Phytochemistry 206 (2023) 113518

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The healing bitterness of *Gentiana lutea* L., phytochemistry and biological activities: A systematic review

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ARTICLE INFO

Keywords:
Gentiana lutea Linn.
Gentianaceae
Yellow gentian or bitterwort
Systematic review
Bitter compounds
Pharmacological activities
Molecular mechanisms

ABSTRACT

Over many years, natural products have been a source of healing agents and have exhibited beneficial uses for treating human diseases. The *Gentiana* genus is the biggest genus in the Gentianaceae, with over 400 species distributed mainly in alpine zones of temperate countries around the world. Plants in the *Gentiana* genus have historically been used to treat a wide range of diseases. Still, only in the last years has particular attention been paid to the biological activities of *Gentiana lutea* Linn., also known as yellow Gentian or bitterwort. Several *in vitro/vivo* investigations and human interventional trials have demonstrated the promising activity of *G. lutea* extracts against oxidative stress, microbial infections, inflammation, obesity, atherosclerosis, etc..

A systematic approach was performed using Pubmed and Scopus databases to update *G. lutea* chemistry and activity. Specifically, this systematic review synthesized the major specialized bitter metabolites and the biological activity data obtained from different cell lines, animal models, and human interventional trials. This review aims to the exaltation of *G. lutea* as a source of bioactive compounds that can prevent and treat several human illnesses.

1. Introduction

The plant kingdom provides a valuable resource for drug formulations with the potential to prevent and/or treat many illnesses. Historically, compounds from natural resources have been considered the backbone of traditional healing worldwide and have also been an integral part of cultures (Salehi et al., 2019). However, their application as characterized and isolated molecules useable for developing new drugs did not begin until the 19th century. From this point on, due to their low production costs and safety, natural compounds have played a key role in the synthesis of modern drugs, particularly antimicrobial and anticancer agents. Among the numerous natural resources existing in nature, *Gentiana lutea* Linn. has been selected for this work.

The Gentianaceae is a cosmopolitan group of more than 1600 species belonging to 87 genera distributed worldwide. *Gentiana* Linn. is the largest genus in the family and comprises more than 360 species, including *Gentiana lutea* L., also known as yellow Gentian or bitterwort (Pérez-García et al., 2012). *G. lutea* is a perennial herb that grows in the mountains of Europe, Western Asia, and Turkey, although it is now cultivated in several areas of the world, including India. Erect stems and yellow flowers characterize the gentian species; however, the official drug consists of dried rhizomes and roots and is included in several pharmacopoeial monographs (Buchwald and Mikołajczak, 2015; Mathew et al., 2004). Gentian root appears as branched, single subcylindrical pieces of different lengths, usually 10–40 mm thick. The rhizomes are more prominent in diameter than the roots and often bear one or more apical buds and surrounding leaf scars. Roots are collected in autumn, and drying them immediately after harvesting is essential to avoid fermentation processes responsible for reducing extract content. The freshly harvested material is yellowish-white on the inside but, during drying, becomes darker and develops its characteristic odor. The raw material is brittle as it is completely dried, but it quickly absorbs moisture from the air and becomes hard (Buchwald and Mikołajczak, 2015).

To date, *G. lutea* is an imperiled species in most European countries due to the uncontrolled harvesting of roots to extract bitter glucosides; for this reason, it is under protection. Gentian roots are a high source of

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https://doi.org/10.1016/j.phytochem.2022.113518
Received 9 August 2022; Received in revised form 17 November 2022; Accepted 18 November 2022
Available online 21 November 2022

