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(54) Title: JOINT CARE COMPOSITION

(57) Abstract: The present invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. It also relates to a method of preventing or treating osteoarthritis in mammals, the method comprising administering to said mammal a composition which comprises curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline.

JOINT CARE COMPOSITION

The present invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. It also relates to a method of preventing or treating osteoarthritis in mammals, the method comprising administering to said mammal a composition which comprises curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline.

Cartilage deterioration can be caused by several reasons such as repeated exercise, instability of the joint, etc., which may result in inflammation of the joints. While a greater portion of humans with arthritis have rheumatoid arthritis, most of the arthritis occurring in companion animals is osteoarthritis.

Nowadays, no cure exists for osteoarthritis, and the pharmacological treatment is limited to alleviating symptoms. The most popular are non-steroidal anti-inflammatory drugs, but these are associated with adverse effects. A safer treatment is desirable.

The first aspect of this invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. Treating osteoarthritis includes ameliorating osteoarthritis symptoms.

The present invention relates, for all aspects, to any mammal, including a human. In particular, the present invention relates to a companion animal such as a dog, a cat or an equine animal (e.g. a horse) or any other such animal that suffers or is prone to suffer from osteoarthritis.

The composition of the present invention comprises curcuminoid. Curcuminoid is curcumin or a derivative of curcumin. The chemical structures of curcuminoids differ in their functional groups.

Curcuminoid includes curcumin, demethoxycurcumin, *bis*-methoxycurcumin and/or

tetrahydrocurcumin.

Curcuminoids are natural phenols that are present, in particular, in the Indian spice turmeric. Turmeric is derived from the roots of the plant *Curcuma longa*. Curcuminoids have also been found in roots of other species in the plant family Zingiberaceae of the *Curcuma* genus. Curcuminoids have a distinctly earthy, bitter, peppery flavour and a mustardy smell.

In particular, turmeric contains 60-80% curcumin, 15-30% demethoxycurcumin and 2-6% *bis*-demethoxycurcumin.

The curcuminoid in the composition of the invention can be of any format, including a powder or lipid extract.

In some embodiments, curcuminoid can be mixed with phospholipids and/or cellulose, starch or derivatives thereof to form complexes. This may assist in stability and/or to further increase solubility and bioavailability of the curcuminoid.

The curcuminoid can be mixed with essential oils, piperine or bromelain. The curcuminoid can be mixed with phosphatidycholine, for example lecithin.

Preferably, the curcuminoid of the present invention is curcumin, which is the most active curcuminoid. Curcumin according to the present invention includes demethoxycurcumin, *bis*-demethoxycurcumin and/or tetrahydrocurcumin.

The composition of the invention comprises curcuminoid and green tea polyphenol.

Tea (*Camellia sinensis*), in particular green tea, has a high content of flavonoids, including polyphenols, in particular catechins. Catechins in tea include epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), catechin, and gallocatechin (GC).

Preferably, the green tea polyphenols include catechin. Preferably, the catechin includes EGCG. Green tea extract usually contains at least about 25% polyphenols, about 12.5% of catechins and about 9.3% of EGCG.

Epigallocatechin gallate (EGCG) is the ester of epigallocatechin and gallic acid. EGCG is the most abundant catechin in tea and is a potent antioxidant. It is particularly found in green tea. EGCG is a major polyphenol of green tea and exhibits anti-oxidant, anti-tumour and anti-mutagenic activities.

The composition of the invention comprises curcumin and a combination of glycine, proline and hydroxyproline.

A combination of glycine, proline and hydroxyproline represents 50% of the total amino acid content of hydrolyzed collagen. Preferably, a combination of glycine, proline and hydroxyproline is hydrolyzed collagen. The amino acid composition of hydrolyzed collagen is as set in the table below;

Table 1

Amino acids	**Percentage**
Proline/Hydroxyproline	25%
Glycine	20%
Glutamic acid	11%
Arginine	8%
Alanine	8%
Other essential amino acids	16%
Other non-essential amino acids	12%

Hydrolyzed collagen is obtained by the enzymatic hydrolysis of collagenous tissues found in the bones, skin, and connective tissue of animals such as cattle, fish, horses, pigs, and rabbits. Hydrolyzed collagen is well digested and is preferentially accumulated in cartilage.

A preferred composition includes curcuminoid, green tea polyphenol and a combination of glycine, proline and hydroxyproline. Preferably, this composition includes curcumin, green tea polyphenol and hydrolyzed collagen.

The invention is preferably a foodstuff. It can be any foodstuff, such as dry, semi moist or wet food product. In particular, the foodstuff may be a pet food product.

