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(54) Title: ANTI-HERPES COMPOSITION AND ANTI-HERPES PHARMACEUTICAL FORMULATION

(57) Abstract: A composition comprising: extract of fenugreek (*Trigonella foenum-graecum*) 0.01 to 90%; extract of *Quercus infectoria* gall 0.1 to 90%; extract of *Pueraria mirifica* 0.1 to 90%; extract of *Hamamelis virginiana* 0.01 to 90%.



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Anti-herpes composition and anti-herpes pharmaceutical formulation

The subject-matter of the present invention is a composition comprising the following extracts: Fenugreek - *Trigonella foenum-graecum*; manjakani - *Quercus*
5 *infectoria* gall; *Pueraria mirifica*; *Hamamelis virginiana*. Also claimed is a pharmaceutical formulation comprising said composition for use in the topical treatment of herpes infections.

The composition of the invention and the pharmaceutical formulation of the invention are particularly but not exclusively suitable for treating herpes simplex 1 (HSV-1)
10 and especially herpes simplex 2 (HSV-2).

Fenugreek, *Trigonella foenum-graecum*, a plant of the Fabaceae family, contains trigonelline as a characteristic component within its seeds. The clinical efficacy of this has been demonstrated for treating diabetes, since it is capable of rapidly reducing blood sugar levels. It also has a demonstrated galactagogue action.

15 *Quercus infectoria*, a tree belonging to the Fagaceae family, is host to a parasite, of the hymenoptera order and Cynipidae family, *Cynips tinctoria*, which infects the tree. The female insect bores into the buds and lays its eggs there. This puncture leads to the formation of the galls, inside which the eggs hatch and from which the larvae emerge before becoming adult insects. The wall of the gall is very strong and
20 contains a large amount of tannins. Because of this, galls are used as astringents and haemostatics.

Pueraria mirifica is a herb with tuberous roots. These have a high phytoestrogen content and are traditionally used in traditional Thai medicine, with what are considered to be anti-aging effects.

25 *Hamamelis virginiana* is a plant of the Hamamelidaceae family. The healing

properties of a decoction of *Hamamelis* have long been known to native Americans. The tannin content of the leaves and bark mean that it can be used as a decongestant, astringent and haemostatic.

Herpes simplex 1 (HSV-1) and herpes simplex 2 (HSV-2) viruses are two members
5 of the Herpesviridae family. HSV generally infects fibroblasts or epithelial cells, causing their lysis, as well as neurons, in latent form in the latter case. Pathological manifestations are herpes labialis, typically caused by HSV-1, and genital herpes, associated with HSV-2. The first, extremely widespread form is responsible for the occurrence of characteristic febrile blisters that normally involve the skin of the face
10 (lips, nose).

The infection caused by HSV-1 can readily recur because the virus survives throughout life within the cells of the trigeminal nerve ganglion and, under specific conditions (stress, lowered immune defences etc.) can be reactivated, migrating via the nerves to the skin.

15 The second form is a genital infection, also known as herpes genitalis. Both are contracted through physical and sexual contact.

Genital herpes (HSV-2) is one of the most common sexually transmitted infections in the world, and is prevalent in women.

Genital herpes can have a significant impact on the interpersonal relationships of
20 patients, as well as considerable public health implications because of the possibility of simultaneous transmission of other sexually transmitted infections. For example it has been seen that anyone suffering from HSV-2 runs a three times greater risk of contagion from the HIV virus.

Furthermore, once infected, the virus remains in the body throughout life and it is not
25 currently possible to get rid of it; the only available therapies are aimed at alleviating

the symptoms and reducing lesion healing times

The best genital herpes treatments available to date are antiviral drugs, though these have to be administered systemically, for long periods and at a high dosage.

The antiviral drugs most frequently used at present are based on aciclovir or its
5 derivatives such as valaciclovir and famciclovir.

However, these drugs have to be administered for long periods and repeatedly, and only act on the symptoms, i.e. on the severity and extent of the herpetic manifestation.

It is not possible, with these drugs, to reduce substantially the risk, frequency or
10 severity of the manifestations of the virus. The limited efficacy of these therapies is particularly evident in the case of herpes simplex 2, whose manifestations last for about 20 days.

What is more, for treating herpes simplex 2 infections, these drugs are administered systemically, which leads to side effects that are often not insignificant.

15 Furthermore, viruses have developed increasing resistance to the drugs in current use.

Therefore, the need to have compositions that can be used for treating herpes infections and do not suffer from the above-mentioned deficiencies is strongly felt.

Another object of the invention is to provide a composition for treating herpes
20 simplex 1 and/or 2 that can be effectively applied locally, i.e. administered topically.

Another objective of the invention is to provide a composition for treating herpes simplex 1 and/or herpes simplex 2 that is both effective in treating the virus and not cytotoxic.

Another objective of the invention is to provide a composition for treating herpes
25 simplex 1 and/or herpes simplex 2 that produces its effects in a short time and

therefore requires shorter administration times.

Description of the invention

According to a first aspect of the invention a composition comprising the following extracts is provided: Fenugreek - *Trigonella foenum-graecum*; manjakani - *Quercus*
5 *infectoria* gall; *Pueraria mirifica*; *Hamamelis virginiana*.

According to another aspect of the invention, a pharmaceutical formulation is provided that comprises the above-mentioned composition for use in treating herpes infections.

According to another aspect of the invention a pharmaceutical formulation is
10 provided containing at least the composition of the invention as an active ingredient, in a quantity such as to produce a significant therapeutic effect, together with one or more pharmaceutically acceptable carriers and/or excipients.

This pharmaceutical formulation can be prepared by conventional technical methods in common practice in the pharmaceutical industry, such as those illustrated in
15 *Remington's Pharmaceutical Sciences* handbook, Mack Pub. N.Y. – latest edition.

