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(54) **Title**: COMPOSITIONS FOR THE TREATMENT OF NEUROPATHIC PAIN

(57) **Abstract**: The present invention relates to the field of compositions for the treatment of neuropathic pain. More specifically, the present invention relates to compositions comprising compounds obtained from plant extracts for the treatment of neuropathic pain caused by chemotherapeutic drugs.

COMPOSITIONS FOR THE TREATMENT OF NEUROPATHIC PAIN

DESCRIPTION

The present invention relates to the field of compositions for the treatment of neuropathic pain. More specifically, the present invention relates to compositions comprising compounds obtained from plant extracts for the treatment of neuropathic pain caused by chemotherapeutic drugs.

PRIOR ART

Pain has been defined by the International Association for the Study of Pain (IASP) as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*". Within this definition, a particular typology of pain linked to neurological anomalies and referred to as neuropathic pain is gaining increasingly in significance as a result of a substantial and growing prevalence on a global level and has been recently redefined by the IASP Special Interest Group on Neuropathic Pain (NeuPSIG) as a "*pain arising as a direct consequence of a lesion or disease affecting the somatosensory system*" (Haanpää et al. 2010).

The NeuPSIG has also proposed a new approach for grading the level of diagnostic certainty of neuropathic pain given the absence of specific "gold standard" criteria. The diagnostic process entails: a diagnostic hypothesis derived from the clinical history of the patient, the presence of somatosensory anomalies during the neurological examination and at least one confirmation test. At the end of the diagnostic process, the neuropathic pain can be graded in accordance with three levels: possible, probable and certain.

In a clinical environment, it is common for neuropathic pain to also be classified based on the etiology and the anatomical area affected, as well as on the basis of its central or peripheral origin. The differentiation is important because lesions of the central and peripheral nervous system differ in terms of underlying physiopathological mechanisms, clinical manifestations and required treatment (Haanpää et al, 2009).

At present, there are no diagnostic instruments available that make it possible to provide an unmistakable diagnosis of neuropathic pain.

The characteristic symptomology of *neuropathic pain* makes it possible to distinguish it from other types of pain commonly reported in which the nervous system is unaltered, including migraine conditions, back pain, and from other types of musculoskeletal pain.

Neuropathic pain comprises a heterogeneous group of conditions that cannot be explained by a single etiopathological mechanism or by a particular anatomical

lesion.

These disorders of the central or peripheral nervous system include various neuropathies (diabetic neuropathy, post herpetic neuropathy, inflammatory neuropathies, neuropathy caused by alcohol abuse, neuropathy associated with HIV/AIDS) and can be caused by the damaging action of various toxins (for example neurotoxins, drugs having neurotoxic action), by acute trauma (including surgery), by chronic trauma (for example repetitive motor disturbances), such as carpal tunnel syndrome, and by diseases of the central nervous system (such as ictus, multiple sclerosis, brain ischemia, Parkinson's disease, lesions of the spinal cord, and cranial trauma).

Diagnosis is not easy since the nerve, although producing continuous surges of pain, is often anatomically intact.

Neuropathic pain is present in various pathological states and is manifested with a variety of symptoms, which are grouped together on the basis of the following characteristics: the pain is perceived in the absence of a process or of a permanent and identifiable tissue lesion;

- unpleasant, abnormal or unusual sensations (dysesthesia) are present, often reported as burning or electric shock;
- brief episodes of paroxystic pain of a shooting or stabbing nature;
- the pain occurs later compared to the provoking lesion;
- the pain is perceived in a less sensitive region;
- even slight stimuli are painful (allodynia);
- there is noticeable build-up and persistent activity after the application of repeated stimuli.

It is believed that neuropathic pain affects up to 3% of the population and that approximately 1 to 5 European adults in 100 are affected by chronic pain.

The literature reports that in the United States neuropathic pain is also a potentially serious problem for the national assistance systems, with a prevalence of 1.5%.

80% of patients of advanced phase neoplasm show symptoms of neuropathic pain. In addition, antineoplastic agents represent the main category of neurotoxic drugs to have significant clinical problems, both in terms of their widespread use and in terms of the potential severity of their toxicity. In fact, the peripheral neurotoxicity of antineoplastic drugs constitutes the main cause of suspension or reduction of the treatment dose. In addition, even if the neurotoxicity is not severe enough to limit the treatment dose, its onset may severely influence the quality of life of the patients affected by tumors and may result in chronic

discomfort.

Among the antineoplastic drugs, the derivatives of platinum (cisplatin and oxaliplatin) are greatly affected by this type of neurological toxicity, which constitutes a factor limiting their use. The toxicity that limits the administration of oxaliplatin is of a neurological type. It comprises a peripheral sensory neuropathy characterized by dysesthesia and/or paresthesia of the extremities, possibly accompanied by cramps, often provoked by the cold. These symptoms have been confirmed in up to 95% of treated patients.

Chronic neuropathic pain is therefore a significant neurological problem, both in terms of its frequency and in terms of the tendency to become chronic and to assume an invalidating nature. It is also a pain that does not respond well to the most common analgesics, such as acetylsalicylic acid and paracetamol, or to the most common non-steroidal anti-inflammatory drugs.

The objective of the pharmacological treatments should be the prevention or the eradication of pain, but in reality at most only a reduction of pain to a tolerable level can be achieved with the currently available treatments.

The effect of drugs on pain is normally quantified by a VAS (visual analogic scale) or a numerical scale having 11 values which enable gradation from “no pain” to “maximum pain”. This is often supplemented by the assessment of the quality of life and of the change perceived by the patient or by the doctor.

To date, no class of drugs has been proven as universally effective for the prevention or resolution of the pain in patients suffering from neuropathic pain.

In general, “off label” drugs belonging to the following categories are used, however these have significant side effects in the long term:

- antidepressants;
- anticonvulsants (gabapentin):
- opioids (methadone - oxycodone);
- tramadol;
- lidocaine;
- anti-inflammatory cytokine inhibitors.

Antidepressants, in particular tricyclics, have a certain positive effect on some types of neuropathic pain. Their efficacy has been demonstrated for neuropathic pain caused by diabetes and trauma, post herpetic neuralgia, and spinal cord damage. However, they are associated with significant side effects, including anomaly of the conduction of the cardiac electrical stimulus, urinary retention, drowsiness, dizziness, orthostatic hypotension, dryness of the mouth (Jensen et al. 2009).

Gabapentinoids with indications approved for neuropathic pain are not compounds

having specific action, but have a general action on neuronal hyperexcitability, a mechanism which is common to many chronic pain conditions, including fibromyalgia and complex regional pain syndrome (CRPS). Gabapentin and pregabalin are generally well tolerated, however their side effects include drowsiness, dizziness and peripheral edema.

Opioids appear to be effective in reducing the intensity of neuropathic pain caused by diabetes, post-amputation and post herpetic neuralgia as well as that caused by spinal cord damage. However, these drugs commonly cause negative effects to the detriment of cognitive ability, constipation and nausea, and their use is also limited by the risk of abuse of the drug and immune-related and hormone-related dysfunctions.

The various drugs, even when effective, achieve only a partial reduction of pain: 25-40% in 40-60% of patients contrasted with the numerous undesirable side effects caused by their continued use.

It is important to underline that there is not currently an effective treatment for preventing or reversing this painful condition. Gabapentin, pregablin and alpha-lipoic acid are almost completely devoid of any antihyperalgesic effect (Nanna, 2007; Bridges, 2001; Andrés J.D 2003, Fernihough J 2004; Jackson KC , 2006; Dworkin RH 2003, Taylor RS).

There is thus a need for a product that has analgesic function, but is devoid of significant and limiting side effects as observed with opioids or antiepileptics.

The use of hypericum extracts containing hypericin in the treatment of neuropathic pain is described in international patent application WO2009106263(A1) .

Neuropathic pain, due to its severity, chronicity, resistance to habitual therapy and severe consequences on the quality of life, therefore constitutes a significant unresolved medical shortcoming.

SUMMARY OF THE INVENTION

The present invention relates to a new mixture of compounds particularly effective for the treatment of neuropathic pain.

The efficacy of the composition of the present invention in the treatment of neuropathic pain has been assessed in various experimental models. In particular, it has been analyzed in an *in vivo* model, as described in Cavalletti et al. 2001 (Cavalletti et al. "Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat" Eur. J. Cancer 37, 2457-2463 2001), in order to assess the ability of a specific compound to reverse neuropathy induced in the rat by means of chemotherapeutic agents. These experiments have demonstrated that the efficacy of the composition in reversing neuropathic pain is enhanced by the

selection of specific components, for example derivatives of phytocomplexes, in specific proportions.

The experimental data indicates that the composition of the present invention is extremely effective in the treatment of neuropathic pain in patients suffering from neuropathic pain in general, including those who have neuropathic pain caused by treatment with chemotherapeutic drugs. In particular, it has been observed and demonstrated that the composition of the invention has the advantage of not inhibiting in any way the antitumor activity of the chemotherapeutic drug in cases of neuropathic pain caused by chemotherapeutic drugs. The present description, in the experimental section, reports *in vitro* experiments (on cell lines) and *in vivo* experiments (on mice) which have demonstrated that the combination of polyphenols (with a particular composition in flavonoids and quercetin) with total anthocyanosides in specific proportions according to the present description (therefore the composition of the invention), although increasing the efficacy in the treatment of neuropathic pain, does not interfere with the activity of the chemotherapeutic drug. This present composition therefore has the basic advantage of enabling effective treatment of neuropathic pain, but without interfering with the antitumor activity of the chemotherapeutic drug.

The present invention relates to a composition for use in the treatment of neuropathic pain, comprising:

a) polyphenols between 3 and 20% by weight of said composition

of which

a') flavonoids, quercetin excluded, account for between 1 and 9% by weight of said composition;

a'') quercetin accounts for between 0.05 and 0.6% by weight of said composition;

b) anthocyanosides account for between 0.1 and 1% by weight of said composition,

wherein the ratio between polyphenols in a) and flavonoids in a') is between 1.5 and 4.5.

The compositions according to the present invention are characterized in that they do not inhibit the antitumor activity of a chemotherapeutic drug.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Levels in % of malondialdehyde (MDA) revealed in lipid peroxidation assay. The figure shows the values of malondialdehyde, assayed on cells treated with the mixtures shown in table 1 (ABO-) compared to a control of cells treated with Fenton's reagent in the absence of compound (oxidation). The values reported, which are proportional to the extent of the lipid peroxidation reaction, are expressed in percentage in relation to the control condition and are reported as a value ± s.e.m.

* p<0.01 compared to the control; ^ p<0.01 compared to the oxidation value. Check the accuracy of this caption.

Figure 2. Levels of NBT oxidized from the superoxide anion produced from the reaction between hypoxanthine and xanthine oxidase. The values of absorbance relative to the oxidation of NBT are expressed as values of absorbance ± s.e.m. * p<0.01 compared to the control; ^ p<0.01 compared to the oxidation value.

Figure 3. Levels of superoxide anion freed in the cultures of rat cortical astrocytes following treatment with oxaliplatin 100 µM. The concentrations of superoxide anion freed in the cell cultures are expressed in µmol ± s.e.m. * p<0.01 compared to the control; ^ p<0.01 compared to the oxidation value.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a composition for use in the treatment of neuropathic pain, comprising:

a) polyphenols between 3 and 20% by weight of said composition

of which

a') flavonoids, quercetin excluded, account for between 1 and 7% by weight of said composition;

a'') quercetin accounts for between 0.05 and 0.6% by weight of said composition;

b) anthocyanosides account for between 0.1 and 1% by weight of said composition,

wherein the ratio between polyphenols in a) and flavonoids in a') is between 1.5 and 4.5.

In an embodiment of the invention, the composition as described in any part of the present description and the claims is a composition for use in the treatment of neuropathic pain.

The polyphenols are ubiquitous compounds in the plant world and have a key role in the physiology of the plant as they contribute to the following aspects :

- resistance to microorganisms and insects;
- pigmentation;
- sensory characteristics.

Fruit and vegetables require a multitude of compounds in order to preserve their integrity since they are continuously exposed to ambient stresses, such as UV rays and high temperatures. These factors stimulate the synthesis of protective compounds, such as anthocyanins; in fact, vegetables and fruits in the Mediterranean area are particularly rich in these compounds due to the specific combination of heat and light.

The term “polyphenols” includes several classes of compounds having a common

chemical structure: these are derivatives of benzene having one or more hydroxyl groups bonded to the ring. This structure allows such compounds to function actively as antioxidants since they chemically stabilize free radicals, also function as chelating agents of pro-oxidizing metals and *quenchers* of the formation of singlet oxygen. Polyphenols constitute the active ingredients of many medicinal plants and mechanisms of action responsible for their pharmacological activity, but are not yet fully known. Generally, they influence the quality, acceptability and stability of food, acting as flavorings, colorants and antioxidants.

In accordance with the present description, "total polyphenols" means all the molecules including at least one phenol function, such as simple phenols and flavonoids.

The total phenols of the composition of the present invention can be obtained from extracts of the following plants: *Hypericum perforatum, Punica granatum, Sylibum marianum, Cynara scolimus, Vitis vinifera, Camelia sinensis, green tea, Rosmarinus officinalis, Malpighia glabra, Curcuma longa, Curcuma xantorrhiza, red tea*.

Such extracts can be hydro alcoholic extracts, hydro alcoholic extracts lyophilized, dry extracts, wet extracts, granulates, or mixtures thereof.

The extracts as defined above can be prepared in accordance with any method commonly used by a person skilled in the art.

The percentage in weight of total polyphenols in the composition of the present invention can vary between 3 and 20%, preferably between 3 and 9% by weight, and for example may be approximately 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 5.7%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 10%, 10.5%, 11%, 11.5%, 12%, 12.5%, 13%, 13.5%, 14%, 14.5%, 15%, 15.5%, 16%, 16.5%, 17%, 17.5%, 18%, 18.5%, 19%, 19.5% and 20% by weight of the composition. It is clear that any other numbers other than those indicated specifically above, including decimals, also included in the range between 3 and 20 are also to be included explicitly in the present invention.

The concentration of total polyphenols in the composition can be expressed as a content of gallic acid and can be determined, for example, by means of the Folin-Ciocalteu reagent, this being a reaction that extracts the phenolic substances in ethanol 45% and reacts the extract with Folin's reagent in an alkaline environment with sodium carbonate 20%. After two hours, absorbance is read at 760 nm.

The polyphenols indicated above correspond to those indicated in a) in the composition for use in the treatment of neuropathic pain as described and claimed here.

This definition of total polyphenols also includes flavonoids and therefore also

quercetin and anthocyanosides that contain at least one phenol group. Some, but not all, anthocyanosides are therefore also included in the indicated values as total polyphenols. As a result, a measure of the total anthocyanosides that also includes anthocyanosides devoid of phenol group is also provided in the present description.

From a chemical point of view, flavonoids are diphenylpropanes separated into various classes in accordance with the degree of oxidation of the heterocyclic ring. They constitute a category of polyfunctional substances having high bioactivity, including polyphenols, that includes more than 5000 compounds.