The pet foodstuff is preferably a commercial pet food product. Such a product is preferably sold as a product for feeding to a pet animal, in particular a pet cat or a pet dog.

A typical pet foodstuff contains about 20-30% crude protein and about 10-20% fat, the remainder being carbohydrate, including dietary fibre and ash. A typical wet or moist product contains (on a dry matter basis) about 40% fat, 50% protein and the remainder being fibre and ash. The foodstuff of the invention may be a dry product (with approximately 5 to approximately 15% moisture), a semi-moist product (with approximately 15 to approximately 70% moisture) or a wet product (with approximately 70 to approximately 90% moisture).

The remaining components of the foodstuff are not essential to the invention and typical standard products can be included. The combined ingredients of the foodstuff according to the invention can provide all of the recommended vitamins and minerals for the particular animal in question (a complete and balanced food).

The foodstuff according to the present invention encompasses any product which a pet consumes in its diet. Thus, the invention covers standard food products including liquids, as well as pet food snacks (for example, snack bars, pet chew, crunchy treat, cereal bars, snacks, biscuits and sweet products) and supplements.

The foodstuff can be provided as a food supplement. The food supplement can be a powder, sauce, topping, biscuit, kibble, pocket or tablet that can be administered with or without an additional foodstuff. Where the food supplement is administered with an additional foodstuff, the food supplement can be administered sequentially simultaneously or separately. The food supplement may be mixed with the foodstuff, sprinkled over the foodstuff or served separately. Alternatively, the food supplement can be added to a liquid provided for drinking such as water or milk.

The foodstuff is preferably a cooked product. It may incorporate meat or animal derived material (such as beef, chicken, turkey, lamb, fish, blood plasma, marrow bone etc. or one or more thereof). The product alternatively may be meat free (preferably including a meat substitute such as soya, maize gluten or a soya product) in order to provide a protein source. The foodstuff may contain additional protein sources such as soya protein concentrate, milk proteins, gluten etc. The foodstuff may also contain a starch source such as one or more grains (e.g. wheat, corn, rice, oats, barley etc.), or may be starch free.

The foodstuff of the invention is preferably produced as a dry product containing from approximately 5% to approximately 15% moisture. The preferred dry food is more preferably presented as a small biscuit – like kibbles.

The table below details the amount of the composition according the present invention and the amount of the composition for the dogs to take according to the present invention:

Table 2

	Diet 1	Diet 2	Diet 3	Energy need (kcal/kg^0,75)		
	3515 kcal	2500 kcal	5600 kcal	80	95	145
	%DM	%DM	%DM	mg/400 kcal	mg/400 kcal	mg/400 kcal
Curcuma (turmeric) extract	0,171	0,07	0,32	210	175	102
Curcuminoids	0,034	0,013	0,065	43	35	22
Curcumin	0,026	0,01	0,05	33	27	15
Green tea extract	0,341	0,155	0,63	414	349	225
Green tea poly	0,085	0,035	0,16	106	87	51
Green tea EGCG	0,032	0,01	0,06	39	32	15
Collagen hydrolyzed	1,706	0,7	3,2	2108	1747	1016
Glycine	1,158	0,53	2,15	1416	1185	767
Proline	1,358	0,63	2,5	1651	1390	911
Hydroxyproline	0,177	0,08	0,33	217	182	116
Total gly+pro+hydroxypro	2,693	1,2	5	3295	2757	1736

The composition in the first aspect of the invention may comprise curcuminoid at an amount ranging from about 0.005 to 1.1% by weight of curcuminoid on an "as is" weight percent of the food. The amount of curcuminoid can be any amount from 0.005 to 1.1% (as is). The amount of curcuminoid can be any amount from 0.1 to 1% (as is). The amount of curcuminoid can be any amount from 0.1 to 0.6% (as is). The amount of curcuminoid can be any amount from 0.3 to 0.6% (as is).

The composition in the first aspect of the invention may comprise curcuminoid at an amount ranging from about 0.005 to 0.15% by weight of curcuminoid on an "as is" weight percent of the food. The amount of curcuminoid can be any amount from 0.005 to 0.15% (as is) (7 to 99 mg/400 kcal).

When the diet is dry the "as is" weight is the same as the "dry matter weight".

Preferably, the amount of curcuminoid in the composition ranges from about 0.01 to 0.07% (as is) (14 to 46 mg/400 kcal). Most preferably, the amount of curcuminoid is 0.035% (as is) (36 mg/400 kcal).

