

Great nutraceutical potential of bioactive compounds from *Beta vulgaris cicla* and *rubra*

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ABSTRACT

Beta vulgaris subsp. *cicla* (BVc, leaf beet) and *Beta vulgaris* var. *rubra* (BVr, red beetroot) belong to the *Amaranthaceae* family and have been used for centuries as food and medicinal plants. The main bioactive phytochemicals of BVr are the betalains, a group of water-soluble pigments derived from betalamic acid, which are divided into two classes: the yellow/orange-coloured betaxanthins (BX) and the red/violet-coloured betacyanins (BC). The seeds, leaves and roots of BVc are rich in phenolic acids and apigenin-derived flavonoids, namely vitexin, vitexin-2-O-rhamnoside (VOR) and vitexina-2-O-xyloside (XVX). We isolated BVc and BVr phytochemicals in our laboratory and tested them individually and in combination for their anticancer and anti-inflammatory activity. In cancer cells, vitexin flavonoids were able to induce intrinsic apoptosis, while betalains induced extrinsic apoptosis. Combinations of two or three molecules exhibited synergistic antioxidant, anti-inflammatory and anticancer activity, particularly towards hepatic, intestinal and urinary bladder tumours.

Keywords

Anticancer
Anti-inflammatory
Antioxidants
Betalains
Nutraceutical products
Vitexin flavonoids

Introduction

Beta vulgaris subsp. *vulgaris* is a herbaceous biennial plant belonging to the order of the *Caryophyllales*, in the family of the *Amaranthaceae*, and in the *Betoideae* subfamily [1]. *Beta vulgaris* has a diploid karyotype with nine pairs of chromosomes [2].

The flowers are produced in large inflorescences in the second year, at the apex of the stem. The fruit is an aggregate of two or more fruits, forming an irregular body which produces irregular brown seeds, with two cotyledons and one embryo (Fig. 1) [1].

The leafy beet group was domesticated first in the Mediterranean region and then introduced into the Middle East, India and China. Beet is now cultivated worldwide in temperate regions, with leafy beet preferring warmer temperatures than beetroot [3].

Taxonomically, *Beta vulgaris* is divided into four important crops (Table 1). *Beta vulgaris* subsp. *cicla* (BVc, leaf beet),

also called chard or spinach beet and grown for its leaves, is an important economic crop in many regions of Italy. *Beta vulgaris* var. *rubra* (BVr, red beetroot) is widely cultivated in Northern and Central Italy for its dark red, yellow or white roots. *Beta vulgaris* var. *altissima* (sugar beet) was widely grown in Italy until the 1980s for table sugar production but was then abandoned as unprofitable, as was fodder beet.

Cultivated and wild-type *Beta vulgaris* species have long been used in folk medicine. Leaf beet and beetroot are used to stimulate the immune system, protect bone, and combat inflammation, liver intoxication and gastrointestinal disease [1].

The juice and leaves of red beetroot are used in traditional medicine for genito-urinary and intestinal tumours, and to protect the gastro-intestinal tract [4]. The naturopath Rudolf Breuss developed a liquid formulation, with depurative and chemopreventive properties, based on red beetroot and other vegetables.

In this review, we describe the phenolic nutrients with pharmacological importance in *Beta vulgaris* subsp. *cicla* (BVc, leaf beet) and *Beta vulgaris* var. *rubra* (BVr, red beetroot) to stress their importance as bioactive phytochemicals for use in nutraceutical products.

