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(54) Title: A PROCESS FOR THE PREPARATION OF CRYSTALLINE AND NON HYGROSCOPIC PHENOLIC RICH COLORED FRACTIONS FROM PLANTS

(57) Abstract: The invention describes a green process for the isolation of non-hygrosopic and crystalline colored fractions from various phenolic rich plants such as namely *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Embllica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arbortristis*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like. The colored fractions are found to be stable at ambient temperatures and against microbial attack. The process comprises of extraction (microwave, ultrasound, percolation, reflux and Soxhlet) of dried powder of plant material with alcoholic/hydroalcoholic solvent. Concentrating the extracted solvent *in vacuo*, diluting the extract with water, filtering and then passing the filtrate through resin column for purification. Column is first washed with water to remove the water soluble hygroscopic components. Thereafter the column is eluted with alcohol to get the crystalline dye which is found to be antioxidative and phenolic rich in nature.



A PROCESS FOR THE PREPARATION OF CRYSTALLINE AND NON HYGROSCOPIC PHENOLIC RICH COLORED FRACTIONS FROM PLANTS

Field of the invention

5 The present invention relates to a process for preparation of phenolic rich crystalline and non-hygroscopic natural colors from plant extracts. Particularly, it relates to a green process for the preparation of natural colors from plant extracts wherein plants containing various phenolic compounds including flavonoids, isoflavonoids, tannins, lignans, neolignans, stilbenoids, quinones, phenolic acids and
10 their derivatives etc. are utilized to obtain colored fractions possessing multi-fold benefits like non-hygroscopic crystalline nature, improved stability, reproducibility in production of colors and solubility in both water and alcohol besides various therapeutic activities including antioxidant activity etc. The developed protocol utilizes green extraction tools like microwave and ultrasound which enhance its ecofriendly
15 nature. The process is suitable for the isolation of colored fractions from various plants namely *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arborescens*, *Quercus*
20 *infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus var. atropurpureus Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like. The colors derived from the method of present invention hold
25 immense potential for application in cosmetics (including hair colors), textiles, food and beverages, agriculture and pharmaceutical industry.

Background of the invention

30 “Natural Dye” refers to colors obtained from plants, insects and mineral substrates and are used for dyeing in cosmetics, pharmaceuticals, textiles and food industry. The plant kingdom offers a vast source of natural dyes/colors obtainable from various plant parts. The main natural dye/color substances used worldwide have been

extracted from the leaves, fruits, seeds, flowers, barks and roots of dye-yielding plants. In prehistoric times, man used to dye furs, textiles, and other objects with natural substances of vegetables, and to a lesser extent also of animal origin. With the influx of synthetic dye substitutes for natural dyes in 18th & 19th centuries, the use of natural dye
5 has ceased in most part of the world. This is due to the fact that synthetic dyes are cheaper and easier to use. However, due to stringent environmental standards imposed by many countries in response to the toxic and allergic reactions associated with synthetic dyes, there is a revival of interest in the use of natural dyes/colors for various applications.

10 Many of the plants are reported for their medicinal properties and find diverse usage in traditional system of medicine. For example:

Rhododendron arboreum (Ericaceae), locally known as Baras is common in western Himalaya, occurring mainly at 5000–8000 feet. The roots, leaves and flowers of *R. arboreum* are important crude source of drugs in traditional and modern system of
15 medicines. The flowers are also used to cure dysentery, fever and headache and are known to possess anti-inflammatory activity [Swaroop et al., Chromatographia, 2005, 62, 649–652].

Similarly, *Hippophae rhamnoides* (Elaeagnaceae) commonly known as Sea buckthorn has been extensively used in traditional medicine system of Tibet, Mongolia
20 and China for the treatment of circulatory disorders, wound healing, skin disorders, hepatic injury etc [Beveridge et al., *J. Agric. Food Chem.*, 1999, 47, 3480-3488; Eccleston et al., *J. Nutr. Biochem.*, 2002, 13, 346-354]. Modern medicinal researches have shown *H. rhamnoides* to be a highly effective radioprotective, hepatoprotective, gastroprotective agent [Sharma R.K., Arora R., in *Herbal Drugs: A Twenty First*
25 *Century perspective*, Jaypee publications, New Delhi, 2006, pp 317-321].

Similarly other plants like *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arbortristis*, *Quercus infectoria*, *Juglans nigra*,
30 *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia*, *Stevia*

rebaudiana, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like are used by locals over centuries as food, dyes and for medicinal purposes.

Natural dyes/colors are friendlier to the environment than synthetic dyes as they exhibit better biodegradability and generally have a higher compatibility with the environment [F.A. Nagia, R.S.R. EL-Mohamedy, Dyes and Pigments, 2006, 1-6]. A number of studies have been reported for the extraction of dyes/colors from the natural resources such as vegetables [US-4156077, US-4358286], marine organisms [US-7012093 B2], fungus [WO9831824] and other plants parts.

The following other prior art references are disclosed as below:

U.S. Patent No. US-4302200 discloses a process for extracting anthocyanin-type colors from natural products.

U.S. Patent No. US-4383833 discloses a process for natural edible dye preparation from bean husks giving red shades.

U.S. Patent No. US-4452822 discloses a process for extraction and intensification of anthocyanins from grape pomace and other material.

WO 2006061847 A1 discloses a process for the preparation of a herbal black dye from natural materials comprising the extracts of *Juglans regia*, *Indigofera tinctoria*, *Terminalia chebula*, *Acacia sinuata*, *Lawsonia inermis*, *Trigonella foenum-graecum*, *Sapindus mukorossi*, *Eclipta alba*, *Embllica officinalis*, *Acacia catechu* and *Piper betle*.

US Patent-6406503 B1 discloses a method for preparing natural black dye from walnut rinds.

US Patent No. US-3963700 discloses a process for the recovery of anthocyanin from plant sources.

US Patent No. US-5789647 discloses a process for the extraction of natural carotenoid dyes.

WO 2004072182 A2 discloses a novel method of dyeing the textile article from medicinally rich herbs.

EP Patent No. EP-0754734 A1 discloses a simple process for the preparation of natural dyes from various parts of plants such as pomegranate, dolu and tesu etc.

Some of other typical prior art references include *Bioresource Tech.*, 2005, 96, 363-372; *Industrial Crops and Products*, 1997, 6, 303-311; *Int. J. Food Sci. Tech.*, 2004, 39, 1087-1091; *Int. J. Food Sci. Tech.*, 2000, 35, 5-22; *J. Cleaner Production*, 2003, 11, 499-509; *Int. J. Pharmaceutics*, 2002, 241, 293-299; *J. Agric. Food Chem.*
5 2003, 51, 2772-2776; *Industrial Crops and Products*, 2003, 17, 199-207.

In most of the prior art, generally water or hydroalcoholic mixture is used for the extraction which on lyophilization or spray drying at high temperatures gives color yielding products. However, a major problem encountered with above methods is that the colors generally become viscous/hygroscopic after some air exposure as extracts are
10 usually prepared using water or hydroalcoholic mixture as a solvent leading to presence of various types of sugar moieties, fatty acids or other primary metabolites along with required color product. Ultimately the colored product becomes highly susceptibility towards microbial attack. So such dyestuffs require additives such as preservatives and pest protective agents as mentioned in European patent application EP 0754734A1 so
15 as to increase shelf life.

The use of acids or bases during extraction of colors from natural sources is also widespread as mentioned in US patent 4156077 and EP 0754734A1. Such harsh extraction procedure might result in degradation of some active components resulting in decreased therapeutic activity and lower interest among consumers to use them in
20 food items and cosmetics.

Moreover, the content of color active compounds in plants generally varies with environmental variations, storage conditions and type of extraction resulting in the variations in color yielding properties of extracts.

Further, it is pertinent to mention that isolation of single molecule based natural
25 colors is usually tedious and hence not cost-effective due to their presence in traces. At the same time, chances of degradation of air, light and heat sensitive molecule will further deteriorate the quality of single molecule based natural dye. To overcome the above problems, it is desirable to have color rich fractions, instead of single molecule based colors, which might have better therapeutic properties and stability due to
30 synergistic effects.

For the convenience and even the necessity of finding new sources where from natural dyes/colors may be extracted, the present invention originates from research

work carried out on isolation of natural colors of plant origin, and more particularly on plants rich in phenolic compounds like flavonoids, isoflavonoids, tannins, lignans, neolignans, stilbenoids, quinones, phenolic acids and their derivatives etc. As the plants used in the invention namely *Rhododendron arboreum*, *Hippophae rhamnoides*,
5 *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycirriza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arborescens*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus
10 var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like are used by locals over centuries for food and medicinal purposes, so obtained color fraction would be safe and non toxic for human use as demonstrated by the toxicity data for dye obtained from *Rhododendron*
15 *arboreum*. Further, these phenolic rich plants have plethora of bioactive properties such as antioxidant, anticancer, antimutagenic, antimicrobial, pesticidal, antimalarial, protein binding capacity and radioprotection which will be imparted in the isolated color fraction of these plants resulting in enhanced therapeutic and commercial value. In this context, our invention discloses a simple and green process for the preparation of non-
20 hygroscopic crystalline natural colors from the extracts of various phenolic rich plants using environmentally benign extraction techniques such as microwave and ultrasound assisted extraction. The colors isolated by the present invention are found to possess several beneficial features like crystalline form, non-hygroscopic nature, improved shelf life, impervious to microbes and antioxidant properties (measured in terms of %
25 inhibition by DPPH assay) which simultaneously enhance the physico-chemical stability and medicinal profile of the isolated dyes. The presence of bioactive phenolic compounds viz. chlorogenic acid, syringic acid, *p*-coumaric acid, quercetin, quercitrin, quercetin-3-*O*-galactoside, rutin, isorhamnetin-3-*O*-glucoside, isorhamnetin and kaempferol will enhance the medicinal benefits of the isolated colors. The process was
30 applied effectively for the isolation of crystalline colored fraction from various commercially important color yielding plants like *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycirriza glabra*,

Coffea arabica, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Embllica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arborescens*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp.,
5 *Daucus carota* ssp. sativus var. atropurpureus Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like.

