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AISA can control the inflammatory facet of SASP

Patrizia A. d'Alessio[a,*], Marie C. Béné[b]

[a] Genopole Entreprises, 4 rue Pierre Fontaine, 91058, Evry, France
[b] Pôle Laboratoires Service d'Hématologie Biologique CHU de Nantes, 9 Quai Moncousu, 44000, Nantes, France

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ABSTRACT

Senescent cells have been suspected, because of their secretory phenotype (SASP or Senescence Associated Secretory Profile), to contribute to the extension of the chronic inflammatory condition leading to unhealthy aging processes. AISA (Anti-Inflammatory Senescence Actives) monoterpens have been characterized as possessing anti-inflammatory capacities in young cells submitted to pro-inflammatory cytokine stimulation. They have also been demonstrated to have the ability to act on senescent cells, reversing their characteristic pro-inflammatory phenotype.

This is due to the fact that AISA act on the cytoskeleton scaffold of cells where actin polymerization induces the expression of adhesion molecules, fueling the infernal inflammatory loop. In comparison to other isoprenoid actives in degenerative diseases, the AISA monoterpene adds a mood-modulating capacity inducing the *vagus nerve* tone and its potent anti-inflammatory role.

1. Introduction

There is an intimate link between inflammation and cancer development, related to neo-vascularization. Yet inflammation is also known to accelerate the loss of cells' replicative capacity. The latter phenomenon, called senescence, increases with aging. Moreover, it is self-sustained by the secretion of inflammatory cytokines by senescent cells. This is at the root of degenerative disorders and age-associated diseases (Campisi and Robert, 2014). The ying/yang effect of inflammation could be the very mechanism leading to a decrease in cancers after 70 years old, through the maintenance/enhancement of senescence in pro-tumoral cells. (Rao and Jackson, 2016; Faget et al., 2019). There is therefore an intriguing paradigm. Are we more and more inflamed, by the growth arrest of pro-inflammatory senescent cells as we age? Or can we slow-down aging through our capacity to get rid of a deleterious chronic inflamed status? Has evolution established some disposable soma barriers (Westendorp and Kirkwood, 1998) so that we cannot escape the descent into a complex inflammaging biochemistry that fatally will shorten our lifespan?

Because of the progressive discovery of the detrimental effects of inflammation, anti-inflammatory drugs were developed. However, at some point around 2004, it became obvious that drastic anti-inflammatory interventions, *i.e. via* COX-2 inhibitors, would not be tolerable because of the threat of cardio-vascular imbalance they were associated to (Simon and White, 2005).

Shortly before, a new paradigm for drug discovery had been validated for cancer (Sager and Lengauer, 2003). It stated that such drugs should be able to interfere with cell signaling. In the case of endothelial cells, this meant affecting the mechano-transduction in vessel walls (Nagel et al., 1999). This would involve the elastic status of the cytoskeleton and appropriate monitoring of actin polymerization in endothelial cells as well as the expression of adhesion molecules (Cheng et al., 1996).

Hence a road was opened to look for compounds impairing the connection between cytoskeleton modifications and adhesion molecules expression, the latter being enhanced under the influence of pro-inflammatory cytokines. Such inhibitory capacities would profoundly affect the diapedesis process elicited by pro-inflammatory cytokines such as TNF-α by which the endothelial cell recruits activated leukocytes (Alon and van Buul, 2017) enhancing the inflammatory status.

2. Inflammatory phenotype and senescence

This new approach of the intertwined notions of inflammation and senescence required confirmation. This was achieved through a model, that we developed (Moutet et al., 1998) of endothelial cells either submitted to extensive senescence-inducing passages or obtained from elderly animals (Bisson et al., 2008). It was indeed demonstrated in these experiments that senescence was accompanied by a polymerization of cytoskeleton actin. Concomitantly, an overexpression of

* Corresponding author.
E-mail address: endocell@aisa-tx.com (P.A. d'Alessio).

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adhesion molecules ICAM-1, VCAM-1 and selectins was observed (Fig. 1).

Both these phenomena, *i.e.* actin polymerization (and therefore cytoskeleton cable formation) and overexpression of adhesion molecules are tightly related. These adhesion molecules then become able to catch the integrins exposed by leukocytes and trigger the diapedesis of such pro-inflammatory cells as neutrophils. The relationship with inflammation was consolidated by showing that TNF-α stimulation yielded the same effects on young cells (Moutet et al., 1998).