Abbreviation

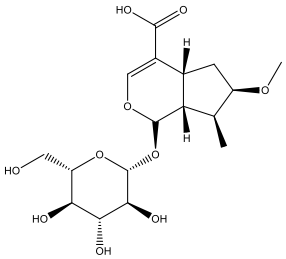
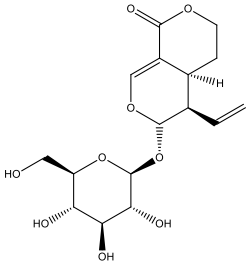
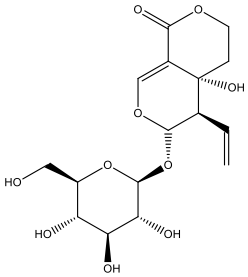
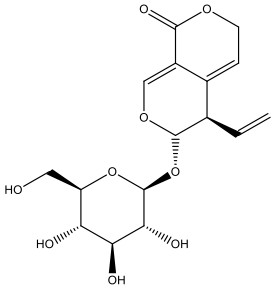
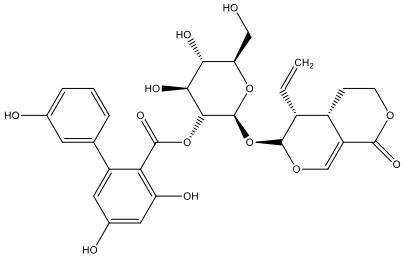
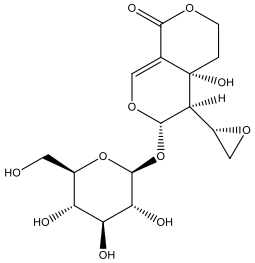
α-RIM	α-subunit-interacting regulator
AA	Arachidonic acid
ACC	Anterior cingulate cortex
Adipoq	Adiponectin
AID	Autoinhibitory domain
ALR2	Human recombinant aldose reductase
AMPK	AMP-activated protein kinase
Bcl-2	Anti-apoptotic protein B-cell lymphoma 2
BHA	Butylhydroxyanisole
CAM	cellular adhesion molecules
CAT	Catalase
CBMN	Cytokinesis-block micronucleus
CBP	CREB binding protein
CDKs	Cyclin-dependent kinase
Cebpα	CCAAT/enhancer-binding protein α
CerS3	Ceramide synthase 3
CNS	Central nervous system
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DEPMPO	5-(diethoxy-phosphoryl)-5-methyl-pyrroline-N-oxide
DMBA	7,12-dimethylbenz(α) anthracene
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELOVL	Elongases
E-NTPDases	Ecto-nucleotide triphosphate diphosphohydrolases
ERK1/2	Extracellular signal-regulated kinase 1/2
ESR	Electron spin resonance
Fabp4	Fatty acid-binding protein 4
FRAP	Ferric reducing antioxidant power
GLUT4	Glucose transporter type 4
GPX	Glutathione peroxidase
GSH	Glutathione
HeLa	Human cervix adenocarcinoma
HOCl	Hypochlorous acid
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intracellular CAM-1
IP_3	Inositol 1,4,5-trisphosphate
KD	Kinase domain
Lpl	Lipoprotein lipase
LPS	Lipopolysaccharides
LS174	Human colon carcinoma
MAO	Monoamine oxidase
MAPKs	Mitogen-activated protein kinases
MCF7	Human breast cancer
MDA	Malondialdehyde
MDP	Muramyldipeptide
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MPO	Myeloperoxidase
NGF	Nerve growth factor
NMDA	GluN2B-containing N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptors
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OVX	Ovariectomy
$P2Y_2$	Purinoceptor 2
PBMC	Peripheral blood mononuclear cell line
PC3	Human prostate cancer
PCNA	Proliferating cell nuclear antigen
PDBu	PKC activator phorbol-12,13-dibutyrate
PDGF-BB	Platelet-derived growth factor-BB
PECAM-1	E-selectin, platelet endothelial CAM-1
PEPCK	Hepatic phosphoenolpyruvate carboxykinase
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLCγ2	Phospholipase C-γ2
Plin1	Perilipin1
pRB	Retinoblastoma protein
RASMCs	Rat aortic smooth muscle cells
RASMCs	Rat aortic smooth muscle cells
ROS	Reactive oxygen species
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SH-SY5Y	Human neuroblastoma cell line
Sirt-1	Sirtuin-1
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant assay
TNF-α	Tumor necrosis factor alpha
TPC	Total phenolic content
TrkA	Tyrosine kinase receptor
VCAM-1	Vascular CAM-1
VSMC	Vascular smooth muscle cells
XO	Xanthine oxidase enzyme

bitter molecules such as amarogentin and gentiopicroside, known for their medicinal properties since ancient times. Gentian is indeed a pleiotropic drug able to exert antioxidant, antimicrobial, anti-inflammatory, anti-atherosclerotic, antihypertensive, antiobesogenic, hepatoprotective, radioprotective, and antidepressant properties. This review explores the research articles available to date to provide a complete overview of the current findings in the field of *G. lutea* compounds' biological activities using a systematic approach.

2. Chemical composition

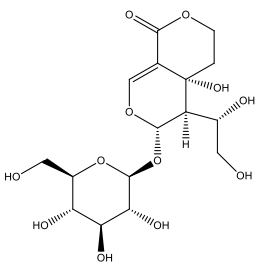
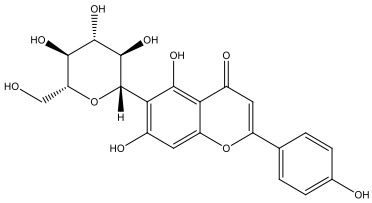
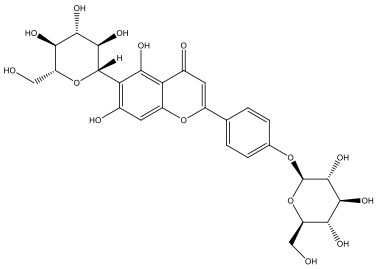
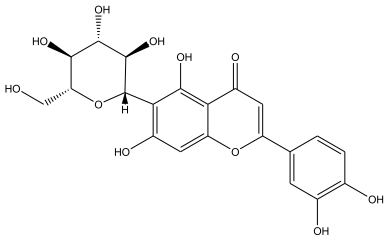
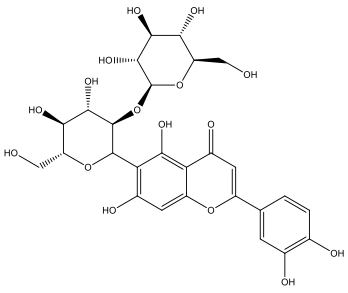
Numerous metabolites have been identified from *G. lutea,* such as iridoids, secoiridoids, xanthones, and flavones represent the main bioactive compound classes (Table 1). Variations in compound levels were observed between aerial and subaerial parts. Gentiopicroside and sweroside were found to be most abundant in root extract, isovitexin was more predominant in leaf extract, while the amount of isogentisin was ten times higher in flowers than in leaves (Šavikin et al., 2009a). The bitter principles are mostly found in the outer layers of the thinner roots. In addition, roots and rhizomes are organs that store different compounds such as lipids (6–7% dry weight), carbohydrates (30–50% dry weight) such as sucrose, gentianose, and a smaller quantity of glucose, gentiobiose, and fructose, pectin, essential oil, and free amino acids. Several studies reported that gentiopicroside is the most dominant compound (1.85–9.53%), followed by loganic acid (0.10–1.30%), swertiamarin (0.08–0.45%), and sweroside (0.05–0.35%). Gentisin, isogentisin (0.03–0.48%), and amarogentin (0.01–0.07%) are found in much lower concentrations (Aberham et al., 2007; Mustafa et al., 2015). The difference in the content of compounds was also evidenced between wild, cultivated, and commercial *G. lutea* samples and may be due to environmental factors, geographical conditions, origins, and methods for the obtainment, as well as the plant's age and stage of development (Mustafa et al., 2015). Plants can also have various heavy metal concentrations based on the different growing areas. Due to the soil's acidity in mountain regions, *G. lutea* roots showed cobalt, nickel, and chrome concentration within their critical concentrations in plants. Usually, heavy metal concentrations are higher in roots than in other parts of the plants (Radanović et al., 2007). In the following section, active

