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Cytochrome p450 and innovative nutraceutical products

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Abstract

Dietary supplements are products that are ingested in addition to the regular diet to provide additional health-promoting nutrients. Dietary supplements are defined and regulated differently in the European Union (EU) and the United States (US). A fundamental aspect, besides the one related to the composition of the various products on the market, is linked to their quality, both from a nutritional and a pharmacological point of view. Concerning the knowledge of the metabolic aspects, the analysis of the interference, as an inductive or an inhibitory effect, of the p450 enzyme on individual preparations of supplements, is crucial. In this study, we present the results of the interference analysis of a new nutraceutical product based on 38% Bergamot Polyphenolic Fraction

BPF® (*Citrus bergamia* Risso et Poit.), Pomegranate (*Punica granatum*) and Citrus fruits (*Citrus aurantium* var. *dulcis*, *Citrus maxima* Burm. Merr, *Citrus paradisi* Macfad) extract with cytochrome p450, showing that the product has limited activity on the cytochromes involved in most of human drug metabolism. This nutraceutical product is to be considered safe and potentially useful in the context of multiple treatments, not interfering with the traditional chronic therapies of patients. These findings open the door to modern "pharma-grade" nutraceuticals, expanding the safety and quality profiles of these new products.

Introduction

The cytochrome p450 (CYP) family is an enzymatic superfamily of membrane-bound hemoproteins, expressed in nearly every cell type in the body, that have significant roles in drug detoxification, cellular metabolism, and maintaining homeostasis, through reactions requiring nicotinamide adenine dinucleotide phosphate (NADPH) and O_2.[1,2] These enzymes play a crucial role in metabolizing a wide range of both natural and foreign compounds, being able to act on several exogenous, such as drugs and toxins of external origin, and endogenous, as waste products of the organism, substrates.

CYPs possess the ability to impact drug responses by modulating drug action, safety, bioavailability, and resistance through metabolism.[3] This influence occurs in metabolic organs as well as specific local sites of action. These enzymes have a fundamental role in metabolic processes leading to drug and compound elimination from the organism. Although p450 expression occurs also in the intestine, lung, kidney, and heart, the highest concentration of most p450s responsible for drug metabolism is in the liver. Considering that diseases can alter (in terms of decreasing or increasing) the levels of certain hepatic CYP enzymes, it causes documented impacts on drug clearance and clinical drug-related toxicity: chronic liver diseases such as cholestasis, hepatitis B and C, alcoholic liver disease, cirrhosis and nonalcoholic fatty liver disease (NAFLD) bring to reduced or increased clearance of known substrates as antipyrine, theophylline, caffeine, halothane, disulfiram, valproic acid, warfarin, losartan, rosiglitazone, fluoxetine, tamoxifen, and diclofenac.[4]

Although there are about 50 enzymes that belong to the family of cytochrome p450, 90% of drugs and waste products are metabolized by 6 enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5, and, among these, CYP2D6 and CYP3A4 are considered the most representative.[5,6] Recent advancements in understanding the structures of CYPs have shed light on drug metabolism mechanisms and highlighted the potential of utilizing CYPs as drug targets.[7]

One fundamental aspect of modern pharmacology is to investigate the interactions among various therapeutic products. At the core of it lie the insights into the mechanisms of action of cytochrome p450 and the ensuing practical implications. Through exploring these aspects, it has become apparent that certain drugs cannot be used concurrently with others, as they may anomalously potentiate or deactivate certain effects.

We have recently initiated a study project involving innovative nutraceutical products with a "pharma-grade" development plan. One of the initial steps in this process has been to examine the interference of some of these products with cytochrome p450. The described study pertains to the determination, in an *in-vitro* model, of the interference of a product based on 38% Bergamot Polyphenolic Fraction BPF® (*Citrus bergamia* Risso et Poit.), Pomegranate (*Punica granatum*) and Citrus fruits (*Citrus aurantium* var. *dulcis, Citrus maxima* Burm. Merr*, Citrus paradisi* Macfad) extract with cytochrome p450.

Cytochrome and elderly

Elderly people represent a unique population, as they undergo changes in their bodies induced by aging and are often subjected to polypharmacy for comorbidities that may arise through time. However, these medications may interfere with each other in their mechanisms of action, especially at the level of cytochromes, altering their effectiveness.