Of interest, as alluded to in the introduction, senescent cells have been demonstrated in numerous studies to self-sustain their inflammation status (Salama et al., 2014). This is occurring *via* their specific secretory profile, called SASP (Young and Narita, 2009). Although IL-6 is clearly the key component of this phenomenon, SASP is in fact a multifaceted phenotype involving a number of interleukins, chemokines, metalloproteins and cellular interactions *via* the regulation of cytokine receptors (reviewed in Coppé et al., 2010). This complex landscape initiates an independent pro-inflammatory stimulation, escaping immune control (Tchkonia et al., 2013). Even stem cells can be affected by such a pro-inflammatory environment, as a physiological regulation (Capone et al., 2018). However, under excessive stress, the production of progenitors could be fundamentally compromised thereby impairing any potential of regeneration (Campisi, 2005; Goligorsky, 2014). Of note, the pluripotent mesenchymal stem cells can also be affected by these situations with the result of impaired capacities of tissue regeneration and accelerated aging (Choudhery et al., 2012; Lunyak et al., 2017).

Thus, as per extrapolation of Gerald Edelmans' legacy, adhesion indeed favors the expansion of inflammation (Edelman, 1993) essentially through its ancestral and conserved capacity of recruiting neutrophils (Schimmel et al., 2017). Would it nonetheless be possible to stop the potentially deleterious recruitment of immune cells without interfering with the beneficial pro-inflammatory stimulation, at the root of protection against infection and necessary for repair? This concept would drive a search for actives fundamentally different from currently proposed biologics such as anti-TNF antibodies or chimeric molecules which, although remarkably efficient, are merely dampening the effect of already secreted cytokines (Monaco et al., 2015) and are not devoid of side-effects.

We embarked on this quest based on the relationship of cytoskeleton and surface adhesion molecules. Indeed, as evoked above, the motility/polymerization of stress fibers favor the expression of the extracellular moiety of adhesion molecules. We tested the possibility of a mechanical alteration of actin polymerization. Could immune regulation thus be envisioned as actin polymerization upon TNF-α stimulation?

2.1. Evidence from cell cultures

Starting form a very large pool of plant extracts (ICSN[1] collection) that had been gathered at the frontier between China and Vietnam and preserved at Hanoi University, we proceeded to a bio-guided selection and eventually narrowed our findings to a special type of molecules, all belonging to the class of monoterpenes. These were dubbed AISA for Anti-Inflammatory Senescence Actives. Consequently, a discovery conjugating the inhibitory effects on actin polymerization and adhesion molecule expression was patented in 2005 (PCT WO 2005/105074 A2; d'Alessio, 2005).

These monoterpens were respectively geranyl-acetate, geraniol, *d*-Limonene, perillyl alcohol, and iso-menthone. In cell culture models of senescent endothelial cells, they were indeed able to deconstruct the actin polymerization scaffold and concomitantly reduce the overexpression of adhesion molecules. Moreover, these actives induced a remarkable change in cells' shape. Senescent cells are large and flattened, resembling a fried egg. Upon treatment *in vitro*, they were able to retrieve a "rejuvenated" 3D structure.

Moreover, AISA was also shown to stop the NF-κB dependent neosynthesis of such adhesion molecules as ICAM-1, VCAM-1 or E- and P-selectin and thus provided a new paradigm for an efficacious anti-inflammatory approach (d'Alessio et al., 2013).

From these experiments, two important conclusions could be consolidated:

- senescence, inflammation, actin polymerization and expression of adhesion molecules are interconnected
- this can be successfully counteracted by natural compounds, demonstrating the reversibility of these mechanisms.

2.2. Evidence from murine pre-clinical studies

The next step was to push the proof of concept in *in vivo* models. Several years were thus spent to explore the toxicity, bioavailability and therapeutic window of the lead active identified, *d*-Limonene. It soon appeared to display no toxic effect, confirming an abundant literature about this widely used compound endowed with multiple capacities (Sun, 2007). Focusing on the suspected inflammation counteractive effect identified *in vitro*, we turned to clinically-relevant models. Oral or topic administration of *d*-Limonene proved extremely efficient in the prevention and cure of a colitis model in rats (d'Alessio et al., 2013) as well as on dermatitis and wound healing models in mice (d'Alessio et al., 2014a). This proof of concept of the anti-inflammatory properties of *d*-Limonene was accompanied by two more surprising and unexpected effects. Wound healing in mice was accelerated by *d*-Limonene topical application, but without neo-angiogenesis.