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| Group | Taxon | Common name | Uses | Italian name |
|-------------|--|------------------------------|--|--------------------------|
| Leaf beet | <i>Beta vulgaris</i> subsp. <i>vulgaris</i> , convar. <i>cicla</i> | Swiss chard | Leaves and stalk boiled or steamed Medicinal plant | Bieta a coste |
| | <i>Beta vulgaris</i> subsp. <i>vulgaris</i> , convar. <i>cicla</i> , var. <i>cicla</i> | Spinach beet | Whole leaves boiled as spinach or raw in salads Medicinal plant | Bieta erbetta da taglio |
| Fodder beet | <i>Beta vulgaris</i> subsp. <i>vulgaris</i> , convar. <i>crassa</i> | Mangelwurzel | Raw as animal food | Bietola da radici |
| Sugar beet | <i>Beta vulgaris</i> subsp. <i>vulgaris</i> , convar. <i>vulgaris</i> , var. <i>altissima</i> | Sugar beet | Commercial crop for industrial sugar production | Barbabietola da zucchero |
| Garden beet | <i>Beta vulgaris</i> subsp. <i>vulgaris</i> , convar. <i>vulgaris</i> , var. <i>vulgaris</i> | Red beetroot, garden beet | Boiled or steamed as a food, centrifuged as a juice Medicinal plant | Bietola rossa |

Table 1 - Taxonomy of beet cultivars with their uses

Nutritional aspects

BVc leaves and BVr roots are usually eaten boiled or steamed. BVc is rich in minerals, such as Fe, K, Ca, P and Mg, and numerous vitamins such as B3, B5, B9, A, C and folic acid. Phenolic acids, flavonoids and carotenoids, fibre and saponins are present as well [3]. BVr is also consumed as a crude juice containing Ca, P, Fe, B vitamins and polyphenols [5]. Steaming allows the retention of most vitamins and polyphenols, while boiling releases most of the nutrients into the cooking water [6–8].

Patients with bladder problems must use caution when consuming BVc and BVr because of the presence of up to 1% oxalates in second-year plants. Variable amounts of geosmin, a bicyclic alcohol, are also present in BVr [9]. Geosmin has an unpleasant earthy flavour, which is mostly lost when the vegetable is boiled in plenty of water [5].

Pharmacology

BVc and BVr contain many phytochemicals providing pharmacological effects. Earlier studies by Ozsoy-Sacan *et al* [10] and Sener *et al* [11] demonstrated that BVc leaf extract exhibited hypoglycaemic properties in diabetic animals. Research to discover the components responsible for this pharmacological effect tentatively identified saponins, which inhibit gluconeogenesis and glycogenolysis [12]. However, the definitive mechanism responsible for the hypoglycaemic effect remains unknown and further investigation is required. In recent years, the wide use of separation technology, in particular automatic medium pressure liquid chromatography (MPLC) and cheap new stationary phases has allowed many laboratories to prepare milligram amounts of individual phytochemicals in order to study their specific biological properties. Using a dereplication process, we extracted the

secondary metabolites using ethanol and then isolated the individual phytochemicals from BVc and BVr using liquid–liquid fractionation and MPLC. We identified their chemical formulas using HPLC-MS and NMR and tested their biological activities in tumour cell lines. We used the following cancer cell lines: breast, MDA-MB-231, MCF-7; colon, LoVo, CaCo-2, RKO; hepatic, HepG2, Hep3B; and urinary bladder, T24. In parallel, we performed similar experiments with normal cell lines, such as human fibroblasts (HF), human keratinocytes (NCTC-2544) and human lymphocytes, in order to determine if the action against cancer cells was replicated in normal cells [5].

The chemical structures of the active phytochemicals of BVc and BVr studied in our laboratory are given in Fig. 1.

The seeds, leaves and roots of leaf beet are rich in phenolic acids such as siringic, caffeic and vanillic, as well as flavonoids derived from apigenin (Fig. 1), such as vitexin, vitexin-2-O-rhamnoside (VOR) and vitexin-2-O-xyloside (VOX) [5], and also catechin, myricetin and quercetin [3].

The main molecules in BVr are betalains, a group of water-soluble phenolic pigments derived from betalamic acid. They are subdivided into two classes: the yellow/orange betaxanthins (BX) and the red/violet betacyanins (BC). The main betacyanin in beetroot is betanin with its aglycone betanidin, while the most abundant betaxanthin is vulgaxanthin I (Fig. 1) [13, 14].

