Abstract:

Title: PURIFIED GRAPE SEEDS EXTRACTS, PRODUCTION AND USE THEREOF FOR THE TREATMENT OF CENTRAL AND PERIPHERAL HUMAN DISEASES

The invention describes the preparation of new grape seed extracts and their use in the treatment of various human pathological conditions, such as vascular diseases, cognitive disorders, depressive disturbances or clinical pictures of physical and mental frailty and weakness.
Purified grape seeds extracts, production and use thereof for the treatment of central and peripheric human diseases

This invention describes new cocoa and grape seed extracts and the process for their production, their use and the use of their mixtures for the treatment of symptoms associated with cognitive, vascular or depressive disorders, or in cases in which there is a need to increase physical strength such as for example in the case of athletes or in the case of asthenic conditions of varying origin.

Thus a first aspect of the invention is a description of a new purified cocoa extract (CE) having a high concentration, above 20% by weight (w/w), of flavan-3-ols (as monomers and dimers) and the flavan-3-ols/xanthine concentration ratio of more than five, together with a method for its preparation from commercial products.

A second aspect of the invention relates to description of the preparation of a new plant extract from grape seeds (GSE), a natural substance which is also rich in polyphenols, like cocoa beans, and has a high concentration of flavan-3-ols of between 16% and 25% by weight, through an extraction and purification process.

A third aspect of the invention relates to characterisation of the pharmacological and pharmacokinetic properties of the cocoa and grape seed extracts obtained according to the processes described in the invention, and also the characterisation of an appropriate mixture thereof to verify any synergistic properties of such an association.

Finally a further aspect of the invention relates to a description of a pharmaceutical composition comprising both purified plant extracts, that is cocoa extract (CE) and grape seed extract (GSE). This pharmaceutical composition is preferably a solid form for oral administration, a preferred formulation comprises a mixture comprising 50% by weight of both the plant extracts according to the invention, or another formulation may relate to a mixture comprising the same quantity of flavan-3-ols for each of the two purified plant extracts.

The polyphenols present in cocoa beans (Theobroma cacao) have been shown to possess beneficial effects, due to their antioxidant action, on various pathological conditions, such as hypertension (Desideri, Hypertension, 2012;60:794-801), vascular diseases (Esser, Faseb Journal, 13-239384, Dec 3,2013), cognitive deficit (Sokolov, Neurosci Biobehav Rev, 2013,37,2445-53), states of prolonged physical exercise (endurance) or asthenia (Davison, Eur J. Nutr, 2012,51, 69-79). These
polyphenols include flavanols and their oligomers, but also their polymers and other inactive or undesired components such as tannins, xanthines, fats, ash, etc., all substances which reduce the pharmacological activity exhibited by the active components, of which there are essentially four, that is (+)-catechin, (-)-epicatechin, and their two oligomers, procyanidin B1 and procyanidin B2.

In general plant extracts which have received very little purification have been used in all these studies; in fact, to our knowledge, methods of extraction and purification which have made it possible to obtain plant extracts with a level of flavan-3-ol concentration similar to that described in this invention have not hitherto been described in the scientific or patent literature, neither has the preparation and use of their mixtures for the treatment of various diseases in man, such as cardiovascular, cognitive or humoral conditions (depression), and situations of physical weakness (asthenia) of various kinds.

In addition to the publications cited above there is an extensive patent literature which describes methods for extracting polyphenols from cocoa beans and grape seeds and their use.