The formulations according to the present invention contain, together with the active composition, at least one pharmaceutically acceptable carrier or excipient. These can be adjuvants with particularly useful formulations, for example solubilising agents, dispersants, suspending agents and emulsifiers.

20 In general, the formulations of the present invention are administered in a “therapeutically effective quantity”. The quantity of formulation actually administered will typically be determined by a doctor, having regard to the relevant circumstances, including the condition to be treated, the chosen route of administration, the formulation actually administered, any combination of drugs, the age-related body
25 weight, and the response of the individual patient, the severity of the patient’s

symptoms and so on. For any formulation, the therapeutically effective dose can initially be estimated either in cell culture assays or on animal models, usually mice, rats, guinea-pigs, rabbits, dogs or pigs. The animal model can also be used for determining the appropriate concentration range and route of administration. This information can then be used for determining doses and routes to be used for administration in humans. When calculating the human equivalent dose (HED) it is advisable to use the conversion table provided in the *Guidance for Industry and Review Staff* document (2002, US Food and Drug Administration, Rockville, Maryland, USA).

As stated, the formulation can also contain a pharmaceutically acceptable carrier for administration of a therapeutic agent.

An in-depth discussion of pharmaceutically acceptable carriers is available in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., N.J. 1991).

Pharmaceutically acceptable formulations in therapeutic compositions can also contain liquids such as water, saline solution, glycerol and ethanol.

Furthermore, additional substances such as wetting agents or emulsifiers, substances for buffering pH and the like, can be present in these formulations.

The pharmaceutical formulations for the invention are preferably formulations for topical use, advantageously in the form of a cream or gel, or also lipstick.

It has surprisingly been demonstrated that a composition of the invention containing the combination of the four extracts fenugreek – *Trigonella foenum-graecum*; manjakani – *Quercus infectoria* gall; *Pueraria mirifica*; *Hamamelis virginiana* has a high antiviral potency against the HSV-2 virus and, although to a slightly lesser extent, on HSV-1.

It has also been demonstrated that the composition containing the combination of

the four extracts fenugreek – *Trigonella foenum-graecum*; manjakani – *Quercus infectoria* gall; *Pueraria mirifica*; *Hamamelis virginiana* has a virucidal effect on the HSV-2 virus and, although to a slightly lesser extent, on HSV-1.

It has also been demonstrated by the applicant that the above-mentioned
5 composition produces its antiviral and virucidal effect in the absence of cytotoxicity.

The synergistic effect observed from combining the four above-mentioned extracts is surprising: whereas the four extracts taken individually and tested under the same experimental conditions lead to a reduction of around 15% in the virus titre, a combination of all four surprisingly leads to a reduction in virus titre of around 93%.

10 Furthermore, the tests carried out and reported below have demonstrated that, while the four extracts taken individually do have some cytotoxicity, the sample comprising all four extracts tested under the same experimental conditions has no cytotoxicity.

In a preferred embodiment the extracts are present as dry extract or glycolic extract.

In one embodiment the composition comprises (the percentages, where not
15 otherwise indicated, as expressed as weight/volume ratios):

- extract of fenugreek (*Trigonella foenum-graecum*) 0.01 to 90%;
- extract of *Quercus infectoria* gall 0.1 to 90%;
- extract of *Pueraria mirifica* 0.1 to 90%;
- extract of *Hamamelis virginiana* 0.01 to 90%;

20 Preferably, said composition comprises:

- dry extract or glycolic extract of fenugreek 0.01 to 30%;
- dry extract or glycolic extract of *Quercus infectoria* gall 0.1 to 30%;
- dry extract or glycolic extract of *Pueraria mirifica* 0.1 to 30%;
- dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 30%;

25 Even more preferably, said composition comprises:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.01 to 10%;
- dry extract or glycolic extract of *Quercus infectoria* gall 0.1 to 10%;
- dry extract or glycolic extract of *Pueraria mirifica* 0.1 to 10%;
- 5 - dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 10%;

In a preferred embodiment, said composition comprises:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.1 to 5%;
- dry extract or glycolic extract of *Quercus infectoria* gall 0.5 to 8%;
- 10 - dry extract or glycolic extract of *Pueraria mirifica* 0.3 to 5%;
- dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 3%;

Even more preferably, said composition comprises:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.2 to 4%;
- 15 - dry extract or glycolic extract of *Quercus infectoria* gall 1 to 5%;
- dry extract or glycolic extract of *Pueraria mirifica* 1 to 3%;
- dry extract or glycolic extract of *Hamamelis virginiana* 0.1 to 2%;

In a preferred embodiment, said composition comprises:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.5%;
- 20 - dry extract or glycolic extract of *Quercus infectoria* gall 3%;
- dry extract or glycolic extract of *Pueraria mirifica* 1.8%;
- dry extract or glycolic extract of *Hamamelis virginiana* 0.6%;

Preferably, said extracts are dry extracts.

In another embodiment, said composition comprises other active substances.

- 25 In another embodiment, said composition also comprises pharmaceutically accepted

excipients.

In a preferred embodiment the pharmaceutical formulation is in cream form.

Preferably, said cream is an oil-in-water emulsion comprising: extract of *Trigonella foenum-graecum*, extract of *Quercus infectoria* gall, *Pueraria mirifica* root extract,
5 extract of *Hamamelis virginiana* and, preferably, one or more emollients, one or more sunscreens, emulsifiers, preservatives, humectants, stabilisers, perfumes. In another embodiment, said cream also comprises one or more further active substances, preferably selected from the group comprising: *Prunus amygdalus dulcis* oil, *Simmondsia chinensis* seed oil, *Persea gratissima* oil, phenoxyethanol,
10 *Panax ginseng* root extract, *Melaleuca alternifolia* oil, extract of *Echinacea angustifolia*.