Table: 1
Main bioactive compounds of *Gentiana lutea* L.

Compound	Molecular Formula	Structure	Part of *G. lutea* L.	Ref.
IRIDOIDS AND SECOIRIDOIDS				
Loganic Acid	$C_{16}H_{24}O_{10}$		Root	(Aberham et al., 2007; Mustafa et al., 2015)
Sweroside	$C_{16}H_{22}O_9$		Root	(Aberham et al., 2007; Mustafa et al., 2015)
Swertiamarin	$C_{16}H_{22}O_{10}$		Leaf Root	(Aberham et al., 2007; Menković et al., 2000; Mustafa et al., 2015)
Gentiopicroside	$C_{16}H_{20}O_9$		Leaf Flower Root	(Aberham et al., 2007; Menković et al., 2000; Mustafa et al., 2015)
Amarogentin	$C_{29}H_{30}O_{13}$		Root	(Aberham et al., 2007; Citová et al., 2008; Mustafa et al., 2015)
Eustomoside	$C_{16}H_{22}O_{11}$		Leaf	Balijagić et al. (2012)
Eustomorusside	$C_{16}H_{24}O_{12}$		Leaf	Balijagić et al. (2012)

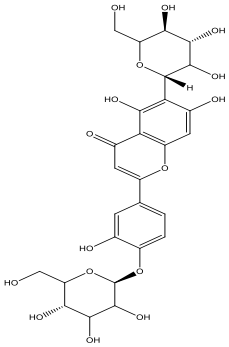
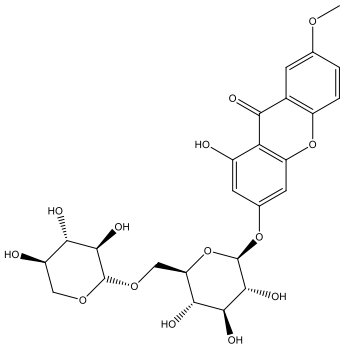
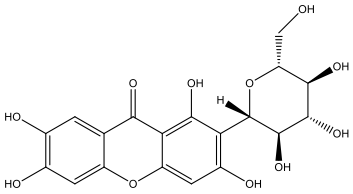
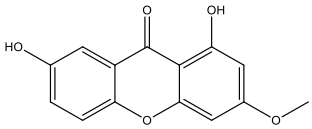
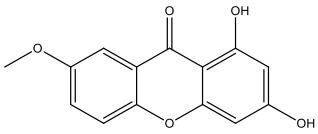
(*continued on next page*)

Table: 1 (*continued*)

Compound	Molecular Formula	Structure	Part of *G. lutea* L.	Ref.
IRIDOIDS AND SECOIRIDOIDS				
Septemfidoside	$C_{32}H_{46}O_{21}$		Leaf	Balijagić et al. (2012)
FLAVONOIDS				
Isovitexin	$C_{21}H_{20}O_{10}$		Leaf	Balijagić et al. (2012)
Isosaponarin	$C_{27}H_{30}O_{15}$		Leaf	Balijagić et al. (2012)
Isoorientin	$C_{21}H_{20}O_{11}$		Leaf	Balijagić et al. (2012)
Isoorientin O-2″-Glucoside	$C_{27}H_{30}O_{16}$		Leaf	Balijagić et al. (2012)

(*continued on next page*)

Table: 1 (*continued*)

Compound	Molecular Formula	Structure	Part of *G. lutea* L.	Ref.
IRIDOIDS AND SECOIRIDOIDS				
Isoorientin O-4′-Glucoside	$C_{27}H_{30}O_{16}$		Leaf	Balijagić et al. (2012)
XANTHONES				
Gentioside	$C_{25}H_{28}O_{14}$		Root	Aberham et al. (2007)
Mangiferin	$C_{19}H_{18}O_{11}$		Leaf, flower	Menković et al. (2000)
Gentisin	$C_{14}H_{10}O_5$		Root	(Aberham et al., 2007; Citová et al., 2008)
Isogentisin	$C_{14}H_{10}O_5$		Leaf, flower Root	(Aberham et al., 2007; Citová et al., 2008; Menković et al., 2000; Mustafa et al., 2015)

molecules from *G. lutea* will be discussed by dividing them into classes of compounds.