By way of example, among the patents relating to cocoa beans we would cite US6015913 (2000), which describes a method for extraction from cocoa beans providing a product in which the polyphenol concentration in the dry product is the same as that in the beans from which it has been derived; patent US6312753B1 describes a method for the preparation of a solid fat-free cocoa extract with a procyanidin pentamer content of at least 50 mg/g of dry product; patent US8632829B2 describes cocoa extracts rich in polyphenols obtained using various extraction processes, but without precisely indicating the percentages of the individual (monomer and dimer) components; US2008/0131565 A1 describes a process for extraction from cocoa beans yielding a product having a polyphenol concentration of approximately 2.5% in the dry extract (25mg/g); CA2615046 describes methods of extraction of polyphenols from cocoa beans, methods which retain the total quantity of polyphenols in the extract that are present in the beans, in an amount of not less than 33 mg/g of fat-free solid cocoa; W099/45797 claims a product comprising a mixture of cocoa polyphenols and arginine, which will bring about an increase in the production of NO and in which the procyanidin content is not less than 0.3% by weight of the product; finally we would also cite patent US2011/0280933 which claims a product for the treatment of pulmonary damage with an inhalatory pharmaceutical composition containing a mixture of epicatechin and its oligomers in a concentration of 10 mg/g of cocoa extract.
As far as the patent literature relating to polyphenol extracts from grape seeds is concerned, we would cite among others WO2014/013122 A1, which describes the preparation of an extract rich in polyphenols obtained from white grapes yielding a product having a gallic acid content of between 8 and 50 \( \mu g/g \) of dry husk, and a catechin and epicatechin content of between 0.3 and 1.28 mg/g and 0.3 and 0.8 mg/g of dry husk respectively, flavan-3-ol concentrations which are appreciably lower than those obtained by the method according to the invention; patent US7767235B2 describes a grape extract having a high polyphenol content, over 80% by weight, which in addition to high concentrations of monomers and dimers nevertheless also contains high concentrations of trimers, tetramers and pentamers; patent US6544581B2 describes a new method for the extraction of polyphenols from grape seeds after these have been caused to germinate, but the composition and quantity of the polyphenols present in the aqueous extract is not described; finally patent US6544581B1 describes a new method for the extraction and purification of polyphenols from grape seeds to yield a product having a polyphenol content of between 5% and 45% by weight, in which the monomer content, as a percentage of the dry powder, is, according to what has been described, nevertheless not much more than 10%.

This invention therefore describes the preparation of purified extracts of cocoa beans and grape seeds using extraction and purification processes on unpurified commercial cocoa bean and grape seed extracts, their chemical/physical and pharmacological characterisation, and their use both alone and in suitable mixtures as active substances for the treatment of various human diseases.

**Method for the preparation of the purified cocoa extract (CE) having a high flavan-3-ols content**

The starting material is a commercial cocoa powder obtained from washed and dried cocoa beans, generally characterised by a total flavan-3-ols content of less than 5% by weight, a total polyphenols content, expressed as gallic acid, of less than 50% by weight and a flavan-3-ols/xanthines ratio of less than 3.

The process comprises various passes which are described below by way of example.

**Extraction stage:**
The commercial cocoa powder (10 kg) is treated with 50-80% w/vol., preferably 65%, aqueous ethanol in a weight/volume ratio of between 5 and 20, preferably 10, with mechanical stirring, at a temperature of between 35°C and 40°C for a time of not less than 2 hours.
Filtration and first concentration stage:
The part of the cocoa powder which is not extracted is removed by filtration using a press filter or by centrifuging. The extraction liquid containing mainly polyphenols and xanthines is concentrated by evaporation under vacuum until a liquid residue containing approximately 30% w/vol. of ethanol and a dry residue of around 4% w/vol. is obtained.

Xanthine content reduction stage:
The concentrated water/alcohol mixture is purified by liquid/liquid extraction with an organic solvent which is immiscible with water, preferably methylene chloride or ethyl acetate, which extracts the xanthines present in the natural product, namely caffeine and theobromine, which might give rise to serious contraindications to use of the product because of their hypertensive activity.

Second concentration stage:
The water/alcohol phase purified from xanthines is concentrated to a volume having a dry residue of approximately 10% w/vol., filtered again to remove the insoluble fraction and then passed to a further purification, which is described in the next paragraph.

Purification stage:
This purification process takes place through chromatography on a column packed with an adsorbent resin, preferably using a moderately polar acrylic, non-ionic resin, such as Amberlite® XAD7HP; the concentrated water/alcohol extract is eluted with ethanol so as to obtain further enrichment of the polyphenols.

Final concentration and drying stage:
The liquid eluted from the chromatography column is concentrated under vacuum to remove the solvent in order to obtain a dry material content of more than 75% by weight and then dried under vacuum to yield approximately 1 kg of highly purified product, which should comply with the analytical purity specifications indicated below. The product prepared in this way has the appearance of a brown powder, with a slightly aromatic smell and a bitter taste.

In the appended drawings, Figure 1 is a graph of UPLC analyses of the purified cocoa extract (CE).