Aging brings about notable effects. They involve a gradual decline in the functional reserve of various organs, potentially impacting pharmacokinetics, and especially drug metabolism. Changes in body composition potentially lead to an expanded volume of distribution for lipophilic drugs and a prolonged half-life.[8] The reduced hepatic first-pass effect, attributed to diminished liver mass and perfusion, can elevate the bioavailability of certain drugs and hepatic drug clearance of specific medications may decline through years.[9]

It is fundamental to know that most elderly patients are in treatment with many drugs, especially those against hypertension, dyslipidemia, and metabolic syndrome, and such patients often take drugs that use the cytochrome p450 system to be fully metabolized. Some of the most commonly used drugs in these health conditions interfere with cytochrome p450 and among them, the most widely used are beta-blockers, sartans, calcium antagonists, and even some diuretics and statins.[10]

In these complex patients, nutraceuticals have recently been used. Considering that, it is evident how critical it will be to have the metabolic profiles of innovative nutraceuticals, including the interference with cytochrome p450 metabolism.[11] A basic pre-requisite for modern quality

nutraceuticals should be a complete knowledge of their metabolic pathways.[12] So, this information is inescapable from knowledge of the interaction with the cytochrome p450 system.

The objective of our study was to test the possible interference with the cytochrome p450 system of a nutraceutical product extracted from Bergamot, Pomegranate, and Citrus fruits. Specifically, the performed study concerns the potential inhibitory effect of the formulation on the activity of CYP2D6 and CYP3A4 enzymes, which belong to the cytochrome p450 family.

Bergamot

Bergamot (*Citrus bergamia* Risso et Poiteau) is a citrus fruit native to southern Italy, that contains an especially high content of flavonoids, the main subclass of polyphenols, including neoeriocitrin, naringin and neohesperidin, interesting for their multiple benefits. They act to improve immune response and cardiovascular function and have shown anti-oxidant, anti-inflammatory, and cholesterol-reducing properties. There is evidence that orally administered bergamot can reduce total cholesterol and low-density lipoprotein (LDL) cholesterol in patients with hypercholesterolemia.[13] These components also suppress inflammation in endothelial cells, inhibiting the formation of plaques and improving the arterial response.[14] Moreover, *in-vitro* studies have provided evidence that polyphenols from bergamot can alter the function of adenosine monophosphate-activated protein kinase (AMPK) and pancreatic cholesterol ester hydrolase.[15]

BPF is a particular product derived from bergamot juice, that contains 40% flavonoids; the remaining 60% are carbohydrates, fatty acids, pectins, and other compounds such as maltodextrins. BPF is obtained from peeled-off fruits by a patented method consisting of industrial wringing, pressing, and squeezing.[16] Then through decanters and centrifuges the juice is separated from the solid part. Once extracted, the essential oil of bergamot, the juice passes clarification and repeated passages in absorbent columns, finally obtaining the mentioned bergamot polyphenolic fraction.[17]

The main polyphenol components of BPF are flavonoids; they are implicated, as told before, in the regulation of several metabolic enzymes expressed in the liver, blood, and endothelial cells.[16] As previously mentioned, polyphenols derived from bergamot help keep cholesterol healthy, and help weight management, so they could represent a valid alternative to statins, especially in patients intolerant to them.

Pomegranate

Pomegranate (*Punica granatum*) juice has a history in traditional medicine and contains primary constituents such as hydrolyzable tannins, anthocyanins, and ellagic acid. The impact of flavonoids found in pomegranate juice is linked to their anti-inflammatory, anti-oxidant, anti-hepatotoxic, anti-atherogenic, and anti-tumoral properties, resulting in the prevention of cardiovascular diseases, obesity, diabetes, and cancer.[18] This is achieved by reducing specific cytokines associated with low-grade inflammation and parameters connected to cardiovascular risk.[19] Studies have shown that pomegranate polyphenols not only have a strong antioxidant capacity but also inhibit the growth of pathogenic bacteria and fungi.[20] Considering these findings, it results that incorporating pomegranate juice into one's diet may serve as a viable supplementary approach to addressing metabolic syndrome.[21-23]

