide Lösung verwendet werden kann.

8. Natürliche Zusammensetzung zur Kontrolle der bakteriellen Infektionen, die die Zusammensetzung nach Anspruch 1 umfasst, **dadurch GEKENNZEICHNET, dass** diese Zusammensetzung mindesten 10% (v/v) sortenreinen Honig auf der Basis von Extrakten aus dem sortenreinen Honig enthält.

Revendications

1. Composition naturelle afin de contrôler les infections bactériennes dans les pommes de terre, laitues, végétaux, coeurs et fleurs en général, **caractérisée en ce qu'**elle est élaborée à partir d'extraits de flavonoïdes et/ou de phénols provenant de miel monofloral organique qui agissent indépendamment comme régulateur des infections bactériennes, ici, le miel en question provient de l'Ulmo (arbre) et est composé de telle manière qu'au moins 50% du total des grains de pollen qu'il contient corresponde à une seule espèce végétale et dont la composition est la suivante :

Composé	Concentration (ug/ml)
Acide gallique	0,236+- 0,005 ug/ml
Acide coumarique	0,221+-0,005 ug/ml
Acide férulique	0,235+-0,005 ug/ml
Acide salicylique	0,435+-0,005 ug/ml
Naringénine	0,456 +-0,005 ug/ml
Kaempherol	0,174+-0,005 ug/ml

avec un PH compris entre 4,9 et 5,5.

2. Procédure pour obtenir un extrait de miel organique monofloral qui soit utile pour contrôler les infections bactériennes dans les pommes de terre, laitues, végétaux, coeurs et fleurs en général, ici, le miel en question provient de l'Ulmo (arbre) et est composé de telle manière qu'au moins 50% du total des grains de pollen qu'il contient corresponde à une seule espèce végétale, **caractérisée en ce qu'**il suit les étapes suivantes :

a. Peser la quantité de miel à utiliser ;
b. Diluer ledit miel dans de l'eau distillée et acidifiée avec HCl (PH=2) ;
c. Verser la solution ainsi obtenue dans un flasque volumétrique et remplir avec de l'eau acide ;
d. Filtrer ladite solution en la faisant passer à travers une colonne d'échange cationique à une vitesse de dégouttement constant afin de retenir les composés phénoliques dans ladite colonne ;
e. Laver la colonne avec de l'eau acide et jeter le liquide restant ;
f. Laver une deuxième fois ladite colonne avec de l'eau neutre distillée et jeter le liquide restant ;
g. Laver une troisième fois avec de l'éthanol pur afin d'éviter les composés phénoliques de la colonne puis recueillir l'extrait et le transvaser dans un ballon pour effectuer une roto-évaporation jusque à séchage sous vide à une température de 45°C, afin d'éliminer le solvant et concentrer les composés phénoliques obtenus ;
h. Resuspendre le résidu dans de l'eau distillée ;
i. Décanter la suspension et rajouter de l'éther diéthylique afin de recueillir une phase éthérée ; extraire à nouveau deux fois avec de l'éther ;
j. Concentrer la solution éthérée ainsi obtenue à sec dans un roto-évaporateur et
k. Resuspendre le résidu dans de l'eau distillée stérilisée pour ensuite filtrer l'extrait à l'aide de filtres seringue en nitrocellulose de 20um puis stocker par -20°C.

3. Procédure conforme à la revendication 2, **caractérisée en ce qu'**en phase d) le processus de filtrage est effectué avec du coton hydrophobe ou du papier filtre de type Watman N°2 et la colonne utilisée peut être sélectionnée comme colonne d'échange cationique avec de la résine Amberlite XAD-2 et la vitesse de dégouttement peut se situer entre 2 +- 0,2 ml par minute de façon constante.

4. Procédure conforme à la revendication 2, **caractérisée en ce que,** de manière alternative, il est possible d'obtenir des extraits d'éthanol de miel en recueillant en phase d) une partie du miel dilué et filtré et en le filtrant afin de le conserver à - 20°C ; ici, dans ladite phase I) resuspendre le résidu dans de l'alcool éthylique à 85°C, filtrer et conserver à -20°C ; et ici, dans ladite phase k) resuspendre le résidu dans de l'alcool éthylique à 85°C, filtrer et conserver à -20°C.