Analysis by means of inverse phase liquid chromatography (UPLC) using a UV detector to determine the flavan-3-ols and xanthines yielded the following results expressed as % by weight
(w/w) concentrations of the corresponding elution peaks, the retention times (in minutes) being indicated in brackets:
theobromine 3.6% (2.8 min); procyanidin B1 1.1% (5.5 min); (+)-catechin 6.1% (6.9 min); caffeine 0.17% (7.5 min); procyanidin B2 6.9% (9.2 min); (-)-epicatechin 9.6% (10.7 min). The total flavan-3-ols therefore amount to 23.7%, total xanthines to 3.77%, and total polyphenols, expressed as gallic acid (UV method), 90.1%.

In comparison, the unpurified starting commercial product had the following chromatography values: theobromine 1%; procyanidin B1 0.05%; (+)-catechin 0.8%; procyanidin B2 1.6%; caffeine 0.3%; (-)-epicatechin 0.96%; total flavan-3-ols 3.4%; total xanthines 1.3%; total polyphenols 31%.

Thus using the purification process described above a % increase in total flavan-3-ols of approximately quite seven times that the starting commercial product was obtained, and the flavan-3-ols/xanthines ratio increased by about two and a half times (approximately 6.3 for the purified product against 2.6 for the starting commercial cocoa powder).

Method for the preparation of purified grape seed extract (GSU) having a high flavan-3-ols content

The starting material is a washed and dried grape seed powder generally characterised by a total flavan-3-ols content of less than 5% by weight, and total polyphenols content, expressed as gallic acid, of less than 50% by weight; in grape seeds, unlike cocoa beans, xanthines are virtually absent.

The process comprises various stages, which will be described below by way of example.

Extraction stage:
The grape seed powder (10 kg) was first treated with deionised water in a weight/volume ratio of between 5 and 20, preferably 10, with mechanical stirring, at a temperature of between 50°C and 70°C, preferably 60°C, for a time of not less than 2 hours.

Filtration and first concentration stage:
The part of the grape seed powder which was not extracted was removed by filtration using a press filter or by centrifuging. The extraction liquid containing mainly polyphenols was concentrated by evaporation under vacuum to obtain a liquid residue containing a dry residue in a quantity of between 25 and 35% weight/volume, preferably around 30% weight/volume.
First purification stage:
Part of the less soluble extract is removed in this stage; thus the concentrate obtained in the previous stage is diluted with deionised water to a dry residue concentration of around 10%. The product is allowed to stand with stirring for approximately ten hours at a temperature of preferably below 20°C, filtered again to remove the insoluble fraction, while the aqueous stage is passed to a further purification described in the next paragraph.

Column purification stage:
This purification stage takes place in a chromatography column packed with an adsorbent resin, preferably using a moderately polar acrylic non-ionic resin, such as Amberlite® XAD7HP; the polyphenols present in the aqueous phase placed in the column are adsorbed by the resin, while other extraneous substances are not retained and are therefore removed by elution. Subsequently the adsorbed polyphenols are recovered by elution with aqueous alcohol, preferably 90% w/vol. alcohol.

Final concentration and drying stage:
The liquid eluted from the chromatography column is concentrated under vacuum to remove the solvent to yield a dry material content of more than 75% by weight and then dried under vacuum to yield approximately 0.8-1 kg of highly purified product, which should comply with the analytical purity specifications indicated below. The product prepared in this way has the appearance of a brown powder, with a slightly bitterish taste.

In the appended drawings, Figure 2 is a graph of the UPLC analysis of the purified grape seed extract (GSE).

Analysis by reverse phase liquid chromatography (UPLC) with a UV detector at 278 nm to determine flavan-3-ols, was carried out using a Lichosphere C-18 column and a mobile phase gradient comprising 0.3% phosphoric acid/acetonitrile at a temperature of 40°C. The following results expressed as % concentration by weight (w/w) of the corresponding elution peaks were obtained, the retention times (in minutes) being indicated in brackets:
procyanidin B1 5.5% (7.5 min); (+)-catechin 5.7% (9.9 min); procyandin B2 3.1% (12.1 min); (-)-epicatechin 4.3% (10.7 min). The total flavan-3-ols therefore amounted to 18.7%. 
In comparison, the unpurified commercial starting product yielded the following chromatography values: procyanidin B1 1.3%; (+)-catechin 1.8%; procyanidin B2 0.9%; (-)-epicatechin 0.7%; total flavan-3-ols 4.7%.