They have biochemical properties of functional interest in the nutritional and therapeutic field.

It has been demonstrated that flavonoids have an important role in cardioprotection, since many studies report that diets rich in flavonoids reduce the risk of cardiovascular diseases.

Flavonoids also promote neuroprotection and additionally function as detoxifying agents since they increase the activity of phase II enzymes, dedicated to the elimination of metabolites which have a toxic action for the organism.

Flavonoids of the composition of the present invention can be obtained from plant extracts, as indicated above, of *Hypericum perforatum, Punica granatum, Sylibum marianum, Cynara scolymus, Vitis vinifera, Camelia sinensis, Rosmarinus officinalis.*

The percentage in weight of flavonoids, quercetin excluded, in the composition of the present invention can vary between 1 and 7% by weight, approximately 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 5.7%, 6%, 6.5%, and 7% by weight of the composition. It is clear that any other numbers other than those indicated specifically above, including decimals, also included in the range between 1 and 7 are also to be included explicitly in the present invention.

The concentration of flavonoids, quercetin excluded, in the composition can be determined for example by reacting a methanol solution containing the flavonoids to be analyzed with a solution of boric acid and oxalic acid in acetic acid and formic acid and by reading the absorbance of the solution containing the flavonoids at 425 nm.

The flavonoids indicated above correspond to those indicated in a') in the composition for use in the treatment of neuropathic pain as described and claimed here.

To provide the advantageous effects described above, the composition according to the present invention must comprise quantities of polyphenols and flavonoids such that the ratio between the polyphenols in a) and the flavonoids in a') present in the composition is between 1.5 and 4.5; for example this ratio being any ratio of

approximately 1.5; 1.6; 1.7 1.8; 1.9; 2; 2.1; 2.2; 2.3; 2.4; 2.5; 2.6; 2.6; 2.8; 2.9; 3; 3.1; 3.2; 3.3; 3.4; 3.5; 3.5; 3.6; 3.8; 3.9; 4; 4.1; 4.2; 4.3; 4.4; and 4.5. Quercetin is a natural component which is widespread in the plant word and forms part of the group of flavonoids classified as flavonols.

It is also called meletin or sofretin and is a flavonol having an antioxidant, anti-inflammatory, antiviral, immunomodulatory, anticancer and gastroprotective action. Antiallergic action and preventative action with regard to secondary complications of diabetes have also been recognized.

The Western diet generally provides a daily share of flavonols between 20-50 mg, the majority of this being constituted by glycosides of quercetin, of kaempferol and of myricetin. Of this, approximately 13.82 mg are constituted by glycosides of quercetin. It is contained in various fruits, for example apples, berries, brassicaceae, tomatoes, tea, onions, etc. and also in extracts (including hydro alcoholic extracts) of *Hypericum perforatum, Camelia sinensis, Sylibum marianum, Vaccinium myrtillus.*

The percentage in weight of quercetin in the composition of the present invention can vary from 0.05% to 0.6% by weight, for example 0.05%, 0.06%, 0.07%, 0.08%, 0.09%.0.1%, 0.11%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, 0.2%, 0.21%, 0.22%, 0.23%, 0.24%, 0.25%, 0.26%, 0.27%, 0.28%, 0.29%, 0.3%, 0.31%, 0.32%, 0.33%, 0.34%, 0.35%, 0.36%, 0.37%, 0.38%, 0.39%, 0.4%, 0.41%, 0.42%, 0.43%, 0.44%, 0.45%, 0.46%, 0.47%, 0.48%, 0.49%; 0.5%, 0.51%, 0.52%, 0.53%, 0.54%, 0.55%, 0.56%, 0.57%, 0.58%, 0.59%, and 0.6% by weight of the composition. In various embodiments, said percentage can be between 0.05% and 0.1%; between 0.05% and 0.2%; between 0.05% and 0.3%; between 0.05% and 0.4%; or between 0.05% and 0.5%,

The concentration of quercetin in the composition can be determined, for example, by means of HPLC in mobile phase and by measuring the wavelength at 370 nm.

Anthocyanosides are a class of polyaromatic, polyoxydrilated plant pigments capable of reacting with oxidants, including molecular oxygen and free radicals, thus reducing the damage that these molecules can cause to cells and to tissues.

They are found in flowers and fruits, for example in shrubs and in the autumnal leaves of some plants. The color of anthocyanosides can vary from red to blue and is dependent on the pH of the medium in which they are found and on the formation of salts with heavy metals present in these tissues.

The anthocyanosides of the composition of the present invention can be obtained from plant extracts, such as hydro alcoholic extracts, of *vitis vinifera*, *Vaccinium myrtillus*, *Hibiscus sabdariffa*.

The percentage by weight of the total anthocyanosides (also including the

anthocyanosides having phenol groups) in the composition of the present invention can vary from 0.1 to 1% by weight, for example 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1% by weight of the composition.

The concentration of the total anthocyanosides in the composition can be determined, for example, by means of a test that extracts the sample with HCl 0.1% in MeOH away from light and by subsequently reading the solution obtained at 540 nm.

The presence of the anthocyanosides, albeit not strictly necessary in order to impede the interference with the chemotherapeutic drug, has been found to be very useful, even at moderate concentrations, in improving the anti-pain effect of the composition.

For preparation of the plant extracts to be used in the composition of the present invention, conventional methods known to a person skilled in the art can be used and therefore do not require any further description.

Such extracts can by hydro alcoholic extracts, hydro alcoholic extracts lyophilized, dry extracts, wet extracts, granulates, or mixtures thereof.

Any extraction method known to a person skilled in the art can be used to prepare the extract according to the invention, for example by means of the preparation of aqueous or alcoholic extracts or of extracts obtainable by means of organic solvents. The term “aqueous solvent” indicates any solvent composed completely or in part of water. This term can therefore define water itself, hydro alcoholic solutions in any proportion, or solvents composed of water and of a compound such as propylene glycols in any proportion. The alcoholic solvents may include ethanol in particular. The final extract can be prepared for example in the form of dry, wet, lyophilized or granulated extract by the method known in the prior art. Lyophilized plant extracts can be prepared by total evaporation (sublimation) of the solvent after concentration of the hydro alcoholic extract and freezing of the extract thus obtained. Said evaporation can be carried out at temperatures below 50°C, for example below 40°C, so as not to alter the active ingredients.

In accordance with the present description, various embodiments of the composition of the invention are therefore possible, some of which will be described hereinafter.

In one embodiment, the total polyphenols are obtained from extracts of one or more of *Hypericum perforatum, Punica granatum, Sylibum marianum, Cynara scolimus, Vitis vinifera, Camelia sinensis, green tea, Rosmarinus officinalis, Malpighia glabra Curcuma longa, Curcuma xantorrhiza, red tea*, wherein the flavonoids are obtained from extracts of one or more of *Hypericum perforatum, Punica granatum, Sylibum marianum, Cynara scolimus, Vitis vinifera, Camelia sinensis, Rosmarinus officinalis*,

the quercetin is obtained from extracts of one or more of *Hypericum perforatum, Camelia sinenis, Sylibum marianum, Vaccinium myrtillus*, and the anthocyanosides are obtained from extracts of one or more of *Vitis vinifera* e/o *Vaccinium myrtillus.*

In another embodiment, the total polyphenols are obtained from extracts of one or more of *Hypericum perforatum, Vitis vinifera, Rosmarinus officinalis*, wherein the flavonoids are obtained from extracts of one or more of *Hypericum perforatum, Vitis vinifera, Rosmarinus officinalis*, the quercetin is obtained from extracts of one or more of *Hypericum perforatum*, and the anthocyanosides are obtained from extracts of one or more of *Vitis vinifera.*

In accordance with a further embodiment, the total polyphenols are obtained from extracts of one or more of *Hypericum perforatum, Rosmarinus officinalis*, wherein the flavonoids are obtained from extracts of one or more of *Hypericum perforatum, Rosmarinus officinalis*, the quercetin is obtained from extracts of *Hypericum perforatum*, and the anthocyanosides are obtained from extracts of *Vitis vinifera.*

In a further embodiment, the total polyphenols are obtained from extracts of one or more of *Hypericum perforatum, Vitis vinifera,* wherein the flavonoids are obtained from extracts of one or more of *Hypericum perforatum, Vitis vinifera*, the quercetin is obtained from extracts of *Hypericum perforatum*, and the anthocyanosides are obtained from extracts of *Vitis vinifera.*

In a further embodiment, the total polyphenols are obtained from extracts of *Hypericum perforatum* and/or *Cynara scolymus* and/or *Rosmarinus officinalis,* wherein the flavonoids are obtained from extracts of *Hypericum perforatum* and/or *Cynara scolymus*, the quercetin is obtained from extracts of *Hypericum perforatum*, and the anthocyanosides are obtained from extracts of *Vitis vinifera* and/or *Vaccinium myrtillus.*

In a further embodiment, said polyphenols are obtained from extracts of *Hypericum perforatum* and *Rosmarinus officinalis,* wherein said flavonoids are obtained from extracts of *Hypericum perforatum*, said quercetin is obtained from extracts of *Hypericum perforatum* and said anthocyanosides are obtained from extracts of *Vitis vinifera.*

Hypericum perforatum, also known as St John's wort, is a species belonging to the *Hypericum* genus and contains a large number of different classes of substances: derivatives of naphthodianthrones such as hypericin, pseudohypericin and isohypericin, and derivatives of phloroglucinol, such as hyperforin.

It also contains flavonoids, such as hyperoxide, rutin, querectin and isoquercetin, procyanidins, essential oils and xanthans.

The studies carried out by the present inventors have shown that lyophilized

extracts of *Hypericum perforatum* contain approximately 10-14 % of polyphenols expressed as % of gallic acid, in particular between 6 and 8 % of the extract of hypericum and represented by total flavonoids, and between 1.2 and 1.7 % of the extract of hypericum and represented by quercetin. The extract does not contain anthocyanosides.

In any case and in all embodiments of the present invention, the polyphenols and the flavonoids of the present invention are not obtained exclusively from extracts of *Hypericum perforatum.*

For the preparation of the extract, both the flower heads and the entire plant belonging to said species are used as starting material. The procedure for drying the plants or parts thereof are known to a person skilled in the art and do not require any further description here. In one embodiment, said extract is obtainable in liquid form using a suitable solvent or mixture of solvents selected from the group comprising ethanol, water, distilled water, esters, ethers, acetone or a hydro alcoholic mixture, for example 80, 75, 70 or 60 % of alcohol in water.

Of course, the extract can be prepared from at least one of the many varieties belonging to the *Hypericum perforatum* species. In the present description, the term "*Hypericum perforatum*" or “hypericum” must therefore be considered as indicating any of the many varieties of plant belonging to the *Hypericum perforatum* species.

The species *Vitis vinifera* comprises a very high number of various vine varieties, some of which, due to the specific coloration of the leaves, are defined as “red vines”. In a specific embodiment of the invention, the term *Vitis vinifera* is to be limited to “red vine” vine varieties. For the preparation of the extract of red vine according to the present invention, plant materials derived from the leaves in all stages of development can be used. The extract can be prepared from at least one of the many varieties belonging to the *Vitis vinifera* species which fall within the red vine definition. In the present description, the term “red vine” or “vine” is therefore to be considered as indicating any of the many varieties of plants belonging to the *Vitis vinifera* species commonly defined as “red vine”.

Rosmarinus officinalis also known as rosemary is a species belonging to the *Rosmarinus* genus and contains different classes of substances, including pinene, camphene, cineole, eucalyptus, camphor, borneol, diterpenes phenols, including carnosol and carnosic acid, hydroxycinnamic derivatives, including rosmarinic acid, flavonoids, including nepetin and triterpenoids, including oleanoic acid.

Of course, the extract can be prepared from at least one of the many varieties belonging to the *Rosmarinus officinalis* species. In the present description, the term "*Rosmarinus officinalis*" or “rosemary” is therefore to be considered as indicating

any one of the many varieties of plants belonging to the *Rosmarinus officinalis* species.

Punica granatum, commonly known as pomegranate, is a deciduous fruit-bearing shrub or small tree that grows to between five and eight meters tall.

Silybum marianum, commonly known as milk thistle, is an annual or biannual plant from the family of Asteraceae. The parts of the plant used to obtain the extracts are semi-mature.

Cynara scolimus is a plant belonging to the species *Cynara cardunculus* (var. *Scolymus)* originating from south Europe. The parts of the plant used to obtain the extracts are the basal leaves, whole or reduced into fragments.

Camelia sinensis is the species of plant whose leaves and leaf buds are used for the production of green tea. It is a plant belonging to the Camelia genus.

The blueberry plant (*Vaccinium myrtillus)* grows in Europe, North America and Asia. The parts of the plant used to obtain the extracts are the berries. The compounds present in the extracts of blueberry include tannins, glycoside flavonoids, phenol acids, pectins, triterpenes, and polyphenols, including procyanidines and anthocyanidins.

Curcuma longa is a perennial herbaceous plant originating from the ginger family, Zingiberaceae. The extract is prepared for extraction with solvents from the dried and ground root.

In one embodiment, the compositions of the present invention further comprise vitamin B1, vitamin B6 and/or vitamin B12. The vitamins of the B complex complement the action of the product given their property of regeneration of the nervous system. The combination of vitamin B1, B6 and B12 has a synergistic effect in the treatment of neuropathic pain with the other compounds of the composition of the present invention, improving allodynia, hyperalgesia and the speed of conduction of the nerve.

In particular, vitamin B1 may be present in a concentration by weight of composition between approximately 0.005% and 0.01%; vitamin B6 may be present in a concentration by weight of composition between approximately 0.005% and 0.01%.

In one embodiment, the compositions of the present also comprise royal jelly and/or brewer's yeast and/or spirulina or derivatives thereof. The presence in the composition of royal jelly has the advantage of providing essential amino acids, the addition of brewer's yeast or of vitamins derived therefrom has the advantage of providing vitamin B1, whilst the addition of spirulina has the advantage of providing vitamin B12. Such nutrients all have beneficial effects on the correct functioning of nerve supply and therefore indirectly in the treatment of neuropathic pain.

Royal jelly may be present, for example, in a percentage between 10 and 30% by weight of the composition. Brewer's yeast may be present, for example, in a percentage between 20 and 40% by weight of the composition. Spirulina may be present in a percentage by weight between 10 and 30% by weight of the composition. The use of royal jelly in these percentages produces a good share of essential and branched amino acids able to contrast the turn-over increase of the proteins and consequent losses of muscular tone caused by the elevated catabolism observed in individuals affected by tumors.

All the compositions comprising compounds a), a'), a'') and b) in the considered relative ratios described above are also included in the scope of protection of the present invention. The compositions according to the present invention are extremely effective in the treatment, also to be understood here as prophylaxis and therapy, of neuropathic pain in general. In particular, they can be used in the treatment of neuropathic pain caused by a treatment with a drug, in particular a chemotherapeutic drug (for example a treatment with oxaliplatin, vincristine, vinblastine, paclitaxel, cisplatin, taxane, epothilones, bortezomib, vinca alkaloids), by chronic diseases, trauma, exposure to toxic chemicals, a current infection, an infection past, altered function of an organ, vascular diseases, metabolic diseases, autoimmune diseases, or when the cause is unknown (idiopathic neuropathic pain).