Preferably, said cream comprises: extract of *Trigonella foenum-graecum*, extract of *Quercus infectoria* gall, *Pueraria mirifica* root extract, extract of *Hamamelis virginiana*, coco-caprylate, aqueous extract of *Hamamelis virginiana* leaves, water,
15 glycerol, *Prunus amygdalus dulcis* oil, *Simmondsia chinensis* seed oil, sodium stearoyl lactylate, tocopheryl acetate, titanium dioxide, caprylic/capric glycerides, ethylhexyl methoxycinnamate, maltodextrin, arginine HCl, caprylic/capric triglycerides, *Persea gratissima* oil, phenoxyethanol, *Panax ginseng* root extract, sodium polyacrylate, xanthan gum, sodium hyaluronate, *Melaleuca alternifolia* oil,
20 extract of *Echinacea angustifolia*.

Preferably, said cream comprises (% weight/volume): extract of *Trigonella foenum-graecum* 0.5%, extract of *Quercus infectoria* gall 3%, *Pueraria mirifica* root extract 1.8%, extract of *Hamamelis virginiana* 0.6%, caprylic/capric triglycerides 1%, ethylhexyl methoxycinnamate 1.25%, *Prunus amygdalus dulcis* oil 2%, tocopheryl
25 acetate 2%, coco-caprylate 38%, *Simmondsia chinensis* seed oil 2%, sodium

stearoyl lactylate 2%, *Persea gratissima* oil 1%, aqueous extract of *Hamamelis virginiana* leaves 24%, glycerol 3%, xanthan gum 0.5%, arginine HCl 1%, *Panax ginseng* root extract 1%, extract of *Echinacea angustifolia* 0.5%, *Melaleuca alternifolia* oil 0.3%, sodium polyacrylate 0.5%, sodium hyaluronate 0.410%. Said
5 formulation also comprises water *q.s.* and preservatives.

The tests carried out have demonstrated that, surprisingly, the cream formulation described above further improves the antiviral and virucidal potency observed with the composition of the present invention comprising fenugreek – *Trigonella foenum-graecum*; manjakani – *Quercus infectoria* gall; *Pueraria mirifica*; *Hamamelis*
10 *virginiana*, showing virucidal activity, i.e. a reduction of some 99.99% with respect to the HSV-2 virus strain.

Another subject-matter of the present invention is formed by a composition comprising fenugreek - *Trigonella foenum-graecum*; manjakani - *Quercus infectoria* gall; *Pueraria mirifica*; *Hamamelis virginiana*, for use in treating HSV-1 and/or HSV-2
15 infections.

Said composition and/or said pharmaceutical formulation is a preferred aspect of the present invention for use in treating HSV-2 infections.

Another aspect of the present invention is formed by a pharmaceutical form comprising said composition and the use thereof in treating HSV-1 and/or HSV-2
20 infections.

Advantageously the pharmaceutical formulation is for topical use.

In one version the pharmaceutical formulation is in gel form.

EXAMPLE 1

In-vitro inhibition of the HSV-2 virus

25 Viral strain used: HSV-2, ATCC 734, amplified in Vero cells, an immortalised cell

line from monkey kidneys.

The Vero cell line is kept at 37°C, 5% CO₂ in Dulbecco's modified minimum essential medium (MEM) (BioWhittaker Europe) with the addition of 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin.

5 PBS (phosphate-buffered saline)

PBS is a Tris-buffered saline solution (TBS) (25 mM Tris): 8 g NaCl, 0.2 g KCl and 3 g Tris base are dissolved in 800 mL distilled H₂O. The pH is brought to 7.4 ± 0.2 by adding HCl. Distilled H₂O is added to make up to a litre. The solution is divided into aliquots and sterilised in an autoclave at 121°C for 20-30 minutes.

10 Each virus suspension was prepared and amplified on a large scale in monolayer cell cultures. After infection and multiplication of the virus, the cell debris was removed by means of double centrifugation at low speed (2550 rpm for 10 min) and the supernatant containing the virus was taken off so as to determine its virus titre. The supernatant was then subdivided into 2-mL aliquots of a known titre and stored
15 at a temperature of -80°C in a freezer.

1.8 mL serum-free MEM was added to 0.2 mL of virus suspension prepared in this manner and serial dilutions from 10⁻² to 10⁻⁹ were performed.

0.1 mL of each dilution was transferred into a 96-well plate containing the cell monolayer at confluence (>90%) after drawing off the culture medium. Each dilution
20 of the virus suspension was plated in sextuple. Twelve control wells were left untreated. After 1 hour's incubation at 37°C with rocking agitation for the time to viral adsorption, 100 µL MEM + 10% FBS was added to the inoculum.

The infections were incubated in 5% CO₂ at 37°C ± 1 for 6 days and observed with an inverted microscope to detect the formation of lysis plaques, caused by the
25 cytopathic effect (CPE) of the virus suspension. The plaques present in the wells at

the countable dilution were counted with an inverted microscope after fixation and staining of the cell monolayer with crystal violet in methanol.

The CPE results (quantitative test) of each dilution are expressed as a percentage of positive results between 100% and 0% and recorded as 0 for no CPE and from 1
5 (25% CPE) to 4 (100% CPE) depending on the degree of cell damage.

The virus titre was calculated by using the Spearman-Kärber method (estimation of ID₅₀).

Preparation of test samples

The applicant has previously tested the activity of samples containing the four
10 extracts separately and then the activity of a sample containing the four extracts together.

In the experimental test the following extracts were tested:

A- dry extract of fenugreek (*Trigonella foenum-graecum*) 0.5%

B- dry extract of *Quercus infectoria* gall 0.3%

15 C- dry extract of *Pueraria mirifica* 1.8%

E- dry extract of *Hamamelis virginiana* 0.6%

The percentages indicated (weight/volume) refer to the weight of the dry extract diluted in the volume of serum-free MEM.

The plant extracts at the above-mentioned tested concentrations were prepared
20 (w/v) by diluting them in serum-free MEM.