Objects of the invention

10

The principal object of the present invention is to provide a simple and green process of the type described in the introduction, by means of which crystalline and non-hygroscopic phenolic rich colored fractions possessing various bioactivities including antioxidant properties can be conveniently produced from different parts of
15 plants using green extraction techniques like microwave and ultrasound so as to eliminate the disadvantages associated with previous such reports.

The main object of the present invention is to provide a green, simple, efficient and commercially feasible process for isolation of color rich fractions from plants.

Yet another object of the invention is to develop a process for the isolation of
20 phenolic rich colored fractions from plant materials and is not restricted to a single plant material or plants only.

Yet another object of the present invention is to employ eco-friendly microwave technique for the extraction of natural colors.

Yet another object of the present invention is to employ eco-friendly ultrasound
25 technique for the extraction of natural colors.

Still another object of the invention is to reduce extraction time in minutes, from days, required in the conventional method for extraction.

Yet another object of the invention is to prepare a composition having enhanced bioactive properties including antioxidant and thus with increased commercial
30 importance.

Yet another object of the invention is to develop a simple purification process for the dye/colors from above mentioned plants.

Still another object of the invention is to develop a process which utilizes less or non-hazardous solvents for the extraction of color rich fractions and which can be reused.

Yet another object of the invention is to develop a simple purification process which is free from acid/base treatment during extraction and column purification.

Yet another object of the invention is to develop natural colors with multifold benefits like crystalline form, non-hygroscopic nature, stable shelf life, impervious to microbes and having antioxidant properties.

10 Summary of the invention

The present invention provides a green process for the isolation of phenolic rich crystalline colored fractions from various plants. These plants were collected from different regions of India and then dried at room temperature. The dried and ground plant parts were extracted in microwave oven, ultrasonic bath, Soxhlet extractor, reflux or simply percolated with solvent selected from a group consisting of water, methanol, ethanol, methanol/water, ethanol/water, isopropanol, butanol, hexanes, chloroform, ethers, dichloromethane, acetone, ethyl acetate, acetonitrile and mixture thereof. The extracts obtained were then concentrated *in vacuo* at temperature 40-50°C to remove the organic solvent and diluted with water. The diluted extract was filtered and passed through column (with or without vacuum) containing a stationary phase of resin selected from a group consisting of Amberlyte XAD-2, XAD-4, XAD-7, XAD-16, IRA 400, IRA 67, Dianion HP-20 and the like. The column was eluted with distilled water or water:alcohol mixture in varying ratio to remove the highly water soluble hygroscopic components which were spray dried or concentrated separately to obtain hygroscopic powder which may contain amino acids, sugars and other primary metabolites. The final elution was performed with an organic solvent selected from a group of alcohols such as methanol, ethanol, propanol and the like. The organic eluent was then concentrated *in vacuo* to obtain the crystalline colored fractions. The obtained color rich fractions were then subjected for bioactivity assay including antioxidant, toxicological evaluation and HPLC analysis.

Brief Description of the Accompanying Drawings

Table 1: Antioxidant activity and total phenolic content of isolated colored fractions and crude extracts of some plants

5 **Table 2:** The toxicological tests for the dye isolated from *Rhododendron arboreum*

Figure 1: HPLC chromatogram of alcoholic extract from *Rhododendron arboreum* (viscous material)

10 **Figure 2:** HPLC chromatogram of color rich fraction from *Rhododendron arboreum* (free flowing material)

Figure 3: HPLC chromatogram of alcoholic extract from *Hippophae rhamnoides* (viscous material)

15 **Figure 4:** HPLC chromatogram of color rich fraction from *Hippophae rhamnoides* (free flowing material)

Figure 5: Thermal stability data of *Rhododendron arboreum* dye up to 12 hours

20 **Figure 6:** Stability data indicating stable shelf life of *Rhododendron arboreum* dye up to six months at room temperature

25 **Figure 7:** Microscopic photograph of *Rhododendron arboreum* colored fractions showing crystals

Detailed description of the Invention

Accordingly, the present invention provides a process for the preparation of phenolic rich crystalline and non hygroscopic natural colors from plant extracts, the
30 above said process comprising the steps of:

- a) drying and grinding of plant material to obtain a powder;
- b) extracting the powdered material obtained in step (a) by microwave or ultrasonication or Soxhlet extraction or percolation or reflux;

- c) concentrating the alcoholic or hydro alcoholic extract as obtained in step (b) *in vacuo* to remove the organic solvent and provide the concentrated extract;
- d) diluting the concentrated extract as obtained in step (c) with water;
- 5 e) filtering the diluted extract as obtained in step (d) to remove water insoluble impurities;
- f) Passing the filtrate over a resin column selected from Amberlyte XAD 2, XAD 4, XAD 7, XAD 16, IRA 400, IRA 67, Dianion HP-20 and the like;
- 10 g) eluting the column first with water to remove highly polar water soluble hygroscopic components and subsequently with an organic solvent preferably an alcohol and collecting the organic solvent layer;
- h) concentrating the organic solvent layer of step (g) *in vacuo* to get crystalline and non-hygroscopic colored fraction which is rich in phenolic compounds;
- 15 i) spray drying or lyophilizing the highly polar water soluble hygroscopic components of step (g) which may contain amino acids, sugars and other primary metabolites.

In one embodiment of the present invention, the isolated colors are obtained from the plant parts selected from leaves, flowers, fruits, rhizomes/roots or whole plant.

20 In another embodiment of the invention, the extraction is carried out employing microwave or ultrasound or conventional methods like Soxhlet, reflux or percolation.

In another embodiment of the invention, the microwave used is selected from monomode or multimode microwaves at an operating frequency of 2450 MHz.

25 In another embodiment of the invention, the operating wattage of microwave irradiation is in the range of 150 to 700 W.

In another embodiment of the invention, extraction time required for microwave assisted extraction is in the range of 2 to 20 min, preferably 5 min.

In another embodiment of the invention, the ultrasound energy level for the ultrasonic bath is in the range of 80 to 100%.

30 In another embodiment of the invention, extraction time required for ultrasound assisted extraction is in the range of 40-100 min, preferably 60 min.

In another embodiment of the invention, the extraction is carried out with solvent selected from a group consisting of alcohol or water or mixture of alcohol/water in varying ratios or mixture of solvents with polarity ranging from non polar to polar with or without ionic liquids;

5 In another embodiment of the invention, the ratio of dry plant material to the extracting solvent is in the range of 1:1 to 1:10 by weight, preferably 1:5.

In another embodiment of the invention, the solvents used for extraction are removed at a temperature in the range of 40-45°C to get a concentrated extract.

10 In another embodiment of the invention, the dilution of concentrated extract with water or its mixture with alcohol is in ratio ranging from 1:10 to 1:50 preferably 1:20.

In another embodiment of the present invention, diluted extract is loaded on resin followed by elution with sufficient amount of water or mixture of water with alcohol in varying ratios till colorless and then finally washing the column with organic
15 solvents preferably alcohol.

In another embodiment of the invention, the eluting solvent consists of water or mixture of water and alcohol in the ratio ranging from 100:0 to 90:10 which upon spray drying or lyophilization or concentration *in vacuo* provides a hygroscopic powder which may contain sugars, amino acids etc.

20 In another embodiment of the invention, alcohol for eluting column is selected from a group consisting of methanol, ethanol, isopropanol or butanol, preferably ethanol.

In another embodiment of the invention, the concentration of alcohol is performed *in vacuo* at temperature in the range of 40-50°C to provide a color fraction
25 which is crystalline and non-hygroscopic in nature.

In another embodiment of the present invention, the term non hygroscopic implies that upon exposure to air at room temperature the colored fraction does not absorb moisture.

In another embodiment of the invention, the isolated color fractions are stable to
30 heat at a temperature up to 80°C for up to 12 hours.

In another embodiment of the invention, the isolated color fractions are a combination of various molecules including phenolic compounds, such as flavonoids and phenolic acids, which are primarily responsible for its color properties.

In another embodiment of the invention, the isolated phenolic rich color
5 fractions also possess some other Nitrogen, Sulfur or Oxygen containing secondary metabolites such as caffeine.

In another embodiment of the invention, the isolated color fractions are soluble in both alcohol and water.

In another embodiment of the invention, the isolated color fraction provides a
10 range of colors with or without the use of mordents and pH adjustment.