The betalains were isolated in the 1930s and intensively studied in the 1980s and 1990s [13, 15–17]. These pigments have recently been receiving increased attention as they are quite stable at the pH values of many foods and are used as an additive dye in various food products, particularly cheese, tomato paste, sauces, ice cream, sweets, breakfast cereals and desserts.

The European Food Safety Authority (EFSA) allows the use of red beetroot dye, which has the e-number E162 [18]. This

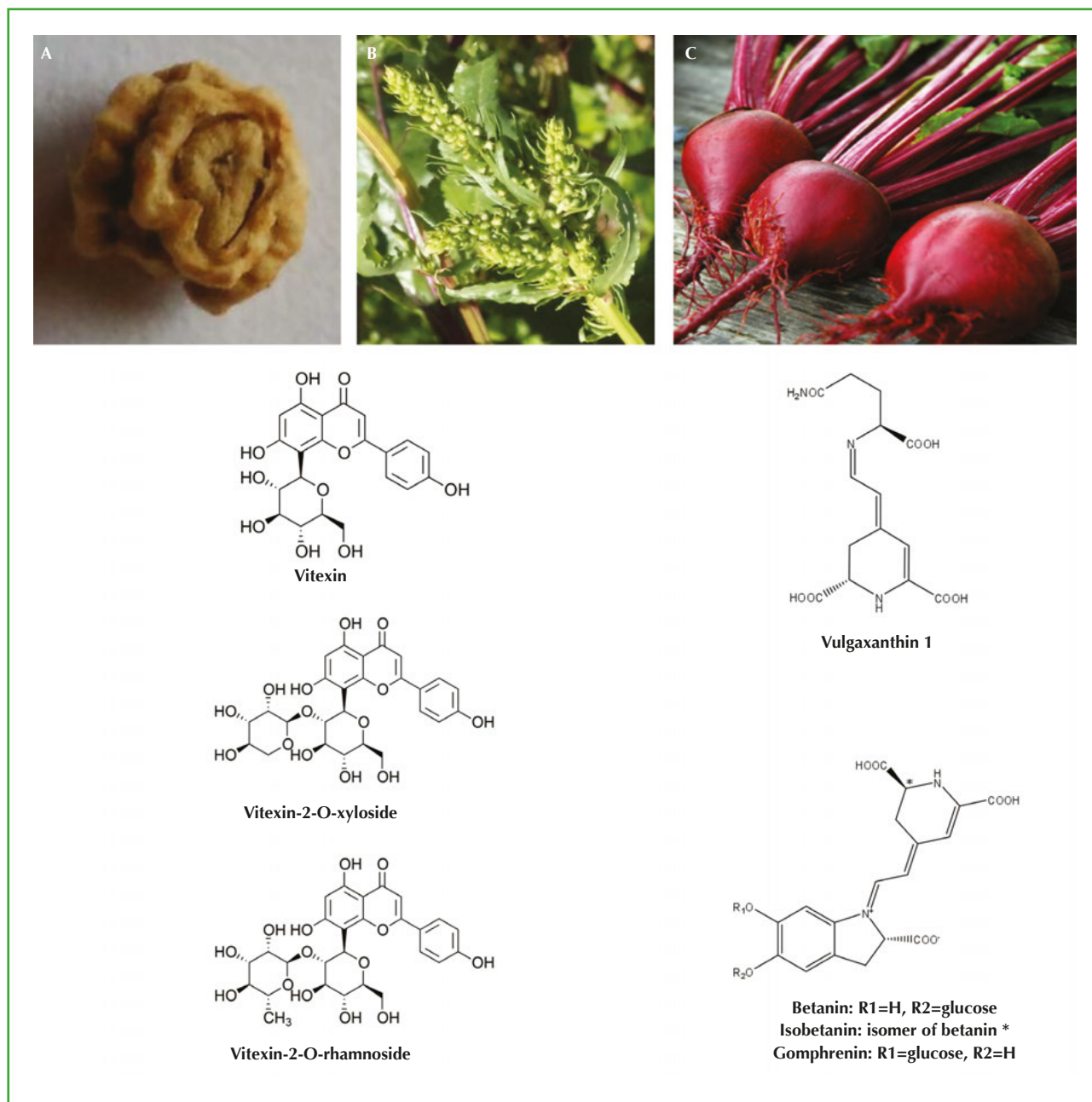


Figure 1 - Seed, flowers and roots of *Beta vulgaris* and structure of vitexin flavonoids and betalains: (A) seed of *Beta vulgaris* subsp. *cicla*; (B) flowers and fruits of *Beta vulgaris* var. *rubra*; (C) roots of *Beta vulgaris* var. *rubra*, cv. Detroit

dye consists of up to 90% betalains, and therefore exerts a healthy effect due to the high antioxidant capacity of the pigment [14]. The amount of dye added to foods reaches 10–15 mg/100 g food, thus increasing antioxidant capacity and protecting the body during post-prandial oxidative stress [19].

The pharmacological effects of the BVC and BVR bioactive molecules are summarized in Figs. 2 and 3.

Figure 2 shows the changes in key molecules involved in different signalling pathways due to vitexin flavonoids. The inhibition of DNA synthesis and cell cycle progression was detected by flow cytometry, showing a reduction in the

number of cells in the S-phase due to arrest of the cell cycle in G1 [20]. Experiments with tritiated thymidine were also performed and results were expressed as % of DNA synthesis inhibition [21].

Induction of apoptosis was evaluated using (a) fluorescent detection of annexin V bound to apoptotic cells, and quantitation by flow cytometry [14, 22], (b) detection of an increase in pro-apoptotic proteins, such as Bax and a decrease in the anti-apoptotic Bcl family proteins [14, 22], (c) detection of an increase in cleaved PARP by measuring PARP1 fragments (PARP is the enzyme which contributes to DNA repair after oxidative stress and is cleaved during apopto-