2.1. Iridoids

Iridoids and secoiridoids are compounds widely diffuse in the plant kingdom, especially in the *Gentiana* genus, and are pyran cyclopentane monoterpenes primarily found in the form of glycosides by reaction with glucose at the C-1 hydroxyl group. Iridoids and secoiridoids are responsible for numerous biological activities such as hepatoprotective, antitumor, and anti-inflammatory (Wang et al., 2020). Phytochemical investigations reported that *G. lutea* is a great source of these compounds responsible for the bitter flavors since it contains loganic acid, sweroside, amarogentin, swertiamarin, gentiopicroside (also known as gentiopicrin) (Menković et al., 2000), and the swertiamarin derivatives, septemfidoside, eustomorusside, and eustomoside, which were detected in *G. lutea* leaves for the first time by Balijagić et al. (2012). Amarogentin has the highest bitterness index (58×10^6), while swertiamarin, gentiopicroside, and sweroside possess a bitterness index of 12×10^3 (Ariño et al., 1997).

2.2. Flavonoids

Flavonoids are a heterogeneous group of active molecules characterized by different phenolic structures that may occur as glycosides and are known for their antioxidant and anti-inflammatory activities (Rana and Gulliya, 2019). Isovitexin, isosaponarin, isoorientin and isoorientin-2″-*O*-glucoside, isoorientin- 4′-*O*-glucoside are the main flavonoids isolated from *G. lutea* (Balijagić et al., 2012).

2.3. Xanthones

Xanthones are principally found as glycosides or mono- or polymethyl ethers. They are compounds of considerable interest due to their

many biological properties, such as antibacterial, antifungal, hepatoprotective, and antioxidant activity (Negi et al., 2013). Isogentisin, gentisin, mangiferin, and gentioside were identified in *G. lutea* (Menković et al., 2000). The first two are positional isomers with the same molecular weight and possess a weak acidic character due to the hydroxyl groups of the aromatic rings (Citová et al., 2008).

3. Results and discussion

3.1. Study characteristics

This systematic review adhered to the PRISMA statement recommendation and included articles published between 2000 and 2022 (Ponticelli et al., 2022). The search was made using PubMed [http://www.ncbi.nlm.nih.gov/pubmed (accessed May 2021)] and Scopus [http://www.scopus.com (accessed May 2021)] as databases and comprise all reports published until June 2022. The keywords used for the search were *Gentiana lutea* paired with the following words: obesity,