In another embodiment of the invention, the solvents and resin are recycled and reused multiple times.

In another embodiment of the invention, the isolated crystalline color fraction may find application in food, beverages, cosmetic, textile and pharmaceutical industry
15 as well as nano encapsulated products.

In another embodiment of the invention, the isolated colored fractions possess enhanced bioactivity including antioxidant properties.

In another embodiment of the invention, the isolated colored fractions are found to be non-allergic and non-toxic to human use.

20 In another embodiment of the invention, the above process is not applicable to plants rich in essential oils or other non polar compounds.

In another embodiment of the invention, the developed process is free from use of acid or base treatment during extraction and column purification thereby maintaining its natural attributes.

25 In another embodiment of the invention, the starting material used is selected from the medicinal plants comprising *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe*
30 *Vera*, *Nyctanthes arborescens*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Schleichera oleosa*, *Berberis* spp., *Tagetes* spp., *Lawsonia inermis*, *Hibiscus* spp., *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and vegetable

plants like *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia* and the like.

In another embodiment of the invention, the starting material used is selected from commercially important beverage plants like *Camellia sinensis*, *Hippophae rhamnoides*, *Embllica officinalis*, *Glycirriza glabra* and *Coffea arabica* which make its useful in instant beverage products.

In another embodiment of the invention, the crystalline color material obtained from tea and coffee possesses high content of caffeine making it useful for high caffeine beverage products.

In another embodiment of the invention, a decaffeinated crystalline color material obtained from tea and coffee is produced by giving washing to the diluted water extract at step 1(d) using organic solvent selected from a group consisting of ethyl acetate or chloroform followed by steps 1(f)-1(h).

In another embodiment of the present invention, the isolated color fractions are stable in air and moisture.

In another embodiment of the present invention, the isolated non-hygroscopic, phenolic rich color fractions are free from the use of preservatives as they are less susceptible towards microbial attack.

The antioxidative properties were measured with 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays and represented in terms % inhibition (Table 1). Total phenol content was estimated as GAE (Gallic acid equivalent). The toxicological data was generated for the colors (Table 2) isolated from *Rhododendron arboreum*. The HPLC analysis shows that the isolated colored fractions contain phenolic compounds such as chlorogenic acid (*Rt* 9.86), syringic acid (*Rt* 11.29), *p*-coumaric acid (*Rt* 13.33), quercetin (*Rt* 17.28), quercitrin (*Rt* 13.63), quercetin-3-*O*-galactoside (*Rt* 12.58), rutin (*Rt* 12.064), isorhamnetin-3-*O*-glucoside (*Rt* 13.58), isorhamnetin (*Rt* 20.043), kaempferol (*Rt* 19.64) that are known to possess various bioactivities. The content of these phenolic compounds was found to be higher in color fraction as compared to their parent extracts which is quite evident from the much higher absorbance of peaks observed in HPLC analysis. Also, the developed process is capable of removing both highly polar glycosides as well as non polar compounds as apparent from the HPLC investigation of methanolic extract and those of isolated colors, which further leads to

free flowing solid crystalline colored fractions. The application of modern extraction methods like microwave and ultrasound results in higher yield of colored fraction.

In view of above, the present invention discloses a simple and clean process for isolation of colored fractions from the extracts of various phenolic containing plants like *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycirriza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arborescens*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like. The colored fractions isolated by the present invention are found to possess several beneficial features like crystalline form, non-hygroscopic nature, stable shelf life, impervious to microbes and antioxidant properties which simultaneously enhance the physico-chemical stability and medicinal profile of the isolated colors. The presence of bioactive phenolic compounds viz. chlorogenic acid, syringic acid, *p*-coumaric acid, quercetin, quercitrin, quercetin-3-*O*-galactoside, rutin, isorhamnetin-3-*O*-glucoside, isorhamnetin and kaempferol is expected to enhance the medicinal benefits of the isolated colored fractions.

Further, the process can be applied to other plants like *Abrus precatorius*, *Adina cordifolia*, *Barringtonia racemosa*, *Caesalpinia sappan*, *Cassia* spp., *Dolichos biflorus*, *Entada rheedii*, *Plumbago* spp., *Mucuna pruriens*, *Garcinia mangostana*, *Mesua ferrea*, *Gymnema sylvestre*, *Helicteres isora*, *Tinospora cordifolia*, *Azadirachta indica*, other *Lawsonia* spp., *Mimusops elengi*, *Murraya* spp., other *Aloe* spp., *Phyllanthus amarus*, *Bombax malabaricum*, *Solanum xanthocarpum*, other *Syzygium* spp., *Terminalia arjuna*, *Terminalia bellirica*, *Withania somnifera*, *Vernonia anthelmintica*, *Tectona* spp., *Berberis* spp., *Urtica* spp., *Wrightia* spp., *Aegle* spp., *Juniperus communis*, other *Rhododendron* spp., other *Hippophae* spp., other *Rheum* spp., *Mangifera indica*, *Bidens* spp., *Dahlia*, *Salix caprea*, *Pterocarpus* spp., *Polygonum multiflorum*, *Hamamelis virginiana*, other *Citrus* spp., *Indigofera* spp., other *Glycirriza* spp., *Adiantum* spp., *Symplocos* spp., *Baccharis* spp., *Betula* spp., *Berginia*

spp., *Ruta graveolens*, *Swertia* spp., *Picrorhiza* spp., other *Ginkgo* spp., *Petunia* spp., *Vitis vinifera*, *Morus* spp., *Potentilla* spp., *Agrimonia* spp., *Alnus sieboldiana*, *Casuarina stricta*, *Psidium guajava*, *Bixa orellana*, *Phyllanthus* spp., *Sapium japonicum*, *Ricinus communis*, *Rhus semialata*, *Rhus succedanea*, *Tellima grandifolia*,
 5 *Dioscorea* spp., *Jatropha* spp., *Piper* spp., *Fuchsia* spp., *Euphorbia* spp., *Acer* spp., *Mallotus japonicus*, *Aleurites cordata*, *Rubus* spp., *Camellia japonica*, *Cotinus* spp., *Stachyurus praecox*, *Asculus hippocastanum*, *Persea gratissima*, *Coriandrum sativum*, *Brassica* spp., *Malus* spp., *Crocus sativus*, *Allium cepa*, *Tamarindus indica*, *Cuminum cyminum*, *Nigella sativa*, *Lonicera* spp., *Solanum lycopersicum*, *Morus* spp., *Boswellia*
 10 spp., *Pyrus* spp. *Eclipta* spp., *Rosa* spp., *Arachis hypogaea*, *Artemisia* spp., *Fagopyrum esculentum*, *Zingiber officinale*, *Vanilla* spp., *Cicer arietinum*, *Phaseolus vulgaris*, *Vaccinium* spp., *Fragaria* spp., *Prunus* spp., *Hypericum perforatum*, *Solanum trilobatum*, *Beta vulgaris*, other *Daucus carota* ssp., *Saccharum* spp. and also
 15 Mushroom, Algae, Microbes, Honey, Lac and the like which are rich in phenolics and additionally contain some other chemical moieties like heterocyclic molecules or terpenoids etc.

EXAMPLES

20 The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of the present invention.

Example 1. Isolation of colored crystalline fraction from *Rhododendron arboreum* with Soxhlet extraction

25 The dried and powdered flowers of *Rhododendron* (100 g) were taken in Soxhlet apparatus and extracted with ethanol (500 mL; sample to solvent ratio of 1:5) for 8 hours at 55-60°C. The extract was concentrated *in vacuo* to remove the solvent (The obtained concentrated extract was viscous in nature). The concentrated extract was then diluted with water (200 mL; extract to water ratio of 1:20) and filtered. The
 30 diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then the column was eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non-hygroscopic and

phenolic rich bioactive reddish brown colored fraction with yield of 9.72%. The stability of this fraction towards heat (inside oven) was also checked up to 80°C for 12 hours followed by HPLC analysis.

5 **Example 2. Isolation of colored crystalline fraction from *Rhododendron arboreum* with percolation**

 The dried and powdered flowers of *Rhododendron* (100 g) were taken in percolator and extracted with ethanol (500 mL; sample to solvent ratio of 1:5) for 48 hours at room temperature. The extract was concentrated *in vacuo* to remove the
10 solvent. The concentrated viscous extract was then diluted with water (200 mL; extract to water ratio of 1:20) and filtered. The diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column is eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain
15 crystalline, non-hygroscopic and phenolic rich bioactive reddish brown colored fraction with yield of 8.56%.

Example 3. Isolation of colored crystalline fraction from *Rhododendron arboreum* employing ultrasound and microwave

20 The dried and powdered flowers of *Rhododendron* (100 g) were taken in flask and extracted with ethanol (500 mL; sample to solvent ratio of 1:5) employing ultrasonic assisted extraction (for 60 min at room temperature at 80% ultrasonic power) and microwave assisted extraction (for 5 min in small cycles of 30 sec, multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W). The
25 extract was concentrated *in vacuo* to remove the solvent. The concentrated viscous extract was then diluted with water (200 mL; extract to water ratio of 1:20) and filtered. The diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column was eluted with ethanol. The organic
30 layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive reddish brown colored fractions with yield of 9.40 % incase of UAE and 9.89% incase of MAE.