The compositions may also be used in the treatment of neuropathic pain described as diabetic peripheral neuropathy, post herpetic neuralgia, trigeminal neuralgia, neuropathic pain of the lower back, reflex sympathetic dystrophy, phantom limb syndrome. Whereas such pains can depend on various causes, it is clear that the composition of the present invention is effective independently of the causes of such symptomatology, and independently of the fact that these pains are to be attributed to the use of chemotherapeutic drugs or to other causes.

The dosage of the compositions can vary depending on the age, sex and general conditions of the patient, and on the nature and severity of the pathology or disorder. In order to determine the dosage, it is necessary to take into account the condition to be treated, the severity of the condition to be treated, the weight and general physical condition of the specific patient, and also other medicines that the patient may be taking, as is well known to experts in the field.

The composition of the present invention can also be administered in combination or in concomitance or sequentially with other drugs, which may be chemotherapeutic drugs such as those listed above or also drugs for the treatment of one or more of the pathological conditions indicated above.

It is therefore clear that said effective quantity may be lowered or increased as

required in accordance with the responses of the treated patient and/or in accordance with the assessment made by the doctor prescribing the combination and/or the compositions of the present invention.

The composition can be administered orally.

Typically, compositions for oral use in solid form may contain, per dosage unit, a quantity of polyphenol between 20 and 50 mg, for example from 30 to 40 mg, a quantity of anthocyanosides between 1 and 4 mg, for example from 2 to 3 mg, a quantity of flavonoids from 10 and 25 mg, for example from 15 to 20 mg, and a quantity of quercetin between 0.1 and 1 mg, for example from 0.3 to 0.5 mg.

Typical dosage regimes may be from 2 to 8 dosage units per day in the above-mentioned quantities, for example administering one or two dosage units every 6-8 hours.

The term “dosage unit” in the present description means the unit formulation for a single administration, for example a tablet, capsule, etc.

Such compositions can be prepared by methods known in the art using the compounds obtained from the above-described extracts and one or more vehicles and/or diluents and/or excipients, for example mixing the single extracts directly during the preparation of the composition and adding a mixture of the compounds prepared previously to the vehicles and/or diluents and/or excipients.

The compositions can be in any formulation prepared by the methods known to a person of average skill in the art, for example solid forms, semi-solid forms, liquid forms, granules, capsules, tablets, lozenges, granulates, powders, syrups, elixirs, hard gelatins, soft gelatins, suspensions, emulsions (oil-in-water or water-in-oil), solutions, or gels.

The composition as described here, in any of the above-mentioned embodiments, can be in the form of a pharmaceutical composition, that is to say may comprise ingredients of pharmaceutical grade or may be, or may be inserted into, a food supplement or into a food for special purposes.

For the preparation of the pharmaceutical compositions, the mixture of the compounds will be formulated in suitable dosage units with one or more pharmaceutically acceptable excipients and additives. Pharmaceutical compositions in the form of tablets and capsules for oral administration can be in the form of a single dose and may contain conventional excipients, including for example binders, for example gum arabic, gelatin, sorbitol, tragacanth, or polyvinylpyrrolidone; fillers, for example lactose, sugar, corn starch, calcium phosphate, sorbitol or glycine; tableting lubricants, for example magnesium stearate, talc, polyethylene glycols or silica; disintegrants, for example potato starch; and pharmaceutically acceptable

wetting agents, for example sodium lauryl sulfate. The tablets can be coated by methods that are well known in standard pharmaceutical practice.

The compositions can be produced in the form of a food supplement, food for special purposes, or in such a form by adding to them one or more excipients and/or food ingredients.

The present description also provides a method for the treatment of neuropathic pain, comprising the administration to patients in need of effective quantities of a composition as described here.

Said neuropathic pain can be pain caused by: a treatment with a drug, a treatment with a chemotherapeutic drug, chronic diseases, trauma, exposure to toxic chemicals, a current infection, an infection past, altered function of an organ, vascular diseases, metabolic diseases, autoimmune diseases; or is described as diabetic peripheral neuropathy, post herpetic neuralgia, trigeminal neuralgia, neuropathic pain of the lower back, reflex sympathetic dystrophy, phantom limb syndrome or it is a neuropathic pain of no known cause (idiopathic neuropathic pain).

In the treatment method, the exact dose and the frequency of administration of the compositions will depend on the particular severity of the condition to be treated, and the age, weight and general physical condition of the particular patient, as is well known to those skilled in the art. Typical dosage regimes to be used in the treatment method can be from 2 to 8 dosage units per day in the above-defined quantities, for example administering one or two dosage units every 6-8 hours.

The present invention has been described here with reference to some of its embodiments. It is understood that other embodiments may also exist which are based on the same inventive concept, without departing from the scope of protection of the claims as specified hereinafter.

It is understood that when the quantity of the components indicated in the composition examples below do not add up to the total quantity indicated, the remaining components can be excipients as understood within the broader and general sense of the term as used commonly and can therefore include excipients in the strict sense, preservatives, thickening agents, binders, vehicles, etc.

As will be seen from the subsequent experimental examples and from the summary of the data obtained, the authors of the present invention have found that, for improved efficacy in the treatment of neuropathic pain in general, combined with an absence of interference of the composition with the activity of chemotherapeutic drugs in the case in which the pain is induced by chemotherapeutic drugs, it is necessary for there to be a particular ratio between the total polyphenols a) and the

flavonoids a') as defined above. The (limited) presence of anthocyanosides, in addition, increases the efficacy of the tested mixtures in many of the experiments conducted.

EXAMPLES OF COMPOSITIONS

Example 1

COMPOUNDS IN THE PRODUCT	% of the mixture of the product
Total polyphenols, such as gallic acid	6
Anthocyanosides	0.5
Total vitamins	0.05
Vitamin B1	0.0078
Vitamin B6	0.0085
Excipients	93.4873

*6% by weight of composition of total polyphenols comprises, expressed in % by weight of the composition,

total flavonoids	4
quercetin	0.1

Example 2

COMPOUNDS IN THE PRODUCT	% of the mixture of the product
Total polyphenols, such as gallic acid of which, in % of the mixture of the product flavonoids 3.9 quercetin 0.52	5.7
Total anthocyanosides	0.52
Total organic acids	0.50
Proteins	23.15
Total free amino acids	1.21
Polysaccharides of which, in % of the mixture of the product soluble dietary fiber 2.38	34.02
Insoluble dietary fiber	6.44
Sugars of which, in % of the mixture of the product monosacchrides 13.74 oligosaccharides 2.39	16.13
Fats	1.90
Total vitamins of which, in % of the mixture of the product Vitamin B1 0.0078 Vitamin B6 0.0085	0.05
Total metals	3.80

Example 3

COMPONENTS	per minimum daily portion 3480 mg (6 op)	per maximum daily portion 4640 mg (8 op)
VITAMINS		
Vitamin B_1	0.23 mg	0.3 mg
Vitamin B_6	0.25 mg	0.33 mg

Other nutritional factors		
Total polyphenols expressed as gallic acid*	165 mg	220 mg
Total anthocyanosides	15 mg	20 mg
ENERGY VALUE	12.25 Kcal	16.33 Kcal
proteins	0.515 g	
carbohydrates	1.075 g	
fats	0.06 g	

*165 mg of total polyphenols, comprising

of which total flavonoids	113 mg	151 mg
of which quercetin	5.8 mg	7.7 mg

Example 4

COMPOUNDS IN THE PRODUCT	mg per 6 capsules	mg per 8 capsules
Total polyphenols expressed as gallic acid*	210	280
Total anthocyanosides	15	20
Total organic acids	14.4	19.2
Proteins	671.0	894.6
Total free amino acids	34.9	46.6
Polysaccharides of which soluble dietary fiber 69.0 mg per 6 capsules and 92.0 mg per 8 capsules	985.9	1314.6
Insoluble dietary fiber	186.6	248.8
Sugars	467.4	623.2
of which monosaccharides	398.2	530.9
of which oligosaccharides	69.2	92.3
Fats	55.2	73.6
Total vitamins Vitamin B1 0.1 and Vitamin B6 0.2 per 6 capsules Vitamin B1 0.2 and Vitamin B6 0.3 per 8 capsules	1.4	1.8
Total metals	109.3	145.8
Loss on drying	180	243

*210 mg of total polyphenols comprising

total flavonoids	113	151
quercetin	2.8	3.7

Example 5

COMPOUNDS IN THE PRODUCT	% in the mixture of the product
Total polyphenols, such as gallic acid	6
Total anthocyanosides	0.5
Total vitamins of which % in the mixture of the product Vitamin B1 0.0078 Vitamin B6 0.0085	0.05
Brewer's yeast	20.8
Royal jelly	25.1
Rice starch	16.7

*6% by weight of composition of total polyphenols comprises, expressed in % by weight of the composition,

total flavonoids	4

quercetin	0.1

Example 6

COMPOUNDS IN THE PRODUCT	% in the mixture of the product
Total polyphenols, such as gallic acid *	6
Total anthocyanosides	0.5
Total vitamins	0.05
Brewer's yeast	20
Royal jelly	25
Rice starch	17
Water	27.25

*6% by weight of composition of total polyphenols comprises, expressed in % by weight of the composition,

total flavonoids	4
quercetin	0.2

Example 7

COMPOUNDS IN THE PRODUCT	% in the mixture of the product
Total polyphenols, such as gallic acid *	6
Anthocyanosides	0.5
Total vitamins of which % in the mixture of the product Vitamin B1 0.0078 Vitamin B6 0.0085 Vitamin B12 0.0080	0.05
Excipients	90

*6% by weight of composition of total polyphenols comprises, expressed in % by weight of the composition,

total flavonoids	4
quercetin	0.2

Example 8

COMPOUNDS IN THE PRODUCT	% in the mixture of the product
Total polyphenols, such as gallic acid *	6
Anthocyanosides	0.5
Total vitamins of which % in the mixture of the product Vitamin B1 0.0078 Vitamin B6 0.0085	0.05
Spirulina	25.1
Rice starch	16.7

*6% by weight of composition of total polyphenols comprises, expressed in % by weight of the composition,

total flavonoids	4
quercetin	0.2

EXPERIMENTAL EXAMPLES

In the experiments described hereinafter, the following compositions are

indicated with the respective codes

The values shown in the table below represent the percentage of each component relative to the analyzed mixture.

Table 1

MIXTURE CODE	PERCENTAGE BY WEIGHT OF THE COMPONENTS IN THE MIXTURE				
	TOTAL POLYPHENOLS expressed as gallic acid	TOTAL FLAVONOIDS, expressed as hyperosides	TOTAL ANTHOCYANOSIDES, expressed as cyanidin	Quercetin	Other relevant active ingredients present in the mixture
ABO -01	1.2	1	not present	not present	
ABO-02	2	1.7	not present	not present	
ABO-05	8	5	not present	0.24	
ABO-06	7.70	4.83	not present	not present	Caffeoylquinic derivatives expressed as chlorogenic acid: 10.9
ABO-07	7	-	0.05	trace	Total procyanidins expressed as cyanidin: 25
ABO-08	2	1.4	0.17 - 0.25	0.2	Total acids expressed as hydroxycitric acid: 27
ABO-09	12.25	7.76	not present	1.52	Total hypericins: 0.284, Hyperforins 0.946
ABO-10	33	-	not present	1	Caffeine: 5, Epigallocatechin gallate: 12
ABO-11	16.12	7.77	not present	not present	Carnosol: 1, Rosmarinic acid: 4
ABO-12	19.90	3-7	1.39	0.5-2	
ABO-13	6.47	1.89	not present	0.092	CURCUMIN: 3.8
ABO -14	0.4	0.006	0.09	0.005	Vitamin C: 6
ABO-15	0.4	0.006	0.09	0.005	Vitamin C: 5
ABO-17	0.018	0.001	not present	not known	Apigenin
ABO-21	35.4	1.7	25.56	0.1	

Data relating to the mixtures in the table above is presented in examples 1 and 2 (see tables 3 and 4 in particular)

The values shown in the table below represent the percentage of each component relative to the combinations presented in the present description.

Table 2

MIXTURE CODE	PERCENTAGE BY WEIGHT OF THE COMPONENTS IN THE MIXTURE			
	TOTAL POLYPHENOLS expressed as gallic acid	TOTAL FLAVONOIDS, expressed as hyperosides	TOTAL ANTHOCYANOSIDES, expressed as cyanidin	Quercetin

MIXTURE CODE	PERCENTAGE BY WEIGHT OF THE COMPONENTS IN THE MIXTURE			
	TOTAL POLYPHENOLS expressed as gallic acid	TOTAL FLAVONOIDS, expressed as hyperosides	TOTAL ANTHOCYANOSIDES, expressed as cyanidin	Quercetin
ABO-09-21/ 10 (ABO-09: 9% - ABO-21: 91%)	33.3	2.24	23.25	0.22
ABO-09-21 / 30 (ABO-09, 23% -ABO-21: 77%)	30.0	3.09	19.68	0.42
ABO-09-21 / 60 (ABO-09: 37.5% – ABO-21: 62.5%);	26.7	3.975	15.97	0.63
ABO-09-11 /10 (ABO-09: 9% - ABO-11: 91%)	15.77	7.76	0	0.13
ABO-09-11/ 30 (ABO-09: 23% - ABO-11: 77%)	15.23	7.76	0	0.34
ABO-09-11/ 60 (ABO-09: 37.5% - ABO-11: 62.5%)	14.67	7.76	0	0.57
ABO-09-10 /10 (ABO-09: 9% - ABO-10: 91%)	31.13	0.69	0	1.04
ABO-09-10/ 30 (ABO-09: 23% - ABO-10: 77%)	28.22	5.98	0	0.23
ABO-09-10/ 60 (ABO-09: 37.5% - ABO-10: 62.5%)	25.22	2.91	0	1.19
ABO-09-12 /10 (ABO-09: 3.2% - ABO-12: 96.8%)	19.65	5.08	1.34	1.25
ABO-09-12/ 30 (ABO-09: 9% - ABO-12: 91%)	19.21	5.24	1.26	1.27
ABO-09-12/ 60 (ABO-09: 16.6% - ABO-12 83.4%)	18.63	5.45	1.15	1.29
ABO-09 60 mg/kg+ABO-12 300 mg/kg* B1 source 200mg/Kg+branched 240 mg/Kg	8.38	2.45	0.52	0.58

Data relating to the mixtures in the table above are presented in example 2 (in particular see table 5 and in example 3 tables 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5, and 6)***1. ASSESSMENT OF THE EFFECT OF SOME FRACTIONS OF PLANT EXTRACTS IN AN OXIDATION MODEL INDUCED BY OXALIPLATIN***

Description of the oxidation model induced by oxaliplatin

The chemotherapeutic agent is able to produce a neurotoxic effect that expresses

itself in the form of morphological and molecular changes to the nervous tissue. Such damage is responsible for producing impairing symptoms of pain that become chronic, depending on the dose of the chemotherapeutic drug.