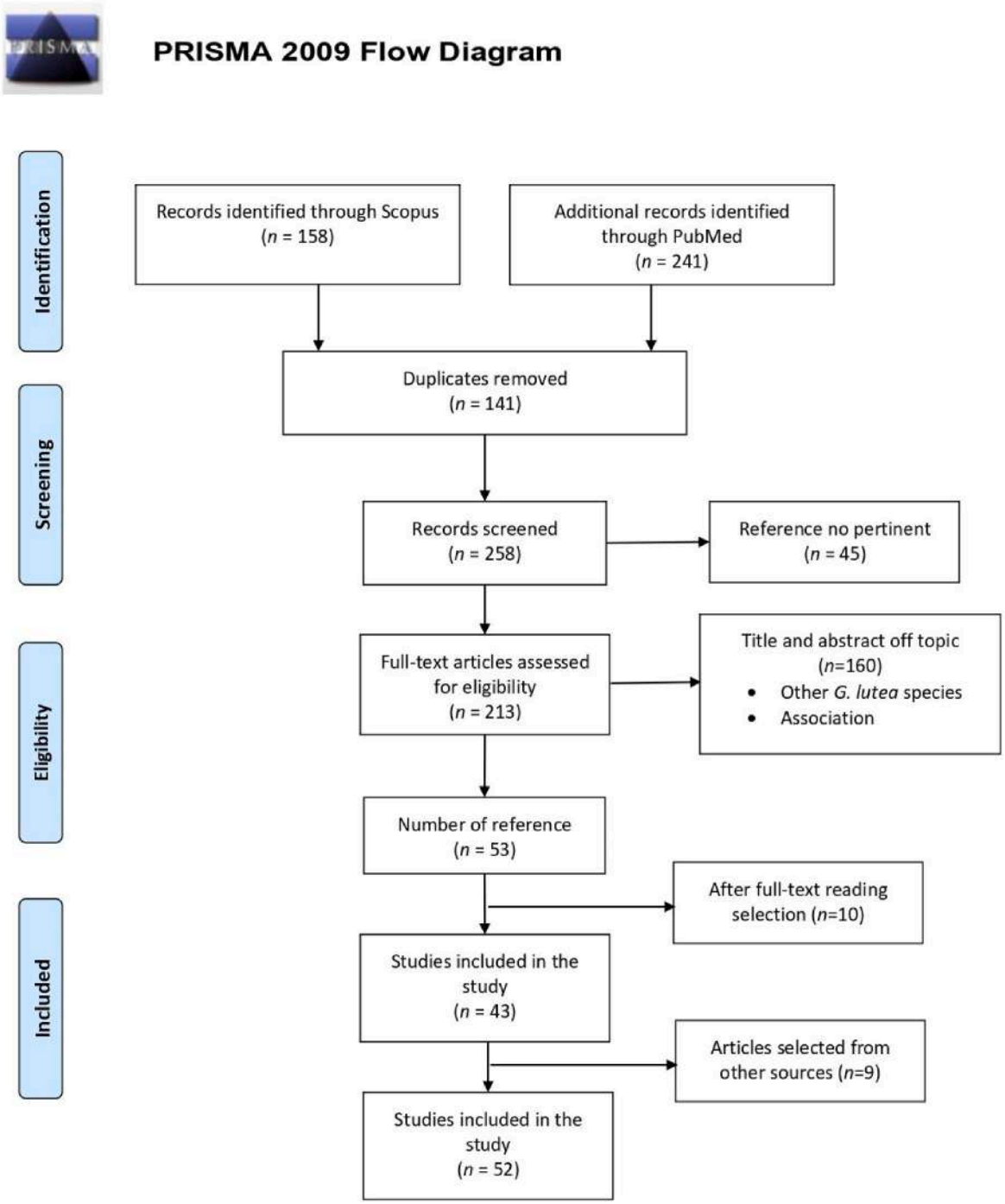


Fig. 1. Diagram of systematic review literature search results based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

antioxidant, inflammation, diabetes, atherosclerosis, cardiovascular diseases, neurological diseases, antimicrobial, antiviral, antibacterial, antifungal, cytotoxicity, and apoptosis. Research papers were restricted to English-language publications.

The initial selection provided 399 articles, of which 158 were found on PubMed and 241 on Scopus. Among the 399 items, only 354 were related to the research subject; from these 354 documents, 141 were duplicates. Of those 213 studies, 160 were off-topic based on the exclusion criteria, while 10 did not contain experimental data congruent with the topic. The final reference list comprises 53 items, of which 9 publications are derived from other sources (Fig. 1). The origin of the selected papers comes from 25 countries (Fig. 2a).

This systematic review included 48 *in vitro/vivo* reports and 4 clinical investigations. For *in vivo* and clinical investigations, dosages, administration frequency, and therapy duration were cited in every case. Risks bias assessment, founded on a checklist from the Cochrane Handbook for Systematic Reviews of Interventions, is reported in Fig. 3.

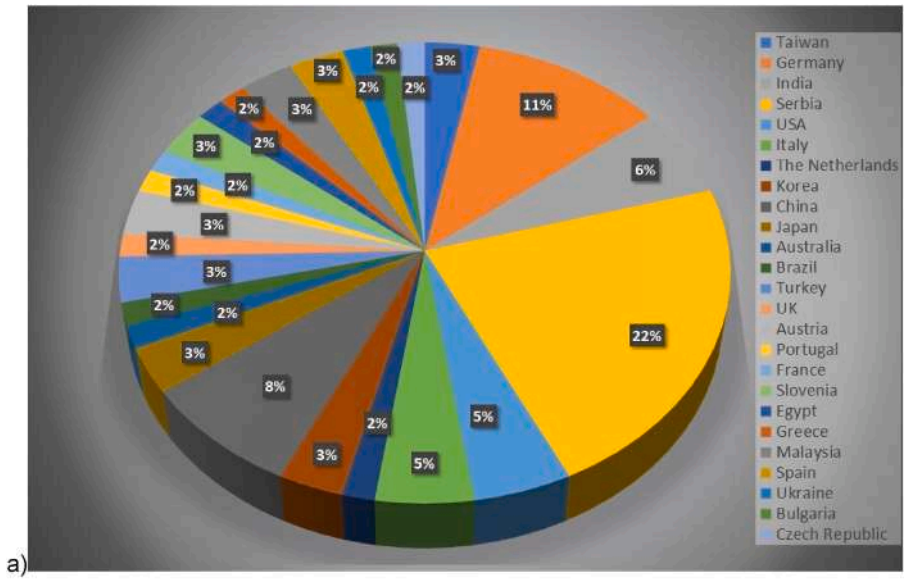
The different studies' results regarding *G. lutea*'s pharmacological activities have been summarized as follows.

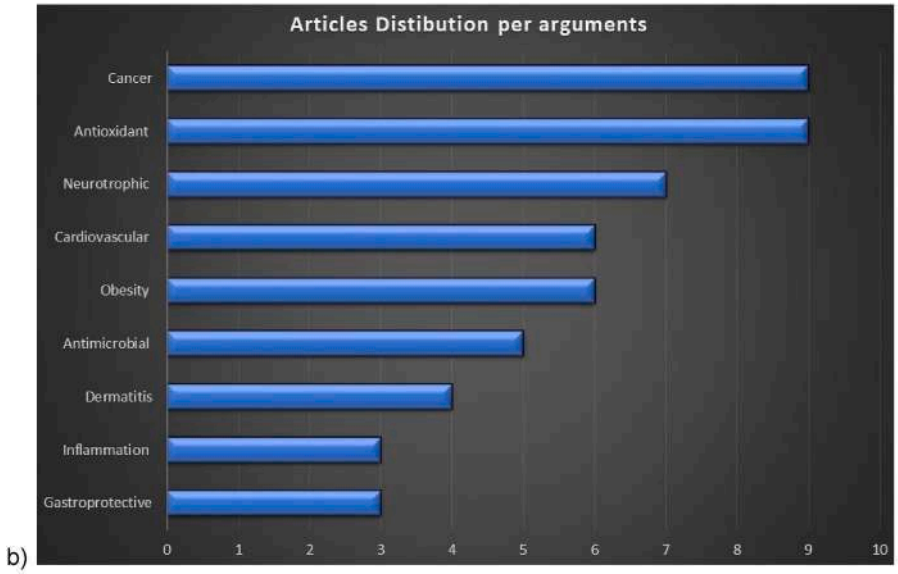
3.2. *Gentiana lutea L. And antioxidant activity*