Example 4. Isolation of colored crystalline fraction from *Rhododendron arboreum* employing other resins

The dried and powdered flowers of *Rhododendron* (5 g) were taken in flask and extracted with water (100 mL; sample to solvent ratio of 1:5) employing microwave assisted extraction (for 5 min in small cycles of 30 sec, multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W). The diluted and filtered extracts were then passed over XAD-16 and XAD-4 resin columns. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column was eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain reddish brown color fractions with the yield of 0.82% in case of XAD-4 and 1.16% incase XAD-16.

Example 5. Isolation of colored crystalline fraction from *Rhododendron arboreum* employing used resins

The dried and powdered flowers of *Rhododendron* (100 g) were taken in percolator and extracted with ethanol (500 mL; sample to solvent ratio of 1:5) for 48 hours at room temperature. The extract was concentrated *in vacuo* to remove the solvent. The concentrated viscous extract was then diluted with water (200 mL; extract to water ratio of 1:20) and filtered. The diluted extract was then passed over previously used XAD-7 resin column (resin was regenerated by washing with alcohol and water respectively). The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column is eluted with recovered ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non-hygroscopic and phenolic rich bioactive reddish brown colored fraction with yield of 8.46%.

Example 6. Isolation of colored crystalline fraction from *Hippophae rhamnoides* employing microwave

100 g dried and powdered fruits of *Hippophae rhamnoides* were extracted in microwave (multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W) with ethanol (500 mL; sample to solvent ratio of 1:5) for 5 min in small cycles of 30 sec. The extract was concentrated *in vacuo* to remove the solvent. The concentrated viscous extract was then diluted with water (200 mL; extract to water

ratio of 1:20) and filtered. The diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column was eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive brown colored fraction with yield of 5.34%.

Example 7. Isolation of colored crystalline fraction from *Rhododendron* and *Hippophae* employing microwave with water as solvent

20 g powdered *Rhododendron*'s flowers were extracted in microwave (multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W) with water (200 mL; sample to solvent ratio of 1:10) for 5 min in small cycles of 30 sec. The extract was then filtered. The filtered extract was then passed over a bed of XAD-7 resin under vacuum to hasten the process. The resin was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Resin bed was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive color fraction with the yield of 7.5%. Similarly, *Hippophae* fruits (20g) were processed with the above method giving yield of 5.2%.

Example 8. Isolation of colored crystalline fraction from *Camilla sinensis*

100 g powdered black/green tea leaves were extracted in microwave (multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W) with water (500 mL; sample to solvent ratio of 1:5) for 5 min in small cycles of 30 sec. The extract was then filtered. The filtered extract was then passed over XAD-7 resin column. The column was first washed with water: ethanol solution (90:10 v/v) to remove the highly water soluble components. This fraction was then concentrated and spray dried to get a colored hygroscopic powder containing amino acids and sugars etc. Column was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive brown colored fraction with yield of 10.34-12.68%. The HPLC analysis of isolated color fraction shows the presence of caffeine as a major constituent making it useful for high caffeine beverage products.

Example 9. Isolation of decaffeinated colored crystalline fraction from *Camilla sinensis*

100 g powdered black/green tea leaves were extracted in microwave (multimode microwave with operating frequency 2450 MHz and operating wattage of 700 W) with water (500 mL; sample to solvent ratio of 1:5) for 5 min in small cycles of 30 sec. The water extract was then filtered and partitioned with ethyl acetate to remove caffeine. The remaining water part was then passed over XAD-7 resin column. The column was first washed with water to remove the water soluble components. Column was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive brown colored fraction with yield of 7-8% which was found to be free from caffeine upon its HPLC analysis.

Example 10. Isolation of colored crystalline fractions from *Emblica officinalis* using water and ethanol as successive extraction solvent

375 g powder of dried *Emblica officinalis* was refluxed with water (3.75 L; sample to solvent ratio of 1:10) for 2 hrs. The extract was then filtered. The filtered extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components. This fraction was then spray dried to get a hygroscopic powder. Column was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non hygroscopic and phenolic rich bioactive brown colored fraction with yield of 10%.

The above remaining plant material (after extraction with water) was air dried and re- extracted with ethanol (2 L). The extract was then filtered and concentrated *in vacuo* to remove the organic solvent. The obtained extract was diluted with 1 L of hot water, cooled and filtered. The filtered extract was then passed over XAD-7 resin column. The column was eluted first with water and finally with ethanol. The organic layer was concentrated to obtain another crystalline, non hygroscopic brown colored fraction with yield of 2-3%.

Example 11. Isolation of colored hygroscopic powder from *Bixa orellana*

5 g powder of Annatto seeds (*Bixa orellana*) was extracted in microwave with water (100 mL; sample to solvent ratio of 1:20) for 5 min (monomode microwave with operating frequency 2450 MHz and operating wattage of 150 W). The extract was then

filtered. The filtered extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components. Column was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline and non hygroscopic orange-red colored fraction with yield of 5%-6%. The water fraction was concentrated and spray dried to get a yellow colored hygroscopic powder with a yield of 20%-25%.

Example 12. Isolation of colored fraction from *Curcuma longa*

5 g powder of *Curcuma longa* was extracted in microwave with water (75 mL; sample to solvent ratio of 1:15) for 3 min (monomode microwave with operating frequency 2450 MHz and operating wattage of 150 W). The extract was then filtered. The filtered extract was then passed over XAD-7 resin column. The column was first washed with water to separate the water soluble components. Column was then eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain viscous colored fraction with yield of 2-3%. The water fraction was concentrated and spray dried to get a shining yellow colored powder with a yield of 7-8%.

Example 13. Isolation of colored crystalline fraction from *Rheum emodi* with ultrasonication

The dried and powdered roots of *Rheum emodi* (5 g) were taken and extracted with ethanol (50 mL; sample to solvent ratio of 1:10) for 60 min at 40°C and 80% ultrasonic power. The extract was concentrated *in vacuo* to remove the solvent. The concentrated viscous extract was then diluted with water (100 mL; extract to water ratio of 1:20) and filtered. The diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components. Then the column was eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non-hygroscopic and phenolic rich bioactive brown colored fraction with yield of 14.98%.

Example 14. Measurement of antioxidant activity and total phenolic content of isolated colored fractions and crude extracts of some plants

The ability of the extract to scavenge DPPH[•] radicals was assessed as described by Brand-Williams et al. (*Lebensm.-Wiss. Technol.* 1995, 28, 25–30) with slight

modification. A 100 μL aliquot of ethanolic extract was mixed with 3.9 ml ethanolic DPPH $\cdot$ (3×10^{-4} mol/L) solution. After a 30 min reaction at 25°C, the absorbance was recorded at 517 nm and the capability to scavenge the DPPH $\cdot$ Radical i.e., percent inhibition was calculated using the following equation:

5 **% Inhibition = $[(A_C - A_S) / A_C] \times 100$**

where A_C is the absorption of the control reaction and A_S is the absorption of the sample solution. A control consisted of 0.1 mL of ethanol and 2.9 mL of DPPH $\cdot$ solution. All readings were taken in triplicate.

Total phenol content was estimated as GAE (Gallic acid equivalent) as described by Singleton et al. 1999 (V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Ed. L. Packer in Methods in enzymology, San Diego, CA: Academic Press, 1999, vol. 299, p. 152–178). Briefly, 200 μL aliquot of dissolved extract was transferred to a 10 ml volumetric flask, to which was subsequently added 1ml of undiluted Folin-Ciocalteu reagent and further after 1 min, 800 μL 7.5 % Na_2CO_3 was added. After 30 min incubation at 25 °C, the absorbance was measured at 765 nm and compared to a GA calibration curve.

20 **Example 15. Isolation of colored crystalline fraction from *Rhododendron* and *Hippophae rhamnoides* on large scale (Kg)**

The dried and powdered plant material of *Rhododendron* and *Hippophae rhamnoides* (1 Kg each) were taken in percolator and extracted with ethanol (5 L; sample to solvent ratio of 1:5) for 48 hours at room temperature. The extract was concentrated *in vacuo* to remove the solvent. The concentrated viscous extract was then diluted with water (1.5 L; extract to water ratio of 1:10) and filtered. The diluted extract was then passed over XAD-7 resin column. The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately. Then column was eluted with ethanol. The organic layer was concentrated *in vacuo* 45°C to obtain crystalline, non-hygroscopic and phenolic rich bioactive color fraction with yield of 8.54% for *Rhododendron arboreum* and 5.26% for *Hippophae rhamnoides*.