In some embodiments, the curcuminoid in the composition is curcumin at an amount ranging from about 0.005 to 0.15% by weight of curcumin on an "as is" weight percent of the food. The amount of curcumin can be any amount from 0.005 to 0.15% (as is) (7 to 99 mg/400 kcal). Preferably, the amount of curcumin ranges from about 0.01 to 0.05% (as is) (14 to 32 mg/400 kcal). Most preferably, the amount of curcumin is 0.026% (as is) (27 mg/400 kcal).

The composition in the first aspect of the invention may comprise green tea polyphenol in an amount ranging from about 0.01 to 1.1 % by weight of green tea polyphenol on an "as is" weight percent of the food. The amount of green tea polyphenol can be any amount from 0.01 to 1.1% (as is). The amount of green tea polyphenol can be any amount from 0.1 to 1% (as is). The amount of green tea polyphenol can be any amount from 0.1 to 0.6% (as is). The amount of green tea polyphenol can be any amount from 0.3 to 0.6% (as is).

The composition in the first aspect of the invention may comprise green tea polyphenol in an amount ranging from about 0.01 to 0.3 % by weight of green tea polyphenol on an “as is” weight percent of the food. The amount of green tea polyphenol can be any amount from 0.01 to 0.3 % (as is) (14 to 197 mg/400 kcal). Preferably, the amount of green tea polyphenol ranges from about 0.03 to 0.17% (as is) (43 to 113 mg/400 kcal). Most preferably, the amount of green tea polyphenol is 0.085% (as is) (87 mg/400 kcal).

In some embodiments, the green tea polyphenol is EGCG at an amount ranging from about 0.005 to 0.2% by weight of EGCG on an “as is” weight percent of the food (7 to 131 mg/400 kcal). The amount of EGCG can be any amount from 0.01 to 0.06% (as is) (14 to 39 mg/400 kcal). Most preferably, the amount of EGCG is 0.032% (as is) (33 mg/400 kcal).

The composition in the first aspect of the invention may comprise a combination of glycine, proline and hydroxyproline in an amount ranging from about 0.5 to 10% by weight of combined glycine, proline and hydroxyproline on an “as is” weight percent of the food. The amount of combined glycine, proline and hydroxyproline can be any amount from 0.5 to 10% (as is) (720 to 6591 mg/400 kcal). Preferably, the amount of combined glycine, proline and hydroxyproline ranges from about 1.2 to 5% (as is) (1736 to 3295 mg/400 kcal). Most preferably, the amount of combined glycine, proline and hydroxyproline is 2.7% (as is) (2780 mg/400 kcal).

In some embodiment, the combination of glycine, proline and hydroxyproline is hydrolyzed collagen in an amount ranging from about 0.5 to 5% by weight of combined glycine, proline and hydroxyproline on an “as is” weight percent of the food. The amount of hydrolyzed collagen can be any amount from 0.5 to 5% (as is) (720 to 3295 mg/400 kcal). Preferably, the amount of hydrolyzed collagen ranges from about 0.7 to 3.2% (as is) (1016 to 2138 mg/400 kcal). Most preferably, the amount of hydrolyzed collagen is 1.7% (as is) (1750 mg/400 kcal).

In other embodiments, the composition may comprise curcumin in an amount of about 27 mg/400 kcal (35 mg/400 kcal of curcuminoids) with about 87 mg/400 kcal of green tea polyphenol and with about 2757 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and

hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 1747mg/400kcal.

In other embodiments, the composition may comprise curcumin in an amount of about 33 mg/400 kcal (43mg/400 kcal of curcuminoids) with about 106 mg/400 kcal of green tea polyphenol and with about 3295 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 2108mg/400kcal.

In other embodiments, the composition may comprise curcumin in an amount of about 15 mg/400 kcal (22mg/400 kcal of curcuminoids) with about 51mg/400 kcal of green tea polyphenol and with about 1736 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 1016mg/400kcal.

These values apply to a composition for feeding to a mammal, in particular a companion animal.

The second aspect of the invention relates to a method of preventing or treating osteoarthritis in mammals.

Osteoarthritis (OA) is a degenerative and inflammatory condition that affects the joints in mammals. It is also known as degenerative arthritis or degenerative joint disease. Osteoarthritis is a group of abnormalities involving degradation of joints, including articular cartilage and sub-chondral bone.

Osteoarthritis is the consequence of an imbalance of catabolism and anabolism, wherein catabolism is increased; anabolism is decreased causing the inflammation of chondrocytes. Chondrocytes are the only cells found in healthy cartilage. They produce and maintain the cartilaginous matrix, which consists mainly of collagen and proteoglycans. The composition of the invention has demonstrated to provide, *inter alia*, a decrease in inflammation, a decrease in catabolism and an increase in anabolism in *in*

vitro inflammation-induced chondrocytes and in *in vitro* healthy chondrocytes. Thus, the composition of the invention prevents and/or treats osteoarthritis in animals.