In neuronal cell cultures, such as cultures of glial cells, oxaliplatin induces the production of oxygen radicals. Incubation with oxaliplatin 30 μM is able to promote the oxidation of a fluorescent probe. This effect is produced immediately subsequently to the addition of the substance to the culture medium, suggesting that oxidative stress is an underlying mechanism for the cell damage induced by oxaliplatin.

Similarly, *in vivo* (rat), repeated daily administration of oxaliplatin 2.4 mg/kg^{-1} intraperitoneally (i.p.) for 21 days induces oxidative stress at both the peripheral nervous system and the central nervous system. This oxidoreductive imbalance is manifested in damage at lipid level (lipid peroxidation), protein level (protein carbonylation) and of the DNA (increase in the levels of 8-OH-dG, 8-hydroxyguanosine).

The model of neuropathy induced by oxaliplatin in the rat by means of the administration protocol described above at the same time promotes oxidative damage, which is a form of neuropathic pain characterized by hyperalgesia and allodynia.

Assessment of the antioxidant effect

The antioxidant profile of extracts and fractions rich in chemical compounds having antioxidant activity (polyphenols, flavonoids, procyanidins, anthocyanosides, catechins, derivatives of carnosic acid, vitamins, etc.) from the non-cytotoxic concentration was assessed in detail in order to isolate possible pharmacological agents which can be used to help the problem of neuropathic pain originating from treatment with chemotherapeutic drugs.

To this end, the antioxidant profile was studied both in terms of the hydroxyl radical (lipid peroxidation), the superoxide anion produced following xanthine and hypoxanthine, and the anion produced following chemotherapeutic treatment with oxaliplatin.

In the laboratory, a neuropathy induced by the chemotherapeutic agent oxaliplatin was characterized, said neuropathy inducing in animals used for experimental purposes a pain syndrome accompanied by morphological and molecular changes to the nervous tissue. These results revealed oxidative damage reproducible in cellular models of toxicity caused by oxaliplatin. In particular, in primary cultures of rat cortical astrocytes, oxaliplatin is able to induce a rise in the production of superoxide anion.

The tests were carried out in two different experiments: the first was carried out on samples of ABO-1 to ABO-17, the second with the sample ABO-21. The results are reported separately, the relative controls also being different.

1.2 MATERIALS AND METHODS

Cell cultures

The primary line of astrocytes was obtained by the method described by *McCarthy* and *De Vellis* (1980). The astrocytes were isolated from the cortex of neonatal rats (P1-P3). The cortexes, after removal of the meninges, were mechanically homogenized and soaked in trysin-EDTA (Sigma - Germany) 0.5% and DNAsi (Sigma, Germany) 1% for 30 minutes at 37°C with moderate stirring. The suspension was then filtered (100 µm filter, Millipore - Italy) and centrifuged at 1200 rpm for 10 minutes. The pellet was resuspended in a suitable medium (high-glucose DMEM (4.5 g/l), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium pyruvate 1mM, glutamine 1%, 20% fetal bovine serum (FBS; Lonza – Belgium), and plated in flasks treated previously with poly-L-lysinine (10 µg/mL of poly-L-lysine; Sigma). Every 2-3 days, the medium described was substituted with another culture medium containing 10% fetal bovine serum. Once confluence was reached, the microglia were eliminated with a shaker at approximately 200 rpm for 1 hour followed by a greater speed overnight in order to separate the oligodendrocytes. The cells remaining adhered to the flask produced a 90% pure astrocyte culture. These were then incubated at 37°C in humid atmosphere containing 5% CO_2 for 21 days before the experiment.

Lipid peroxidation

$FeCl_3$ and ascorbic acid produce a hydroxyl radical in accordance with Fenton's reaction. This radical is able to interact with the lipids present in the cerebral tissue, generating malonyl dialdehyde. To this end, rat cerebral material (male Sprague-Dawley rats, Harlan, Italy) was homogenized in PBS so as to obtain a final concentration of 10% w/v. The compounds under examination at a concentration of 50 µg/mL were added to 100 µL of the homogenate of nervous tissue and were incubated for 30 minutes at 37 C° together with $FeCl_3$ (20 µM) and ascorbic acid (100 µM). At the end of the incubation, 4 mL of thiobarbituric acid (Sigma-Aldrich, Germany) 36 Mm were added (dissolved in a solution of 10 % acetic acid and therefore brought to pH 4.0 with NaOH). The samples were then boiled for 1 hour and the reaction was blocked by placing the tubes in ice. After centrifugation at 1600xg at 4°C for 10 minutes, the levels of malonyl dialdehyde in the supernatant were measured by means of spectrophotometry (550 nm).

Nitroblue tetrazolium (NBT) oxidation test

The experiments were conducted in accordance with the method described by *Ciuffi et al., 1998*. The assay includes the production of superoxide anion by means of the reaction between hypoxanthine (600 mM) and xanthine oxidase (10 mU/ml). The antioxidant profile of the compounds was assessed in the test, monitoring the kinetics of oxidation of nitroblue tetrazolium (NBT, 10 mM) in the presence of a concentration of 50 µg/mL of single extracts. The antioxidant activity was measured by means of spectrophotometry at a wavelength of 560 nm. The values reported were recorded after 60 minutes of reaction and are expressed as absorbance units (AU).

1.3 RESULTS

Lipid peroxidation

The reaction between $FeCl_3$ and ascorbic acid, specifically for generation of hydroxyl radical, significantly increases the levels of lipid oxidation, raising the base value of malonyldialdehyde from 23.40 ± 2.20 µmol/mg protein (100%) to 44.10 ± 2.5 µmol/mg protein (188.50± 10.89%), (figure 1). The analyses of the antioxidant profile of the extracted vegetables has revealed an optimum efficacy of the extracts enriched in anthocyanosides **ABO-12** (27.84 ± 0.99%), catechins **ABO-10** (26.71 ± 0.87%) and derivatives of carnosic acid **ABO-11** (26.12 ±1.84%). These substances not only fully prevent the oxidation caused by Fenton's reaction, but are quite able to reduce the base levels below 100%. The extracts ABO-13 (56.27 ± 1.61%), ABO-05 (88.26 ± 0.04%) and ABO-07 (99.40 ± 1.34%) also have pronounced antioxidant properties, revealing levels of malonyldialdehyde at control levels. The extract rich in polyphenols, in particular flavonoids and querectin, **ABO-09** significantly hinders the generation of the product of oxidation by approximately 60% (124.91±7.05%). The extract ABO-08 limits the generation of malonyldialdehyde, although to a lesser extent (149.27 ± 4.96%). Moderate antioxidant properties (reduction of approximately 20 %) were also revealed for the extract of ABO-01 (160.01 ± 1.12%), for the extract ABO-06 (158.87±1.36%) and for ABO-02 (160.01±1.12%). No antioxidant activity was recorded for the extracts of ABO-03, ABO-14, ABO-17 and for ABO-15, which are the only extracts not to be statistically significant (figure 1).

Nitroblue tetrazolium (NBT) oxidation test

The base level of oxidation of nitroblue tetrazolium is 0.15 ± 0.02 AU. Following the reaction between xanthine and hypoxanthine, thanks to which superoxide anion is produced, the oxidation value of NBT is significantly increased, 5.35 ± 0.56 AU. (figure 2).

The assessment of the antioxidant profile of the different extracts (figure 2) made it possible to observe pronounced antioxidant properties for the extract **ABO-12** (1.62

± 0.09 AU), for the extract **ABO-10** (0.82 ± 0.04 AU) and for the extract **ABO-11** (1.70 ± 0.07 AU), which considerably reduce the maximum oxidation of nitroblue tetrazolium, bringing it back almost to the control levels. A significant antioxidant effect was also recorded for the extract ABO-05 (1.93 ± 0.17 AU), ABO-13 (3.17 ± 0.03 AU) and **ABO-09** (1.81 ± 0.03 AU). Such an antioxidant effect reflects the values recorded in the lipid peroxidation experiments. The extract ABO-09 is more effective in limiting the production of superoxide anion compared to the hydroxyl radical (figure 2 and figure 3). The extracts ABO-06, ABO-08 and ABO-07 (2.20 ± 0.17 AU) also significantly reduce the levels of superoxide anion freed by the reaction between hypoxanthine and xanthine oxidase (respectively 2.34 ± 0.13 AU and 2.11 ± 0.03 AU). Such data, if compared with that collated from the lipid peroxidation experiments, indicates that both ABO-06 and ABO-08 are able to selectively reduce the levels of superoxide anion compared to the hydroxyl radical. An antioxidant effect has also been observed for ABO-14 (4.07 ± 0.1 AU).

No significant antioxidant effect was revealed for the extract of ABO-01, ABO-02, ABO-03, ABO-17. In the second batch of samples, the most powerful and significant was ABO-21.

Oxidative damage caused by oxaliplatin. Measurement of the superoxide anion in cultures of rat cortical astrocytes by means of cytochrome C.

In the astrocyte culture, oxaliplatin 100 μM induces, after 4 hours of incubation, oxidative stress measured as a rise in the levels of superoxide anion from 154.9 ±13.1 μmol O_2^-, base value, to 380.1 ± 11.4 μmol O_2^-. Even in this complex system, the antioxidant capabilities of the extracts under examination were comparable with those outlined in the NBT biochemical test, this also being relative to the measurement of the superoxide anion (figure 3). The extract **ABO-12** (145.4 ± 11.4 μmol of O_2^-), the extract **ABO-10** (110.3 ± 17.8 μmol of O_2^-), and the extract **ABO-11** (148.8 ± 22.9 μmol of O_2^-) are able to prevent oxidation, maintaining the levels of superoxide anion at the control value (figure 3). A significant antioxidant effect was also recorded for ABO-07 (203.6 ± 22.4 μmol of O_2^-), ABO-05 (198.5 ± 25.6 μmol of O_2^-), ABO-08 (201.7 ± 33.7 μmol of O_2^-), ABO-06 (216.8 ± 24.2 μmol of O_2^-), ABO-09 (184.3 ± 15.7 μmol of O_2^-) and ABO-13 (254.9 ± 24.6 μmol of O_2). The extracts ABO-01, ABO-02, ABO-03, ABO-15, ABO-14 and ABO-17 were not able to reduce the levels of superoxide anion produced following treatment of the cultures with oxaliplatin. In the second batch of samples, the most powerful and significant was ABO-21.

Oxidative damage caused by oxaliplatin. Measurement of the superoxide anion in cultures of rat cortical astrocytes by means of cytochrome C.

The method makes it possible to reveal the extent of the oxidative damage induced by the chemotherapeutic agent oxaliplatin by means of the measurement of the superoxide anion produced in primary cultures of rat cortical astrocytes. The cells were plated in 6-well multiwells ($5 \cdot 10^5$/well). Once confluence was reached, the cells were starved for at least 12 hours. To produce the superoxide anion, the cultures were then incubated for 4 hours at 37°C with oxaliplatin 100 µM (in a medium without phenol red containing 0.1% FBS) and cytochrome C (Sigma - Germany) (1mg/mL), in the presence of the extracts of plant origin to be tested (50 µg/mL). At the end of incubation, the supernatant θ was centrifuged. The amount of superoxide anion present in the solution (Tyrian purple) was measured by means of spectrophotometry at 550 nm. The production of superoxide anion θ was expressed as µmol/h/well using a molar extinction coefficient of $2.1 \cdot 10^4\ M^{-1}cm^{-1}$.

1.4 STATISTICAL ANALYSIS

All the experimental results are expressed as mean ± S.E.M. A one-way analysis of variance (ANOVA) followed by a Bonferroni test was conducted in order to verify the significance between two media. The analyses of the variance and the Bonferroni test were carried out using the statistical program OriginPro 8.1. Values of $P < 0.05$ or $P<0.01$ were considered significant.

2 EFFECT ON HYPERALGESIA BY OXALIPLATIN

Experiments of induction of neuropathic pain induced by chemotherapeutic agent were conducted (Cavalletti et al, 2001).

Oxaliplatin in the rat at a dose of 2.4 mg/kg^{-1} i.p. induces, on day 21, a neuropathy characterized by hyperalgesia and allodynia in accordance with the information provided by *Cavaletti et al*. The post-mortem examination of the spinal cord of the rat treated with oxaliplatin reveals a component of oxidative damage that is also expressed at lipid level.

2.1 *MATERIALS AND METHODS*

Induction of hyperalgesia with oxaliplatin in the rat

The reduction of the pain threshold is induced by the administration of oxaliplatin 2.4 mg/kg i.p., previously dissolved in 5% glucosate solution for 5 consecutive days for a total of 2 weeks (10 administrations). The total dose of injected oxaliplatin is then equal to 24 mg/kg i.p. Only the glucosate solution was administrated to the controls.

Paw-pressure test

The test was carried out on rats. The equipment used was an analgesimeter which exerts a force, expressed in grams, which is applied at constant velocity (32 g/s) via a conical punch to the upper surface of the rear paw of the animal. The magnitude of the force is displayed continuously by an indicator that moves along a linear

scale. The animal was held closed in the palm of the hand and the nociceptive threshold was expressed as the force at which the animal responded by retracting the paw or stiffening its body or squeaking. Even in this case, in order to reveal variations induced by the drug, the nociceptive threshold of the animals was assessed both before and after the treatment. When the force reached the value of 240 g, the animal was killed, even if it had not yet responded to the stimulus. *Leighton et al., Br. J.Pharmacol., 93, 553-560. 1988.*

2.2 RESULTS

Table 3 shows the results concerning the effect of a single administration of the extracts of: ABO-10. ABO-07, ABO-11 and ABO-12 (30-300 mg kg^{-1}p.o.) with respect to hyperalgesia induced by repeated treatment with the chemotherapeutic agent oxaliplatin assessed in the rat in the presence of a pressure stimulus (*paw-pressure test*). All the assayed extracts were devoid of efficacy at does of 30 and 60 mg kg^{-1}p.o. For the extracts of ABO-12, even the dose of 100 mg kg^{-1}p.o.was ineffective. By contrast, the dose of 300 mg kg^{-1}p.o. for all the assayed extracts was able to reduce the lowering of the pain threshold induced by oxaliplatin. Such activity is obtained from 15 min. after administration, reaches the maximum effect after 30 min, and then declines until disappearing around 120 min.

Only the fraction ABO-09 demonstrated significant activity even at doses of 30 and 60 mg/kg from 15 minutes, thus constituting the most powerful extract.