Example 16. HPLC analysis of colored fraction isolated from *Rhododendron arboreum* and *Hippophae rhamnoides*

HPLC analysis of the isolated colored fractions was performed on Shimadzu LC-20 instrument equipped with PDA detector (SPD M20A) using reverse phase Phenomenex Luna RP-18 column (4.6 mm i.d. × 250 mm, 5 μm). A gradient elution comprising of acidified water (0.1% acetic acid) and acetonitrile was run at a flow rate of 1 mL/min with the following program, 10 to 40% acetonitrile in 0 to 10 min, 40 to 50% acetonitrile in 10 to 15 min, 50 to 70% acetonitrile 15 to 20 min and 70 to 100% acetonitrile in 20 to 25 min. PDA detection was done at 290 nm for *Rhododendron* and 355 nm for *Hippophae*. Following molecules were identified by matching their retention time and UV spectra with reference standards: chlorogenic acid (*Rt* 9.86), syringic acid (*Rt* 11.29), *p*-coumaric acid (*Rt* 13.33), quercetin (*Rt* 17.28), quercitrin (*Rt* 13.63), quercetin-3-*O*-galactoside (*Rt* 12.58), rutin (*Rt* 12.064), isorhamnetin-3-*O*-glucoside (*Rt* 13.58), isorhamnetin (*Rt* 20.043), kaempferol (*Rt* 19.64).

15

Example 17. Experiment for stability against attack by microbes

The viscous Hippophae extract (1.0 g) and its isolated dye (1.0 g) were kept at room temperature in open atmosphere. It was observed that fungal growth started appearing on the extract after a week while no such fungal growth was observed in isolated dye material.

20

Example 18. Application of above described process to other plants species

For the application of above described process to other dye yielding plants, various plant species such as *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*, *Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arbortristis*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Berberis* spp., *Tagetes* spp., *Schleichera oleosa*, *Lawsonia inermis*, *Hibiscus* spp., *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Stevia rebaudiana*, *Acacia catechu*, *Butea* spp., *Rumex* spp., *Terminalia chebula* and the like were selected. The dried and powdered plant material (100 g; sample to solvent ratio of 1:5) was extracted in ultrasonicator with ethanol (500 mL) for 60 min at 40°C and 100% ultrasonication power. The extract was concentrated *in vacuo* to remove the

30

solvent. The concentrated extract was then diluted with water (200 mL; extract to water ratio of 1:20) and filtered. The diluted extract was then passed over XAD-7 resin column (with or without vacuum). The column was first washed with water to remove the highly water soluble components which was spray dried or concentrated separately to give hygroscopic colored powder. Then column was finally eluted with ethanol. The organic layer was concentrated *in vacuo* at 45°C to obtain crystalline, non-hygroscopic colored fractions in good yield with color ranging from light yellow to reddish brown. The yield for different plants varies between 2.38-32.16%.

Example 19: The toxicological tests* undertaken for the dye isolated from *Rhododendron arboreum*

The toxicological tests conducted at Shriram Institute for Industrial Research, Delhi undertaken for the dye isolated from *Rhododendron arboreum* were as under:

The main advantages of the present invention

The main advantages of the present invention are that it provides:

1. A novel, cost effective and industrially feasible process for the isolation of non-hygroscopic, crystalline and anti-oxidative colored fractions from different plants.
2. A process to employ eco-friendly microwave and ultrasound technique for the rapid and efficient extraction.
3. A process wherein shorter extraction time is required ranging from 5 to 40 min for microwave assisted extraction and 30-120 min for ultrasound assisted extraction, which is a remarkable reduction from the conventional extraction techniques (in days).
4. A process which is free from acid-base treatment.
5. A process in which isolated color rich fraction have multifold benefits like crystalline form, non-hygroscopic nature, stable shelf life, impervious to microbes and antioxidant properties.
6. A process in which isolated color rich fraction is rich in bioactive phenolic compounds.

7. A process for extremely easy column purification employing single eluent in remarkably short period.
 8. An ecofriendly and economical industrial process for the separation of phenolic enriched fraction from water soluble compounds.
 - 5 9. A process in which water soluble part upon concentration and spray drying provides hygroscopic colored powder.
 10. A process which utilizes less or non-hazardous chemicals.
 11. A process which allows the reuse of solvents and resins.
- 10 These advantages are of significant economic value and easy to perform on a large commercial scale production of non hygroscopic, crystalline and phenolic rich bioactive color fractions.

Table 1. Antioxidant activity and total phenolic content of isolated colored fractions and crude extracts of some plants

Name of Plant	Total Phenolic content (mg/GA equivalent)		Antioxidant activity (DPPH radical scavenging activity) % Inhibition (after 30 min of incubation)	
	Crude Extract	Dye	Crude Extract	Dye
<i>Rhododendron arboreum</i>	0.125	0.478	90.27	94.42
<i>Hippophae rhamnoides</i>	0.074	0.295	19.79	28.09
<i>Camellia sinensis</i>	0.217	0.416	90.52	91.75
<i>Quercus infectoria</i>	0.677	0.857	94.64	95.01
<i>Daucus carota</i> (Black carrot)*	0.063	0.085	8.83	20.98
<i>Rheum emodi</i>	0.266	0.337	40.56	54.36

* The % inhibition increased up to 80% after 120 min of incubation

Table 2: The toxicological tests for the dye isolated from *Rhododendron arboreum*

S. No.	Toxicological test	Result
1.	Sub Chronic oral toxicity study in rats (90 days oral dose administration) Under the conditions of this study, the 90 days repeated oral administration of "Natural Dye (obtained from <i>R. arboreum</i>)" at the dose level of 1000 mg/kg Body weight (maximum recommended dose) to albino rats did not induce any observable toxic effects when compared to control group of animals. Hence, No Observable Adverse Effect Level (N.O.A.E.L) = >1000mg/kg Body Weight.	Non toxic
2.	Passive cutaneous anaphylaxis test	Non allergenic
3.	Prausnitz-Kustner test	Non allergenic
4.	Enzyme linked immuno sorbent assay (ELISA) test	Non allergenic

WE CLAIM:

1. A process for the preparation of crystalline and non hygroscopic phenolic rich colored fractions from plants/plant parts, that exhibit enhanced bioactivity and are less susceptible towards microbial attack; the above said process comprising the steps of:
 - a) drying and grinding of plant material to obtain a powder;
 - b) extracting the powdered material obtained in step (a) by microwave or ultrasonication or Soxhlet extraction or percolation or reflux;
 - 10 c) concentrating the alcoholic or hydro alcoholic extract as obtained in step (b) *in vacuo* to remove the organic solvent and provide the concentrated extract;
 - d) diluting the concentrated extract as obtained in step (c) with water;
 - e) filtering the diluted extract as obtained in step (d) to remove water insoluble impurities;
 - 15 f) Passing the filtrate over a resin column selected from Amberlyte XAD 2, XAD 4, XAD 7, XAD 16, IRA 400, IRA 67, Dianion HP-20 and the like;
 - g) eluting the column first with water to remove highly polar water soluble hygroscopic components and subsequently with an organic solvent preferably an alcohol and collecting the organic solvent layer;
 - 20 h) concentrating the organic solvent layer of step (g) *in vacuo* to get crystalline and non-hygroscopic colored fractions rich in phenolic compounds;
 - i) spray drying or lyophilizing the highly polar water soluble hygroscopic components of step (g) which may contain amino acids, sugars and other primary metabolites.
2. A process as claimed in claim 1, wherein the colored fractions are obtained from whole plant or the plant parts selected from leaves, flowers, fruits, rhizomes/roots.
- 30 3. A process as claimed in claim 1, wherein the microwave used is selected from monomode or multimode microwaves at an operating frequency of 2450 MHz

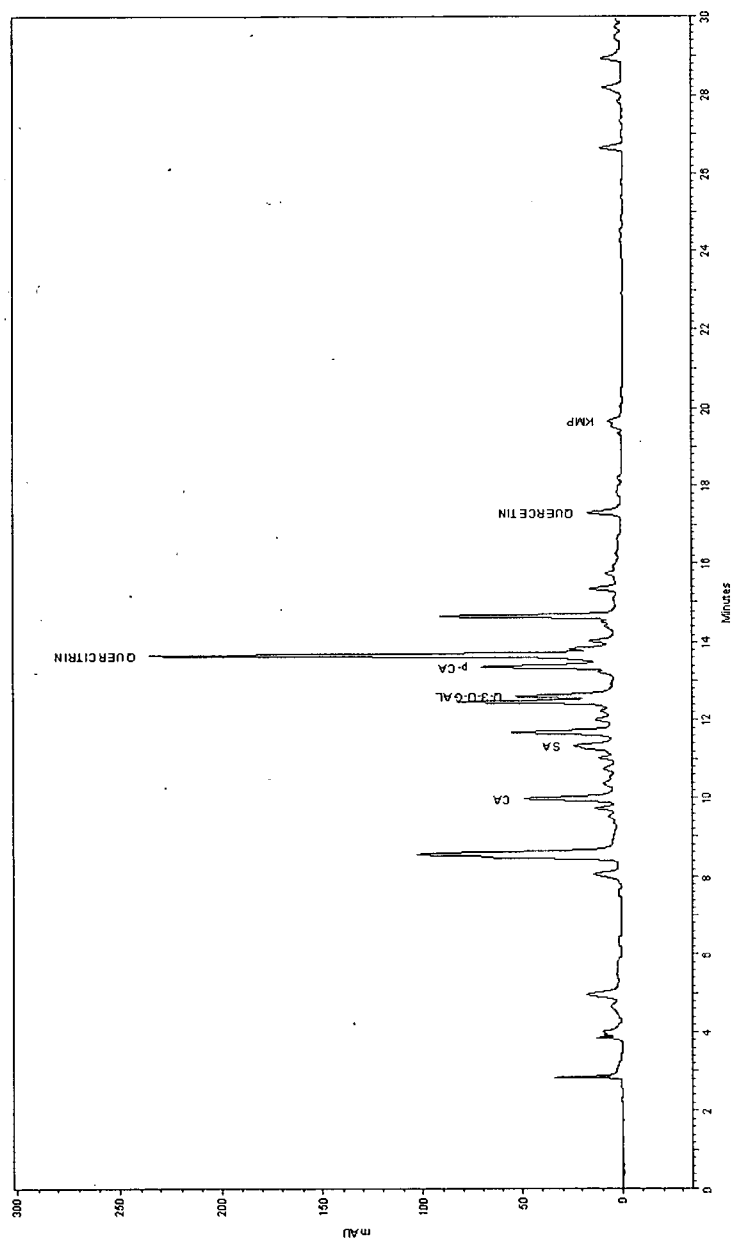
and an operating wattage of microwave irradiation in the range of 150 to 700 W,