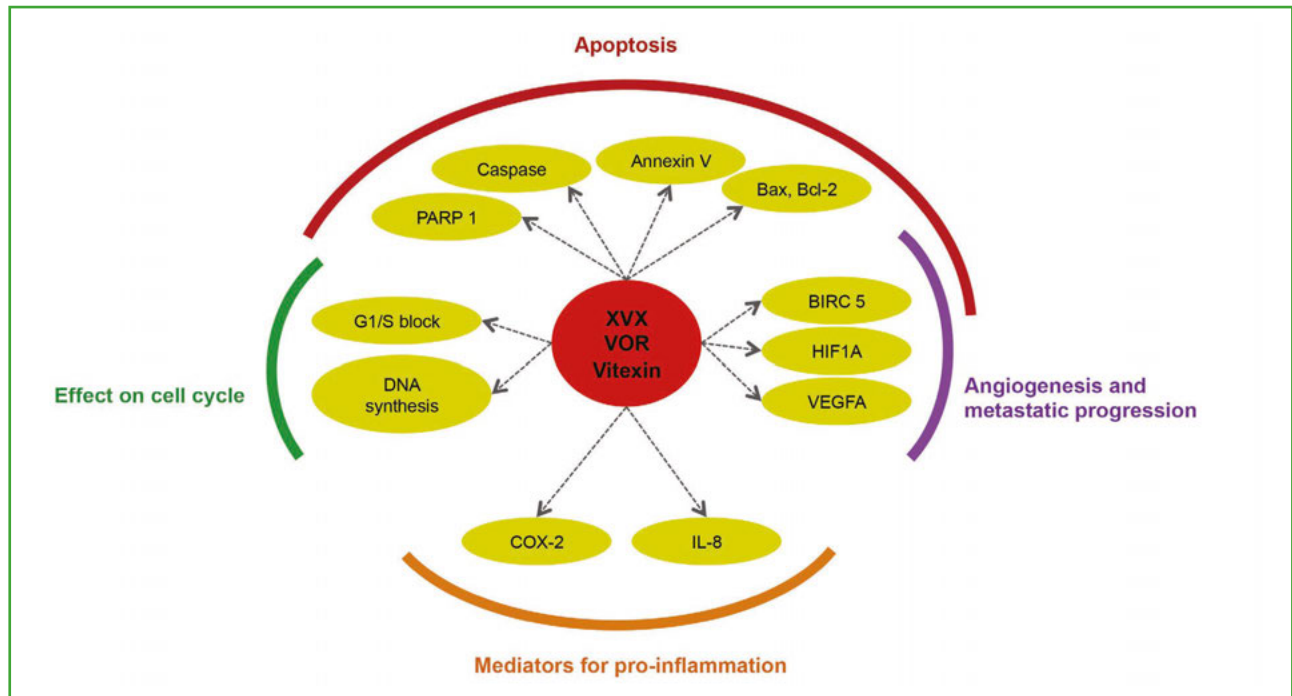


Figure 2 - Regulation of signalling pathways due to the combination of vitexin flavonoids in tumour cell lines. VOR vitexin, XVX vitexina-2-O-xyloside