Table 3

Effect of some extracts on hyperalgesia induced in the rat by oxaliplatin (*Paw-pressure test*)

	Paw-pressure test (g)				
Treatment mg/kg ,p,o,	**Before treatment**	**After treatment**			
CMC	*62.7 ± 2.8*	*58.1 ± 3.3*	*62.7 ± 4.4*	*61.2 ± 3.8*	*63.9 ± 4.6*
OXALIPLATIN	*29.5 ± 2.7*	*33.4± 3.4*	*30.9 ± 3.6*	*32.3 ± 3.5*	*29.8 ± 3.0*
OXA + ABO-10 30 mg/kg	*32.4± 2.6*	*36.8 ± 3.5*	*38.2 ± 4.2*	*34.5 ± 4.1*	*30.7 ± 3.5*
OXA + ABO-10 60 mg/kg	*31.2 ± 2.5*	*37.2 ± 3.4*	*41.1 ± 3.7*	*36.2 ± 4.1*	*37.5 ± 3.9*
OXA + ABO-10 100 mg/kg	*33.4 ± 2.8*	***45.8 ± 3.1****	***46.2 ± 3.3****	***41.8 ± 3.0^***	*35.8 ± 2.5*
OXA + ABO-10 300 mg/kg	*30.9 ± 3.1*	***44.9 ± 3.9****	***47.1 ± 3.1****	***39.5 ± 3.6^***	*34.7 ± 3.1*
OXA + ABO-07 30 mg/kg	*31.4 ± 2.5*	*35.5 ± 3.3*	*36.2 ± 4.9*	*36.5 ± 3.7*	*31.4 ± 3.3*

Effect of some extracts on hyperalgesia induced in the rat by oxaliplatin (*Paw-pressure test*)

	Paw-pressure test (g)				
Treatment mg/kg ,p,o,	**Before treatment**	**After treatment**			
OXA + ABO-07 60 mg/kg	*33.1 ± 2.8*	*38.9 ± 4.2*	*39.7 ± 3.1*	*39.8 ± 2.6*	*36.7 ± 4.0*
OXA + ABO-07 100 mg/kg	*32.2 ± 2.6* ***5.2*** *± 3.2*	*35.5 ± 3.1*	*39.1 ± 4.4*	*36.2 ± 3.1*	*35.9 ± 3.4*
OXA + ABO-07 300 mg/kg	*29.8 ± 2.2* ***5.2*** *± 3.2*	***43.2 ± 3.7****	***45.3 ± 2.9****	***42.5 ± 3.6****	*37.3 ± 3.5*
OXA + ABO-11 30 mg/kg	*32.9 ± 2.9*	*33.4 ± 2.7*	*28.7 ± 2.5*	*31.1 ± 3.2*	*35.6 ± 3.1*
OXA + ABO-11 60 mg/kg	*31.4 ± 3.1*	*37.5 ± 3.9*	*39.1 ± 3.4*	*36.3 ± 3.6*	*32.8 ± 4.1*
OXA + ABO-11 100 mg/kg	*33.2 ± 3.0*	***44.2 ± 3.1****	***45.2 ± 3.2****	***42.6 ± 3.7****	*31.5 ± 3.0*
OXA + ABO-11 300 mg/kg	*29.5 ± 2.9*	***41.8 ± 4.0****	***48.5 ± 3.4****	***43.6 ± 4.1****	*33.1 ± 4.7*
OXA + ABO-12 30 mg/kg	*32.3 ± 2.6*	*31.5 ± 2.4*	*33.6 ± 2.8*	*30.6 ± 3.4*	*33.0 ± 2.8*
OXA + ABO-12 60 mg/kg	*28.5 ± 3.1*	*32.5 ± 3.1*	*29.6 ± 3.0*	*33.9 ± 3.5*	*36.4 ± 4.5*
OXA + ABO-12 100 mg/kg	*30.7 ± 3.5*	*35.3 ± 3.8*	*39.1 ± 3.4*	*34.5 ± 2.6*	*32.9 ± 3.7*
OXA + ABO-12 300 mg/kg	*32.2 ± 3.4*	***39.5 ± 2.2^***	***46.7 ± 2.6****	***45.1 ± 3.9****	***41.5 ± 4.3^***

*^P< 0.05; *P< 0.01 in vs CMC Each value represents the mean of 8 rats,*

Table 4

Dose/response curve of the extract of ABO-09 on hyperlagesia induced by oxaliplatin in the paw-pressure test in the rat					
		Paw-pressure in the rats (g)			
TREATMENT i.p.	***TREATMENT per os***	***Pre-test***	***15 min.***	***30 min***	***45 min***
SALINE	*CMC*	*57.4 ± 3.5*	*61.8 ± 5.4*	*67.4 ± 6.3*	*62.3 ± 5.4*
OXA	*CMC*	*22.6 ± 4.6*	*25.1 ± 6.1*	*26.9 ± 5.4*	*24.3 ± 6.6*
OXA	*ABO-09 30 mg/kg*	*21.7 ± 1.2*	***58.1 ± 3.5****	***67.8 ± 2.0****	*29.1 ± 2.0*
OXA	*ABO-09 60 mg/kg*	*24.5 ± 1.3*	***48.2 ± 4.6****	*25.7 ± 2.4*	*21.5 ± 0.0*
*Oxaliplatin 2.4 mg/kg for 5 consecutive days of the week (15 injections i.p. – cumulative dose 36 mg/kg) ^ P< 0.05 * p> 0.01*					

The sample ABO-07 (particularly rich in procyanthocyanosides) was rejected from the analyses of successive in-depth studies because it is effective in only one dose (the highest): 300 mg/kg.

The samples ABO-10 (rich in polyphenols, particularly catechins), ABO-11 (rich in antioxidant compounds derived from carnosol) at doses of 100 and 300 mg/kg up to 90 minutes after administration and ABO-12 (increased concentration of anthocyanosides) at a dose of 300 mg/kg, the only active dose, but at all assessed times, up to 120 minutes after administration.

The sample ABO-09 was by far the most powerful, being effective even at very low doses: 30 mg/kg.

Summary of the results obtained

To summarize, these are the compounds selected by means of *in vitro* screening for their antioxidant power in respect of the superoxide anion produced by oxaliplatin that were revealed to be individually effective, even in the *in vivo* model, to a more or less comparable extent, with the exception of ABO-09, which was the most powerful:

- ABO-09: extract characterized by an increased content of flavonoids;
- ABO-10: extract with increased content of catechins;
- ABO-11: extract with an increased concentration of carsonol derivatives;
- ABO-12: extract with increased concentration of anthocyanosides;

The experiment was continued by assessing the efficacy *in vivo* of compositions obtained from a mixture of some of the samples (ABO-10. ABO-11 at the fixed dose of 100 mg/kg and ABO-12 at the fixed dose of 300 mg/kg) with the sample ABO-09 at variable doses of 10. 30 and 60 mg/kg. The results are illustrated below.

Table 5

Assessment in the rat of the antihyperalgesic action in the neuropathy model induced by oxaliplatin of mixtures of single extracts with Abo-09

Effect of some mixtures of single extracts on hyperalgesia induced in the rat by oxaliplatin (paw-pressure test)					
	Paw-pressure test (g)				
TREATMENT mg/kg ,p.o.	***Before treatment***	***After treatment***			
		30 min	***60 min***	***90 min***	***120 min***
CMC	*60.5 ± 3.0*	***59.4 ± 3.1***	***63.1 ± 3.5***	***58.8 ± 4.2***	*61.7 ± 4.3*
OXALIPLATIN	*30.4 ± 2.5*	*31.3± 3.5*	*32.4 ± 2.9*	*31.6 ± 3.4*	*28.8 ± 3.1*
OXA + ABO-09 10 mg/kg + ABO-10 100 mg/kg	*31.9± 2.8*	***44.3 ± 3.7****	***45.3 ± 4.1****	***40.6 ± 3.9****	*34.0 ± 3.5*
OXA + ABO-09 30 mg/kg + ABO-10 100 mg/kg	*28.4 ± 2.6*	***57.1 ± 4.4****	***61.1 ± 3.8****	***55.2 ± 4.6****	***39.6 ± 3.5^***
OXA + ABO-09 60 mg/kg + ABO-10 100 mg/kg	*30.5 ±3.1*	***58.3 ± 4.1****	***59.5 ± 3.4****	***53.8 ± 3.8****	*36.2 ± 4.5*
OXA + ABO-09 10 mg/kg + ABO-12 300 mg/kg	*31.7 ± 2.8*	***41.6 ± 3.5****	***43.5 ± 3.9****	***39.8 ± 3.2^***	*32.3 ± 3.9*
OXA + ABO-09 30 mg/kg + ABO-12 300 mg/kg	*32.5 ± 3.1*	***44.5 ± 4.0^***	***42.3 ± 4.1****	***40.9 ± 3.8^***	*35.7 ± 3.1*
OXA + ABO-09 60 mg/kg + ABO-12 300 mg/kg	*29.1 ± 2.9*	***45.7 ± 3.1****	***59.2 ± 4.2****	***57.2 ± 3.3****	*42.9 ± 3.5*
OXA + ABO-09 10 mg/kg	*28.4 ± 2.5*	***46.3 ± 3.2****	***48.7 ± 3.5****	***41.9 ± 3.0****	*36.6 ± 3.0*

Effect of some mixtures of single extracts on hyperalgesia induced in the rat by oxaliplatin (paw-pressure test)

	Paw-pressure test (g)				
TREATMENT mg/kg ,p.o.	***Before treatment***	***After treatment***			
+ ABO-11 100 mg/kg					
OXA + ABO-09 30 mg/kg + ABO-11 100 mg/kg	*32.3 ± 3.0*	***52.5 ± 3.8****	***51.2 ± 3.9****	***46.3 ± 3.6****	*38.8 ± 4.4*
OXA + ABO-09 60 mg/kg + ABO-11 100 mg/kg	*30.1 ± 3.1*	***59.1 ± 3.2****	***58.1 ± 4.2****	***51.6 ± 3.7****	*44.5 ± 3.6*
OXA + ABO-09 10 mg/kg + ABO-21 100 mg/kg	*30.5 ± 2.7*	*39.2 ± 2.5*	*37.5 ± 3.4*	*35.6 ± 3.5*	*35.1 ± 2.7*
OXA + ABO-09 30 mg/kg + ABO-21 100 mg/kg	*29.4 ± 3.2*	***41.8 ± 3.0****	***43.6 ± 2.9****	*38.8 ± 3.1*	*32.9 ± 2.5*
OXA + ABO-09 60 mg/kg + ABO-21 100 mg/kg	*32.8 ± 3.4*	***40.5 ± 3.2^***	***44.7 ± 3.2****	*37.1 ± 2.5*	*30.6 ± 2.2*

*^P< 0.05; *P< 0.01 in vs CMC, Each value represents the mean of 8 rats,*

The results demonstrate that the combinations produced improved results, even reaching values equal to the negative control with just CMC.

3. IN VITRO ASSESSMENT OF THE ABILITY *OF MIXTURES OF THE SINGLE EXTRACTS* TO MODULATE THE APOPTOTIC PROCESSES INDUCED BY OXALIPLATIN

The combinations of plant extracts found to be effective in reversing in the animal used for experimental purposes the neuropathic pain induced by oxaliplatin were examined *in vitro* so as to rule out the fact that the neuroprotective action exhibited by these combinations with respect to the chemotherapeutic agent under examination also causes the survival of tumor cells, thus nullifying the action of the oxaliplatin.

Within the scope of this study, we focused our attention on a line of human colon carcinoma tumor cells HT-29 (neoplasia particularly sensitive to oxaliplatin) where

we induced damage caused by the chemotherapeutic agent under examination. The vitality of the cells incubated with oxaliplatin was assessed in the presence and in the absence of the preparations under examination. In addition, the pro-apoptotic capability of the antitumor agent was also assessed on the same culture line, again both in the presence and in the absence of the combinations of plant extracts. In particular, we assessed the effect of these combinations on the enzyme activity of caspase 3, a central effector of the apoptotic cascade (*Springer, J Biochem Mol Biol, 35: 94-105, 2002*) by means of a fluorescent assay. In practice, we measured the apoptotic action of oxaliplatin and compared this with that expressed by the chemotherapeutic agent in the presence of the aforementioned combinations. In this way, we confirmed that any anti-apoptotic effect of the extracts does not nullify the antineoplastic effects of oxaliplatin.

3.1 COMBINATIONS:

On the basis of the above, the following four combinations were tested within the scope of the study in a human colorectal tumor cell line (HT 29), in the presence of oxaliplatin: ABO-09 with ABO-21 or ABO-11 or ABO-10 or ABO-12 combined in various ways, and referred to as:

Combination ABO-09 and ABO-21

ABO-09-21/ 10 (ABO-09: 9% - ABO-21: 91%)

ABO-09-21 / 30 (ABO-09, 23% -ABO-21: 77%)

ABO-09-21 / 60 (ABO-09: 37.5% – ABO-21: 62.5%);

Combination ABO-09 and ABO-11

ABO-09-11 /10 (ABO-09: 9% - ABO-11: 91%)

ABO-09-11/ 30 (ABO-09: 23% - ABO-11: 77%)

ABO-09-11/ 60 (ABO-09: 37.5% - ABO-11: 62.5%)

Combination ABO-09 and ABO-10

ABO-09-10 /10 (ABO-09: 9% - ABO-10: 91%)

ABO-09-10/ 30 (ABO-09: 23% - ABO-10: 77%)

ABO-09-10/ 60 (ABO-09: 37.5% - ABO-10: 62.5%)

Combination ABO-09 and ABO-12

ABO-09-12 /10 (ABO-09: 3.2% - ABO-12: 96.8%)

ABO-09-12/ 30 (ABO-09: 9% - ABO-12: 91%)

ABO-09-12/ 60 (ABO-09: 16.6% - ABO-12: 83.4%)

3.2 RESULTS:

The combination ABO-09-ABO-12 does not interfere with the action of oxaliplatin on the tumor line HT-29, either in terms of cell vitality or in terms of programmed cell death. The combination ABO-09-ABO-11 reduces the oxaliplatin-dependent anti-

tumor action only in terms of mortality at 48h and only with respect to the highest concentration of oxaliplatin. The apoptotic phenomenon is unchanged. The combinations ABO-09-21/30 and 60 reduce the mortality induced by oxaliplatin after 48h of incubation and its apoptotic action. The combinations ABO-09-10/30 and 60 reduce the mortality induced by oxaliplatin after 24h of incubation as well as its apoptotic action.