Thus through the purification process described above a % increase in total flavan-3-ols of approximately four times that of the starting commercial product was obtained.

Pharmacological activities of the plant extracts according to the invention:

CARDIOVASCULAR ACTIVITY

The flavan-3-ols present in the cocoa and grape seed extracts are powerful antioxidants and "scavengers" of species reacting with oxygen, and can therefore help to improve endothelial function in the hypertensive animals.

Effect of various cocoa, grape seed extracts and their mixtures on the "ex-vivo" vascular function of spontaneously hypertensive rats

The vascular effects of the purified extracts were studied "ex-vivo" on the isolated aortas of spontaneously hypertensive rats. The animals were treated with the compounds under investigation, cocoa extract (CE), grape seed extract (GSE) and their 1:1 mixture by weight (CAGR), 50 mg/kg/5ml, orally for fifteen days, or with distilled water (control group).

After the animals had been sacrificed under ether anaesthesia, the aortas were sampled, cut to a ring, attached to an isometric pressure transducer in a bath for isolated organs at 37°C under a flow of 95% O₂ - 5% CO₂ and exposed to a contracting concentration of 0.3 μmolar phenylephrine (PE) and subsequently to a cumulative dose-response curve of acetylcholine (Ach) in the range (10⁻⁹ - 3:10⁻⁶ mol/L).

The relaxing activity of the acetylcholine was expressed as a % of the pre-contraction induced by the PE. The maximum relaxing activity induced by Ach in the groups treated with the purified plant extracts was always significantly greater than that of the controls (71%) and specifically 95% for the mixture (CAGR), against 92% for the CE group and 80% for the GSE group.
Effect of the plant extracts according to the invention on arterial pressure in spontaneously hypertensive rats.

The intention was to investigate the effect of the purified CE and GSE extracts and their 1:1 mixture by weight (CAGR) following sub-chronic oral treatment (15 days) with a dose of 10 mg/kg/day in spontaneously hypertensive rats in comparison with a group of control animals treated with an equivalent volume of distilled water. Systolic arterial pressure was measured on day 1 of the treatment at different times following administration, while the sub-chronic effect was assessed 24 hours after the last treatment.

5/6 animals were used per group, and all had to have a mean basal arterial pressure of not less than a 200 mmHg. No significant effect on arterial pressure was observed after the first day of treatment, but after 15 days of treatment there was a fall in systolic pressure from the basal values of between 10% and 15% in all the groups of animals treated with the plant extracts according to the invention. For example, in the EC treatment group the basal value of 230 mmHg changed to a value of 203 mmHg after 15 days' treatment, while in the group of control animals there was a slight increase in pressure: 220 mmHg against an initial value of 214 mmHg.

**ACTIVITY POTENTIATING MUSCULAR STRENGTH**

It has been described how epicatechin increases performance in physical exercise (endurance), probably because of its antioxidant activity. We therefore wished to examine endurance in animals treated with the plant extracts according to the invention in comparison with epicatechin in a series of experiments performed ex-vivo after two weeks of oral treatment with the compounds under investigation.

**Ex-vivo experiments and mechanism of action**

Thus four groups of animals were treated orally with 30 mg/kg/day of purified cocoa extract CE and the CAGR mixture dissolved in 5ml/kg of H₂O, in comparison with a group of control animals and a group of animals treated with 10 mg/kg of epicatechin.

Fatigue resistance was assessed on the "digitorum longus" extensor muscle (DLE); the muscle was placed in a bath for isolated organs and attached to a pressure transducer; on being placed between
two platinum electrodes it was stimulated with a current of 500 mA at a frequency of 100 Hz for 1 second every two seconds, for a total time of 30 seconds.

At the end of the experiment, that is after 30 seconds of stimulation, the following results were obtained, expressed as % of the original contraction force:
Control group 28%; CE group 36%; Epicatechin group 38%; CAGR group 48%.

From a statistical analysis performed on the areas subtended beneath the curve, described by the parameters % strength against time (in seconds) during the 0-30 seconds interval, a significant difference (P<0.05) was only obtained for the comparison between the control groups and the CAGR group, while the difference between the control group and the other two groups under investigation, which was obviously different, did not however reach an acceptable level of significance (P>0.05).