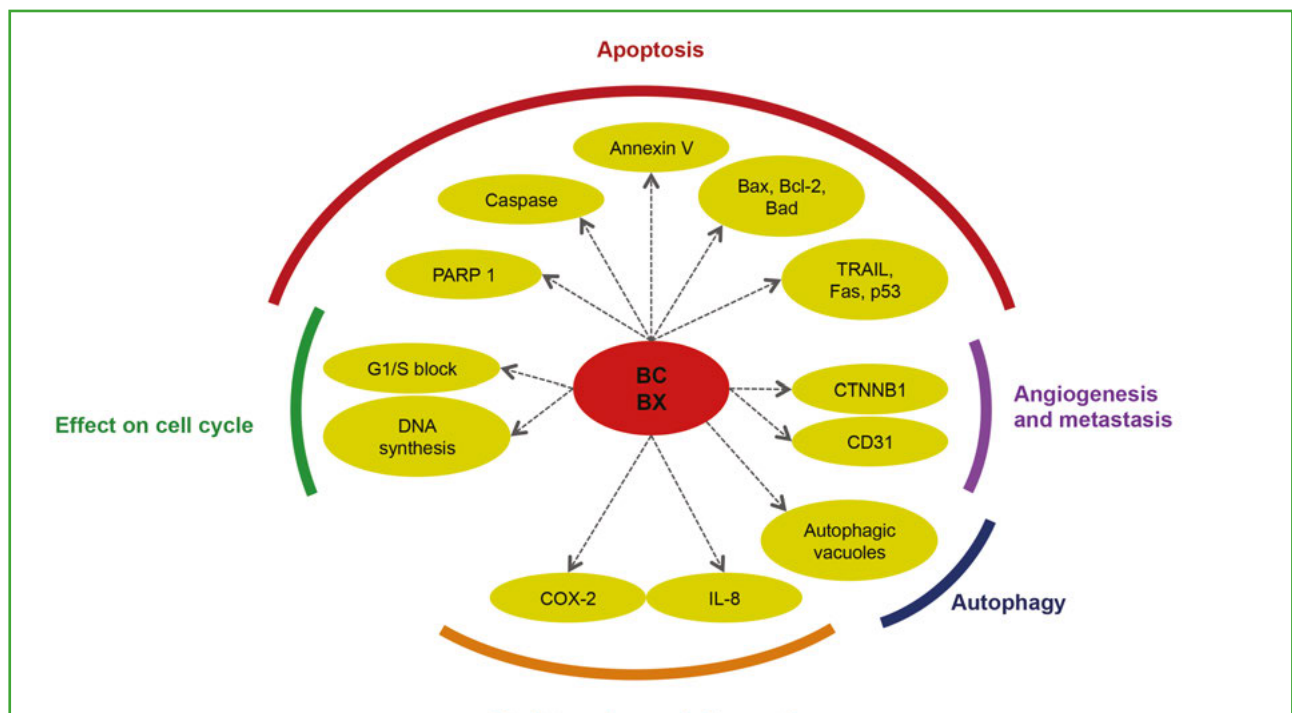


Figure 3 - Regulation of signalling pathways due to the combination of betacyanins (BC) and betaxanthins (BX) in tumour cell lines