A fifth sample, F, was obtained by mixing the four extracts A, B, C, E at the above-mentioned concentrations, obtaining a sample F with the following composition:

F- dry extract of fenugreek (*Trigonella foenum-graecum*) 0.5% + dry extract of
Quercus infectoria gall 0.3% + dry extract of *Pueraria mirifica* 1.8% + dry extract of
25 *Hamamelis virginiana* 0.6%.

Subsequently the samples of the four extracts A, B, C, E were then diluted 1:2, still in MEM, giving the following samples:

A- dry extract of fenugreek (*Trigonella foenum-graecum*) 0.25%

B- dry extract of *Quercus infectoria* gall 0.15%

5 C- dry extract of *Pueraria mirifica* 0.8%

E- dry extract of *Hamamelis virginiana* 0.3%

F- dry extract of fenugreek (*Trigonella foenum-graecum*) 0.25% + dry extract of *Quercus infectoria* gall 0.15% + dry extract of *Pueraria mirifica* 0.8% + dry extract of *Hamamelis virginiana* 0.3%.

10 Any turbidity present, in particular for samples C and E, was eliminated by filtration through a membrane, so as to prevent said residue from being able to compromise cell viability in any way.

The test concentrations of the above-mentioned samples were obtained by dilution using serum-free MEM as a diluent, which had absolutely no influence on the
15 results, being the culture medium of the cells.

An O/W pharmaceutical preparation, called sample G, was also prepared, comprising: extract of *Trigonella foenum-graecum* 0.5%, extract of *Quercus infectoria* gall 3%, *Pueraria mirifica* root extract 1.8%, extract of *Hamamelis virginiana* 0.6%, caprylic/capric triglycerides 1%, ethylhexyl methoxycinnamate
20 1.25%, *Prunus amygdalus dulcis* oil 2%, tocopheryl acetate 2%, coco-caprylate 38%, *Simmondsia chinensis* seed oil 2%, sodium stearoyl 25 lactylate 2%, *Persea gratissima* oil 1%, aqueous extract of *Hamamelis virginiana* leaves 24%, glycerol 3%, xanthan gum 0.5%, arginine HCl 1%, *Panax ginseng* root extract 1%, extract of *Echinacea angustifolia* 0.5%, *Melaleuca alternifolia* oil 0.3%, sodium polyacrylate
25 0.5%, sodium hyaluronate 0.410%. Said formulation also comprises water *q.s.* and

preservatives.

Samples A, B, C, E, F and G are tested to check cytotoxicity and then antiviral potency and virucidal efficacy.

Cytotoxicity check

5 Tests were first conducted to check the cytotoxicity effect of the samples previously prepared.

To check any possible morphological impairment of the cells, caused by the samples referred to above, a cytotoxicity test was performed by mixing 1 mL serum-free MEM with 8 mL of each sample, A to F, at the following concentrations: 10 mg,
10 1 mg, 100 µg, 10 µg, 1 µg/mL. For each one, serial dilutions from 10^{-2} to 10^{-6} (1:10) were prepared, taking 0.2 mL of the mixture obtained and combining it with 1.8 mL serum-free MEM.

0.1 mL (100 µL) of each dilution was plated in sextuple in the monolayer cell cultures at confluence (>90%). The mixture was not added to 6 wells, which served
15 as controls for the cell line. After 1 hour at 37°C 100 µL MEM + 10% FBS was added and the cell culture was placed in an incubator with 5% CO₂ at 37°C ± 1 and observed with an inverted microscope constantly for the next 6 days, to detect any cytopathic effect (CPE), caused by the cytotoxic action of the test substance.

Check of cell sensitivity to the virus

20 Samples A-C, E, F and G, taken at the respective proven non-cytotoxic concentrations, were subjected to further tests to check cell sensitivity to the virus.

10 µg of the non-cytotoxic concentration of each of the samples tested were seeded in 48 wells containing the cell culture at confluence. The remaining 498 control wells were treated with 0.1 mL PBS.

25 After 1 hour's incubation at 37°C ± 1 the solution of each sample and PBS were

removed and the viral inoculum was performed in the 0.1-mL volume of each dilution obtained by preparing dilutions from 10^{-2} to 10^{-9} of the mixture obtained by taking 0.5 mL of virus suspension and adding to it 4.5 mL MEM + 2% FBS.

The infections were incubated in 5% CO₂ at 37°C ± 1 for 6 days and observed with
5 an inverted microscope to detect the formation of lysis plaques, caused by the cytopathic effect (CPE) of the virus suspension. The plaques present in the wells at the countable dilution were counted with an inverted microscope after fixation and staining of the cell monolayer with crystal violet in methanol.

Virucidal test

10 1 mL of virus suspension, at initial titre 10^8 , corresponding to the virus titre of the initial stock, was placed in contact with the cell culture at confluence. After a contact time of 1 hour at 20°C, said sample (1 mL) was transferred into 4.5 mL MEM + 2% FBS and inactivated on ice. Virus quantification was performed by preparing serial dilutions from 10^{-2} to 10^{-9} . For each dilution, 0.1 mL was inoculated into 4 wells in
15 96-well microplates containing the cell culture at confluence (>90%). The test mixture was not added to 4 wells, but just the virus suspension, and another 4 wells of the plate did not receive the inoculum but served as controls.

The infection of the cells was assisted by putting them at 37°C for 1 hour with rocking movement. 100 µL MEM + 10% FBS was then added to each well and the
20 cultures placed in an incubator with 5% CO₂ at 37°C + 1 for 6 days and observed microscopically.

The CPE results of each dilution are expressed as a percentage of positive results depending on the degree of cell damage.

The virus titre was calculated by using the Spearman-Kärber method (estimation of
25 ID₅₀).

$$-\text{Log}_{10} \text{ID}_{50} = -(x_0) - [(R/100) - 0.5] \times \log_{10} \text{dilution factor}$$

in which

x_0 = $\log_{10}$ of the lowest dilution with a 100% positive CPE reaction

R = summation (%) of positive cultures

- 5 The results obtained for each sample tested are shown in the following tables.