Nitric Oxide (NO) is a fundamental endothelial mediator and, depending upon its concentration and the site of production, mainly gives rise to an important function on cellular homeostasis, giving rise to powerful anti-inflammatory, antioxidant and vasodilative activities.

It was therefore the intention to assess levels of the expression of neuronal Nitric Oxide Synthase (n-NOS) in both the cytoplasm fraction and the sarcolemma fraction in these groups.

Briefly, the DLE extensor muscles were homogenised in buffer at pH 7.4 and then centrifuged to yield a soluble fraction (cytosol) and an insoluble pellet, which as a result of successive purifications and centrifuging yielded the sarcolemma, that is the membrane covering the fibres of the muscular tissue. All the samples which had been suitably treated with multipurpose rabbit anti-n-NOS were then analysed by means of Western Blotting. The results were expressed as the intensity of response in comparison with controls.

In general there was an increased expression of n-NOS in all the groups in comparison with the controls; the greatest differences were found with the cytosol fraction. Thus the CAGR group demonstrated a three-fold increase in n-NOS in both the cytosol and sarcolemma fractions, the CE demonstrated a two-fold increase in the expression of n-NOS in the cytosol and 1.5 times in the sarcolemma, while epicatechin was only active in the cytosol fraction with an approximately 2-fold increase in the expression of n-NOS.
In further confirmation of the endothelial activity demonstrated by the plant extracts according to their invention and their possible mechanism of action we intended to assess the vaso-relaxing effect of CAGR extract on isolated rat aorta.

In brief, thoracic rat aortas were removed and cut to a ring with or without functional endothelium; they were then mounted as previously described in a bath of isolated organs, fixed to an isometric pressure transducer and held at 37°C with a flow of O2/CO2 (95%-5%). After a period of stabilisation the aortic rings were caused to contract with phenylephrine and subsequently treated with increasing concentrations of CAGR.

Also in order to characterise the involvement of endothelial NO, some arteries with intact functional endothelium were exposed first to the addition of CAGR in a 10 \( \mu \text{mol} \) concentration of L-Name, an inhibitor of NO-Synthase.

In aortas provided with an intact endothelium CAGR in a concentration of 10 \( \mu \text{g/ml} \) produced a relaxation of 80% in the precontracted tone induced by phenylephrine, while it was completely inactive in the experiments performed with aortas without endothelium. In addition to this, blocking of the expression of NO-Synthase induced by L-Name on intact aortas, that is those provided with endothelium, also in this case caused the CAGR to be completely inactive, confirming that its vaso-relaxing activity is largely associated with activation of NO-Synthase expression.

Finally it is interesting to note that a sample of commercial cocoa (Barry Collabaut) in the same concentration of 10 \( \mu \text{g/ml} \) produced only a weak vaso-relaxing effect (10%).

**Determination of endurance in vivo: mouse swimming test**

In order to confirm the activity of plant extracts according to the invention demonstrated on muscular force in vitro in the experiments described above, we intended to evaluate their effect in an in vivo experiment, that is their resistance in the swimming test.

Mice which were individually placed in a plastic tank (44 x 60 x 34 cm) thermostated to 27°C were used; a weight equal to 5% of their body weight was attached to their tails in order to reduce swimming time, which would otherwise be needlessly excessively prolonged. The animals were considered to be exhausted and therefore recovered when they no longer succeeded in rising to the
surface to breathe for a period of seven seconds. The experiment was performed on the first, seventh and fourteenth days of treatment, that is three trials for each animal.

The treatment groups, 5 animals per group, comprised distilled water (control group) and the three groups of plant extracts according to the invention, administered in a dose of 40 mg/kg/day. The results obtained are summarised in the table below, which shows that all the groups relating to plant extracts according to the invention increased endurance in comparison with the controls; this increase was significant on the fourteenth day of treatment, but no significant difference in the effects of treatments was achieved between the extracts, perhaps in this case because of the relatively small number of animals used in the experiment, although the difference between the CE and the CAGR groups was almost on the limit of significance.

| Endurance: time (sec) of forced endurance during swimming |
| Groups | Day 1 | Day 7 | Day 14 |
| Controls | 740 | 600 | 445 |
| CE | 745 | 620 | 540 |
| GSE | 710 | 630 | 630 |
| CAGR | 770 | 695 | 670 |

*The values are the averages for 5 animals/group*

COGNITIVE ACTIVITIES

It was intended to study the effects of treatment with the plant extracts according to the invention on cognitive process in rats.