The present invention relates, for all aspects, to any mammal, including a human. In particular the present invention relates to a companion animal such as a dog, a cat or an equine animal (e.g. a horse) or any other such animal that suffers or is prone to suffer from osteoarthritis.

In particular, it is a desire in the area of pet foodstuff and companion animal health to provide foodstuff including supplements suitable to support the health of the companion animals. In particular, it is desire to provide diets suitable to promote or maintain the health of already healthy companion animals.

In particular, the second aspect of the invention provides a method for preventing and treating osteoarthritis in mammals, including ameliorating the symptoms of osteoarthritis, in particular companion animals. The method comprises administering to said animal a composition which comprises curcumin with green tea polyphenol or with a combination of glycine, proline and hydroxyproline. The animal may be in need thereof. Since a significant number of dogs suffer from osteoarthritis in their lifetime, all dogs can be considered as in need of prevention.

In particular embodiments, the method comprises administering to said animal a composition comprising curcumin, green tea polyphenol and a combination of glycine, proline and hydroxyproline. Most preferably, the combination of glycine, proline and hydroxyproline is hydrolysed collagen.

Further, the method is preferably administered to an animal, in particular a companion animal, that suffers from osteoarthritis and is in need of ameliorating the symptoms of osteoarthritis or in need of preventing further symptoms of osteoarthritis or in need of treatment of osteoarthritis. This may be to, for example a young pet animal, such as a puppy, or an older companion animal. Where the composition is a foodstuff, the foodstuff may be administered in a dietary regime in accordance with the usual dietary regime of the companion animal. The foodstuff may comprise 100% of the diet of the companion animal or a lesser proportion, depending on the level of prevention or treatment required. The foodstuff allows the composition to be administered with ease thus avoiding a need to supplement the companion animal's food. In addition, the foodstuff can be administered

by the animal's owner thus avoiding constant veterinary supervision. The foodstuff may be available at any outlet selling pet food products or may be available from a veterinarian. The foodstuff may be as described above according to the first aspect of the invention.

As used herein, the term "administration" also includes feeding or any other method of oral administration. Other means of administration may include tablets, capsules, injection, suppositories or any other suitable means.

Preferred features for the second aspect of the invention apply as for the first aspect *mutatis mutandis*.

The present description includes a method for preparing the composition of the first aspect of the invention.

The foodstuff can be made according to any method known in the art such as in Waltham Book of Dog and Cat Nutrition, Ed. ATB Edney, Chapter by A. Rainbird, entitled "A Balanced Diet" in pages 57 to 74 Pergamon Press Oxford.

For example, a process for the manufacture of a foodstuff as defined herein comprises mixing together ingredients with the composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline and forming a foodstuff, in particular a pet foodstuff. Heating/cooking may be applied to any one or more of the ingredients prior to, during or following the mixing.

The composition can be sprayed onto the foodstuff, mixed in with the foodstuff or incorporated into the foodstuff in a matrix. Methods of inclusion of the composition are known in the art.

The importance of the present invention is the beneficial properties of curcuminoid with either green tea polyphenol or with a combination of glycine, proline and hydroxyproline (optionally as hydrolyzed collagen). In particular, an effect which is more than the cumulative effect is seen.

A further benefit is seen with the triple combination of ingredients of: curcuminoid, green tea polyphenol and glycine, proline and hydroxyproline (optionally as hydrolyzed collagen).

The combination of the compounds of the composition of the present invention can provide a synergistic effect in terms of one or more of decreasing inflammation, decreasing catabolism and increasing anabolism.

The invention will now be further described by way of reference to the following Examples, which are provided for the purpose of illustration only and are not to be construed as being limiting on the invention.

Example 1: Individual screening of compounds

Experiments were carried out to assess the effect of several compounds on primary culture of bovine chondrocytes, in which inflammatory and catabolic processes are induced by interleukin-1beta to mimic the effect of arthritic chondrocytes.

The table below details the biomarkers that were measured throughout the experiments to show the effect of the compounds of the three metabolic pathways on chondrocytes.