4. A process as claimed in claim 1, wherein extraction time required for microwave assisted extraction is in the range of 2 to 20 min, preferably 5 min.
- 5 5. A process as claimed in claim 1, wherein the ultrasound energy level for the ultrasonic bath is in the range of 80 to 100%.
6. A process as claimed in claim 1, wherein extraction time required for ultrasound assisted extraction is in the range of 40-100 min, preferably 60 min.
- 10 7. A process as claimed in claim 1, wherein the extraction is carried out with solvent selected from a group consisting of alcohol or water or mixture of alcohol/water or mixture of solvents with polarity ranging from non polar to polar with or without ionic liquids;
8. A process as claimed in claim 1, wherein the ratio of dry plant material to the extracting solvent is in the range of 1:1 to 1:10 by weight, preferably 1:5.
- 15 9. A process as claimed in claim 1, wherein the solvents used for extraction are removed at a temperature in the range of 40-45°C to get a concentrated extract.
10. A process as claimed in claim 1, wherein the dilution of concentrated extract with water or its mixture with alcohol is in ratio ranging from 1:10 to 1:50 preferably 1:20.
- 20 11. A process as claimed in claim 1, wherein the eluting solvent consists of water or mixture of water and alcohol in the ratio ranging from 100:0 to 90:10 which upon spray drying or lyophilization or concentration *in vacuo* provides a hygroscopic powder which may contain sugars, amino acids etc.
- 25 12. A process as claimed in claim 1, wherein alcohol used for eluting column is selected from a group consisting of methanol, ethanol, isopropanol or butanol, preferably ethanol.
13. A process as claimed in claim 1, wherein the concentration of alcohol is performed *in vacuo* at temperature in the range of 40-50 °C to provide a color fraction which is crystalline and non-hygroscopic in nature.
- 30

14. A process as claimed in claim 1, wherein the isolated colored fractions are stable to heat at a temperature up to 80 °C for up to 12 hours.
15. A process as claimed in claim 1, wherein the isolated colored fractions are a combination of various molecules including phenolic compounds, such as
5 flavonoids and phenolic acids, which are primarily responsible for its color properties.
16. A process as claimed in claim 1, wherein the isolated phenolic rich colored fractions also possess some other Nitrogen, Sulfur or Oxygen containing secondary metabolites such as caffeine.
- 10 17. A process as claimed in claim 1, wherein the isolated colored fractions are soluble in both alcohol and water.
18. A process as claimed in claim 1, wherein the isolated colored fractions provide a range of colors with or without the use of mordents and pH adjustment.
- 15 19. A process as claimed in claim 1, wherein the solvents and resin are recycled and reused multiple times.
20. A process as claimed in claim 1, wherein the isolated crystalline colored fractions may find application in food, beverages, cosmetic, textile and pharmaceutical industry as well as nano encapsulated products.
- 20 21. The isolated colored fractions by the process as claimed in claim 1, that possess enhanced bioactivity, including antioxidant properties.
22. The isolated colored fractions from *Rhododendron arboreum* by the process as claimed in claim 21, that are non-allergic and non-toxic to human use.
23. The isolated colored fractions by the process as claimed in claim 1, that are
25 less susceptible to microbial attack.
24. A process as claimed in claim 1, wherein the developed process is free from use of acid or base treatment during extraction and column purification thereby maintaining its natural attributes.
25. A process as claimed in claim 1, wherein the plant/plant part is selected from
30 the medicinal plants comprising *Rhododendron arboreum*, *Hippophae rhamnoides*, *Camellia sinensis*, *Rheum emodi*, *Ginkgo biloba*, *Glycyrrhiza glabra*, *Coffea arabica*, *Rubia tinctorum*, *Rubia cordifolia*, *Punica granatum*,

- Emblica officinalis*, *Capsicum* spp., *Citrus sinensis* peel, *Syzygium cumini*, *Curcuma* spp., *Aloe Vera*, *Nyctanthes arbortristis*, *Quercus infectoria*, *Juglans nigra*, *Acacia nilotica*, *Schleichera oleosa*, *Berberis* spp., *Tagetes* spp., *Lawsonia inermis*, *Hibiscus* spp. *Stevia rebaudiana*, *Acacia catechu*,
5 *Butea* spp., *Rumex* spp., *Terminalia chebula* and vegetable plants like *Daucus carota* ssp. sativus var. atrorubens Alef. (black carrot), *Trigonella foenum-graecum*, *Spinacia oleracea*, *Momordica charantia* and the like.
26. A process as claimed in claim 1, wherein the plant/plant part is selected from commercially important beverage plants like *Camellia sinensis*, *Hippophae*
10 *rhamnoides*, *Emblica officinalis*, *Glycyrrhiza glabra* and *Coffea arabica* which make its useful in instant beverage products.
27. A process as claimed in claim 26, wherein the crystalline colored material obtained from tea and coffee possesses high content of caffeine making it useful for high caffeine beverage products.
- 15 28. A process as claimed in claim 26, wherein a decaffeinated crystalline colored material obtained from tea and coffee is produced by giving washing to the diluted water extract at step 1(d) using organic solvent selected from a group consisting of ethyl acetate or chloroform followed by steps 1(f)-1(h).
- 20 29. A process as claimed in claim 1, wherein the isolated non-hygroscopic, phenolic rich color fractions are free from the use of preservatives.

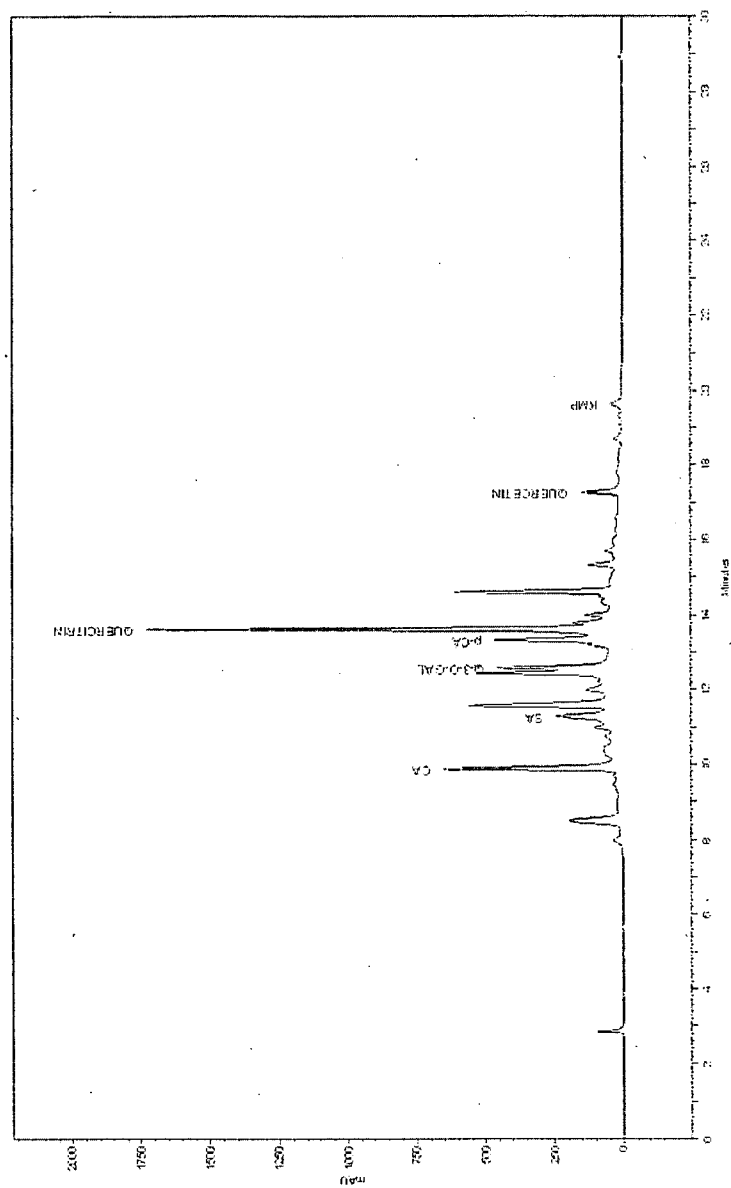
Figure 1



HPLC chromatogram of alcoholic extract from *Rhododendron arboreum* (viscous material)

Peaks: CA= chlorogenic acid, SA= syringic acid, Q-3-O-GAL= quercetin-3-O-galactoside, p-CA= *p*-coumaric acid, KMP= kaempferol.