sis into two fragments, one of which, called PARP1, is an excellent marker of apoptosis [14]), and (d) detection of an increase in the caspases, particularly caspase 3, which is the executioner caspase responsible for the cleavage of many key proteins. The enzymatic assay of caspase 3 measures the progression of the apoptotic pathways in tumour cells [14, 22].

As apoptosis can be blocked by an increase in the concentration of inhibitor of apoptotic proteins (IAP), downregulation of these pro-survival markers in tumour cells means that ultimately apoptosis is accelerated. We observed downregulation of the mRNA expression level of the BIRC5 gene which encodes an IAP protein called Survivin. Moreover, vitexin flavonoids were found to downregulate mRNA levels

of HIF1 α and VEGF-A, which are regulators of angiogenesis and controllers of tumour cell proliferation and invasiveness [23]. We also observed depletion of the pro-inflammatory markers COX-2 and IL-8, which are important for triggering apoptosis. To summarize, vitexin flavonoids show multi-targeted anticancer activity, which triggers apoptosis and downregulates proliferation, survival and angiogenesis factors in tumour cells [5].

Figure 3 shows the key molecules involved in the signalling pathways regulated by betalains. The inhibition of proliferation and the induction of apoptosis were detected using the same methods as reported above for vitexin flavonoids. In the presence of BC and BX, the apoptosis occurred through the extrinsic pathway and in addition there was a dramatic decrease in the mRNA expression levels of COX-2 and IL-8, which was greater than the decrease caused by the vitexin flavonoids [14]. Inhibition of angiogenesis has also been found by other authors [24] with depletion of the expression levels of cluster of differentiation 31, also called CD31, which is involved in angiogenesis and tumour progression. We also detected downregulation of the mRNA levels of CTNBN1 which expresses β -catenin, a protein involved in pro-survival mechanisms [22].

Finally, additive pro-apoptotic signals due to betalains, which modified Bad, TRAIL, Fas and p53 expression levels, as well as the formation of autophagic vacuoles, were described by Nowacki *et al* [25].

In summary, BC and BX and their combination exert multiple anticancer effects by targeting several signalling pathways with resulting inhibition of tumour cell proliferation and inflammation.

Using the ORAC method, we determined that the chemical antioxidant capacity of vitexin flavonoids was approximately 20,000 $\mu\text{molTE/g}$ [5], while the ORAC values of BX and BC were approximately 10,000 $\mu\text{molTE/g}$ [14].

The values of the key markers involved in tumour cell progression indicate that the phytochemical combination XVX+BC+BX is the most efficacious followed by XVX+BC, due to the synergistic effects among the pathways described in Figs. 2 and 3.

Interestingly, our studies and those of other authors [5] showed that in cancer cell lines, vitexin flavonoids trigger the production of reactive oxygen species (ROS) and induce apoptosis through the intrinsic apoptosis pathway, which involves mitochondrial damage and cytochrome c release. The betalains behave as antioxidants within the cells, but trigger the extrinsic apoptosis pathway, which involves Fas and Fas ligand, with recruitment of Fas-associated proteins and acti-

vation of caspase 8, which in turn activates the executioner caspase 3 [5].

This means that a cancer prevention strategy using non-toxic phytochemicals (i.e., chemoprevention) could use a combination of vitexin flavonoids and betalains which activate both the intrinsic and extrinsic apoptotic pathways and block the salvage pathway of tumour cell lines.