Reactive oxygen species (ROS) are typical cellular metabolism products with critical physiological roles in cell signaling; however, an alteration of the balance between ROS production and ROS elimination causes damage to cellular structures (DNA, lipids, and proteins), leading to a condition known as oxidative stress (Da Pozzo et al., 2018). This situation is related to several pathological conditions such as neurological disorders, cardiovascular disease, ischemia/reperfusion, etc. ROS are also responsible for food deterioration leading to the necessity for using synthetic antioxidants. The more used in the food industry are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydeoquinone (TBHQ), which, however, should be related to the increased incidence of carcinogenic illness (Schieber and Chandel, 2014). For this reason, there is a continuous search for undescribed natural molecules with antioxidant action useable for preventing or treating human disease and preserving foods from lipid peroxidation and rapid deterioration (Faraone et al., 2019). Specifically, *G. lutea* seems to be a promising source of antioxidant molecules; this section treats the knowledge about the scavenging activity of this natural source.

3.2.1. *In vitro studies*

The reviewed literature on antioxidant activities of *G. lutea* extracts comprised a wide variety of *in vitro* tests taking into account the advantages and disadvantages of each. Different results between the various studies can be due to numerous factors like different plant ages, methods, and experimental conditions. One of the methods used for determining Gentian's antioxidant activity is electron spin resonance spectrometry (ERS) (Kusšar et al., 2006). Specifically, Kusšar et al. applied ERS through two different methods. One of these is the 2, 2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical with a specific electron spin resonance (ESR) signal. In the presence of substances that can either transfer an electron or donate hydrogen, the DPPH becomes an ESR non-visible complex with a consequent reduction of the ESR spectra intensity. Both the extracts showed a dose-dependent activity, but the leaf extract showed higher activity than the root extract (at 15 mg/mL and 20 mg/mL, root extract inhibited 38–51% of the reference signal while leaf extract inhibited the 95% of reference signal). However, both extracts were less active than the synthetic antioxidant BHA, as evidenced by IC_{50} values (IC_{50} 0.5 mg/mL, 7.2 mg/mL, and 19.0 mg/mL for BHA, leaf, and root extract, respectively). The second method used was the superoxide anion assay; the radical is generated during the oxidation of hypoxanthine to xanthine catalyzed by the xanthine oxidase enzyme (XO). DEPMPO (5-(diethoxy-phosphoryl)-5-methyl-pyrroline-N-oxide) was the spin trap used to create a stable adduct with the superoxide anion producing a characteristic ESR spectrum. Gentian leaf extract showed the best activity against $O_2\bullet^-$ reporting an IC_{50} of 8.2 mg/mL lower than root (IC_{50} 11.1 mg/mL) and the standard BHA (IC_{50} 14.3 mg/mL) (Kusšar et al., 2006). The different results obtained when the extract was tested in the two different assays probably occurred because, in the case of the enzymatic test, the XO could be inhibited by some compounds of the gentian, and therefore there would be less radical production. Thus it is possible to assume that these tests are unsuitable for analyzing *G lutea* extracts. However, the higher antioxidant activity of leaves than roots extract was confirmed by Kintzios et al. through the evaluation of the DPPH radical scavenging activity (70% and 15% for leaves and roots, respectively). To corroborate these results, the same research team investigated the antioxidant activity through a cell-based biosensor assay (a test able to evaluate membrane integrity and, therefore, the cell membrane potential), demonstrating that the methanol extract of leaves showed a protective effect (80% and 60% for leaves and roots, respectively) against the oxidation of immobilized cells with a consequent increase in the cell membrane potential (Kintzios et al., 2010). Despite these results, the most studied part of the plant is that of the roots due to the presence of the characteristic secoiridoids glucosides. The *G. lutea* root antioxidant activity was evaluated through the use of different assays, the total phenolic content (TPC), DPPH, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC), demonstrating that better results were obtained when 50% EtOH/H_2O was used as extractive solvent while the worst with H_2O (Azman et al., 2015; Nastasijević et al., 2012a). In the same way as ethanol, methanol (50 % MeOH/H2O) proved to be a solvent able to generate extract with a higher antioxidant activity than water (Azman et al., 2014). In fact, the methanolic extract of *G. lutea* root exhibited the highest amounts of polyphenols (92.46 ± 6.69 mg tannic acid equivalent/g extract), flavonoids (8.74 ± 0.49 mg quercetin equivalent/g extract) and flavonols (0.98 ± 0.27 mg quercetin equivalent/g dry extract) and also showed the highest antioxidant activity in DPPH assay with an IC_{50} value of 59.0 ± 13.2 μg/mL compared to aqueous extract (Cafaro et al., 2020). These results could be explicated by the polyphenolic nature of *G. lutea* active compounds. It is indeed known that polyphenols in plants formed hydrophobic or hydrogen-bound whit polysaccharides and/or proteins such as inulin and pectin highly present in *G. lutea* roots. Therfore, to ensure the extraction of poliphinols from *G. lutea* roots it is necessary to use solvent able to cleavage these bound and it was demonstrated that binary sistems formed by water and ethanol are the most efficient (Chew et al., 2011). As previously mentioned, both methanol and ethanol provided extract with the highest activity; however, it is important to consider that methanol is toxic, so not applicable for extract destinated to the production of drugs for human use. Contrarily ethanol is safe and green-friendly and should be the first choice for producing extracts from natural resources. In addition to the solvent, the pH also plays an important role in antioxidant activity; for this reason, Bayliak et al. (2016) evaluated the different behavior of gentian in radical H_2O_2 activity under acidic or alkaline pH. They reported a higher activity in acidic conditions probably because many phenolic compounds are unstable at alkaline pH; contrarily, in alkaline pH, it increases the pro-oxidant action may be due to the deprotonation of the phenols conferring stronger electron-donating property. In addition, the same results were confirmed in yeast *Saccharomyces cerevisiae* cells, where the protective effect of *G. lutea* H_2O extract (50 μL/mL) against H_2O_2 (10 mM) stress was observed in acidic pH but not in alkaline pH (Bayliak et al., 2016). Finally, another factor considered is the method of cultivation. Petrova et al. reported that the plants obtained from the cultivation with specific soils added by regulatory factors such as Zeatin (2 mg/L) and auxin IAA (0.2 mg/L) and extracted with methanol (96% *v/v*) have been shown to possess better antioxidant properties than the species classically cultivated (Petrova et al., 2019). Considering this activity, the question arose as to which active compounds could be traced back to antioxidant action. This was done through the ABTS assay, also