Citrus fruits

Sweet oranges (*Citrus aurantium* var. *dulcis*) are rich in hesperidin and naringin, which represent more than 90% of the flavonoids in sweet oranges. As mentioned previously, these flavanones, mainly present in citrus fruits, have emerged as a potential therapeutic agent able to modulate several risk factors of cardiovascular diseases, showing glucose-lowering and anti-inflammatory properties, dyslipidemia-, atherosclerosis-, and obesity-preventing functions and also anti-hypertensive, anti-oxidant and anti-tumoral effects.[24-27]

Shaddock (*Citrus maxima*) and grapefruit (*Citrus paradisi*) components, mainly phenols and flavonoids, have shown antioxidant and antimicrobial activities, in addition to hypocholesterolemic and anti-hypertensive properties through inhibition of angiotensin-converting enzyme (ACE), helping management of cardiovascular diseases.[28,29] Also antigenotoxic and chemopreventive effects of grapefruit compounds have been shown.[30]

Materials and Methods

All experiments were performed by the ECSIN-ECAMRICERT SRL Laboratory, Padova, Italy. The potential inhibitory effect of the formulation on the enzymes CYP2D6 and CYP3A4 was evaluated by a luminometric assay.

Regarding the formulation tested, it consists of a capsule containing a mixture of: i) Bergamot BPF [botanical species and origin: *Citrus bergamia* Risso et Poit., Calabria origin; used plant part: fruit; drying process through spray dry (for organic solvent/water evaporation); quantification of active

ingredients as neoeriocitrin, naringin, neohesperidin, melitidin, brutieridin through high-performance liquid chromatography (HPLC), with a specific value obtained of 38±2%, that expresses the exact quantity of active components producing the activity]; ii) Pomegranate titrated to ellagic acid, that is, knowing the exact quantity of this active component [botanical species: *Punica granatum* L.; used plant part: fruit, harvested in autumn by manual collection; primary processing: washing and drying; extraction in water:ethanol 50:50 v:v]; iii) Citrus titrated to hesperidin, that is, knowing the exact quantity of this active component [botanical species: *Citrus maxima* Burm. Merr, *Citrus paradisi* Macfad, *Citrus aurantium* var. *dulcis*; used plant part: *Citrus maxima* (seeds); *Citrus paradisi* (seeds); *Citrus aurantium* var. *dulcis* (pericarp)/fruit;primary processing: drugs obtained from cultivated plants, with both manual and mechanical harvesting; the drugs are obtained from mature fruits collected from winter to spring; they are washed, dried, and ground; water: ethanol extraction (60:40 v:v); title: total flavonoids calculated as hesperidin 60% minimum (HPLC)].

A product capsule was opened and the contents were resuspended in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL (stock solution). Then, to remove any undissolved particulate matter, the resulting suspension was centrifuged (2000 × g for 5 min at room temperature). After centrifugation, the supernatant was serially diluted in deionized water to obtain a concentration range of 0.0025 to 2500 μg/mL.

The used assays, suitable for assessing the impacts of drugs and novel chemical substances on CYP enzyme functions, entail utilizing a membrane preparation that contains recombinant human cytochrome p450 (CYP). Another membrane without the cytochrome serves as a negative control. These tests leverage a luminogenic reaction to measure enzyme activity.[31] The CYP reaction is carried out by incubating a luminogenic CYP substrate with a CYP enzyme - specifically either CYP2D6 or CYP3A4 - and a NADPH regeneration system.

The employed luminogenic substrates do not interact with luciferase; instead, they are transformed by CYP enzymes into a luciferin derivative. This luciferin product then interacts with a luciferin-sensing reagent, generating light. Monitoring CYP activity is based on the intensity of the light produced, directly correlating with the quantity of luciferin product generated following the CYP reaction.

To test the specificity of the assays, we evaluated the inhibitory activity of known inhibitors of the enzymes CYP2D6 (quinidine) and CYP3A4 (ketoconazole). Quinidine and ketoconazole were resuspended in DMSO and acetonitrile, respectively, at initial concentrations of 13 and 2.7 mg/mL.

The different tested concentrations of the inhibitors were obtained by serial dilution in deionized water.