The animals in all models also appeared serene and relaxed, though curious, suggesting a neurological effect of the monoterpene on the mood.

It was thus tempting to explore the wholly different field of stress management problematics and ethology. The net outcome of this passionate period was that, if there certainly exists a link between cancer and inflammation, an even stronger link relates stress and inflammation. Of course, this could not have been suspected from endothelial cell cultures. Setting up an experiment on Functional Observational Battery (FOB) in rats, we could confirm that supplementing diet with *d*-Limonene significantly improved the animals' behavior in stress conditions (d'Alessio et al., 2014b; Fig. 2).

2.3. Evidence from clinical studies

Having assessed or confirmed the innocuity of the compound and its amazing capacities, we applied for and obtained a grant for a multicenter trial in humans. The RISTOMED (Ostan et al., 2016) project proposed to four groups of healthy aged (65–85 years old) individuals of both genders a Mediterranean diet supplemented for three of them by various nutraceutics, including AISA molecule soft gel capsules in group C. The results were stunning. AISA supplementation displayed a significant anti-inflammatory effect in individuals with a baseline inflammatory status. It restored normal levels of IL-6 (Fig. 3), HOMA-IR, fibrinogen, insulin, and glycemia. Moreover, analysis of the Quality of Life (QoL) questionnaires included in the project disclosed a profound improvement of the scores of Center for Epidemiologic Studies-Depression Scale (CES-D) and grip tests. Last but not least, AISA also acted on microbiota strains by modifying the ratio between coliforms and Clostridia.

3. Mechanisms of action of AISA (Anti-Inflammatory-Senescence Actives)

The studies reported above indicate a broad spectrum of activity for *d*-Limonene. Part of this activity is likely to be linked to the lipophilic

[1] ICSN, Institut Chimie Substances Naturelles, CNRS, Gif-sur-Yvette, France.

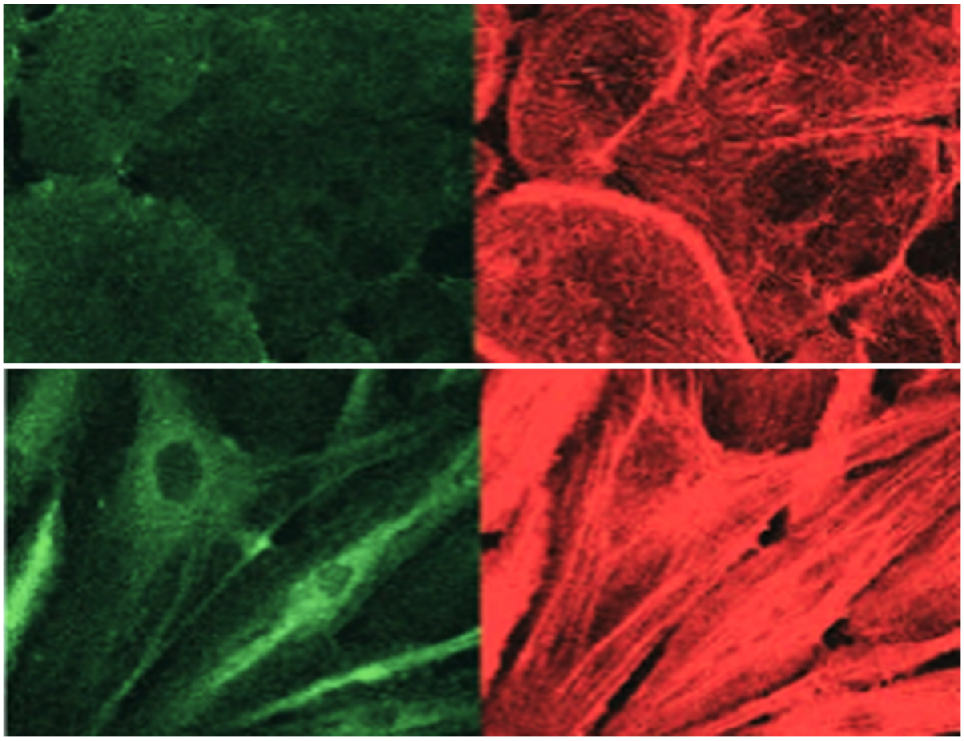

Fig. 1. Human dermis endothelial cells patterns of ICAM-1 (left) and actin (right) expression. The above panels show the absence of ICAM-1 and loose actin in unstressed cells. The below panels exemplify the drastic changes induced by inflammation brought by TNF-α stimulation.

