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The anti-proliferative effect of a bergamot juice extract and its flavanones in leukemic THP-1 cell line involves SIRT2

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Abstract

Plant kingdom provides a wide plethora of remedies which can be exploited to treat prevent and manage human ailments, among which cancer. In these regards, there is consistent supporting evidence of the beneficial properties of *Citrus* fruits, which are consumed worldwide. Among these, pharmacological effects of *Citrus bergamia* Risso (bergamot) have been extensively demonstrated, including anticancer ones, which are due to its elevated flavonoid content. Recently, the flavonoid-rich extract of bergamot juice (BJe) its main flavanones have been investigated for their anti-leukemic activity in THP-1 cells, a model of acute myeloid leukaemia (AML). Specifically, it was shown that BJe and its main flavanones were able to hamper viability of leukemic cells, along with blocking cell cycle in triggering apoptosis. Noteworthy, it has been suggested that in AML there is an over-expression of SIRT2, an enzyme belonging to the family of sirtuins. Interestingly, it has been shown that BJe and its main flavanones can inhibit the deacetylase activity of SIRT2 in the isolated enzyme and in THP-1 cells, where they also reduced its gene expression. Moreover, docking simulations clarified that the main flavanones, namely naringenin and hesperetin, were able to interact with the catalytic core of SIRT2 in a similar manner as the synthetic inhibitor SirReal2. These results support the anti-leukemic potentiality of BJe, along with its main flavanones, highlighting that SIRT2 is involved in these effects.

Key Words: *Citrus bergamia*; bergamot; flavonoids; cancer; acute myeloid leukaemia; sirtuins; SIRT2

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Introduction

According to Newman and Cragg, the plant kingdom provides an inconceivable number of active principles capable of regulating, inhibiting, or boosting several cellular pathways involved in a wide range of physio-pathological conditions, especially in cancer (1). Given the multi-factorial pathogenesis of this disease, phytotherapy can represent an option since its therapeutic success is based on the combined action of a mixture of compounds (2,3). Indeed, plant secondary metabolites can act by contemporarily targeting and disrupting the cell membrane, binding and inhibiting certain proteins, or adhering to or intercalating into RNA or DNA. Moreover, they can interfere with cellular transport processes, activate pro-drugs or transform active compounds to inactive metabolites, act as synergistic partners at different points of the same signalling cascade (multi-target effects) (4).

Among plant secondary metabolites, flavonoids are naturally occurring polyphenols found in plants, fruits, vegetables, teas, and medicinal herbs. According to studies, approximately 10,000 flavonoids have been identified and classified into many subclasses, including flavonols, anthocyanins, flavanones, flavones, isoflavones, and chalcones (5). Dietary flavonoids stand out for their

pharmacological properties, such as antioxidant (6), anti-inflammatory (7), anti-infective (8), neuro-, cardio- and hepatoprotective (9). Flavonoids appear also to have an anticancer impact primarily because of their antioxidant and anti-inflammatory properties, as well as their ability to alter molecular targets and signalling pathways involved in cell survival, proliferation, differentiation, migration, angiogenesis, and hormone activity (10-12).

Citrus fruits (CFs) are primary source of dietary flavonoids, and their presence confers well-known defensive properties that are used to protect human health, proving to be allies against neurodegeneration (13), inflammation and immunity (14). CFs and their derivatives have long been explored for their role in cancer (15-17).

Citrus bergamia Risso (bergamot) and cancer

Citrus bergamia Risso (bergamot; Rutaceae family) is a characteristic plant growing in Calabria, in the south of Italy. Its fruits are primarily used to extract the essential oil (BEO), which is widely used in the preparation of perfumes, cosmetics and food. On the contrary, bergamot juice (BJ), which is produced by squeezing the fruit endocarp, has hitherto been considered a byproduct with waste disposal issues. Nevertheless, in the last decades, BJ has drawn the attention for being plenty of flavonoids, including naringin, hesperetin, neohesperidin, neoeriocitrin, melitidin and brutieridin (18). This has led to the investigation of its beneficial properties, as well as those of the flavonoid-rich extract of BJ, namely BJe.

In recent years, both BJ and BJe proved to possess interesting anti-cancer properties, among others. On this line, they have shown to reduce the growth rate of different cancer cell lines through various molecular mechanisms depending on cancer types (19-21). Furthermore, we found that BJ might inhibit the growth rate of human neuroblastoma SH-SY5Y cells by causing a cell cycle stop in G1 phase and a loss of adhesive ability (22). This latter appeared to be responsible for its anti-migratory impact, as it reduced lung metastatic colonization in a SCID mice model of spontaneous neuroblastoma metastasis development (23). BJ has been demonstrated to suppress the development of human hepatocellular carcinoma HepG2 cells via p53, p21, and NF-κB pathways (20). Following that, we focused on the flavonoid-rich BJ extract, BJe, to investigate its anticancer potential. *In vitro*, it inhibited the growth of human colorectal carcinoma HT-29 cells through a variety of mechanisms, including an increase in reactive oxygen species production, a decrease in mitochondrial membrane potential, and oxidative damage to DNA at high concentrations, while inhibiting MAPK pathways and modulating apoptosis- and cell cycle-related proteins occurred at low concentrations (24). *In vivo*, BJe was able to inhibit the spontaneous tumorigenesis in Pirc rats (F344/NTac-Apc^{am1137}), a genetic model of colorectal cancer, via an anti-inflammatory and pro-apoptotic mechanism (25).