Sample A at 0.5% and 0.25% concentration

Herpes simplex virus type 2: $\text{ID}_{50} = -8.00$

The results of log reduction are summarised in the following table:

<i>Experimental conditions</i>	$-\log_{10} \text{ID}_{50}$			% REDUCTION AFTER TEST	
	<i>After contact time</i>			<i>CONTACT TIME</i>	
	<i>Initial virus titre</i>	<i>Treatment</i> <i>Conc. 0.25%</i>	<i>Treatment</i> <i>Conc. 0.5%</i>	<i>Treatment</i> <i>Conc. 0.25%</i>	<i>Treatment</i> <i>Conc. 0.5%</i>
Test temperature 20°C					
<i>Herpes simplex virus</i> type 2:					
<i>Test Product</i> <i>Extract "A"</i>	7.80*	6.50	1.89	17%	-
	7.83	6.83	1.70	13%	-
	7.90	6.85	2.00	13%	-
	7.92	6.00	1.80	24%	-

* The data express the logarithm of plaque forming units (PFU) relative to 1 mL of test viral suspension.

Table 1

The results obtained demonstrate that sample A at 0.5% concentration is cytotoxic.

The results obtained demonstrate that sample A has an inhibitory potential against the herpes simplex virus type 2 at 0.25% concentration.

At this concentration (0.25%) slight antiviral activity is detected in sample A; in particular it is seen that, after treatment with sample A, viral plaques are much smaller than those formed by the viral control (untreated cells).

This phenomenon of formation of viral plaques with a much smaller diameter than the diameter of those obtained in the control demonstrates that sample A has an antiviral inhibitory effect that takes the form of preventing the replication and cellular propagation of the virus.

Sample A at 0.25% concentration has an antiviral effect of approximately 16%.

Sample B at 3% and 1.5% concentration

<i>Experimental conditions</i>	$-\log_{10} ID_{50}$			% REDUCTION AFTER TEST	
	<i>After contact time</i>			<i>CONTACT TIME</i>	
	<i>Initial virus titre</i>	<i>Treatment Conc. 1.5%</i>	<i>Treatment Conc. 3%</i>	<i>Treatment Conc. 1.5%</i>	<i>Treatment Conc. 3%</i>
Test temperature 20°C					
<i>Herpes simplex virus type 2:</i>					
<i>Test Product</i> <i>Extract "B"</i>	7.80*	7.00	2.19	10%	-
	7.83	6.90	2.00	12%	-
	7.90	7.2	1.50	9%	-
	7.92	6.80	2.00	14%	-

* The data express the logarithm of plaque forming units (PFU) relative to 1 mL of test viral suspension.

Table 2

The results obtained demonstrate that sample B at 3% concentration is cytotoxic.

The results obtained demonstrate that sample B has an inhibitory potential against
 5 the herpes simplex virus type 2 at 1.5% concentration. At this 1.5% concentration a
 slight antiviral effect, approximately 11%, is evident.

As with sample A, it is also seen that, after treatment with sample B, viral plaques
 after treatment are smaller than those formed by the viral control (untreated cells).

Sample C at 1.8% and 0.9% concentration

<i>Experimental conditions</i>	$-\log_{10} ID_{50}$			% REDUCTION AFTER TEST	
	<i>After contact time</i>			<i>CONTACT TIME</i>	
	Test temperature				
20°C	<i>Initial virus titre</i>	<i>Treatment</i> <i>Conc. 0.9%</i>	<i>Treatment</i> <i>Conc. 1.8%</i>	<i>Treatment</i> <i>Conc. 0.9%</i>	<i>Treatment</i> <i>Conc. 1.8%</i>
<i>Herpes simplex virus type 2:</i>					
<i>Test Product</i> <i>Extract "C"</i>	7.80*	7.40	2.10	5%	-
	7.83	6.75	2.90	14%	-
	7.90	7.0	2.70	11%	-
	7.92	6.80	2.30	14%	-

10 * The data express the logarithm of plaque forming units (PFU) relative to 1 mL of test
 viral suspension.

Table 3

The results obtained from the tests performed on sample C are similar to those obtained for sample A. Sample C at 1.8% concentration is cytotoxic, but demonstrates an inhibitory potential against the herpes simplex virus type 2 at 0.9% concentration.

At the 0.9% concentration (as with sample A) slight antiviral activity is detected; in particular it is observed that, after treatment, viral plaques are much smaller than those formed by the viral control (untreated cells).

Sample C at 0.9% concentration has an inhibitory effect of approximately 11%.

Sample E at 1.8% and 0.9% concentration

Table 4

<i>Experimental conditions</i>	$-\log_{10} \text{ID}_{50}$			% REDUCTION AFTER TEST	
	<i>After contact time</i>			<i>CONTACT TIME</i>	
	Test temperature				
20°C	<i>Initial virus titre</i>	<i>Treatment Conc. 0.3%</i>	<i>Treatment Conc. 0.6%</i>	<i>Treatment Conc. 0.3%</i>	<i>Treatment Conc. 0.6%</i>
<i>Herpes simplex virus type 2:</i>					
<i>Test Product Extract "E"</i>	7.80*	3.40	2.10	15%	-
	7.83	6.75	2.90	14%	-
	7.90	7.0	2.70	15%	-
	7.92	6.80	2.30	22%	-

* The data express the logarithm of plaque forming units (PFU) relative to 1 mL of test

viral suspension.

The results obtained for sample E are similar to those obtained for sample A. The results obtained demonstrate that extract E at 0.6% concentration is cytotoxic. This sample has an inhibitory potential against HSV-2 at 0.3% concentration.

At the 0.3% concentration slight antiviral activity is detected; in particular, after treatment, the viral plaques obtained are much smaller than those formed by the viral control (untreated cells).

Sample E at 0.3% concentration has an inhibitory effect of approximately 16%.