Table 1 A: *CELL VITALITY IN % AFTER* ***24H*** *OF INCUBATION WITH OXALIPLATIN AT GROWING CONCENTRATIONS:*

ABO-09-21 *(50 µg/mL)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-21/ 10*	*ABO-09-21/ 30*	***ABO-09-21/60***
0	*100.0 ± 2.6*	***100.0 ± 2.6***	***100.0 ± 2.6***	*100.0 ± 2.6*
0.3	*87.5 ± 7.3*	***89.7 ± 3.2***	***90.2 ± 2.6***	*91.1 ± 1.6*
1	*84.0 ± 4.5*	***86.6 ± 0.9***	***89.3 ± 1.2***	*89.6 ± 1.4*
3	*84.0 ± 2.9*	***85.3 ± 2.0***	***84.8 ± 1.7***	*87.5 ± 4.5*
10	*78.7 ± 3.5*	***81.8 ± 4.0***	***83.6 ± 2.2***	*82.7 ± 3.3*
30	*73.9 ± 3.4*	***80.1 ± 3.3***	***79.8 ± 1.9***	*80.1 ± 2.3*
100	*62.4 ± 2.5*	***68.3 ± 4.5***	***69.1 ± 4.3***	*69.4 ± 3.4*
Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-21 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%				

Table 1 B*: Cell vitality in % after* ***48h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-21*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-21/10*	*ABO-09-21/ 30*	***ABO-09-21/60***
0	*100.0 ± 1.5*	***100.0 ± 1.5***	***100.0 ± 1.5***	*100.0 ± 1.5*
0.3	*89.1 ± 2.7*	***89.8 ± 2.8***	***89.2 ± 3.4***	*89.8 ± 3.1*
1	*79.2 ± 3.8*	***82.5 ± 2.5***	***84.8 ± 2.2***	*83.7 ± 2.3*
3	*76.2 ± 4.3*	***77.8 ± 2.3***	***82.4 ± 4.0***	*82.6 ± 1.6*
10	*74.3 ± 3.2*	***76.5 ± 1.9***	***79.2 ± 2.0***	*79.7 ± 1.1*
30	*67.7 ± 4.2*	***65.7 ± 1.3***	***75.3 ± 1.7***	***73.6 ± 2.5 ****

Cell vitality in % Treatment with oxaliplatin				
100	*44.0 ± 1.7*	***47.5 ± 1.0***	*52.8 ± 1.6 **	***54.2 ± 1.7 ****
*Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of the extract of IPE-MYRTYL (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%. *P <0.05 compared to the control.*				

Table 2 A: *Cell vitality in % after* ***24h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***Abo-09-11*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-11/ 10*	*ABO-09-11/ 30*	***ABO-09-11/ 60***
0	*100.0 ± 2.6*	***100.0 ± 2.6***	***100.0 ± 2.6***	*100.0 ± 2.6*
0.3	*87.5 ± 7.3*	***99.3 ± 4.1***	***90.2 ± 2.7***	*91.1 ± 1.6*
1	*84.0 ± 5.5*	***90.9 ± 2.4***	***89.3 ± 1.2***	*89.6 ± 1.4*
3	*84.0 ± 2.9*	***90.2 ± 3.4***	***84.9 ± 1.7***	*87.5 ± 1.3*
10	*78.7 ± 3.5*	***84.6 ± 4.3***	***83.6 ± 2.2***	*82.6 ± 3.3*
30	*73.9 ± 3.4*	***78.8 ± 3.1***	***79.0 ± 2.2***	*80.1 ± 2.9*
100	*62.4 ± 2.5*	***68.0 ± 5.3***	***68.3 ± 3.3***	*69.1 ± 4.3*
Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-11 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%.				

Table 2 B: *Cell vitality in % after* ***48h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-11*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-11/ 10*	*ABO-09-11/ 30*	***ABO-09-11/ 60***
0	*100.0 ± 1.5*	***100.0 ± 1.5***	***100.0 ± 1.5***	*100.0 ± 1.5*
0.3	*89.1 ± 2.7*	***80.4 ± 1.4***	***86.2 ± 2.4***	*88.1 ± 3.6*
1	*79.2 ± 3.8*	***77.4 ± 0.8***	***81.6 ± 1.2***	*81.2 ± 4.2*
3	*76.2 ± 4.3*	***75.1 ± 1.9***	***80.3 ± 2.1***	*77.8 ± 2.8*
10	*74.3 ± 3.2*	***70.7 ± 2.1***	***73.7 ± 1.6***	*77.7 ± 6.0*

30	*67.7 ± 4.2*	***67.0 ± 1.3***	***71.2 ± 1.8***	*73.1 ± 5.0*
100	*44.0 ± 1.7*	***49.5 ± 1.7***	*57.9 ± 1.5**	***59.4 ± 2.1****

*Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-11 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%. *P <0.05 compared to control.*

Table 3 A: *Cell vitality in % after* ***24h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-10*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-10/ 10*	*ABO-09-10/ 30*	***ABO-09-10/ 60***
0	*100.0 ± 2.6*	***100.0 ± 2.6***	***100.0 ± 2.6***	*100.0 ± 2.6*
0.3	*87.5 ± 7.3*	***85.2 ± 6.1***	***80.6 ± 9.4***	*85.3 ± 5.7*
1	*84.0 ± 5.5*	***83.2 ± 8.3***	***81.7 ± 7.5***	*84.1 ± 9.5*
3	*84.0 ± 2.9*	***80.9 ± 4.0***	***80.7 ± 9.0***	*77.2 ± 3.2*
10	*78.7 ± 3.5*	***79.5 ± 2.6***	***80.2 ± 1.9***	*77.1 ± 2.3*
30	*73.9 ± 3.4*	***70.7 ± 3.0***	***76.9 ± 6.5***	*74.0 ± 3.0*
100	*62.4 ± 2.5*	***69.2 ± 4.1***	*72.4 ± 1.9 **	***72.8 ± 1.0 ****

*Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-10 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%. *P < 0.05 compared to the control.*

Table 3 B: *Cell vitality in % after* ***48h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-10*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	*ABO-09-10/ 10*	*ABO-09-10/ 30*	***ABO-09-10/ 60***
0	*100.0 ± 1.5*	*100.0 ± 1.5*	*100.0 ± 1.5*	*100.0 ± 1.5*
0.3	*89.1 ± 2.7*	*88.3 ± 6.6*	*85.6 ± 7.1*	*89.6 ± 2.8*
1	*79.2 ± 3.8*	*79.2 ± 3.9*	*72.0 ± 2.3*	*75.6 ± 1.6*
3	*76.2 ± 4.3*	*70.3 ± 3.4*	*58.2 ± 1.6*	*65.6 ± 6.6*

10	*74.3 ± 3.2*	*65.3 ± 2.5*	*57.2 ± 2.8*	*69.9 ± 5.2*
30	*67.7 ± 4.2*	*62.8 ± 1.1*	*55.6 ± 1.6*	*60.4 ± 3.1*
100	*44.0 ± 1.7*	*46.8 ± 2.7*	*46.0 ± 1.0*	*48.2 ± 2.7*
Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-10 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%.				

Table 4 A: *Cell vitality in % after* ***24h*** *of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-12*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	***ABO-09-12/10***	***ABO-09-12/30***	***ABO-09-12/60***
0	*100.0 ± 2.6*	*100.0 ± 2.6*	*100.0 ± 2.6*	*100.0 ± 2.6*
0.3	*87.5 ± 7.3*	*91.0 ± 1.6*	*93.6 ± 2.2*	*96.1 ± 2.6*
1	*84.0 ± 5.5*	*90.5 ± 2.5*	*89.7 ± 2.3*	*92.5 ± 2.3*
3	*84.0 ± 2.9*	*87.4 ± 2.2*	*87.0 ± 0.4*	*88.6 ± 5.0*
10	*78.7 ± 3.5*	*84.6 ± 0.5*	*85.1 ± 2.2*	*87.3 ± 3.5*
30	*73.9 ± 3.4*	*75.1 ± 2.0*	*76.9 ± 1.3*	*81.3 ± 1.4*
100	*62.4 ± 2.5*	*62.7 ± 1.1*	*63.2 ± 2.8*	*65.5 ± 2.6*
Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-12 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%.				

Table 4 B: *Cell vitality in % after 48h of incubation with oxaliplatin at growing concentrations: effect of the extract of* ***abo-09-12*** *(50 µg/ml)*

Cell vitality in % Treatment with oxaliplatin				
Concentration of oxaliplatin (µM)	***Control***	***ABO-09-12/10***	***ABO-09-12/30***	***ABO-09-12/60***
0	*100.0 ± 1.5*	*100.0 ± 1.5*	*100.0 ± 1.5*	*100.0 ± 1.5*
0.3	*89.1 ± 2.7*	*92.1 ± 3.0*	*91.8 ± 4.5*	*94.9 ± 3.1*
1	*79.2 ± 3.8*	*89.2 ± 6.4*	*85.9 ± 3.6*	*83.3 ± 3.5*

3	*76.2 ± 4.3*	*81.9 ± 2.5*	*80.0 ± 3.6*	*80.1 ± 3.9*
10	*74.3 ± 3.2*	*80.4 ± 3.6*	*76.3 ± 2.0*	*76.9 ± 5.9*
30	*67.7 ± 4.2*	***69.0 ± 2.6***	***68.6 ± 3.0***	*71.1 ± 2.0*
100	*44.0 ± 1.7*	***43.2 ± 2.0***	***40.5 ± 1.2***	*42.3 ± 1.9*
Human colorectal tumor cells (HT-29) were exposed to growing concentrations of oxaliplatin (0.3 - 100 µM) in the presence or in the absence of ABO-09-12 (50 µg/mL), The values are expressed in percentage as mean ± s.e.m of 6 experiments, The control condition is fixed at 100%.				

Table 5A

ACTIVITY IN % OF CASPASE 3 AFTER 4H OF INCUBATION WITH OXALIPLATIN 100 µM: EFFECT OF COMBINATION OF EXTRACTS (50 MG/ML)

		Oxaliplatin 100 µM			
Treatment	Control	**Oxaliplatin 100 µM**	**ABO-09- 10 mg/kg**	**ABO-09- 30 mg/kg**	**ABO-09- 60 mg/kg**
ABO-09 – ABO-21	100.0 ± 9.0	142.1 ± .8.1*	105.0 ± 6.4^	100.9 ± 2.4^	115.3 ± 5.5^
ABO-09-ABO-11			142.5 ± 2.7	135.8 ± 2.2	133.3 ± 10.1
ABO-09-ABO-10			100.1 ± 9.2^	117.9 ± 7.3^	118.5 ± 3.3^
ABO-09-ABO-12			138.3 ± 8.0	144.9 ± 1.6	145.3 ± 0.7
Human colorectal tumor cells (HT-29) were incubated with oxaliplatin 100 µM in the presence or in the absence of the combinations of extracts described at a concentration of 50 µg/mL. The base condition, that is to say the control, was fixed arbitrarily as 100%. The values are expressed as mean ± s.e.m of 6 experiments. *P<0.05 compared to the control, ^P<0.05 compared to the treatment with oxaliplatin alone.					

The data concerning the interference with the activity of oxaliplatin has indicated that ABO-10 (rich in catechins) and ABO-21 (rich in anthocyanosides) significantly reduced the cytotoxic activity of oxaliplatin, thus causing pharmacodynamic interferences. These were therefore excluded from further in-depth study.

To summarize, on the basis of all the results of the preclinical studies carried out, ABO-09 was selected as a source of flavonoids, and ABO-12 was selected as a source of anthocyanosides, but also of vitamin B6.

In addition to the antioxidant effect in the formulation, a source of vitamin B1

(brewer's yeast) and a source of essential amino acids (royal jelly) were added, whereas the share of vitamin B6 was provided by the extract of red vine.

4. IN VITRO ASSESSMENT OF THE ABILITY OF THE COMBINATION OF TOTAL POLYPHENOLS (WITH FLAVONOIDS AND WITH QUERCETIN), ANTHOCYANOSIDES AND VITAMIN B1, B6 AND ESSENTIAL AMINO ACIDS (ABO-09+ ABO-21 + SOURCE OF ESSENTIAL AND BRANCHED AAs) TO MODULATE APOPTOTIC PROCESSES INDUCED BY OXALIPLATIN

The formulation based on ABO-09, ABO-12, yeast (B1) and royal jelly was tested in 3 cell lines (breast MDA-MB231, lung H1299, colon HCT116) treated for 72h with growing doses of chemotherapeutic drug, neurodol or both.

4.1 MATERIALS AND METHODS

Cell culture conditions: the cells were cultivated in medium DMEM/F-12 Glutamax (Invitrogen), supplemented with FBS 10% (Invitrogen) and insulin 5µgr/ml (SIGMA), temperature 37°C, 5% CO2 and constant humidity.

Assay of cell vitality: the kit ATPlite 1step (PerkinElmer) was used in accordance with the instructions advised by the PerkinElmer protocol.

Reagents: Pemetrexed (ALIMTA, Lilly), Paclitaxel (Mylan), Oxaliplatin (Eloxatin, Sanofi Aventis), 5 Fluorouracil (Teva), Aboca combination.

The three lines were treated for 72h with growing doses of chemotherapeutic drug, neurodol or both.

4.2 RESULTS

Breast tumor line: Cell vitality of the mda-mb-231 breast line recorded at 72 h after treatment with growing doses of the combination (from 150 µg/ml to 8 mg/ml) (A), Paclitaxel (0.15nM to 50 nM) (B) and combination with fixed dose of the combination (50 ug/ml) and growing doses of Paclitaxel (0.15 nM to 12 nM) (C).

LUNG LINE: Cell vitality of the lung line H1299 recorded at 72 h after treatment with growing doses of the combination Aboca (from 150ug/ml to 8 mg/ml) (D), Paclitaxel (0.15nM to 50 nM) (E) and combination with fixed dose of the combination Aboca (50 ug/ml) and growing doses of Paclitaxel (0.15 nM to 50 nM) (F).

In this case too, the interference of neurodol on the cytotoxic action of Paclitaxel was excluded.

In the specific case, interference of neurodol on the cytotoxic action of Paclitaxel was excluded. COLON LINE: Cell vitality of the colon line HCT116 recorded at 72h after treatment with growing doses of the combination Aboca (from 150ug/ml to 8 mg/ml) (A), Oxaliplatin (6 uM to 400 uM) (B), combination with fixed dose of the combination Aboca (50 ug/ml) and growing doses of Oxaliplatin (1.5 uM to 25 uM) (C), 5 Fluoruracil (0.75 uM to 50 uM) (D) and combination with fixed dose of the

combination Aboca (50ug/ml) and growing doses of 5 Fluoruracil (0.15 uM to 25 uM) (E).

In the combination experiment between ABO-09+ABO-12 + essential and branched AAs + vitamin B6 + vitamin B1 and oxaliplatin and 5 FU, lower doses of chemotherapeutic drug were used given the very cytotoxic action of the compounds at the doses used previously in the single treatment. In this case too, it is evident that the product Aboca does not interfere with the cytotoxic action of the two chemotherapeutic drugs.

5. ASSESSMENT IN THE RAT OF THE ANTIHYPERALGESIA OF COMBINATIONS OF PLANT EXTRACTS IN THE NEUROPATHY MODEL INDUCED BY PACLITAXEL

Table 6

Effect of the combination of extract of abo-09, abo-12, royal jelly and brewer's yeast on hyperalgesia induced in the rat by paclitaxel (paw-pressure test)

	Paw-pressure test (g)				
Treatment mg/kg	Before treatment	After treatment			
		30 min	*60 min*	*90 min*	*120*
CMC	57.9 ± 2.8	62.9 ± 3.0	58.2 ± 3.3	55.9 ± 3.6	62.4 ± 2.9
PACLITAXEL	34.5 ± 2.9	33.8 ± 2.9	35.8 ± 3.4	32.3 ± 2.5	33.8 ± 3.1
PACLITAXEL + ABO-09 60 mg/kg ABO-12 300 mg/kg source of essential and branched amino acids 240 mg/kg source of vitamin B1 200 mg/kg	35.1 ± 2.8	**51.2 ± 3.5***	**48.2 ± 3.8***	**41.8 ± 3.6^**	38.5 ± 3.7

Effect of the combination of extract of abo-09, abo-12, royal jelly and brewer's yeast on hyperalgesia induced in the rat by paclitaxel (paw-pressure test)		
	Paw-pressure test (g)	
Treatment mg/kg	Before treatment	After treatment
Treatment: Paclitaxel 0.5 mg kg^{-1} was injected for 4 days (days 1, 3, 5, 8). Cumulative dose of Paclitaxel: 2.0 mg kg^{-1}. The test was carried out 14-18 days after the last injection of Paclitaxel. *P< 0.01 ^ P< 0.05 compared to the group treated with Paclitaxel. Each value represents the mean of 8 rats.		