BJe and its flavanones in acute myeloid leukaemia (AML)

Acute myeloid leukaemia (AML) is a haematological disease defined by the uncontrolled growth of immature myeloid cells (blasts), which accumulate in the bone marrow and alter normal haematopoiesis (26). AML prevalence rises with while being the most prevalent kind of leukaemia in adults, it is linked with the lowest survival rate of any leukaemia (27). Recent developments have shed insight on the pathophysiology of AML, revealing significant genetic and clinical variability, which explains the ongoing need to develop innovative treatment regimens to achieve satisfactory therapeutic outcomes and improved quality of life for AML patients (28,29).

The acetylation status of histones governs epigenetic processes by influencing transcription factor access to DNA, hence controlling gene expression. Histone deacetylases (HDACs) are involved in this mechanism, which leads to increased DNA/histone complex compaction (30). Among HDACs, human sirtuins, NAD^+-dependent enzymes, are the focus of research study because they regulate a wide variety of cellular pathways implicated in cellular aging and age-related illnesses, including cancer (31). SIRT2, one of seven human sirtuins, is a cytoplasmic enzyme that deacetylates histones, α-tubulin, and other transcriptional factors (e.g., p53, NF-κB)(32). Interestingly, SIRT2 mRNA levels are higher in AML patients' blasts than in healthy persons. It is specifically over-expressed in both intermediate- and poor-risk patients when compared to favourable-risk individuals, and it is associated with a considerably shorter overall and event-free survival. Furthermore, SIRT2 overexpression was particularly prominent in AML patients with the M5 subtype, as defined by the French American-British (FAB) classification of AML, thus being considered an unfavourable marker for this disease (33). Nowadays, histone deacetylase inhibitors (HDACi) appear to be a potential therapeutic for cancer treatment and an emerging situation for AML patients who are refractory to conventional chemotherapy (34). Moreover, it was shown that flavonoids, such as quercetin and derivatives, have already proved their potential in inhibiting SIRT2 (35).

On this line, we also investigated whether BJe can be a candidate for the prevention of AML. Indeed, we showed that BJe was able to block the cell cycle in S phase and induce apoptosis in THP-1 cells, an in vitro model of AML, while having no effect on the proliferation of non-tumoral peripheral blood mononuclear cells (PBMCs). From a molecular point of view, the cleavage of caspase-8 and -9 was seen to initiate both intrinsic and extrinsic apoptotic pathways, subsequently activating caspase-3 and PARP (36).

The anti-leukemic properties of BJe were a direct reflection of those of its components. On these regards, the quali-quantitative composition of the BJe employed in these studies showed that the most abundant flavanone glycosides were naringin (NRG) and neohesperidin (NHP), representing about the 83% of the whole extract. Therefore, we assessed their effects in THP-1 cells, along with their

aglycone counterparts, namely naringenin (NAR) and hesperetin (HSP), respectively. Notably, the glycosides were not able to induce any significant alteration of cell viability, whereas both NAR and HSP exerted interesting antiproliferative activity in THP-1 cells, without any toxicity in PMBCs. The differences between BJe and the glycosylated flavanones can be explained since phytocomplexes allow to obtain higher water concentrations of single constituents, and hence greater biological effects. Moreover, the differences in efficacy between glycosides and aglycones are acknowledged since the rutinoside in C-7, the carbohydrate moiety present in both NRG and NHP, hampers the induction of antiproliferative activity (37).

Moreover, the investigation of the mode of death elicited by these flavanones showed that both NAR and HSP were able to block the cell cycle, increasing cells in the S phase, as the whole BJe (38). In addition, these flavanones were able to decrease the gene expression of both p21 and cyclin E1, which are pivotal factors in the progression of cell cycle, specific for the S phase. The pro-apoptotic effects of both NAR and HSP have been previously demonstrated, though in different experimental models for the latter (39,40). The anti-leukemic effects of BJe and its flavanones are resumed in Table 1.

Table 1. Effects of BJe and its flavanones in THP-1 cells and in PBMCs.