Sample F at 1 mg and 10 mg concentration

Table 5

Experimental conditions	-log₁₀ ID₅₀			% REDUCTION AFTER TEST	
	After contact time			CONTACT TIME	
	Initial virus titre	Treatment Conc. 1 mg	Treatment Conc. 10 mg	Treatment Conc. 1 mg	Treatment Conc. 10 mg
Test temperature 20°C					
Herpes simplex virus type 2:					
Test Product <i>Sample "F"</i> (O/W emulsion)	7.90*	6.40	0.6	19%	92%
	8.00	6.00	0.5	25%	94%
	7.90	5.90	0.6	25%	92%
	7.90	5.80	0.6	27%	92%

* The data express the logarithm of plaque forming units (PFU) relative to 1 mL of test

viral suspension.

As can be seen immediately from the table shown above, surprisingly, the cytotoxicity observed when treating the cells with the four extracts mixed together (sample F) is a great deal less than that observed for the individual extracts.

5 The results obtained from sample F demonstrate excellent inhibitory capacity of the mixture against the herpes simplex virus type 2, amounting to a mean reduction of 93%, showing that the composition has a clear synergistic effect when compared with the data shown in Tables 1 to 4 for the individual components.

Furthermore, this virucidal effect is obtained with a 1-hour contact time, i.e. an
10 extremely limited contact time.

Furthermore, no cytotoxicity phenomena were detected at the concentrations tested.

The results shown demonstrate that when sample F is placed in direct contact with the virus it is possible not only to reduce replication and cellular propagation of the virus but also to inactivate the viral particles before the viral attack and replication
15 phase, giving it a virucidal effect.

Further tests conducted by the applicant have demonstrated that the above-mentioned effects – virucidal effect and low cytotoxicity – are also obtained and maintained when the concentrations of the individual extracts in the composition of the invention are varied.

20 In particular, tests performed by the applicant have demonstrated that the above-mentioned effects are obtained with a composition containing dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.2 to 4%; dry extract or glycolic extract of *Quercus infectoria* gall 1 to 5%; dry extract or glycolic extract of *Pueraria mirifica* 1 to 3%; dry extract or glycolic extract of *Hamamelis virginiana* 0.1 to 2%.

25 Further tests performed by the applicant have demonstrated that the above-

mentioned effects are obtained with a composition containing dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.1 to 5%; dry extract or glycolic extract of *Quercus infectoria* gall 0.5 to 8%; dry extract or glycolic extract of *Pueraria mirifica* 0.3 to 5%; dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 3%.

5 EXAMPLE 2

Quantitative test in suspension to assess virucidal activity against the herpes simplex type 2 virus.

The test was conducted on the sample G formulation according to the Italian standard UNI EN 14476:2007: Quantitative test in suspension to assess the
10 virucidal activity of chemical disinfectants and antiseptics used in human medicine.

3 g of formulation G was added to 7 mL serum-free MEM. Serial dilutions up to 10^{-3} (dilution factor 1:10) were prepared, taking 1 mL of the mixture obtained and adding it to 9 mL serum-free MEM.

0.1 mL of each dilution was plated in sextuple in the monolayer cell cultures at
15 confluence (>90%). The mixture was not added to 6 wells, and these served as controls for the cell line.

After 1 hour at 37°C the following was added: 100 µL MEM + 10% FBS, and the cell culture was placed in an incubator with 5% CO₂ at 37°C ± 1°C and observed with an inverted microscope constantly for the next 9 days, to detect any cytopathic effect
20 (CPE), caused by the cytotoxic action of the test substance.

To check whether the test product modifies cell sensitivity to viral infection, the following procedure was carried out: 0.1 mL of the proven non-cytotoxic test concentration of the test product was seeded into 12 wells of the microplates containing the cell culture at confluence and the other 12 wells were treated with
25 0.1 mL PBS. After 1 hour's incubation at 37°C ± 1°C the solution of the test

substance and PBS were removed and the viral inoculum was performed in the 0.1-mL volume of each dilution obtained by preparing dilutions from 10^{-2} to 10^{-9} (1:10) of the mixture obtained by taking 0.5 mL of 5 test virus suspension + 4.5 mL MEM + 2% FBS. After incubation with 5% CO₂ at 37°C ± 1°C it was observed with
5 an inverted microscope constantly for the next 6 days, to detect any CPE.

Inactivation check, test with formaldehyde

2 mL of virus suspension (herpes simplex type 1) was mixed with 8 mL PBS and 10 mL 1.4% (w/v) formaldehyde solution to check the validity of the system. Immediately after a contact time of 5 min., 15 min., 30 min. and 60 minutes, 0.2 mL
10 of this solution was mixed with 1.8 mL MEM + 2% FBS on ice. Serial dilutions from 10^{-2} to 10^{-6} (1:10) were made with PBS + 2% FBS kept on ice. For each dilution, 100 µL was distributed among 6 wells of a 24-well microplate and this was placed in an incubator at 37°C for 1 hour. At the end, 100 µL MEM + 10% FBS was added.

The cell culture was placed in an incubator with 5% CO₂ at 37°C ± 1°C for 6 days,
15 and observed with an inverted microscope to detect any CPE. The plaques present in the wells at the countable dilution were counted after fixation and staining of the cell monolayer with crystal violet solution in methanol. The CPE results of each dilution are expressed as a percentage of positive results between 100% and 0% and recorded as "0" for no CPE and from "1" (25% CPE) to "4" (100% CPE)
20 depending on the degree of cell damage. The virus titre was calculated by using the Spearman-Kärber method (estimation of ID₅₀).

Cytotoxicity of the test solution of formaldehyde

1 mL of 1.4% (w/v) formaldehyde was added to 1 mL PBS. From this dilution, serial dilutions from 10^{-2} to 10^{-4} (1:10) were prepared, taking 0.2 mL of the mixture
25 obtained + 1.8 mL serum-free MEM.