6. ASSESSMENT IN THE RAT OF THE HYPERALGESIC ACTION OF COMBINATIONS OF PLANT EXTRACTS IN THE NEUROPATHY MODEL INDUCED BY VINCRISTINE

Table 7

EFFECT OF THE COMBINATION OF EXTRACT OF ABO-09, ABO-12, ROYAL JELLY AND BREWER'S YEAST ON HYPERALGESIA INDUCED IN THE RAT BY VINCRISTINE (PAW-PRESSURE TEST)					
	Paw-pressure test (g)				
TREATMENT mg/kg ,p,o,	Before treatment	After treatment			
		30 min	*60*	*90 min*	*120 min*
CMC	58.3 ± 2.7	56.4 ± 3.5	62.6 ± 3.1	60.3 ± 3.8	63.5 ± 3.8
VINCRISTINE	35.6 ± 2.7	34.0 ± 2.7	36.7 ± 4.1	31.2 ± 3.7	35.3 ± 3.4
VINCRISTINE + ABO-09 60 mg/kg ABO-12 300 mg/kg source of essential and branched AAs 240 mg/kg source of vitamin B1 200	33.7 ± 3.5	**49.6 ± 3.0***	**44.4 ± 3.5***	38.2 ± 3.3	33.6 ± 2.8

EFFECT OF THE COMBINATION OF EXTRACT OF ABO-09, ABO-12, ROYAL JELLY AND BREWER'S YEAST ON HYPERALGESIA INDUCED IN THE RAT BY VINCRISTINE (PAW-PRESSURE TEST)	
	Paw-pressure test (g)
Treatment: Vincristine was injected e,v, at a dose of 150 µg kg^{-1} for 5 days every 2 days, Cumulative dose 750 mg kg^{-1} e.v. The test was carried out 4 days after the last administration, *P< 0.01 compared to the group treated with Vincristine. Each value represents the mean of 8 rats.	

7. ASSESSMENT IN THE RAT OF THE PHARMACOKINETICS OF OXALIPLATIN IN THE PRESENCE OF EXTRACTS OF ABO-09 AND ABO-12

The possible interference *in vivo* on the bioavailability with the drug oxaliplatin due to possible interferences of red vine and hypericum with the hepatic metabolic systems (cytochrome) was assessed *in vivo* in the rat.

The possible alteration of the plasma concentrations of platinum caused by the endovenous administration of oxaliplatin at a dose of 5 mg kg-1 subsequent to pre-treatment with the plant extracts reported above was investigated. The plant extracts were injected 30 min before oxaliplatin i.p. so that the peaks of activity of the extracts would coincide with the maximum plasma concentration of the chemotherapeutic drug.

The selection of the dose of oxaliplatin used in this study is based not only on the recommended dosage in humans (130 mg/m2), but also so as to have a concentration in the examined samples greater than the minimum definable concentrations.

7.1 EXPERIMENTAL PROTOCOL

The rats were divided into 5 groups, each containing 6 animals.

Group no. 1: CMC p.o. + oxaliplatin 5 mg kg-1 e.v.

Group no. 2: extract of hypericum 60 mg kg-1 p.o. + oxaliplatin 5 mg kg-1 e.v.

Group no. 3: extract formed by: hypericum 60 mg kg-1 p.o and red vine 300 mg kg-1 p.o. + oxaliplatin 5 mg kg-1 e.v.

Table 8

	Time at which the samples were taken (min)					
	0	*5 min*	*15 min*	*30 min*	*60 min*	*90 min*
CMC +OXA e.v.	*38.9 ± 13.3*	*20.4 ± 9.5*	*6.6 ± 1.2*	*4.3 ± 1.2*	*2.7 ± 1.1*	*2.6. ± 0.9*
ABO-09 + OXA e.v.	*36. 3± 12.6*	*22.0 ± 10.7*	*6.7 ± 1.4*	*3.9 ± 1.5*	*2.6 ± 0.9*	*2.3 ± 0.4*

ABO-09 + ABO-12 + OXA E.V.	*39.2 ± 14.7*	*19.8 ± 8.0*	*7.1 ± 2.5*	*4.4 ± 1.5*	*2.3 ± 0.9*	*2.61± 0.7*

Tab. 7 . *Concentration of platinum (mean ± SD) in the plasma after administration e.v. of oxaliplatin*

Materials and methods

Animals

Male Sprague Dawley rats weighing approximately 200 grams provided by Harlan, Italy. The animals were housed in groups of 4 in cages having the following dimensions: approximately 26x41cm with a circadian rhythm of 12 hours with water and food "ad libitum". The rats were fed with a standard diet and were housed at a temperature of 23±1°C. The experimental procedure was approved by the local committee for the control of experimentation on laboratory animals. All the experiments were carried out in accordance with the EC Council Directives of November 1986 (DL 116/92; 86/609/EEC) regarding the handling of animals used for experimental purposes and in accordance with the "*National Institute of Health Guide for the Care and Use of Laboratory Animals*", having acknowledged the Guidelines of the *"International Association for the Study of Pain",* all the measures necessary for minimizing the number of animals and also the suffering caused thereto were taken.

Administration of oxaliplatin

The neoplastic agent was injected as a dose of 5 mg kg-1 e.v. solubilized in a volume of 10 ml kg-1 of 5% glucosate solution, similarly to all the behavioral experiments carried out beforehand.

Dosage

The samples were diluted 1:4 with HCl (v/v) and analyzed for the content of platinum at 306.4 nm using Spectraspan III (*Applied Research Laboratories, Sunland, CA*) in accordance with the method described by Pestieau et al. (*J. Surgical Oncology, 2001: 76: 106-114*). The linearity in the emission of the signal was obtained for a calibration curve of the platinum in the range 0-2.0 mg/L with a correlation efficient > 99%. A blank of 10% HCl (v/v) was analyzed between each sample.

8. ASSESSMENT IN THE RAT OF THE ANTIHYPERALGESIC ACTION IN NUEROPATHY MODELS INDUCED BY STREPTOZOTOCIN AND BY LIGATION OF THE SCIATIC NERVE

The antihyperalgesic efficacy shown by a combination formed by ABO-09 (30 mg kg^{-1} p.o.), ABO-11 (100 mg kg^{-1} p.o.) and ABO-12 (300 mg kg^{-1} p.o.) was assessed in the rat in two models of neuropathy: one induced by loose ligation of the sciatic nerve, and one caused by streptozotocin (abbreviated STZ).

8.1 DIABETIC HYPERALGESIA INDUCED BY STREPTOZOTOCIN

The lowering of the pain threshold was obtained by administration i.p. of streptozotocin (50 mg/kg) Malcangio & Tomlison Pain, 76: 151-157, 1998. The rats developed hyperalgesia from the fourth week after the administration with STZ.

8.2 RESULTS

The combination completely reverses the hyperalgesia induced by STZ up to 120 minutes after injection.

Table 9

Effect of ABO-09, ABO-11 and ABO-12 on hyperlagesia induced in the rat by streptozotocin

	Paw-pressure test (g)				
Treatment mg/kg .p.o.	*Before treatment*	*After treatment*			
		30 min	*60 min*	*90 min*	*120 min*
CMC + CMC	61.6 ± 2.1	62.8 ± 3.5	59.4 ± 3.1	63.9 ± 3.8	58.5 ± 3.5
STZ + CMC	39.3 ± 2.6	36.5 ± 2.8	38.2 ± 3.2	35.4 ± 3.0	37.7 ± 3.5
STZ + ABO-09 + ABO-11 + ABO-12	37.2 ± 2.9	65.9 ± 3.0*	63.8 ± 3.1*	66.2 ± 4.1*	59.8 ± 3.4*

^P< 0.05; *P< 0.01 compared to mice treated with STZ. Each value represents the mean of 8 rats.

9. MODEL OF PERIPHERAL MONONEUROPATHY IN THE RAT

Neuropathic pain is characterized by the development of an altered perception of pain that manifests itself as spontaneous continuous pain and hyperalgesia. The rats were anesthetized and then the sciatic nerve was exposed at the thigh, with the femoral biceps spread apart. Approximately 7 mm of the nerve was freed from the membranes close to the trifurcation, and 4 ligatures were made inside the nerve, approximately 1 mm from another. In another group of animals, the same incision was made, but without the nerve ligation (sham operation). The neuropathy developed in 14 days. The tests with the potentially analgesic substances were carried out on the 14th day after the operation using the “paw-pressure” test (Bennett & Xie, *Pai*n, 33, 87-107, 1988)

9.1 RESULTS

The combination of the three completely reverses, in a statistically significant manner, the hyperlagesia induced by the ligation of the sciatic nerve up to 120 minutes.

Table 10

Effect of ABO-09+ABO-11 +ABO-12 on hyperalgesia induced in the rat by loose ligation of the sciatic nerve

		Paw-pressure test (g)				
Treatment mg/kg .p.o.	*paw*	*Before treatment*	*After treatment*			
			30 min	*60 min*	*90 min*	*120 min*
CMC	sn	60.5 ± 3.0	58.6 ± 2.9	62.6 ± 3.6	61.7 ± 3.3	57.1 ± 3.4
CMC	dx	26.2 ± 2.5	28.1 ± 2.6	25.3 ± 3.0	28.4 ± 3.1	26.7 ± 2.5
ABO-09+ABO-11 +ABO-12	sn	57.3 ± 2.6	71.3 ± 3.1^	68.4 ± 3.2	65.5 ± 2.9	60.3 ± 3.9
ABO-09+ABO-11 +ABO-12	dx	30.1 ± 3.1	50.3 ± 4.4*	46.6 ± 3.7*	44.2 ± 3.0*	37.5 ± 3.3

^P< 0.05; *P< 0.01 compared with mice treated with CMC. Each value represents the mean of 8 rats

10. SUMMARY OF THE EXPERIMENTAL RESULTS

The experiments described above indicate that it is possible, by combining different mixtures, to obtain an increase in the efficacy of the treatment of neuropathic pain.

In particular, the mixtures obtained by combining, at different concentrations, ABO-09 with ABO-10, ABO-11, ABO-12 or ABO-21 were the most effective of the assay *in vivo* of hyperalgesia induced in the rat by oxaliplatin (paw-pressure test).

The combinations proving to be the most effective were therefore tested for the assessment *in vitro* of the capability to modulate the apoptotic processes induced by chemotherapeutic drugs, in particular by oxaliplatin. The objective of these experiments was to assess possible interferences of the selected combinations with the chemotherapeutic drug. The results, described in detail in the paragraphs above, are summarized in the grid shown below.

The data in the grid shown below indicates clearly that there is no interference with the chemotherapeutic drug only when the ratio between total polyphenols and total flavonoids is between 1.5 and 4.5 and the relative concentrations of anthocyanosides in the mixture are not elevated.

Grid 1

MIXTURE CODE	TOTAL POLYPHENOLS expressed as gallic acid	TOTAL FLAVONOIDS, expressed as hyperosides	TOTAL ANTHOCYANOSIDES, expressed as cyanidin	Ratio TOTAL POLYPHENOLS/TOTAL FLAVONOIDS	Interference with the chemotherapeutic drug
ABO-09-21/ 10 (ABO-09: 9% - ABO-21: 91%)	33.3	2.24	23.25	14.84	**YES**
ABO-09-21 / 30 (ABO-09, 23% - ABO-21: 77%)	30.0	3.09	19.68	9.72	**YES**
ABO-09-21 / 60 (ABO-09: 37.5% – ABO-21: 62.5%);	26.7	3.975	15.97	6.73	**YES**
ABO-09-11 /10 (ABO-09: 9% - ABO-11: 91%)	15.77	7.76	0	2.03	**NO**
ABO-09-11/ 30 (ABO-09: 23% - ABO-11: 77%)	15.22	7.76	0	1.96	**NO**
ABO-09-11/ 60 (ABO-09: 37.5% - ABO-11: 62.5%)	14.66	7.76	0	1.89	**NO**
ABO-09-10 /10 (ABO-09: 9% - ABO-10: 91%)	31.13	0.69	0	44.58	**YES**
ABO-09-10/ 30 (ABO-09: 23% - ABO-10: 77%)	28.22	5.98	0	4.72	**YES**
ABO-09-10/ 60 (ABO-09: 37.5% - ABO-10: 62.5%)	25.21	2.91	0	8.67	**YES**
ABO-09-12 /10 (ABO-09: 9% - ABO-12: 91%)	19.65	5.08	1.34	3.86	**NO**
ABO-09-12/ 30 (ABO-09: 23% - ABO-12: 77%)	19.21	5.24	1.26	3.66	**NO**
ABO-09-12/ 60 (ABO-09: 37.5% - ABO-12: 62.5%)	18.63	5.45	1.15	3.41	**NO**
ABO-09 60 mg/kg+ABO-12 300 mg/kg*B1	8.38	2.45	0.52	3.41	**NO**

MIXTURE CODE	TOTAL POLYPHENOLS expressed as gallic acid	TOTAL FLAVONOIDS, expressed as hyperosides	TOTAL ANTHOCYANOSIDES, expressed as cyanidin	Ratio TOTAL POLYPHENOLS/TOTAL FLAVONOIDS	Interference with the chemotherapeutic drug
source 200mg/Kg+branched 240 mg/Kg					

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CLAIMS

1. A composition for use in the treatment of neuropathic pain, comprising:

a) polyphenols between 3 and 20% by weight of said composition

of which

a') flavonoids, quercetin excluded, account for between 1 and 7% by weight of said composition

a'') quercetin accounts for between 0.05 and 0.6% by weight of said composition;

b) anthocyanosides account for between 0.1 and 1% by weight of said composition,

wherein the ratio between polyphenols in a) and flavonoids in a') is between 1.5 and 4.5.

2. The composition according to claim 1, wherein said polyphenols are between 3 and 9% by weight of said composition.

3. The composition according to claim 1 or 2 wherein said a), a '), a'') and/or b) are obtained from plant extracts.

4. The composition according to claim 3 wherein said extracts are hydro alcoholic extracts lyophilized.

5. The composition according to any one of claims 1 to 4 wherein said neuropathic pain is caused by: a treatment with a drug, a treatment with a chemotherapeutic drug, chronic diseases, trauma, exposure to toxic chemicals, a current infection, an infection past, altered function of an organ, vascular diseases, metabolic diseases, autoimmune diseases; or is described as diabetic peripheral neuropathy, post herpetic neuralgia, trigeminal neuralgia, neuropathic pain of the lower back, reflex sympathetic dystrophy, phantom limb syndrome or it is a neuropathic pain of no known cause (idiopathic neuropathic pain).