The inhibitory power of the formulation and specific inhibitors is expressed as half maximal inhibitory concentration (IC50), which is the concentration at which a 50% inhibition of the activity of the considered enzyme is observed, indicating how much drug is needed to inhibit a biological process by half, as a measure of drug's efficacy and potency. Based on the scientific data in the literature, an inhibitor of enzymes belonging to the cytochrome p450 family can be classified as potent, moderate, or weak according to the IC50 values obtained.

Specifically, based on these data, a potent inhibitor is considered to be a substance that has an IC50 < 10 μg/mL, a moderate inhibitor has an IC50 value between 10 μg/mL and 100 μg/mL, and a weak inhibitor has an IC50 > 100 μg/mL; finally, we presume that if the substance has an IC50 much greater than 100 μg/mL it has no inhibitory effect because an excessively high plasma concentration of the substance would be needed to observe effects on the enzyme isoform, so at standard concentrations, it shows no inhibitory effect.[32]

Results

Regarding the bergamot, pomegranate, and citrus fruits derivative product tested, we found that this mixture of extracts has no inhibitory activity against cytochrome CYP2D6 (IC50 243.7 μg/mL), while it appears to be a moderate inhibitor of cytochrome CYP3A4 (IC50 61.9 μg/mL), according to the classification previously mentioned.

This positive finding, resulting in a limited activity on cytochromes involved in drug metabolism, allows us to state that this nutraceutical product is to be considered safe and potentially useful in the context of patients who simultaneously suffer from multiple diseases and need a combination of treatments, even in those with initial metabolic syndrome.[11,33,34]

Discussion

The use of multiple therapies in combination especially in elderly patients due to their multimorbidity is a key theme of modern pharmacotherapy.[35] It is essential to keep in mind that many drugs used especially in widely spread diseases, such as hypertension and dyslipidemia, interfere in their metabolic process with cytochrome systems.

This quality consideration will also need to be extended to modern nutraceuticals, which in recent decades have complemented historical drug therapy by implementing its effectiveness and in some cases allowing us to reduce the frequency of use and decrease the dosage of individual drugs. Nutraceuticals are frequently used as background therapy in chronic conditions such as dyslipidaemias, metabolic syndrome, and cardiovascular diseases, and in these conditions, they are associated with the use of "traditional" drugs: both of them can interfere with cytochromes.[36-38]

One of the distinct aspects of the interaction between drugs is the understanding of their relationships with cytochrome p450, and our observation, based on identifying the role of a nutraceutical in the interaction with cytochrome p450, opens the door to modern "pharma-grade" nutraceuticals, expanding the safety and quality profiles of these products. The concept of “pharma-grade” nutraceuticals expresses a development plan akin to pharmaceuticals that requires preclinical knowledge (compared to products lacking preclinical data), based on characterizing excipients and active ingredients and titrating the individual components of nutraceuticals.

A key feature of innovative quality nutraceutical products must be related to the exact knowledge of the individual components capable of generating not only nutritional adequacy but also potential interference with drug metabolism, especially in elderly people, with their potential age-related cytochrome activity reduction, exposure to a higher risk of adverse reactions. From this point of view, the nutraceutical product we tested showed to be safe, with limited activity on the CYP subfamilies involved in most of human drug metabolism as CYP2D6 (with no inhibitory effect shown) and CYP3A4 (with a moderate inhibitory effect shown), that are considered the most representative of this metabolic role.

This is a pharmaceutical approach to supplements that, through the analysis of the possible interference of nutraceuticals with human metabolic pathways and also with “traditional” drugs, could be considered in the evaluation of all nutraceutical products to minimize any potential side effects and consequently include their use in many clinical conditions. It is significant for determining whether nutraceuticals, when used as background therapy, may not interfere with the traditional chronic therapies of patients. Therefore, the knowledge of traditional drugs must also be applied to this emerging nutraceutical therapy.

The evidence of the importance of preclinical data in nutraceutical production is demonstrated by the observation that even these products, by causing drug-like effects, can induce adverse effects and potentially harmful interferences.[39,40]

In conclusion, the discovery of the interaction of nutraceutical products with cytochromes is fundamental to enhancing knowledge about their safety profiles of them, to create innovative "pharma-grade" products. We hope that such an approach, through the study of the interaction of nutraceuticals with cytochromes and through their characterization in terms of exact quantification and titration of the active ingredients, will be applied to all future products, aiming to improve their quality and safety.[41]

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