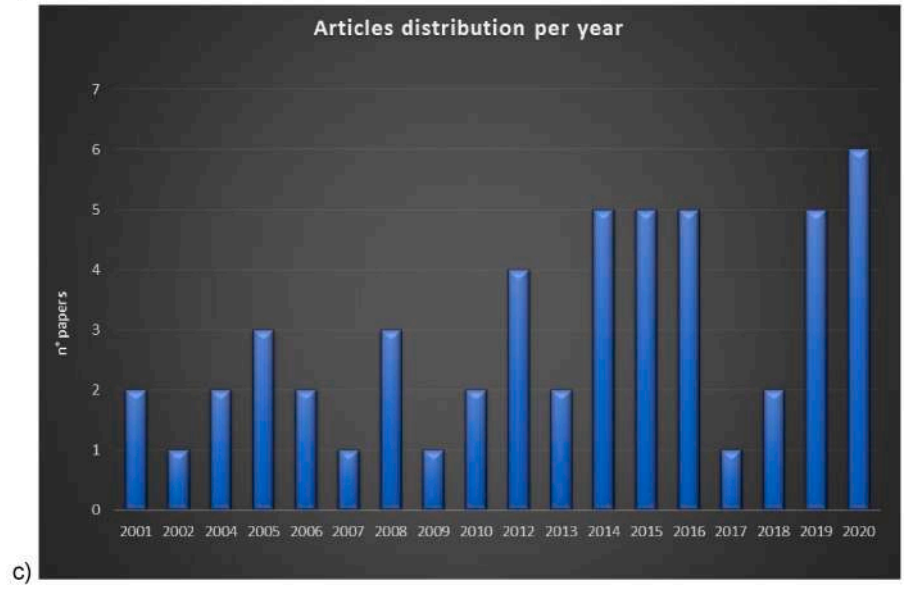


Fig. 2. (a) Schematic representation of author origin country distribution, (b) number of articles per argument, (c) distribution of the selected studies by year of publication.

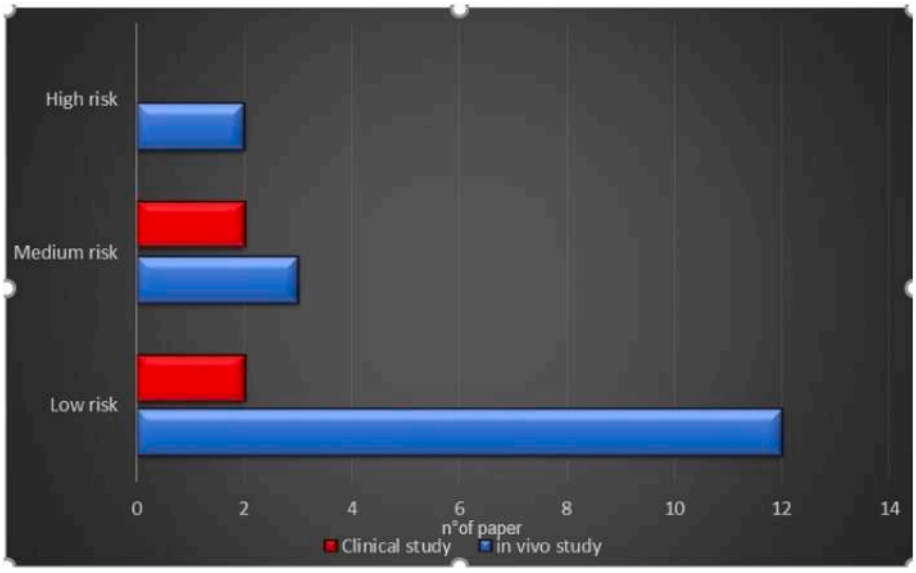


Fig. 3. The quality assessment is based on a checklist adapted from the Cochrane Handbook for Systematic Reviews of Interventions. The *in vivo* (indicated by blue bar) and clinical studies (indicated by red bar) have been classified in high, medium, and low risk of bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

known as Trolox equivalent antioxidant capacity (TEAC) assay, evaluated by HPLC analysis. It was demonstrated that gentiopicroside and sweroside were not involved in the antioxidant activity; however, two unknown compounds were detected as a negative peak at 734 nm, typical of the reduction reaction, indicating that the compounds associated with the antiradical scavenging activity are probably xanthones-glycosides (Azman et al., 2014).