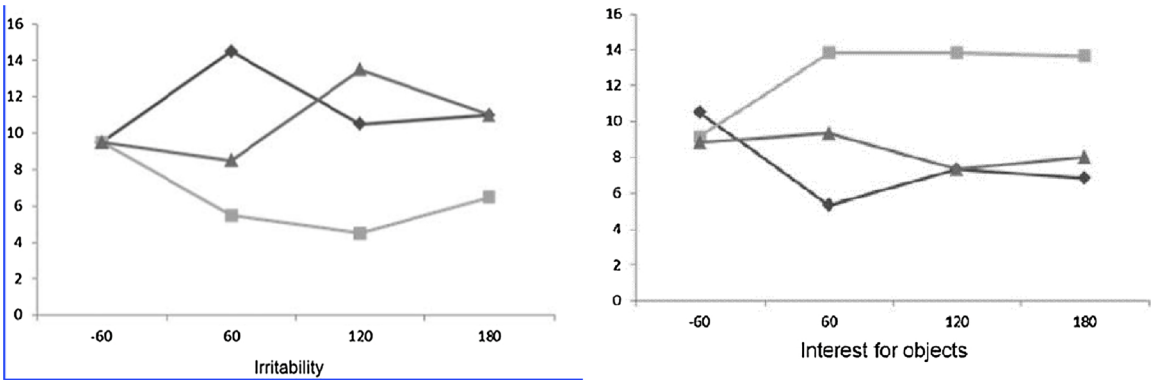

Fig. 2. (d'Alessio, 2014b). Functional Observational Battery tests in rats feeding (diamonds), *d*-Limonene (squares) or the metabolite perillyl alcohol (triangles). Ordinate: arbitrary units of activity; abscissa time span in minutes. The animals were submitted to mild stress, which is shown here to increase irritability and induce a loss of interest in objects. These effects were completely erased by prior feeding with *d*-Limonene one hour before the experiment.

characteristics of this molecule, able to passively cross the double phospholipidic layer of cell membranes (Sapra et al., 2008). This indicated that, because there is no need for a specific receptor, *d*-Limonene may act in an array of different cell types. In the models just described, this indeed stands true for at least endothelial cells (in culture), skin and gut epithelia, as well as some components of the nervous system involved in mood control.

In a series of *in vitro* experiments on mouse fibroblasts, a crucial role of *d*-Limonene on the inhibition of NF-κB translocation upon stimulation by TNF-α could be demonstrated (d'Alessio et al., 2013). The key position of NF-κB in numerous signaling pathways thus likely accounts for the pleomorphic effect of *d*-Limonene. Another molecular mechanism of action had been demonstrated earlier in cancer research. In these studies, *d*-Limonene was shown to inhibit the iso-prenylation of rho, involving molecular motors (Shojaei et al., 2014; Rottner et al., 2017) (Fig. 4).

In her work, Pamela Crowell at the Wisconsin University had focused on a signaling cascade operating in cancer formation and dissemination (Crowell et al., 1996). These findings were applied to phase 3 clinical trials in pancreatic, colon and breast cancer. Without any side effect (Vigushin et al., 1998), it appeared possible to reduce the tumor mass as well as inhibit substantially all metastatic process. Of note, these results were obtained at dosages up to 10 times higher than those we had identified for their anti-inflammatory effect.

Another interesting aspect of the interfering role of *d*-Limonene can be found in apparently unrelated studies examining the importance of prenylation inhibitors in the improvement of such neurodegenerative disorders as Alzheimer's disease (Jeong et al., 2018). This points indirectly to the capacity of *d*-Limonene to impact neuronal circuits involved in mood regulation and control of the reward system. The latter could involve the dopamine-release system, a notion demonstrated in a Japanese rat model where *d*-Limonene significantly modified the production of this neuro-mediator (Fukumoto et al., 2006).