5. L'utilisation d'une composition élaborée sur la base d'extraits de flavonoïdes et/ou de phénols provenant de miel organique monofloral où le miel en question provient de l'Ulmo (arbre) et est composé de telle manière qu'au moins 50% du total des grains de pollen qu'il contient corresponde à une seule espèce végétale, **caractérisée en ce qu'**elle est utile dans le contrôle des infections bactériennes dans les pommes de terre, laitues, végétaux, coeurs et fleurs en général.

6. Utilisation d'une composition conforme à la revendication 5, **caractérisée en ce qu'**elle sert à contrôler la croissance de la bactérie *Erwinia carotovora pv. Carotovora,* qui provoque la maladie du pied noir et pourriture molle de la pomme de terre.

7. Composition naturelle afin de contrôler les infections bactériennes, ce qui comprend la composition de la revendication 1, **caractérisée en ce que** sa formulation se base sur la dissolution dans l'eau ce qui permet de la présenter sous forme de spray (atomisation) ou de solution bactéricide.

8. Composition naturelle qui permet de contrôler les infections bactériennes, ce qui comprend la composition de la revendication 1, **caractérisée par le fait que** cette composition comprenne au moins10% v/v de miel monofloral basé sur des extraits de miel monofloral.

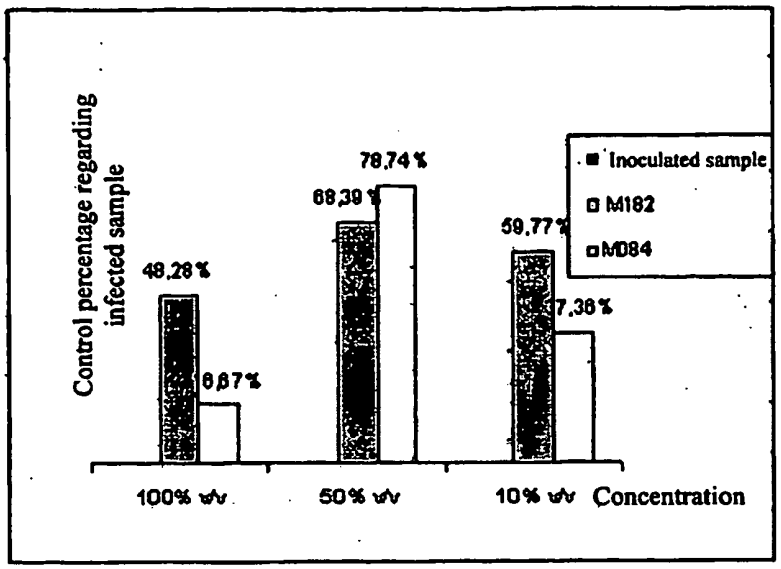

Figure 1

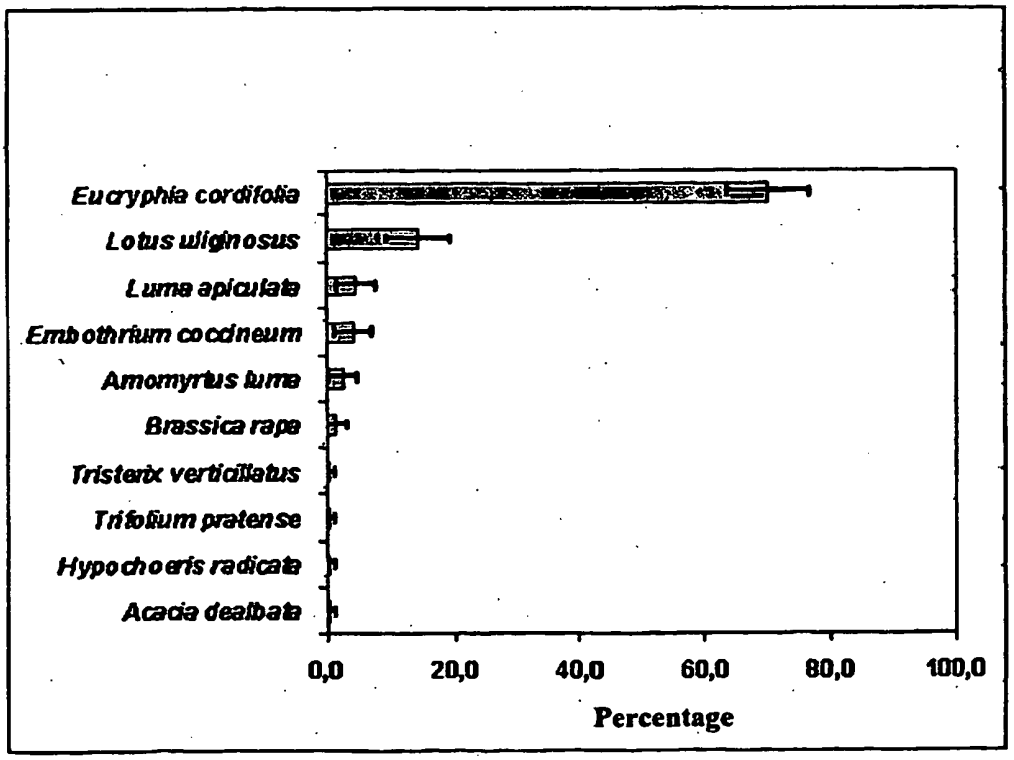

Figure 2

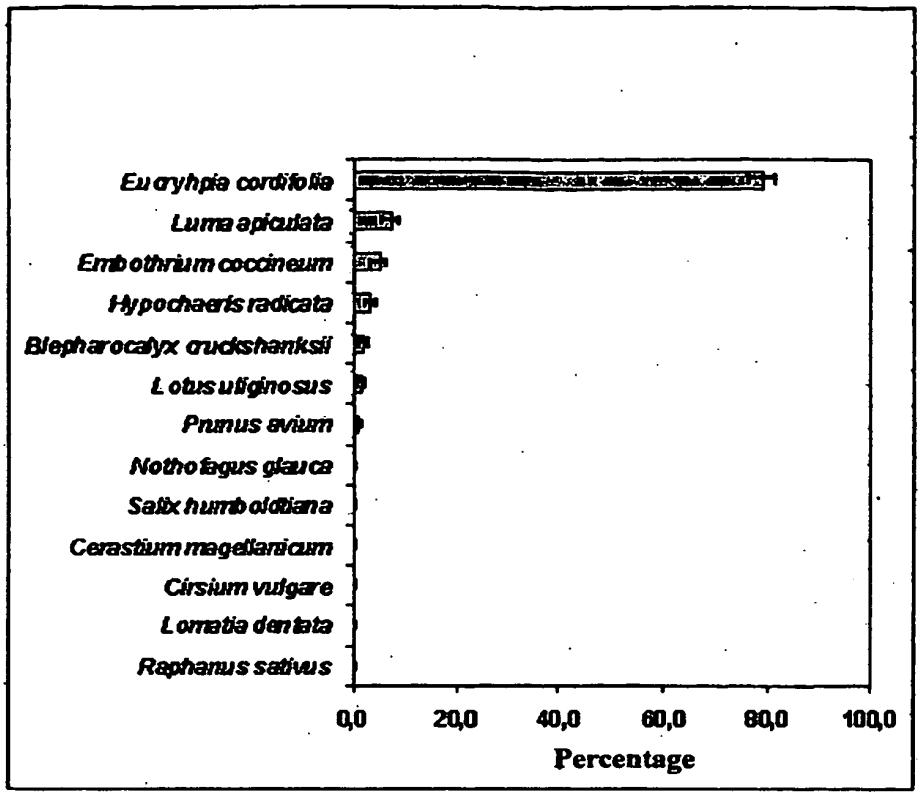

Figure 3

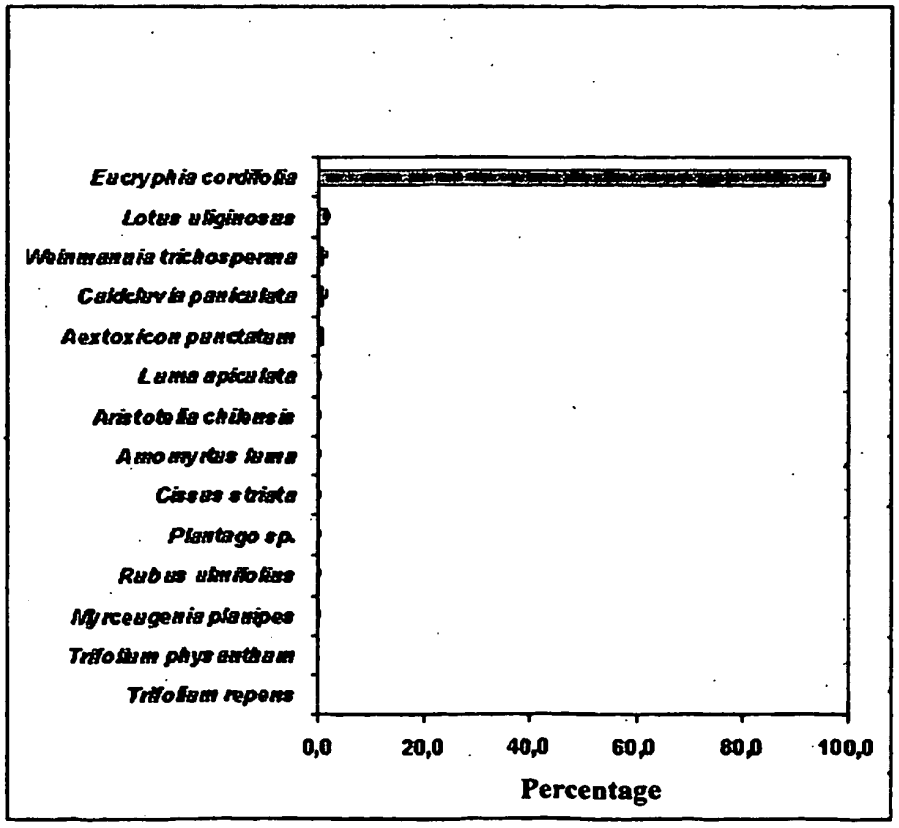

Figure 4

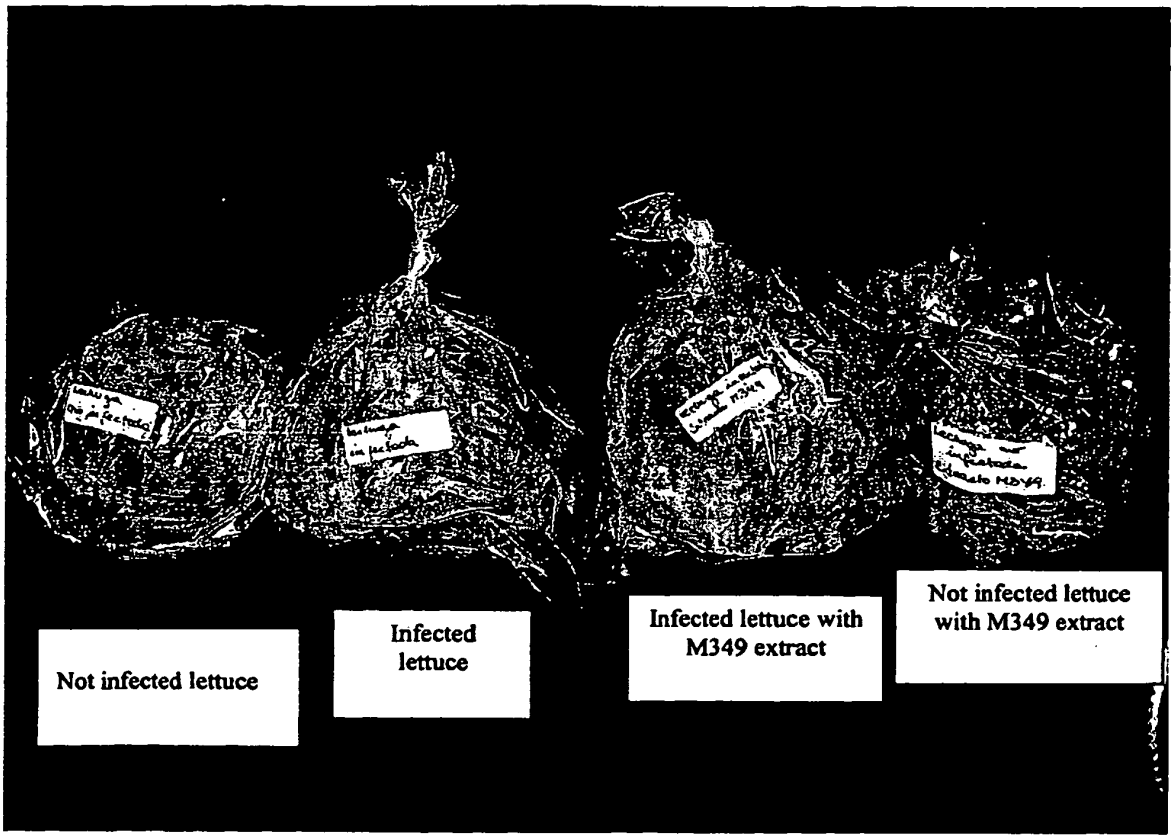
Not infected lettuce
Infected lettuce
Infected lettuce with M349 extract
Not infected lettuce with M349 extract