6. The composition according to claim 5 wherein said chemotherapeutic drug is oxaliplatin, vincristine, vinblastine, paclitaxel, cisplatin, taxane, epothilones, bortemozide, vinca alkaloids.

7. The composition according to any one of claims 1 to 6 wherein said polyphenols are obtained from extracts of *Hypericum perforatum, Rooibos tes, Punica granatum, Sylibum marianum, Cynara scolimus, Vitis vinifera, green tea, Rosmarinus officinalis, Malpighia glabra Curcuma longa, Curcuma xantorrhiza*.

8. The composition according to any one of claims 1 to 7 wherein said flavonoids are obtained from extracts of *Hypericum perforatum, Punica granatum, Sylibum marianum, Cynara scolymus, Vitis vinifera, Camelia sinensis green, Rosmarinus officinalis.*

9. The composition according to any of claims 1 to 8 wherein said anthocyanosides are obtained from extracts of *Vitis vinifera, Vaccinium myrtillus, Hibiscus sabdariffa*.

10. The composition according to any of claims 1 to 9 wherein said quercetin is obtained from extracts of *Hypericum perforatum, green tea, Aspalathus linearis, Sylibum marianum, Vaccinium myrtillus.*

11. The composition according to any one of claims 1 to 10 further comprising vitamin B1, vitamin B6 and/or vitamin B12.

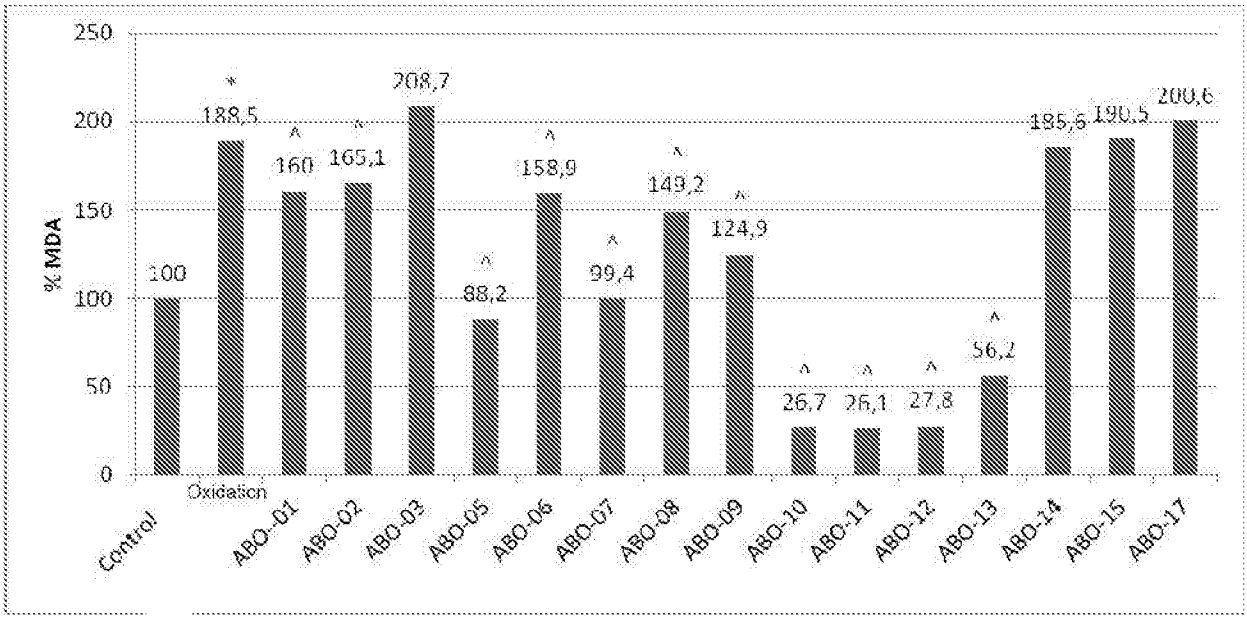
12. The composition according to any one of claims 1 to 11 further comprising royal jelly and/or brewer's yeast.

13. The composition according to any one of claims 1 to 12 wherein said composition is in the form of capsule, tablet, lozenge, granule, powder, syrup, elixir, hard gelatin, soft gelatin, suspension, emulsion, solution, gel.

14. The composition according to any one of claims 1 to 13 wherein said composition is comprised in or consists of a food supplement, food for special medical purposes, a pharmaceutical composition.

15. The composition according to any one of claims 1 to 14 characterized in that it does not inhibit the activity of an antitumor chemotherapeutic agent.

16. Nutraceutical, food supplement or food for special medical purposes comprising a composition according to any one of claims 1 to 15.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ABO-02
ABO-03
ABO-05
ABO-06
ABO-07
ABO-08
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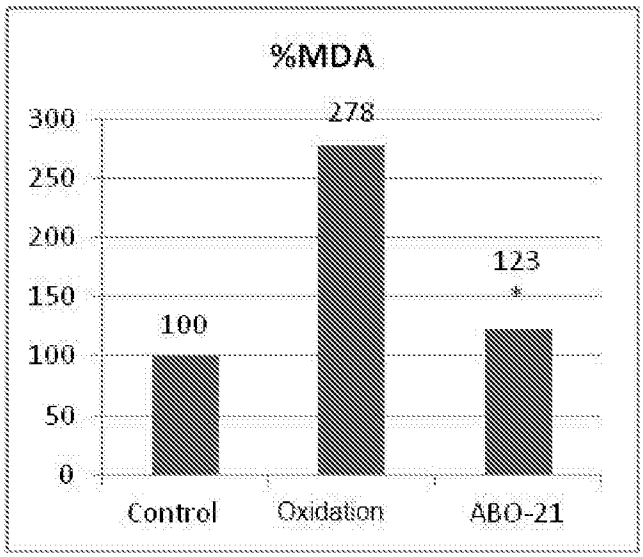
ABO-10
ABO-11
ABO-12
ABO-13
ABO-14
ABO-15
ABO-17

FIG. 1A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Control
Oxidation
ABO-21

FIG. 1B

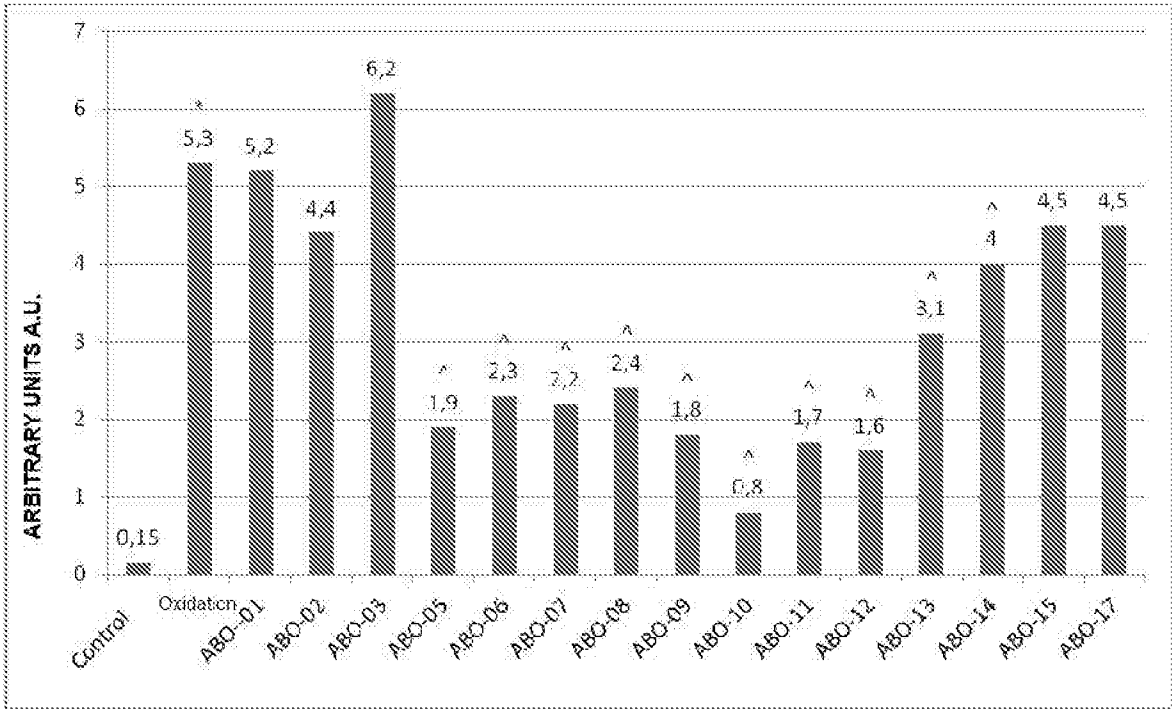


FIG. 2A

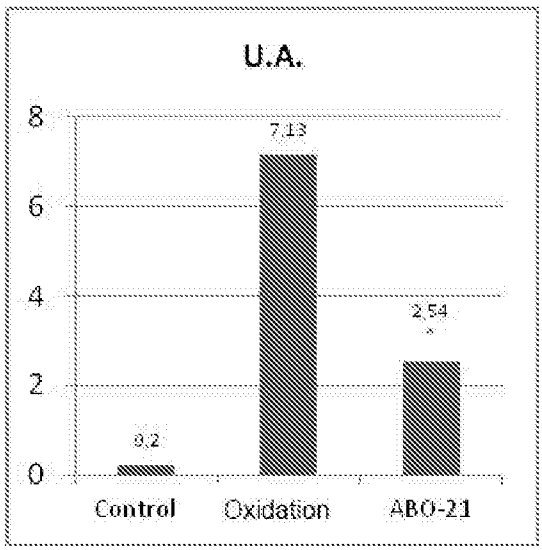
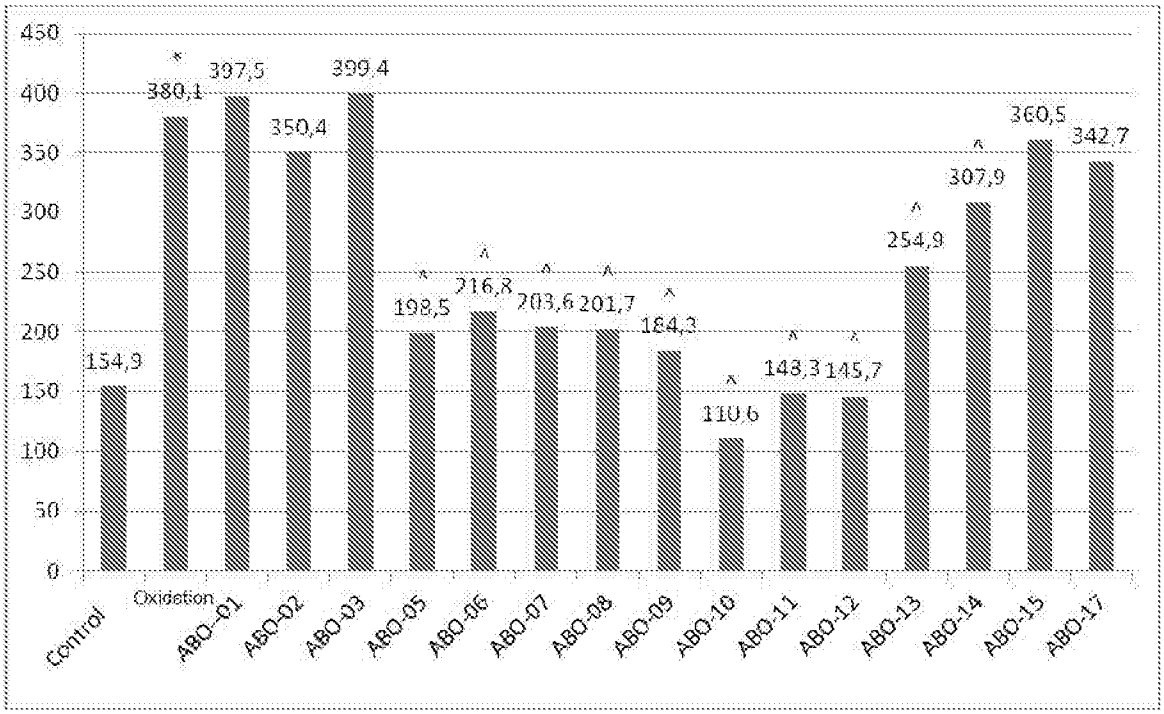


FIG. 2B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145,7
254,9
307,9
360,5
342,7
Control
Oxidation
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ABO-02
ABO-03
ABO-05
ABO-06
ABO-07
ABO-08
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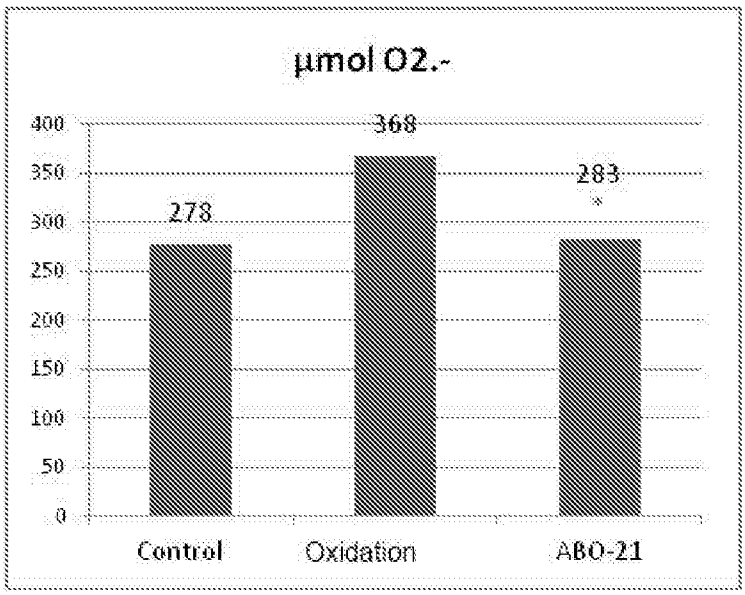
ABO-10
ABO-11
ABO-12
ABO-13
ABO-14
ABO-15
ABO-17

FIG. 3A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Control
Oxidation
ABO-21

FIG. 3B

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2013/055823

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/352 A61K31/05 A61P25/02
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

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, WPI Data, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 2008/052256 A1 (NOVOGEN RES PTY LTD [AU]; BROWN DAVID [AU]; HUSBAND ALAN JAMES [AU]; T) 8 May 2008 (2008-05-08) page 1, paragraph 1 claim 1	1-16
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Y	WO 03/053336 A2 (THE QUIGLEY CORP [US]; ROSENBLOOM RICHARD A [US]) 3 July 2003 (2003-07-03) claims 1,4,7,8,23,25,30	1-16

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 2013	19/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bonzano, Camilla

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2013/055823

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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