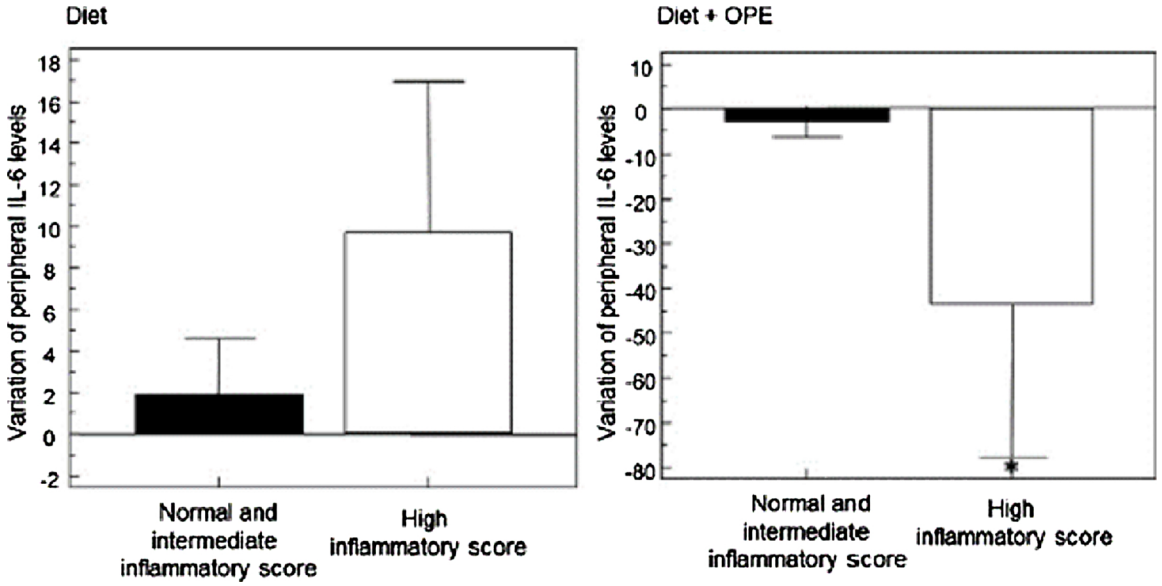

Fig. 3. RISTOMED results on IL-6 inhibition (d'Alessio, 2013) the left panel shows the increase in IL-6 in the group of subjects with a high inflammation baseline score only submitted to an adapted diet. On the right panel, the reverse is displayed, for individuals who followed the same diet supplemented by *d*-Limonene, the major decrease in serum IL-6 being observed in patients with high inflammatory baseline score.

4. Influence of AISA on microbiota

Microbial species harbored by any individual are known under the generic name of microbiota. These microorganisms are recognized to represent at least one log more of any human being's cells (Blum, 2017). The microbiota contributes greatly to homeostasis towards the environment, provision of essential metabolites and assimilation of nutrients. In this acception, diet can favor microbial strains able to induce epigenetic changes relevant for healthy aging (Bhat et al., 2017). The microbiota is moreover exquisitely sensitive to stress. As a relationship to aging, it has recently been shown that the variety of bacterial strains in the microbiota is determinant. Some strains decrease significantly over time in most individuals, with the exception of centenarians. This has been shown in as different cultures as Italy and China, indicating that a greater variety and a higher amount of microbiota are a consistently observed in healthy aging centenarians (Santoro et al., 2018; Deng et al., 2019).

Of crucial relevance, it must be noted that some microbiota strains are specialized in the production of short chain fatty acids (SCFAs). The latter have been extensively characterized for their role in protecting the barrier function of the gut (Louis et al., 2017). Relevant to this matter of intestinal barrier integrity, we demonstrated that *d*-Limonene strengthens the enterocyte barrier in a dose dependent manner, that its action is nearly immediate and lasts for at least 26 h when using the native form of the molecule (d'Alessio et al., 2013).

Moreover, SCFAs are liable to induce the production of histone deacetylases inhibitors, thus liberating the transduction of, notably, anti-tumor molecules such as p53 (Khan et al., 2014).