Passive avoidance

We evaluated the improvements in memory in both naïve rats and rats with a memory deficit induced by treatment with scopolamine through passive avoidance experiments, which comprise evaluating the degree of memory in animals after they had been taught the following.

The equipment used was a box divided into two by a guillotine door; one compartment was lined with black while the other was illuminated by a 60 W bulb. An animal placed in the illuminated part tends to move into the dark: at this point the door closes and the animal receives an irritating
shock of 0.3 mA for a time of 3 seconds (foot shock = FS). The retest to evaluate memory was carried out after 72 hours or 24 hours in the case of the animals treated with scopolamine; a retest comprised placing the animal in the illuminated compartment and recording how long it took to move into the dark (step through latency = STL) up to a maximum time of 120 seconds (cut-off time). If the animals remember that they received a shock in the dark they will not move from the intense light, even though this is an unpleasant stimulus.

The results are expressed as the percentage change in STL, in seconds, in the treated groups in comparison with the control groups, using the following formula:

\[ \% \text{effect} = 100 \times \frac{\text{(treated-controls)}}{\text{120-controls}} \]

The compounds under investigation were administered orally in a dose of 40mg/kg/day for three weeks before training and using the same dose but twice a day during the period between training and retest. In the experiments with scopolamine this was administered in a dose of 1 mg/kg i.p. thirty minutes before training.

On retest, that is 72 hours after FS, the following results, expressed as \% of effect for the various groups treated with the plant extracts in comparison with controls, were obtained:

As will be seen from the data shown above, again in this case all the groups belonging to the plant extracts proved to be more active than the controls in potentiating the memory of the animals and in particular this potentiation proved to be significantly greater (P<0.05) for the CAGR Group.

In the case of the amnesic treatment induced by scopolamine (S), in which the treatment groups in each individual experiment were a control group (FS), a control group (FS+S) and a treated group (T+S = TS), the results were analysed using the following formula:

\[ \% \text{effect} = 100 \times \frac{\text{(TS - controls (FS+S))}}{\text{FS - controls(FS+S)}} \]

Again in this case the most effective treatment was that with the CAGR group, which showed a remarkable 65\% increase in memory in comparison with the untreated controls, followed by the CE Group (53\% increase) and the GSE Group (41\% increase).
These results suggest that treatment with the plant extracts according to the invention and specifically the treatment with the combined CAGR mixture may be useful particularly in the case of cognitive disorders induced by malfunctions of the cholinergic system.

**Object Recognition Test**

In order to confirm these results obtained in rats through passive avoidance experiments we studied the effect of the products on amnesia induced by scopolamine in a second experimental model carried out on mice, which comprises discriminating two objects, an already known old one from a new one (object recognition test).

In brief, the experiment comprises causing animals individually placed in a wooden box illuminated with 120 lux to get to know two identical objects, in this case two wooden spheres 8 cm in diameter, allowing them to explore the objects for 20 minutes; this is the so-called acquisition stage, in which explorative behaviour is defined as behaviour which keeps the animal at a distance of not more than 1 cm from the object. Subsequently, after 24 hours, one of the two objects was replaced by a new different object, in this case a rectangular piece of wood (6x6x1 1 cm) and the animal was allowed to explore the old object and the new object again under the same conditions for 20 minutes: recognition stage.

Before the experiment the animals were divided into 5 groups of 6; two groups both treated with distilled water for two weeks (control groups) and the other three groups for the same period of time with 40 mg/kg/day per os, with each of the plant extracts according to the invention. Thirty minutes before the retest, that is of the recognition stage, four groups, except one of the two control groups, were treated with 1 mg/kg i.p. scopolamine in order to deteriorate their memory performance.