0.1 mL of each dilution was plated in sextuple in the monolayer cell cultures at confluence (>90%). The mixture was not added to 6 wells, which served as controls for the cell line. After 1 hour at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ 100 μL MEM + 10% FBS was added and the cell culture was placed in an incubator with 5% CO_2 at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and observed with an inverted microscope constantly for the next 9 days, to detect any CPE.

Virus check

The infectivity of the test virus suspension must be determined at a contact time of 0 min. and 60 min., replacing the solution of the test product by water as follows: 1 mL virus suspension; 8 mL PBS.

After vortexing, 0.5 mL was drawn off and added to 4.5 mL MEM + 2% FBS held on ice and respectively at time 0 (0') and after 60 min. virus quantification was performed by preparing serial dilutions from 10^{-2} to 10^{-9} (1:10). At each dilution 6 wells in a 24-well plate were inoculated. The infection of the cells was assisted by placing them at 37°C for 1 hour with rocking movement (adsorption time). After this period 100 μL MEM + 10% FBS was added to each well. After incubation in a temperature-controlled incubator with 5% CO_2 at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ the culture was observed with an inverted microscope constantly for the next 6 days, to detect any CPE, caused by the cytotoxic action of the test substance.

Virucidal test

The virucidal test was performed at 20°C The test mixture containing the virus suspension and the test product was prepared as follows: 1 mL virus suspension; 8 mL dilution of the test product (separately, all the dilutions of the sample product previously prepared).

Dilutions of sample to be tested 1:10.

Samples prepared: C1=1.00 g/mL, C2=0.50 g/mL, C3=0.25 g/mL.

Contact time: 60 minutes.

Test temperature: 20°C ±1°C

Experimental micro-organisms:

Herpes simplex virus type 2 (ATCC VR-734)

5 Herpes simplex virus type 1: KOS strain

Each virus suspension was prepared and amplified on a large scale in monolayer cell cultures. After infection and multiplication of the virus, the cell debris was removed by means of double centrifugation at low speed (2500 rpm for 10 min) and the supernatant, containing the virus, was taken off in order to determine its virus
10 titre.

It was subdivided into 2-mL volume aliquots of a known titre in Eppendorf tubes and stored in a freezer at a temperature of -80°C.

Vero cells from monkey kidneys were used.

Immediately after the test contact time, checked with a stopwatch, 0.5 mL of the test
15 mixture was taken after agitation and added to 4.5 mL MEM + 2% FBS, and held on ice, in order to inactivate the activity of the test solution.

Virus quantification was performed by preparing serial dilutions from 10⁻² to 10⁻⁹ (1:10). At each dilution, 6 wells were inoculated in 24-well microplates containing the cell culture at confluence (>90%). The test mixture was not added to 6 wells, but just
20 the virus suspension, and another 6 wells of the plate did not receive the inoculum but served as controls for the cell line.

The infection of the cells was assisted by putting them at 37°C for 1 hour with rocking movement. 100 µL MEM + 10% FBS was added to each well. After incubation in a temperature-controlled incubator with 5% CO₂ at 37°C ± 1°C the
25 culture was observed with an inverted microscope constantly for the next 6 days, to

detect any CPE.

The test product is considered VIRUCIDAL when, at 20°C, after the contact time used for the test, it demonstrates a reduction in viability of at least 10^4 , corresponding to a 4 log reduction (99.99%) against the test viral strain, based on the method and acceptability criteria of Italian standard UNI EN 14476:2007.

The virucidal activity test is valid when the following parameters are detected in the preliminary tests:

- the test viral suspension must have a viral concentration that will lead to at least a 4 log reduction of the initial virus titre: $ID_{50} = 10^8$ /mL;
- the difference in the ID_{50} value of the virus titre between the cell cultures treated with the test product and 5 those treated with test PBS must be $<1 \log_{10}$.

RESULTS:

METHOD TESTING

VIRUS TITRE

Herpes simplex virus type 1, KOS 10 strain

$ID_{50} = -8.16$ TEST VALID

Herpes simplex virus type 2: ATCC 734

$ID_{50} = -8.00$ TEST VALID

CHECK OF CELL SENSITIVITY TO THE VIRUS ($<1 \log_{10}$)

Herpes simplex virus type 1: KOS strain

Virus quantification on cells treated with the test product (concentration C3)

$ID_{50} = -7.66$

Virus quantification on cells treated with PBS:

$ID_{50} = -7.83$

Difference = 0.17 TEST VALID

“O/W formulation based on plant extracts”	C3	2.00	Serum-free MEN	/	/	/	/	4.33	3.83 log₁₀
Formaldehyde	0.7% (w/v)	3.50	PBS	/	5.83	5.66	5.50	4.00	
Control virus	n/a	n/a	PBS	8.00	n.d.	n.d.	n.d.	8.00	n/a
Control virus	n/a	n/a	Serum-free MEN	8.17	n.d.	n.d.	n.d.	8.00	n/a
<u>Herpes simplex virus type 2: ATCC VR 734</u>									
“O/W formulation based on plant extracts”			Serum-free MEN	/	/	/	/	3.83	4.17 log₁₀
Control virus	n/a	n/a	Serum-free MEN	8.00	n.d.	n.d.	n.d.	8.00	n/a

The results demonstrate that the O/W formulation (sample G) is VIRUCIDAL against the herpes simplex virus type 2 (ATCC VR 734), after 60 min. contact, at the 0.25 g/mL concentration, demonstrating a reduction in viability of more than 10⁴ (equal to a 99.99% reduction), when conducted according to the test methods and

requirements of Italian standard UNI EN 14476:2007 – Phase 2 / Stage 1.

Against the herpes simplex virus type 1: KOS strain, after 60 min. contact, a 3.83 log reduction in viability from the initial titre was shown, from which it can be inferred that the test formulation has inhibitory action also against herpes simplex virus type 1, though less than the results for the type 2 strain.

It should be noted that this virucidal effect is achieved by sample G without any cytotoxicity effect.