Table 3: Biomarkers tested

Biomarkers produced by chondrocytes		NO
		PGE2
Genes expressed by chondrocytes	Inflammation	IL-6
		COX2
		iNOS
	Catabolism	MMP3
		ADAMTS4
		ADAMTS5
	Anabolism	COL2
		AGG

Primary culture of bovine chondrocytes in monolayer

Normal bovine articular cartilage was obtained from the metacarpal-phalangeal joint of 1 to 2 year old steers shortly after death. Full-depth articular cartilage was excised and immersed in Dulbecco's Modified Eagle Medium (DMEM) (with phenol red and 4.5 g/L glucose) supplemented with N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

(HEPES) 10 mM, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (all from Lonza, Verviers, Belgium). After three washings, chondrocytes were released from cartilage by sequential enzymatic digestions with 0.5 mg/ml hyaluronidase type IV S (Sigma-Aldrich, Bornem, Belgium) for 30 min at 37 °C, 1 mg/ml pronase E (Merck, Leuven, Belgium) for 1 h at 37 °C and 0.5 mg/ml clostridial collagenase IA (Sigma-Aldrich, Bornem, Belgium) for 16 to 20 h at 37 °C. The enzymatically isolated cells were then filtered through a nylon mesh (70 µm), washed three times, counted and filled to the density of 0.25 x 106 cells/ml of DMEM (with phenol red and 4.5 g/L glucose) supplemented with 10 % foetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine (all from Lonza, Verviers, Belgium) and 20 µg/ml proline (Sigma-Aldrich, Bornem, Belgium). Cells were seeded in a 6-well plate at 0.5 X 106 cells/well by adding 2 ml of the previously described culture medium/well and cultured in monolayer for 5 days. Chondrocytes were then cultured in monolayer until confluence (for about 2 days) in DMEM (phenol red-free and containing only 1 g/L glucose) (Lonza, Verviers, Belgium) supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline. Only primary cultures were used to ensure the stability of chondrocyte phenotype.

When cells achieved confluence, the culture medium was removed and replaced by fresh culture medium (DMEM phenol red-free and containing only 1 g/L glucose supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline) containing some nutraceuticals (12.5 µg/ml of each of them) and in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M) (RD System, Abingdon, UK).

The anti-inflammatory power of the compounds (firstly individually and then in combination) was tested by measuring the chondrocyte viability and the production of PGE2 and NO.

The compounds were added in the culture medium either before inflammation (prevention effect measurement), either simultaneously of the inflammation (treatment effect measurement).

List of compounds screened:

1) Fish oil: 18%EPA+10%DHA (DSM)
2) EPA 99% (Sigma):
3) DHA 99% (Sigma):

4) Aloe Vera (Naturex)
5) Nettle leaf extract (Naturex)
6) Resvida: 99% Resvératrol (DSM)
7) Green tea extract: 25% polyphenols of which 12.5 % are catéchines and 8% is EGCG: (Naturex)
8) Pine bark extract: Pycnogenol: 65-75% procyanidines (Biolandes)
9) Premix of vitamins including vitamin D3
10) GLM (AromaNZ)
11) collagen hydrolysate (Fortigel de Gelita [3,3kDa] : hydrolyzed pork collagen)
12) ASU (Sochim)
13) Curcuma powder : 85% curcuminoids (Naturex)

The results showed the 3 compounds to use were curcuma extract, hydrolysate collagen and green tea extract, which showed significant effects on different parameters. After that, these 3 compounds were tested in response-dose.

Example 2: Dose responses

The methodology of example 1 was followed. Four different concentrations were tested to cover the range of concentrations corresponding to 10^{-5} M, depending on the molecular weight: 0.5 µg/ml, 2.5 µg/ml, 12.5 µg/ml and 62.5 µg/ml.

The results showed that the concentration which gave the best effects without giving toxic effects was 12.5µg/ml for each compound. This is why the concentration of 12.5µg/ml was used for testing compounds in combination with each other.

Example 3: Testing particular combinations of the compounds and the synergistic effects

The method of example 1 was followed.

Supplementation with compounds

When cells achieved confluence, the culture medium was removed and replaced by fresh culture medium (DMEM phenol red-free and containing only 1 g/L glucose supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline) containing some compounds (12.5 µg/ml of each of

them) and in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M) (RD System, Abingdon, UK).

The three compounds were tested namely, curcuma extract (Naturex, Avignon, France), hydrolysate collagen (Gelita, Eberbach, Germany) and green tea extract (Naturex, Avignon, France). Curcuma extract was prepared as a 12.5 mg/ml solution in tetrahydrofuran (Merck, Leuven, Belgium) and then further diluted 1000 times in cell culture medium. Hydrolysate collagen and green tea extract were dissolved in water at the concentration of 12.5 mg/ml, filtered through a sterile mesh (0.20 µm) and then further diluted 1000 times in cell culture medium. The compounds were tested alone at the final concentration of 12.5 µg/ml or in combination (12.5 µg/ml curcuma extract + 12,5 µg/ml hydrolysate collagen; 12.5 µg/ml curcuma extract + 12.5 µg/ml green tea extract; 12.5 µg/ml curcuma extract + 12.5 µg/ml hydrolysate collagen + 12.5 µg/ml green tea extract) in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M). The effects of the compounds were compared to controls: DMEM alone or DMEM + IL-1β.