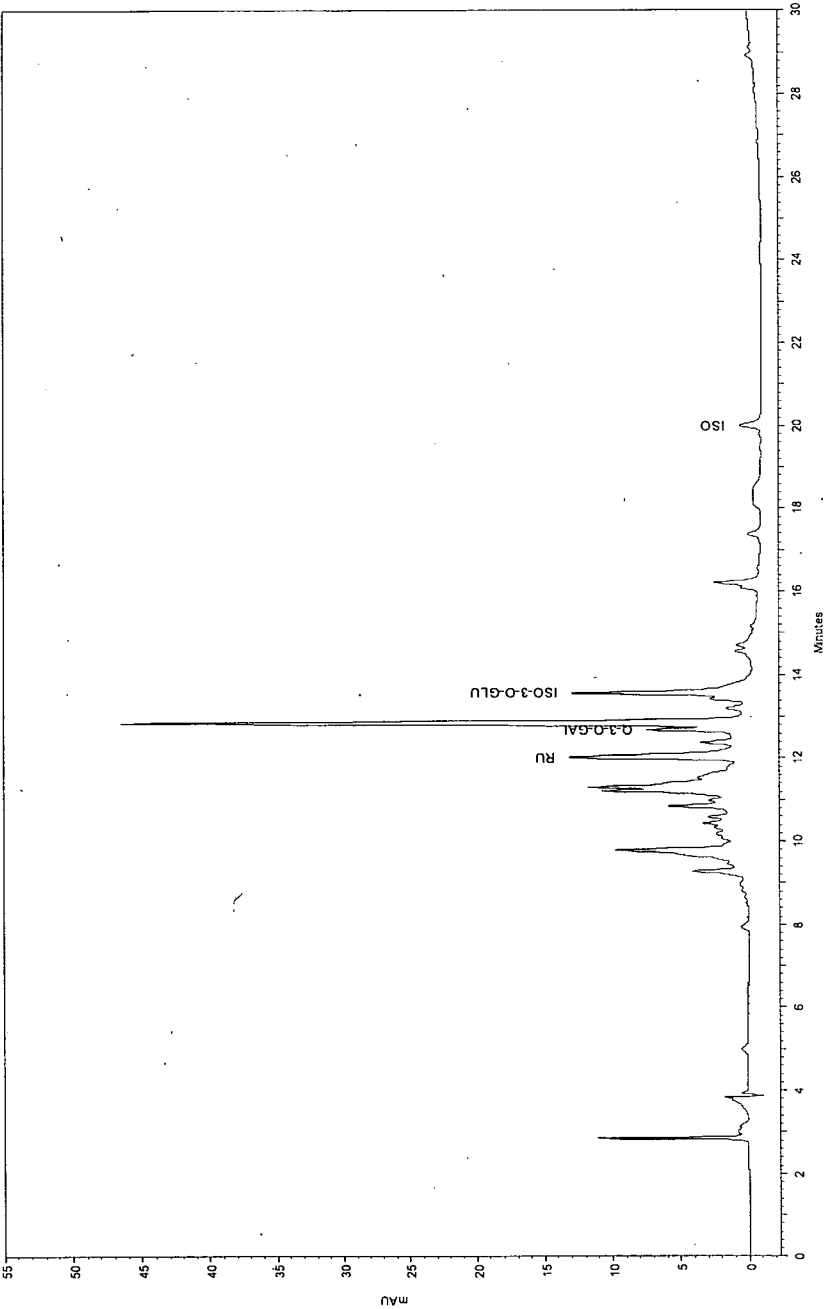
Figure 2



HPLC chromatogram of color rich fraction from *Rhododendron arboreum* (free flowing material)

Peaks: CA= chlorogenic acid, SA= syringic acid, Q-3-O-GAL= quercetin-3-*O*-galactoside, p-CA= *p*-coumaric acid, KMP= kaempferol.

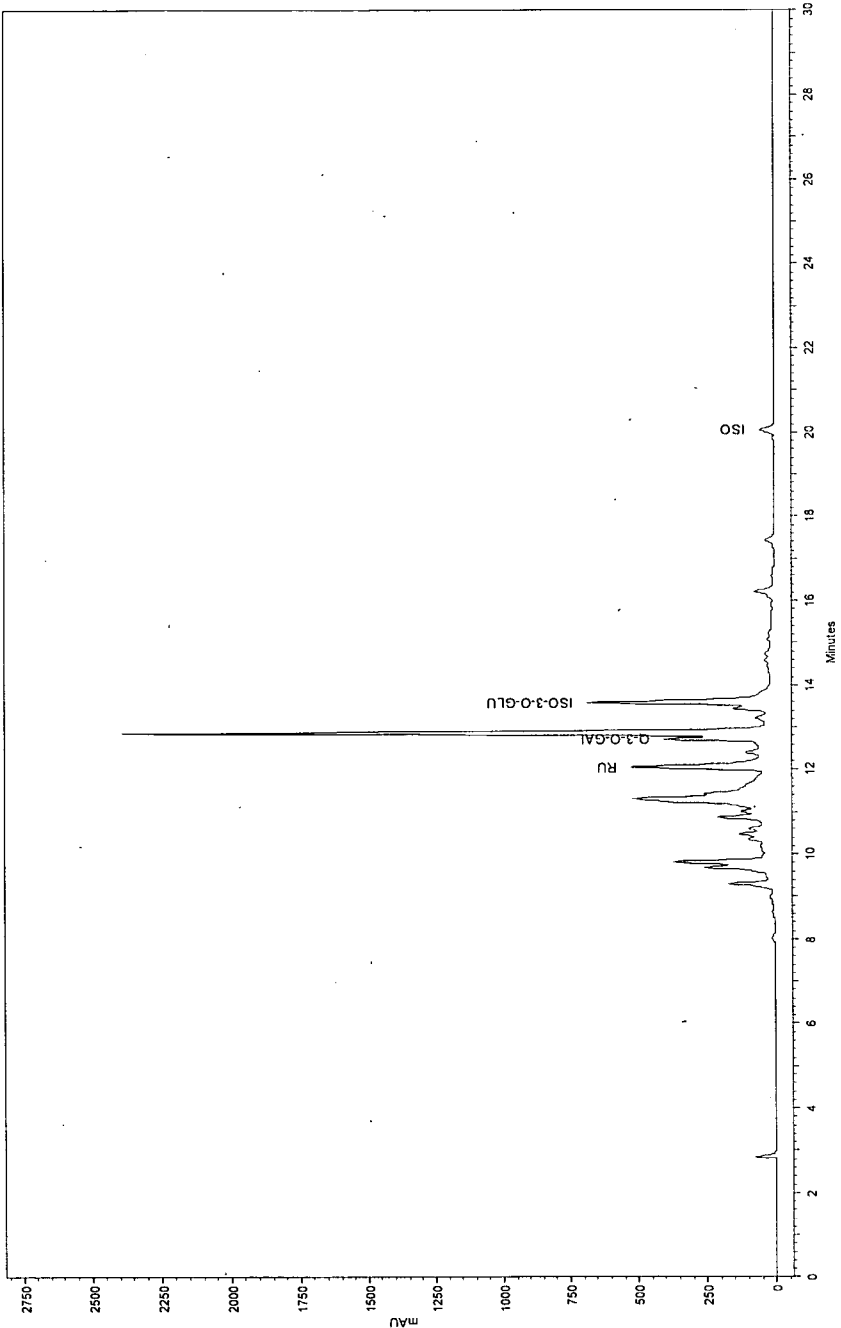
Figure 3



HPLC chromatogram of alcoholic extract from *Hippophae rhamnoides* (viscous material)

Peaks: RU= rutin, Q-3-O-GAL= quercetin-3-O-galactoside, ISO-3-O-GLU, isorhamnetin-3-O-glucoside, ISO= isorhamnetin

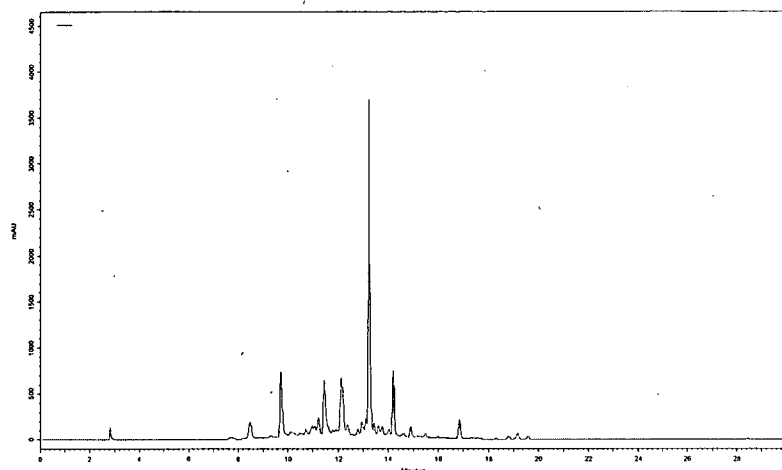
Figure 4



HPLC chromatogram of color rich fraction from *Hippophae rhamnoides* (free flowing material)