The applicant has carried out further tests, which have demonstrated that the virucidal effect is also obtained and maintained when varying the concentrations of the individual extracts in test sample G, i.e. in the pharmaceutical formulation of the invention.

In particular, tests performed by the applicant have demonstrated that the above-mentioned effects are obtained with a sample G containing dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.2 to 4%; dry extract or glycolic extract of *Quercus infectoria* gall 1 to 5%; dry extract or glycolic extract of *Pueraria mirifica* 1 to 3%; dry extract or glycolic extract of *Hamamelis virginiana* 0.1 to 2%.

Further tests performed by the applicant have demonstrated that the above-mentioned effects are obtained with a sample containing dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.1 to 5%; dry extract or glycolic extract of *Quercus infectoria* gall 0.5 to 8%; dry extract or glycolic extract of *Pueraria mirifica* 0.3 to 5%; dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 3%.

Furthermore, the tests conducted demonstrate that the high virucidal activity are realised by the test sample with extremely short contact times, of 60 min.

CLAIMS

1. Composition comprising:

- extract of fenugreek (*Trigonella foenum-graecum*) 0.01 to 90%;
- 5 - extract of *Quercus infectoria* gall 0.1 to 90%;
- extract of *Pueraria mirifica* 0.1 to 90%;
- extract of *Hamamelis virginiana* 0.01 to 90%;

2. Composition according to claim 1 and comprising:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.01
- 10 to 10%, preferably 0.2 to 4%, even more preferably 0.5%;
- dry extract or glycolic extract of *Quercus infectoria* gall 0.1 to 10%,
preferably 1 to 5%, even more preferably 3%;
- dry extract or glycolic extract of *Pueraria mirifica* 0.1 to 10%, preferably 1 to
3%, even more preferably 1.8%;
- 15 - dry extract or glycolic extract of *Hamamelis virginiana* 0.01 to 10%,
preferably 0.2 to 2%, even more preferably 0.6%.

3. Composition according to claim 1 and comprising:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*) 0.2
- 20 to 4%,
- dry extract or glycolic extract of *Quercus infectoria* gall 1 to 5%;
- dry extract or glycolic extract of *Pueraria mirifica* 1 to 3%;
- dry extract or glycolic extract of *Hamamelis virginiana* 0.2 to 2%.

4. Composition according to claim 1 and comprising:

- dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*)
- 25 approximately 0.5%;

- dry extract or glycolic extract of *Quercus infectoria* gall approximately 3%;
- dry extract or glycolic extract of *Pueraria mirifica*, approximately 1.8%;
- dry extract or glycolic extract of *Hamamelis virginiana* approximately 0.6%.

5. Composition according to claim 1 and comprising:

- 5 - 0.5% dry extract or glycolic extract of fenugreek (*Trigonella foenum-graecum*);
- 3% dry extract or glycolic extract of *Quercus infectoria* gall, approximately;
- 1.8% dry extract or glycolic extract of *Pueraria mirifica*;
- 0.6% dry extract or glycolic extract of *Hamamelis virginiana*.

10 6. Composition according to any one of the preceding claims, wherein said extracts are dry extracts.

7. Pharmaceutical formulation for topical use comprising a composition according to any one of claims 1 to 6.

8. Pharmaceutical formulation according to claim 7 comprising: extract of
15 *Trigonella foenum-graecum*, extract of *Quercus infectoria* gall, *Pueraria mirifica* root extract, extract of *Hamamelis virginiana* and, preferably, one or more emollients, one or more sunscreens, emulsifiers, preservatives, humectants, stabilisers, perfumes.

9. Pharmaceutical formulation according to claim 5, comprising one or more further
20 active substances, preferably selected from the group comprising: *Prunus amygdalus dulcis* oil, *Simmondsia chinensis* seed oil, *Persea gratissima* oil, phenoxyethanol, *Panax ginseng* root extract, *Melaleuca alternifolia* oil, extract of *Echinacea angustifolia*.

10. Formulation according to one of claims 7 to 9 that is an oil-in-water cream.

25 11. Formulation according to one of claims 7 to 9, comprising: extract of *Trigonella*

foenum-graecum 0.5%, extract of *Quercus infectoria* gall 3%, *Pueraria mirifica* root extract 1.8%, extract of *Hamamelis virginiana* 0.6%, caprylic/capric triglycerides 1%, ethylhexyl methoxycinnamate 1.25%, *Prunus amygdalus dulcis* oil 2%, tocopheryl acetate 2%, coco-caprylate 38%, *Simmondsia chinensis* seed oil 2%, sodium stearyl lactylate 2%, *Persea gratissima* oil 1%, aqueous extract of *Hamamelis virginiana* leaves 24%, glycerol 3%, xanthan gum 0.5%, arginine HCl 1%, *Panax ginseng* root extract 1%, extract of *Echinacea angustifolia* 0.5%, *Melaleuca alternifolia* oil 0.3%, sodium polyacrylate 0.5%, sodium hyaluronate 0.410%, water and preservatives.

- 10 12. Composition according to any one of claims 1 to 6 for use in treating herpes simplex type 1 or type 2 infections.
13. Formulation according to any one of claims 7 to 9 for use in treating herpes simplex type 1 or type 2 infections.
14. Lipstick comprising the composition according to any one of claims 1 to 6.
- 15 15. Lipstick comprising the composition according to any one of claims 7 to 9.
16. Gel for topical use comprising the composition according to any one of claims 1 to 6.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/054262

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K36/185 A61K36/28 A61K36/48 A61K36/488 A61K36/49
A61K36/54 A61K36/61 A61K36/736 A61P31/22

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 2014/075676 A1 (BIOTEK HOLDING APS V [DK]) 22 May 2014 (2014-05-22) page 1, paragraph 1; claims 1-11 -----	1,2,7,8, 10,12-16
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/054262

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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International application No

PCT/IB2016/054262

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2016/054262

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