	THP-1 cells	**PBMCs**
BJe (1, 2.5 and 5 mg/mL)	– Inhibition of cell viability – Induction of cell death – Apoptosis (increase of caspases 3, 8, 9 and PARP) – Block of cell cycle (S phase)	Not effective
NAR - HSP (100, 200 and 400 µM)	– Inhibition of cell viability – Induction of cell death – Block of cell cycle (S phase) – Increase of cyclin E1 and p21	Not effective

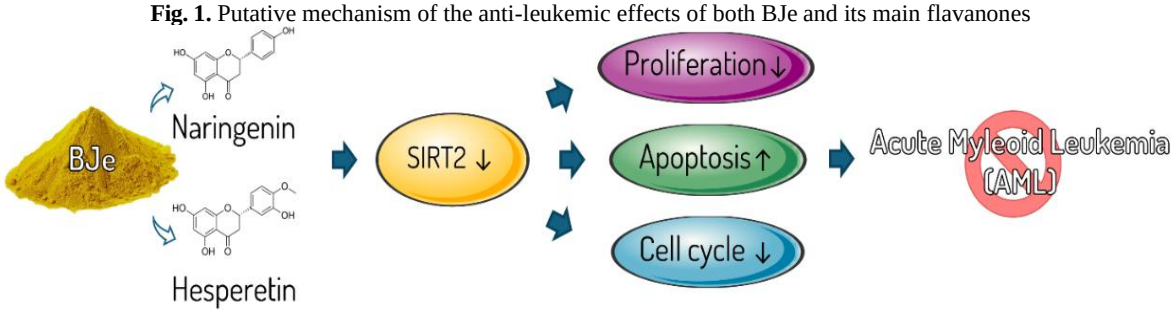
Based on the premises of the role of SIRT2 in AML progression (33) and flavonoids as modulators of this sirtuin (35), we investigated the possible involvement of SIRT2 in the anti-leukemic effect mediated by BJe. On this line, we assessed the potential inhibitory activity of BJe against the isolated recombinant SIRT2 enzyme, proving that this extract was able to significantly hamper the deacetylase activity of the enzyme to a greater extent than the specific inhibitor (i.e. SirReal2) (36). Predictably, the flavanones NAR and HSP were able to inhibit the enzymatic activity of the isolated SIRT2, as happened with the whole BJe (38). To study whether BJe was able to hamper SIRT2 activity cellular setting, we then moved to THP-1 cells. In this model, we showed that BJe dramatically increased the level of acetylated p53, a known SIRT2 target, in THP-1 cells meaning that this enzyme was inhibited (36). Interestingly, we observed that BJe elicited a stronger effect than the specific SIRT2 inhibitor (i.e., SirReal2), whereas comparable to the non-specific

one (i.e., nicotinamide - NAM), suggesting that the high concentrations of flavonoids of BJe might simultaneously inhibit further sirtuins prompting THP-1 cell death via the increase of p53 acetylation, as previously suggested (41). Regarding the main components of BJe, we observed that, in the same model, both NAR and HSP significantly hindered the deacetylation of p53, as the equimolar association of the two, supporting the hypothesis of the synergism among active principles in BJe (38). Apart from the direct inhibition of the enzymatic activity of SIRT2, we also wondered whether BJe and its flavanones were able to alter the gene expression of this enzyme in THP-1 cells. Indeed, we showed that both BJe, NAR and HSP were able to induce an early reduction of mRNA levels of SIRT2 (36,38).

Lastly, we used computational to study the potential binding mechanism of the two flavanones NAR and HSP with SIRT2. This enzyme presents a catalytic core bound to NAD^+, along with N- and C-terminal extensions. The deacetylation activity occurs in large lipophilic area between a Rossmann fold and a zinc domain (42,43). By computational studies, we suggested that NAR and HSP displayed a similar network of interactions like SirReal2 in the lipophilic pocket of SIRT2. Specifically, the main hydrophilic bonds between the synthetic inhibitor and SIRT2 were present in the docking poses of both NAR and HSP, as well as the π-T-shaped interaction. The absence of the crucial π-π stacking interaction within the selectivity pocket of SIRT2 might explain the minor inhibitory activity of the flavanones respect the synthetic inhibitor (38).

In Figure 1, the putative mechanism of action underlying the involvement of SIRT2 in the anti-leukemic activity of BJe and its main flavanones is depicted.

Fig. 1. Putative mechanism of the anti-leukemic effects of both BJe and its main flavanones



Conclusion

There are many kinds of bioactive substances found in nature and, among these, flavonoids are particularly remarkable. The primary dietary source of flavonoids CFs, which are grown, processed, and eaten all over the world. Given the great burden of cancer, the search for new medications, including exploring the world of complementary and alternative medicine is necessary. Among CFs, bergamot proved its highly valuable anti-cancer properties which can be exploited in the prevention of several types of tumours. In the field of haematological disorders, namely AML, we recently showed that BJe, along with its main flavanones, is

able to inhibit malignant cell proliferation via inducing apoptosis and altering cell cycle. Moreover, these mechanisms seem linked to the inhibition of SIRT2, a known marker of proliferation in relapsing AML. These results support the potentiality of these natural remedies, rendering BJe and its flavanones attractive candidates for the prevention of AML.

Ethical Disclosures

The Authors declare no conflict of interest

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