Culture stop

After 24 h in these conditions, conditioned culture medium of three wells of each condition was collected and stored at -20°C. The cells of these corresponding wells were scrapped, an RNA extraction was made using RNeasy mini kit (Qiagen, Venlo, Netherlands), a reverse transcriptase polymerase chain reaction was realised and then a quantitative real time polymerase chain reaction was realised, using the LightCycler 480 (Roche, Vilvoorde, Belgium) to analyse gene expression.

After 48 h in these conditions, conditioned culture medium of the remaining wells (3 of each condition) was collected (lactate dehydrogenase release assay) and stored at -20°C until analysis (nitrite and prostaglandin E2 assays). Cells were scrapped and homogenized in 500 µl of Tris-HCl buffer by ultrasonic dissociation for 20 s at 4 °C, to measure DNA content.

Lactate dehydrogenase release assay

Cell viability was estimated by quantifying the release of lactate dehydrogenase (LDH) in the culture supernatant. A 100 µl sample of the supernatant or dilutions of standard solution (LDH from rabbit muscle) was mixed with 50 µl of Tris buffer (10 mM Tris-HCl (pH 8.5), 0.1 % bovine serum albumin) containing 800 mM lactate. Then, 50 µl of colorimetric

reagent, 1.6 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich, Bornem, Belgium), 4 mg/ml nicotinamide adenine dinucleotide (Roche Diagnostics, Brussels, Belgium), and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) were added, and the absorbance at 492 nm was read after 10 min of incubation at room temperature.

DNA assay

Chondrocytes were homogenized in 500 µl of Tris-HCl buffer by ultrasonic dissociation for 15 s at 4 °C. DNA content was measured in the cell extracts using the fluorimetric method of Hoechst.

Nitrite assay

Nitric oxide (NO) production was determined by quantifying its derived product, nitrite, in the culture supernatant using a spectrophotometric method based upon the Griess reaction. Briefly, 100 µl of the supernatant or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 µl of Griess reagent (0.5 % sulphanilamide, 0.05 % naphtyl ethylene diamine dihydrochloride, 2.5 % H_3PO_4). The absorption was measured at 540 nm. The production of NO was expressed per microgram of DNA.

PGE2 assay

Prostaglandin E2 (PGE2) production was measured in the culture supernatant using the DetectX PGE2 High Sensitivity Immunoassay kit (Arbor Assays, Michigan, USA). Briefly, 100 µl of the supernatant or PGE2 standard dilutions were pipetted into a clear microtiter plate coated with an antibody to capture mouse IgG. A PGE2-peroxidase conjugate (25 µl) is added to the standards and supernatants in the wells. The binding reaction is initiated by the addition of 25 µl of a monoclonal antibody to PGE2. After an overnight incubation at 4°C, the plate is washed and 100 µl of substrate is added. The substrate reacts with the bound PGE2-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated colour is detected at 450 nm wavelength. The production of PGE2 was expressed per microgram of DNA.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT PCR)

RNA from cells from 3 wells of each condition was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands). Then, RNA was reverse transcribed. Quantitative real time

Polymerase Chain Reaction (PCR) was performed by using the SYBR Premix Ex Taq (Tli RNaseH Plus) (Westburg, Leusden, Netherlands). The PCR template source was either first-strand cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA were as follows: bovine HPRT forward and reverse primers: 5'-AGTTTGGAAATACCTGGCG-3' and 5'-AGTCTTTAGGCTCGTAGTGC-3'; bovine interleukin (IL)-6 forward and reverse primers: 5'- TGGTGATGACTTCTGCTTTCC-3' and 5'- TGCCAGTGTCTCCTTGC-3'; bovine cyclooxygenase (COX)2 forward and reverse primers: 5'-GTCTGATGATGTATGCCACC-3' and 5'-ACGTAGTCTTCAATCACAATCT-3'; bovine induced nitric oxide synthase (iNOS) forward and reverse primers: 5'-GGCAAGCACCACATTGAGA-3' and 5'- TGCGGCTGGATTTCGGA-3'; bovine aggrecans (AGG) forward and reverse primers: 5'-TGCCTTTGACGTGAGC-3' and 5'-GCATTGTTGTTGACAAACT-3'; bovine type II collagen (COL2) forward and reverse primers: 5'-CTGCGTCTACCCCAAC-3' and 5'-GGGTGCAATGTCAATGAT-3'; bovine metalloproteinase (MMP)-3 forward and reverse primers: 5'-TCTATGAAGGAGAAGCTGACATAAT-3' and 5'-TTCATGGGCAGCAACAAG-3'; bovine A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) 4 forward and reverse primers: 5'- CTTTCAATGTCCCACAGGC-3' and 5'-CAGGAACGGAAGCGGGTA-3'; bovine ADAMTS 5 forward and reverse primers: 5'-GACACCCTGGGAATGGCA-3' and 5'- CACAGAACTTGGAATCGTCA-3'.
Amplification was performed with a spectrofluorometric thermal cycler (LightCycler 480, Roche Diagnostics, Vilvoorde, Belgium). To standardize mRNA levels, we amplified HPRT, a housekeeping gene, as an internal control. Gene expression was normalized by calculating the ratio between the number of cDNA copies of IL-6, COX2, iNOS, AGG, COL2, MMP-3, ADAMTS4, ADAMTS5, and that of HPRT.