SCFAs also display a significant anti-inflammation activity. Therefore, enriching or inducing the selection of SCFA producers might reduce systemic inflammation and contribute to healthy aging.

Finally, *d*-Limonene was shown in our RISTOMED study to modify the ratio between *Clostridii* and *E.coli*, suggesting an influence on the microbiota strains. As confirmation, a cohort of long-living people (Santoro et al., 2018), was shown to possess a more diverse gut microbiota than younger adults, contradicting conventional views of a loss of microbiota diversity with age (Nagpal et al., 2018) This study also found that SCFA producers such as *Clostridium* cluster XIVa, are

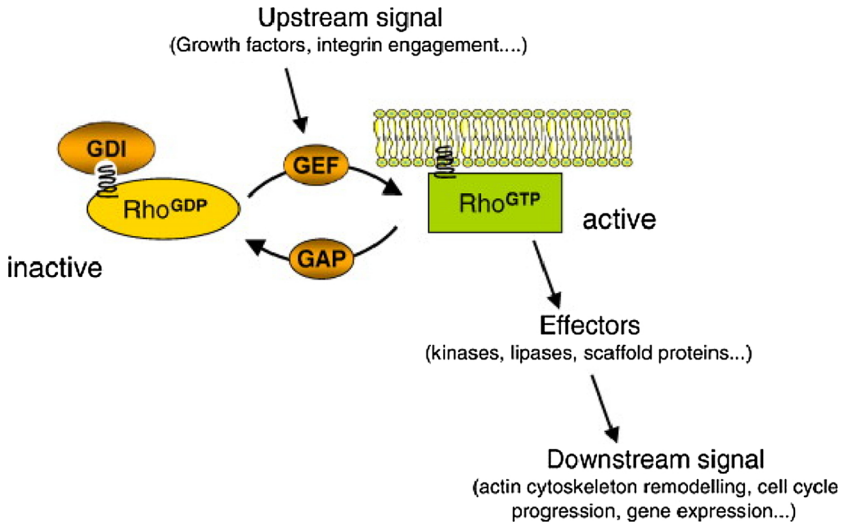

Fig. 4. Iso-prenylation of rho and molecular motors (from Grise et al., 2009).

enriched in long-living Chinese individuals (Kong et al., 2016).

5. Conclusion

By 2050, elderly people (>65 years old) will be 1.6 billion worldwide (He et al., 2015) which encourages the search for new solutions to mitigate age-related health problems. The role of inflammation, it being acute, chronic, silent or cold, is not to be argued upon any more. It is capital to take it into account in the process of unhealthy aging. Our work has contributed evidence about the mechanisms of the anti-inflammatory action of AISA monoterpenes on enterocytes, fibroblasts and endothelial cells. These compounds stop diapedesis, act on rho farnesylation, modulate actin cytoskeleton and adhesion molecules expression. They were also shown to interfere with the NF-κB pathway and increase enterocytes' resistivity (d'Alessio et al., 2013). These effects are also sustained by the demonstration, by others, of the role of monoterpens on the expression of p16 and/or p21 (Bardon et al., 2002; Koyama et al., 2013). In animal models, a broad multi-organ activity has been demonstrated, including neurological effects on behavior (d'Alessio et al., 2014a). These results have been confirmed in humans, both about inflammation- and mood-control (Ostan et al., 2016). An interesting trait was the additional potential regulation of the microbiota, placing AISA at the root of aging-related silent inflammation control.

d-Limonene could thus alleviate inflammation and its consequences, allowing for an enjoyable inflammation-free aging. Moreover, through its anti-neoangiogenic properties demonstrated in *in vitro* and *in vivo* models, it might eliminate the threats of cancer invasion and dissemination.

The unexpected but documented effect of AISA compounds as mood modulating agents involving their role in neuro-endocrine regulation (dopamine enhancement) has to be considered. Indeed, anti-stress biochemical and neuro-immune regulations potently lower the endogenous process of inflammation, thus defusing the accelerating mechanism towards a senescent phenotype.

The circle closes with recent published evidence of the role of isoprenoids in Alzheimer prevention, through an inhibition of iso-prenylation (Jeong et al., 2018), a mode of action also recognized to monoterpens. The great advantage of the latter in this field is their capacity to also address mood improvement (d'Alessio, 2018).

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