In the control group which was not treated with scopolamine a difference of 29% was recorded in exploration time between the old object and the new object, while in the control group plus scopolamine this difference was almost eliminated (5%). All the three other groups treated with the plant extracts plus scopolamine significantly increased the percentage difference in exploration between the two objects in comparison with the controls, 24% for the CE group, 18% for the GSE group, and 25% for the CAGR group, confirming that treatment with the plant extracts according to the invention may be useful in the treatment of cognitive disorders due to malfunction of the cholinergic system.
ANTIDEPRESSIVE ACTIVITY

The potential antidepressive activity of the plant extracts according to the invention and their mixture (CAGR) was evaluated in CD1 mice in the experiment known as "tail suspension (TST)".

The animals were suspended by the tail using adhesive tape at a height of 35 cm above floor level in groups of five. After a first minute of settling down, which was ignored, during which the animals were almost continuously active attempting to regain a horizontal posture, they then tended to hang more or less passively, entering into a state of resignation; the state of immobility, in seconds, during the total observation period (240 seconds) was then measured.

Before the experiment the plant extracts dissolved in water were administered orally in a dose of 20 mg/kg/10ml for three weeks in comparison with a control group treated with distilled water.

Again in this case the more effective treatment was obtained with the CAGR mixture, with an immobility period of 34 seconds, that is approximately half that achieved with the control group (72 seconds of immobility); the CE and GSE groups proved to be almost equally effective with an immobility time of 50 and 42 seconds respectively.

BIOAVAILABILITY EXPERIMENTS

In addition to the efficacy demonstrated in vitro and in vivo on the models provided by way of example above, the characteristic and important advantage of these bean and seed extracts to which this invention relates is their optimum oral bioavailability. Thus, for example, we evaluated the oral absorption of grape seed extract GSE, the mixture of extracts, CAGR, and a commercial cocoa powder (Barry Collabaut) all administered orally, dissolved in distilled water, in a dose of 30 mg/kg in rats. The absorption of epicatechin, catechin and procyanidin B2 present in the extracts was determined by calculating the corresponding plasma concentrations over time after administration, determining the value of the areas subtended beneath the concentration/time curve (AUC). The results so obtained are summarised in Table 2 below.
From the kinetic data provided above it is apparent that the purified grape seed extract GSE and the CAGR mixture revealed a high plasma concentration of flavan-3-ols, especially catechin, which explains the high in vivo activity brought about by the compounds to which the invention relates, while the comparison commercial compound revealed very low level of flavan-3-ols, or levels which were even below the instruments' detection levels.

In general we can say that in all the experiments performed both "in vitro" and "in vivo" the CAGR mixture proved to be more effective, probably also due to a favourable synergistic reaction between the components, than the other groups, that is the CE and GSE plant extracts, which in turn were generally significantly better than the controls, distilled water, or in some cases, epicatechin or commercial cocoa, with a slight prevalence of CE over GSE in the cognitive tests and the opposite in the endurance experiments.

Having regard to all the experimental results stated above, that is the various pharmacological activities such as cardiovascular activity, muscular strength potentiating activity, cognitive and antidepressive activities and their favourable pharmacokinetic characteristics, it is permissible to conclude that use of the purified plant extracts according to the invention, and above all use of their appropriate mixtures such as for example a 50% by weight mixture of each extract, may be of assistance in the treatment of various human diseases, such as for example hypertensive and vascular diseases, or cognitive disorders, or above all those syndromes characterised by a general condition of physical weakness which may also be correlated with depressive or cognitive deficit conditions, which can be grouped together with that which is now recognised as a proper clinical picture of weakness and physical and mental frailty expressed by the scientific term of "physical frailty", and which is particularly of use in geriatric medicine.

Preparation of a prototype tablet with a CAGR mixture of extracts

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<th>Epicatechin</th>
<th>Catechin</th>
<th>Procyanidin B2</th>
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<tr>
<td>GSE</td>
<td>28</td>
<td>46.4</td>
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<tr>
<td>CAGR</td>
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<td>B. Collabaut</td>
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Table 2: AUC values expressed as (ng*h)/ml
It was intended to establish whether a mixture of extracts and cocoa beans and grape seeds according to the invention prepared in a ratio such that the provision of flavan-3-ols was the same for each component could be adapted to the preparation of tablets by means of simple direct compression of the powders.