Figure 5

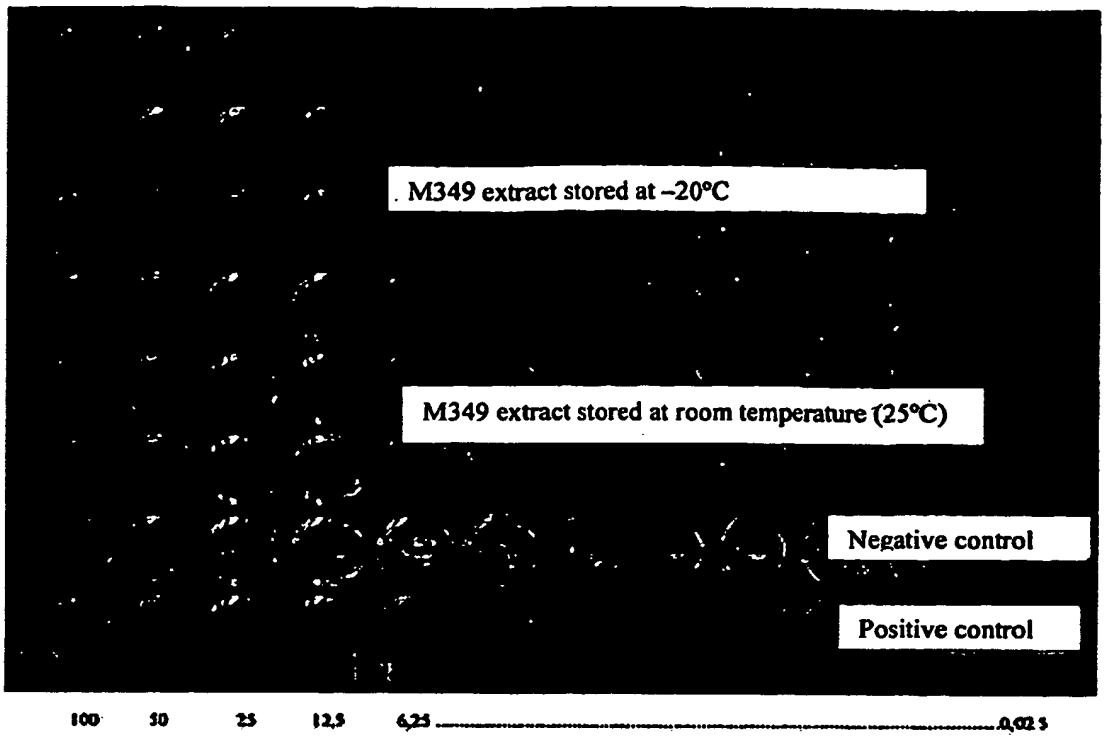

Figure 6

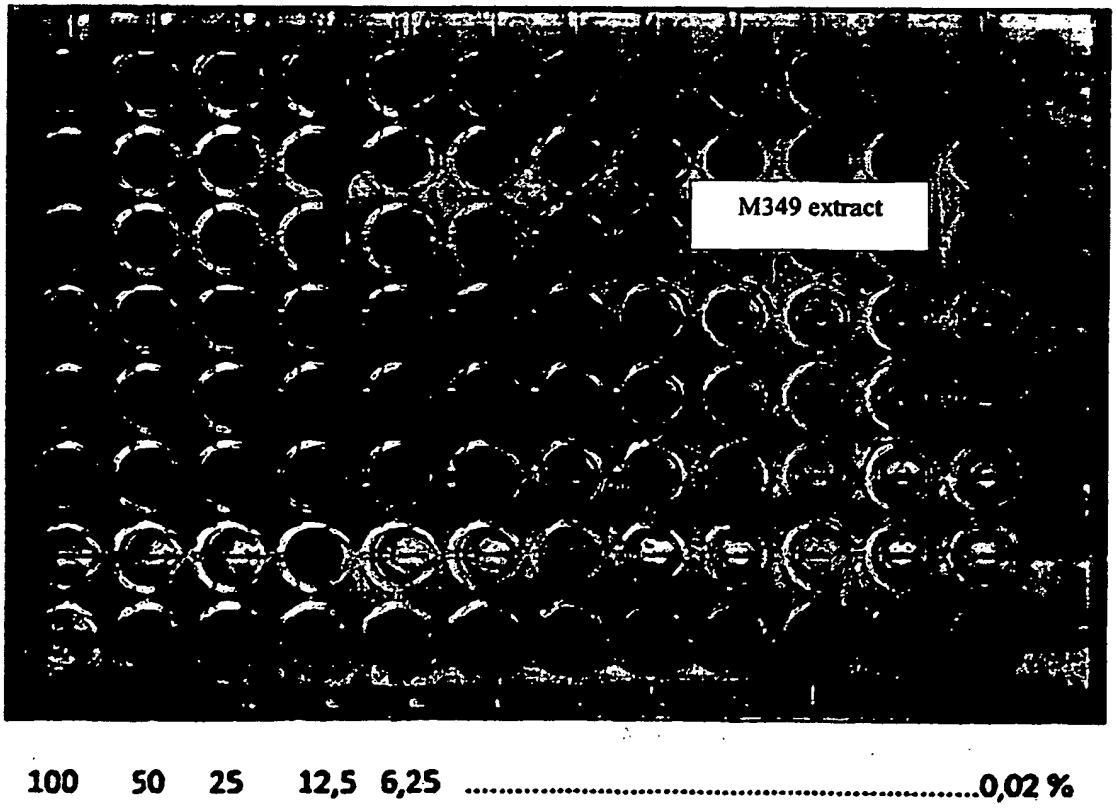

Figure 7

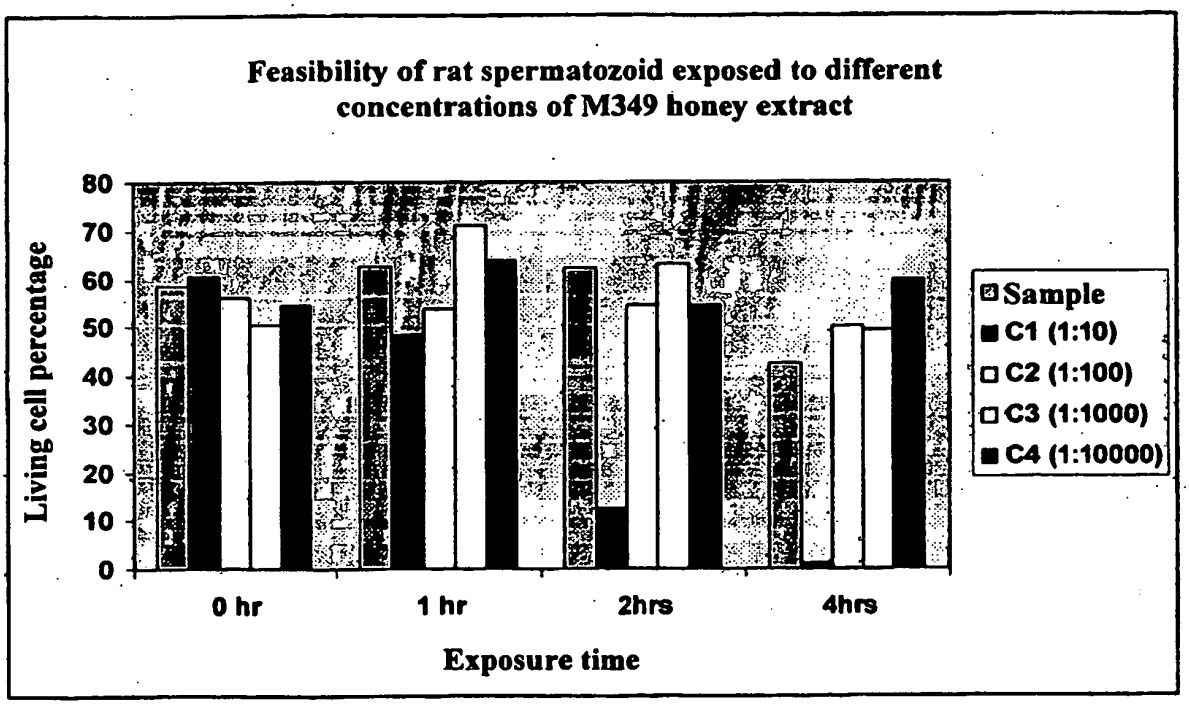
Feasibility of rat spermatozoid exposed to different concentrations of M349 honey extract
Living cell percentage
80
70
60
50
40
30
20
10
0
0 hr
1 hr
2hrs
4hrs
Exposure time
Sample
C1 (1:10)
C2 (1:100)
C3 (1:1000)
C4 (1:10000)

Figure 8

REFERENCES CITED IN THE DESCRIPTION

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