Results were expressed as the mean percentage of increase compared to the control. Statistical significance was assessed using the t-test. Differences were considered statistically significant at $p < 0.05$. Table below details the results provided when combining the compounds and the synergistic effects observed.

Table 4

			Control	Curcumin (=C)	Hydrolyzed collagen (=O)	Green tea (=T)	CO	CT	COT
Chondrocyte produced biomarkers	Inflammation	NO	0	0	0	0	0	0	0
		NO+IL1B	100	-96*	23**	-14*	-100***	-100*	-100***
		PGE2	0	0	0	0	0	0	0
		PGE2+ IL1B	100	-99*	1**	363**	-100*	-100***	-100***
Genes expressed by chondrocytes	Inflammation	IL-6	100	-82*	307**	252**	-43*	-38*	-65*
		IL-6+ IL1B	100	-84*	-1*	-8*	-89*	-100*	-99*
		COX2	100	49**	54**	86**	378**	84**	27**
		COX2+IL1B	100	-51*	-24*	-26*	-58*	-87*	-92*
		iNOS	100	-76*	123**	207**	-65***	-27***	-25***
		iNOS+IL1B	100	-86*	-13*	15**	-91*	-96*	-97*
	Catabolism	MMP3	100	40**	109**	161**	91*	-10***	-15***
		MMP3+IL1B	100	-58*	-20*	-22*	-85*	-99*	-99*
		ADAMTS4	100	-21*	12**	9**	16**	-14*	-58***
		ADAMTS4 + IL1B	100	-55*	-23*	-28*	-68*	-83*	-84*
		ADAMTS5	100	-16*	2**	32**	-31***	-28***	-41***
		ADAMTS5+ IL1B	100	-47*	-13*	22**	-52*	-76***	-71***
	Anabolism	COL2	100	-77**	13*	18*	-84**	-62**	-74**
		COL2+IL1B	100	-57**	-2**	55*	-67**	29***	67***
		AGG	100	-77**	54*	33*	-78**	-30**	-53**
		AGG+ IL1B	100	-77**	-4**	123*	-82**	186***	337***

(* Beneficial effect; ** Negative effect; *** More beneficial than expected)

Discussion

The results of the combinations were better than the additive effect of each compound. An explanation is that because compounds act on different metabolic ways which are related, when there is inflammation, catabolism increases and anabolism decreases. Thus, our non-limiting hypothesis is that curcumin inhibits inflammation induced by IL-1 β (and also induced by collagen and green tea). Once the inflammation is inhibited, catabolism decreases and collagen and green tea polyphenols can have their positive effect on anabolism. Given arthrosis is a vicious circle (inflammation induces catabolism which induces inflammation, etc), when catabolism decreases (and anabolism increases), there is a decrease of inflammation and we recover a virtuous circle.

Moreover, in general, in healthy cells there is always a balance between catabolism and anabolism. We saw that the combinations could have positive effects on the metabolism

of healthy cells (with no induction of inflammation by IL-1β). It is very interesting because, in case of arthrosis or before arthrosis, cells which are still in good health can be protected by our combinations.

CLAIMS

1. A composition comprising curcuminoid with green tea polyphenol and with a combination of glycine, proline and hydroxyproline for use in the prevention and/or treatment of osteoarthritis.

2. A composition for use as claimed in claim 1, wherein the prevention and/or treatment is for a human or a pet companion such as a cat, a dog or a horse.

3. A composition for use as claimed in any one of claims 1 to 2, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen.

4. A composition for use as claimed in any one of claims 1 to 3, wherein the curcuminoid is curcumin.

5. A composition for use as claimed in any one of claims 1 to 4, wherein the composition is in the form of a foodstuff.

6. A composition for use as claimed in any one of claims 1 to 5, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

7. A composition for use as claimed in any of claims 1 to 6, wherein the green tea polyphenol is present at an amount of about 0.01% to 1.1% on a dry matter basis.