Prototype tablets containing 1017 mg of active ingredients, that is the plant extracts according to the invention, equal to 432 mg of CE and 585 mg of GSE, corresponding to a flavan-3-ol content of 200 mg per tablet and a total unit weight of 1300 mg, were therefore prepared. Microcrystalline cellulose PH200 (215 mg) was used as excipient, cross-linked sodium carboxymethyl cellulose (32 mg) was used as the disaggregating agent, and talc (13 mg), colloidal silica (10 mg) and magnesium stearate (13 mg) were used as lubricants.

Thus approximately 10,000 tablets in 21 x 9.8 mm format were prepared by direct compression in accordance with above formulation without any particular problems of adhesion to metal surfaces or problems with uniformity of the weight of the tablets.

In vitro bioavailability tests using these tablets, carried out by means of the dissolution test according to USP method 2 (Paddle) in 0.05 M phosphate buffer, pH 7.4, 37°C, stirring speed 50 rpm, yielded satisfactory results, in that more than 85% of the active ingredients dissolved within the limit time of 30 minutes.

In conclusion, the mixture of plant extracts according to the invention has suitable characteristics for the preparation of oral pharmaceutical forms in the form of tablets.
CLAIMS

1. Process for the preparation of a purified grape seed extract, characterized in that it comprises the following steps:
   a) extraction step in which a raw grape seed powder is treated with deionized water, in a volume/weight ratio between 5 and 20 times and at a temperature ranging between 50°C and 70°C;
   b) filtering and first concentration step in which the product of extraction obtained in a) is subjected to filtration for the removal of exhausted powder to obtain an extraction liquid which is subsequently concentrated by evaporation under vacuum;
   c) first purification step in which the concentrate obtained in b) is diluted with water, left under stirring at a temperature of less than 25°C and re-filtered to obtain an aqueous phase from which the insoluble phase has been substantially removed;
   d) purification step of the aqueous phase obtained in c) carried out on column packed with adsorbent resin able to adsorb the polyphenols and subsequent recovery of said polyphenols by elution with a hydro-alcoholic eluent, preferably ethanol;
   e) final step which consists in concentrating under vacuum the hydro-alcoholic eluate obtained in d) to obtain a product with a dry content exceeding 75% by weight, which is finally dried to give a product containing an amount of flavan-3-ols between 16% and 25%.

2. Process for the preparation of a purified grape seed extract according to claim 4, wherein in the extraction step a) a water/raw grape seed powder ratio of 10 times is preferably used at an extraction temperature of 60°C, in the first purification step c) the concentrate diluted with water is left under stirring at a temperature of 10°C-15°C for a time not less than 10 hours and in which the adsorbent resin used in purification step d) is a moderately polar acrylic resin.

3. Process according to claims 1 or 2, wherein in the filtration and first concentration step b), the concentration is carried out to obtain a liquid having a content of dry solid residue from 25 to 35% weight/volume.

4. Purified grape seed extract obtainable by the process according to one of claims 1 to 3, containing a quantity of flavan-3-ols between 16 and 25% by weight and with a content of catechin and epicatechin exceeding 15% by weight.

5. Grape seed extract according to claim 4 for use in the treatment of cognitive and vascular disorders, depressive syndromes or forms of asthenia in oral administration to an individual suffering from one of said disorders.
6. Grape seed extract according to claim 5, wherein the treatment comprises the administration of said purified extract to an individual suffering from one of said disorders, in a quantity corresponding to a dose of 100 to 400 mg of flavan-3-ols.

7. Pharmaceutical composition comprising a purified grape seed extract according to claim 4 in association with pharmaceutically acceptable excipients for oral administration.

8. Pharmaceutical composition according to claim 7 for use in the treatment of cognitive and vascular disorders, depressive syndromes or forms of asthenia.

9. Pharmaceutical composition according to claim 8 wherein said treatment comprises the administration of a dose of 100 to 400 mg of flavan-3-ols contained in said composition.
FIG. 1: UPLC analysis of purified cocoa extract CE

FIG. 2: UPLC analysis of purified grape seed extract GSE
According to International Patent Classification (IPC) or to both national classification and IPC

A61K36/87 A61K36/185 A61P1/14 A61P25/28

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

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

EPO-Internal, BIOSIS, INSPEC, BEILSTEIN Data, CHEMABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search

23 October 2015

Date of mailing of the international search report

30/10/2015

Name and mailing address of the ISA

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