Bioavailability

Bioavailability and bioaccessibility are both important for the study of the biological effects of phytochemicals. These topics are widely discussed in the literature and are the first factors to be considered when the results from *in vitro* experiments are translated to *in vivo* models.

In our laboratory, we evaluated the bioavailability of vitexin flavonoids using an *ex vivo* system, where a flavonoid was incubated with red blood cells in the presence of 2',7'-dichlorofluorescein diacetate (DCFH-DA), with subsequent measurement of cellular antioxidant activity [26]. This experiment showed that the flavonoid is able to enter red blood cells. A second method utilized polyclonal antibodies for the detection of vitexin flavonoids and their metabolites in the blood of Balb/c mice. Vitexin flavonoids were found in the plasma of mice fed 170 mg/kg of VOX [3].

Wang *et al* demonstrated that after oral administration of vitexin to experimental animals, the compound and its metabolites were found in the intestine and liver, as well as at very low concentrations in the brain and adipose tissue [27]. Similar results were found by Yan *et al* [28] and Liang *et al* [29]. These and other experiments [5], based on oral dose administration, concluded that the bioavailability of the vitexin flavonoid was approximately 3%.

Studies on the intra-caecal administration of XVX in a rat model demonstrated that it was quickly absorbed from the enterocytes and inserted into the entero-hepatic circulation [30]. Other authors evaluated the contribution of bacteria to the transformation of flavonoid C-glycosides by incubating these phytochemicals in the presence of isolated bacterial strains. The authors showed that the same bacteria are able to deglycosylate the C-glycosidic bond, making the molecular fragments bioavailable [31].

To summarize, vitexin is absorbed well by the intestine, likely by passive diffusion. The sugar moiety of XVX and VOR probably limits absorption, but bacterial metabolism and entero-hepatic recirculation prolong the time the molecules remain in the liver and intestines, possibly enhancing their pharmacological effects in these two compartments.

Most phytochemicals have poor bioavailability due to their low capacity for absorption through the cell membrane or for extrusion from cells because of the multidrug resistance (MDR) system. Therefore, combination with other phytochemicals able to inhibit the MDR system should increase the bioavailability of the vitexin flavonoids.

Betalain bioavailability was studied in both animals and humans after the ingestion of beetroot juice [32, 33]. Betacyanins were found in the urine, mostly as metabolized products, but bioavailability was low [34, 35]. Tesoriere *et al* demonstrated in an *in vitro* model which mimics the gut functional barrier, that betanin and indicaxanthin were absorbed unmetabolized via paracellular transport [15].

To summarize, most of the experiments cited above sought to identify the few known metabolites of betalains and vitexin flavonoids in blood and organs. Only after complete elucidation of their metabolic pattern, through phase 1 and 2 reactions, will it be possible to draw definitive conclusions on the amount of molecules absorbed.

Conclusions

The combination of vitexin flavonoids and betalains has been shown to be very efficient for combating inflammation and tumour cell proliferation. The future development of functional foods and nutraceutical products with chemopreventive efficacy must be based on the selection of precise BVC and BVr cultivars and standardized automatic methods to obtain ethanolic extracts with high titres of vitexin flavonoids and betalains. Purification methods used in the laboratory to increase the percentage of the bioactive molecules should be scaled up and also adapted for use under food grade conditions. Toxicity tests and studies on animal models receiving long-term treatment should be carried out, and pharmacokinetic studies to detect metabolites in the different organs must also be conducted. These steps could result in an application being made to the EFSA for the listing of vitexin flavonoids and betalains as novel foods. Combinations of these molecules could be used to develop nutraceutical products to reduce the social and economic burden of cancer and other disorders associated with aging. The development of such products could lead to the production, industrial transformation and commercialization of beet, thus offering economic opportunities to the agri-food sector.

Conflict of interest

The authors declare that they have no conflict of interest.

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