Peaks: RU= rutin, Q-3-O-GAL= quercetin-3-O-galactoside, ISO-3-O-GLU, isorhamnetin-3-O-glucoside, ISO= isorhamnetin

Figure 5 (a to d) : Thermal stability data of *Rhododendron arboreum* colored fraction up to 12 hrs



Control

Figure 5 (a): Thermal stability data of *Rhododendron arboreum* colored fraction Control (0 hrs)

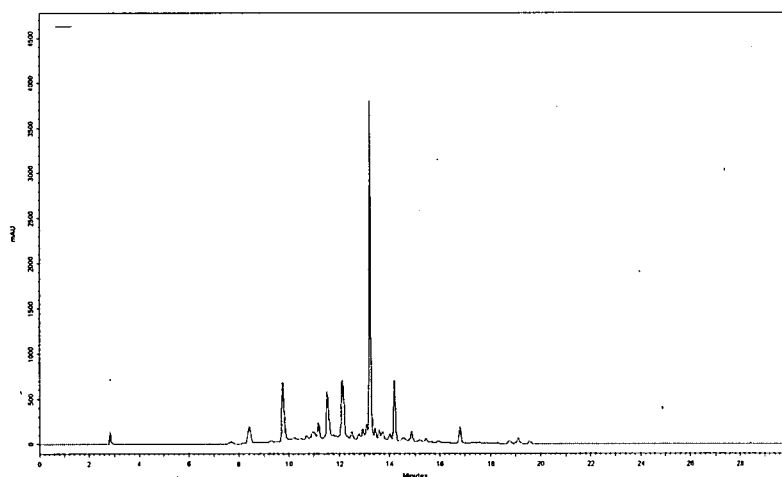


Figure 5 (b): Thermal stability data of *Rhododendron arboreum* colored fraction (after 2 hrs)

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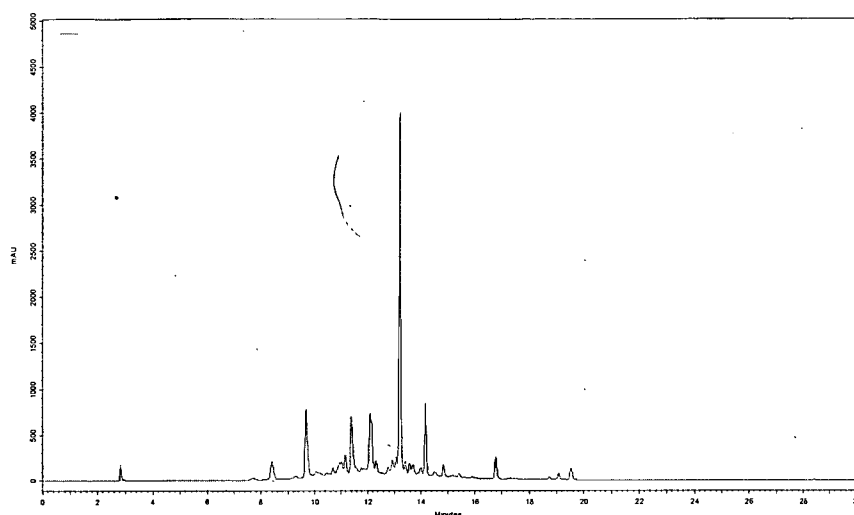


Figure 5 (c): Thermal stability data of *Rhododendron arboreum* colored fraction (after 6 hrs)

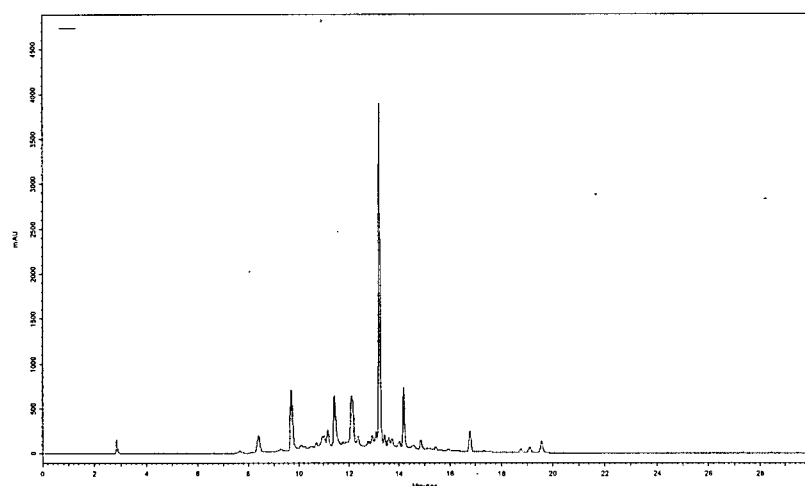
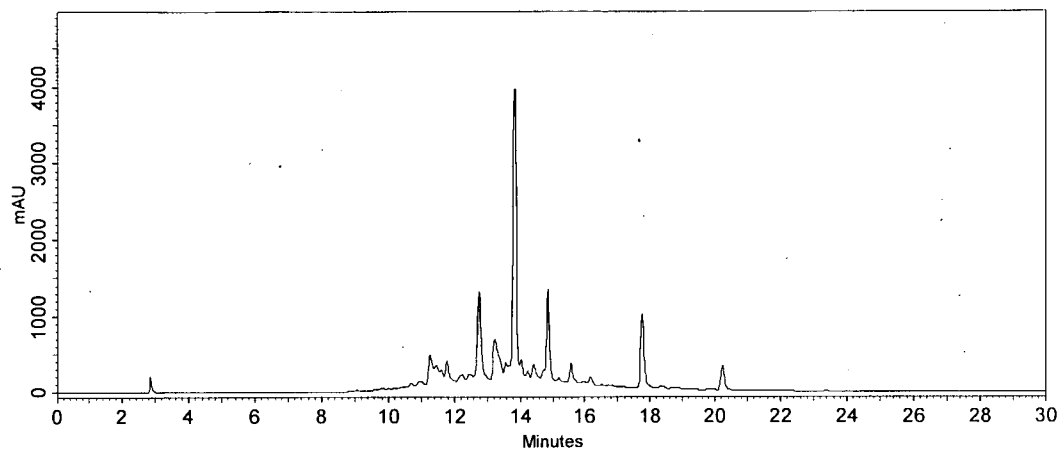


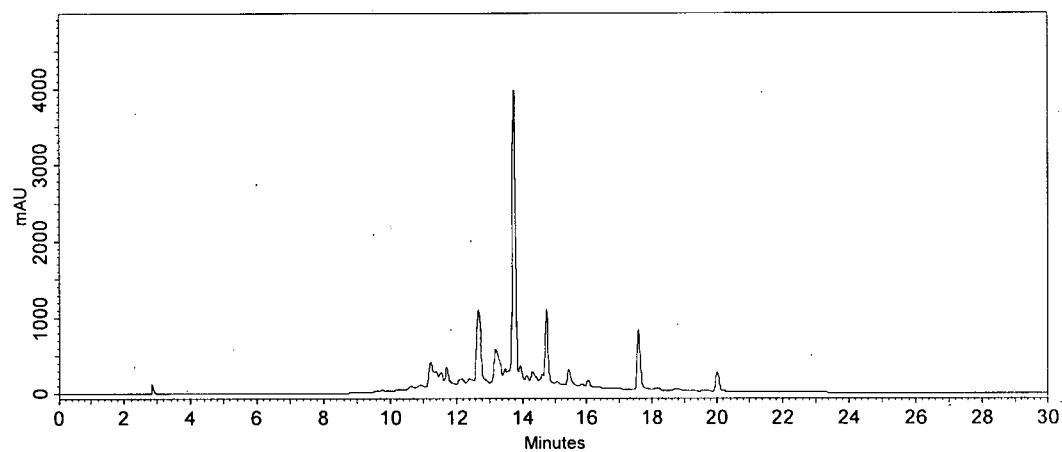
Figure 5 (d): Thermal stability data of *Rhododendron arboreum* colored fraction (after 12 hrs)

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Figure 6 (a & b) : Stability data indicating stable shelf life of *Rhododendron arboreum* dye up to six months at room temperature



(a) Control



(b) After six months

Figure 7: Microscopic photograph of *Rhododendron arboreum* colored fractions showing crystals (at 4x magnification)



INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2010/000519

A. CLASSIFICATION OF SUBJECT MATTER

INV. C09B61/00 C09B67/54

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)

C09B

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 practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A.DIMAURO ET AL.: "Recovery of Anthocyanines from Pulp Wash of Pigmented Oranges by Concentration on Resins" J.AGRIC.FOOD CHEM., vol. 50, 2002, pages 5968-5974, XP002597283	1,2, 7-24,29
Y	figures 2,3; table 1	1-29
Y	WO 02/078652 A1 (COUNCIL SCIENT IND RES [IN]; PUSHPANGADAN PALPU [IN]; MEHROTRA SHANTA) 10 October 2002 (2002-10-10) claim 49	1-29
Y	FR 1 291 155 A (BENCKISER GMBH JOH A) 20 April 1962 (1962-04-20) the whole document	1-29
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

20 August 2010

Date of mailing of the international search report

06/09/2010

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2010/000519

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International application No

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