8. A composition for use as claimed in any of claims 1 to 7, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5% to 10% on dry matter basis.

9. A composition for use as claimed in any one of claims 1 to 8, wherein the curcuminoid is curcumin and is present at an amount of about 0.005% to 0.15% on a dry matter basis.

10. A method of preventing or treating osteoarthritis in a mammal, comprising the step of administering to said mammal a composition comprising curcuminoid with green tea polyphenol and with a combination of glycine, proline and hydroxyproline.

11. The method of any one of claim 10, wherein the mammal is a human or pet companion such as a cat, a dog or a horse.

12. The method of any one of claims 10 to 11, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen.

13. The method of any one of claims 10 to 12, wherein curcuminoid is curcumin.

14. The method of any one of claims 10 to 13, wherein the composition is in the form of a foodstuff.

15. The method of any one of claims 10 to 14, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

16. The method of any one of claims 10 to 15, wherein the green tea polyphenol is present at an amount of about 0.01 to 1.1% on a dry matter basis.

17. The method of any one of claims 10 to 16, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5 to 10% on a dry matter basis.

18. The method of any one of claims 10 to 17, wherein the curcuminoid is curcumin and is present at an amount of 0.005% to 0.15% on a dry matter basis.

19. A composition comprising curcuminoid, green tea polyphenol and a combination of glycine, proline and hydroxyproline.

20. A composition as claimed in claim 19, wherein the combination of glycine, proline and hydroxyproline is hydrolysed collagen.

21. A composition as claimed in claim 19 or claim 20, wherein the curcuminoid is curcumin.

22. A composition as claimed in any one If claims 19 to 21, wherein the composition is in the form of a foodstuff.

23. A composition as claimed in any one of claims 19 to 22, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

24. A composition as claimed in any one of claims 19 to 23, wherein the green tea polyphenol is present at an amount of about 0.01% to 1.1% on a dry matter basis.

25. A composition as claimed in any one of claims 19 to 24, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5% to 10% on dry matter basis.

26. A composition as claimed in any one of claims 19 to 25, wherein the curcuminoid is curcumin and is present at an amount of about 0.005% to 0.15% on a dry matter basis.

27. A process for the preparation of a composition as claimed in any one of claims 19 to 26 comprising mixing together the ingredients and optionally presenting the mixture in a form suitable for composition and administration.

28. A process as claimed in claim 27, wherein the composition is added to the mixture in the form of a spray, coating or in a starch pocket matrix.

29. A composition as claimed in any one of claims 19 to 26, for use in medicine.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2014/059850

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/059850

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/82 A61K38/00 A61K45/06 A61K31/05 A61K31/353
A61P19/02
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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/106191 A1 (BIOXTRACT S A [BE]; PRIEM FABIAN [BE]; JACQUEMOND-COLLET INGRID [BE]) 23 September 2010 (2010-09-23) page 18 - page 19; example 1 page 26 - page 28; example 6 claims 1, 16, 20, 21 -----	1-29
Y	KR 2012 0033633 A (UNIV ULSAN FOUND FOR IND COOP [KR]) 9 April 2012 (2012-04-09) claims 1, 6-8 paragraph [0018] - paragraph [0022] -----	1-29
Y	WO 00/74662 A2 (UNIV SHEFFIELD [GB]; BUTTLE DAVID [GB]; ADCOCKS CLAIR [GB]; COLLIN PET) 14 December 2000 (2000-12-14) page 8 claims 1, 2, 6 -----	1-29

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2014	14/08/2014

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	Uryga-Polowy, V

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	BENITO-RUIZ P ET AL: "A randomized controlled trial on the efficacy and safety of a food ingredient, collagen hydrolysate, for improving joint comfort", INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION, CARFAX PUBLISHING LTD, GB, vol. 60, no. Suppl. 2, 1 January 2009 (2009-01-01), pages 99-113, XP009139049, ISSN: 0963-7486, DOI: 10.1080/09637480802498820 abstract page 111	1-29

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOMÃ AA TRÄ ET AL: "Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs. glucosamine sulphate (GS) in the treatment of knee osteoarthritis (KOA)", INTERNATIONAL ORTHOPAEDICS, SPRINGER, BERLIN, DE, vol. 35, no. 3, 19 April 2010 (2010-04-19) , pages 341-348, XP019887381, ISSN: 1432-5195, DOI: 10.1007/S00264-010-1010-Z abstract page 341, column 2, paragraph 2 page 345 page 348 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/059850

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