However, *G. lutea* showed lower antioxidant activity compared to other plant species. For example, *G. lutea* aqueous rhizomes showed a total antioxidant capacity lower than the *Rhodiola rosea* Linn. aqueous rhizomes extract, probably due to the higher flavonoid amount present in *R. rosea*. *G. lutea*, indeed, mainly consists of secoiridoids and xanthones (Bayliak et al., 2016).

3.2.2. *In vivo studies*

Gentian antioxidant activity was also evaluated in an *in vivo* study. In particular. *G. lutea* root extract was tested for the protective effect against ketoconazole-induced testicular damage in rat models by measuring the activities of antioxidant enzymes: superoxide dismutase (SOD) and catalase (CAT). Together with glutathione peroxidase (GPX), these enzymes constitute the first line of antioxidant defense and are very important since they neutralize and prevent the production of free radicals (Ighodaro and Akinloye, 2018). SOD activity was measured by inhibiting the auto-oxidation of epinephrine to adrenochrome, and CAT activity was measured by obtaining H_2O_2 degradation at 240 nm. As reported by Amin et al. the treatment with only ketoconazole induced depletion in SOD activity and an increase in CAT activity, probably due to an overproduction of H_2O_2 in the testicular tissues. In contrast, the co-treatment of *G. lutea* (1 g/kg of body weight administered orally by gavage) and ketoconazole (100 mg/kg of body weight administered by intraperitoneal injection) did not alter the levels of the considered markers from the control indicating a possible protective action of the *G. lutea* root extract (Amin, 2008).

Based on these analyzed data, it is possible to define *G. lutea* as a promising source of antioxidant molecules. However, it is also true that most of the results obtained derive from *in vitro* studies that do not always indicate *in vivo* activity due to the complexity of organisms. To date, only an article evaluates the antioxidant activity *in vivo;* hence the need for further investigations remains.

3.3. *Gentiana lutea L. And obesity*

Overweight and obesity are complex, multifactorial, and largely preventable diseases affecting over a third of the world's population today. They derive from an energy imbalance that promotes excessive growth and expansion of the adipose tissue, leading to metabolic dysfunction and subsequent complications. Under this condition, it is possible to witness hypertrophy of adipocytes for increased lipid storage, followed by a hypertrophic state. In these conditions, there is an increase in adipogenesis and so in the number of adipose cells. Many studies have investigated the role of *G. lutea* and its constituents in treating obesity.

3.3.1. *In vitro studies*

During preadipocyte differentiation, adipogenesis plays an important role in adipocyte fat accumulation. Based on this knowledge, Park and his working group have investigated how *G. lutea* can regulate adipogenesis. I*n vitro* study assessed on 3T3-L1 preadipocytes demonstrated that treatment with *G. lutea* extract (2, 10, and 50 μg/mL) for 8 days may prevent adipocyte differentiation by downregulated adipogenic-inducing gene expression. *G. lutea* treatment, indeed, significantly inhibited the messenger ribonucleic acid (mRNA) involved in the regulation of genes, including CCAAT/enhancer-binding protein *α* (*Cebpα*), adiponectin (*Adipoq*), glucose transporter type 4 (GLUT4), and lipoprotein lipase (*Lpl*) (Park et al., 2018, 2020). *Cebpα* is a key component of the adipogenic cascade. It was seen that 3T3-L1 preadipocytes, after hormonal induction, could not differentiate if *Cebpα* antisense RNA was expressed into the cells. Likewise, *Cebpα* deficient mice cannot induce lipid accumulation in white and brown adipose tissue (Erickson et al., 2001). *Adipoq* is an anti-inflammatory adipokine almost exclusively synthesized by adipocytes (Dall'Aglio et al., 2021; Palanivel et al., 2007), and its low circulating levels have been associated with obesity, type 2 diabetes, insulin resistance, and cardiovascular disease. However, when *Adipoq* is overexpressed, rapid cells differentiation into adipocytes and a prolonged gene expression for transcriptional factors like *Cebpα* and peroxisome proliferator-activated receptor *γ* (PPAR*γ*) were observed (Fu et al., 2005). GLUT4 is primarily expressed in adipose tissue and controls adipose tissue mass through insulin-mediated glucose transport (Shepherd et al., 1993). *Lpl* is highly distributed in adipose tissue and is implied in releasing fatty acid from

lipoproteins leading to their uptake and storage into adipocytes (Gonzales and Orlando, 2007). Considering *G. lutea*'s ability to reduce the expression of these adipogenic-inducing genes, the question arose as to which compound was related to this activity. The fractionation of *G. lutea* root extract identified a single compound responsible for the anti-obesity effect, loganic acid. It was seen that 3T3-L1 preadipocyte treatment with 2, 10, and 50 μg/mL of loganic acid significantly reduced the mRNA expression of adipogenesis-related genes in a dose-dependent manner. In fact, loganic acid decreased not only the expression of *Cebpα*, *Adipoq,* GLUT4, and *Lpl*, but also that of PPARγ, perilipin1 (*Plin1*), fatty acid-binding protein 4 (*Fabp4*), and tumor necrosis factor (TNF)-alpha (TNF-α) (Park et al., 2018) (Fig. 4). PPARγ is a nuclear transcriptor factor highly expressed in adipose tissue, implied in regulating genes involved in lipid storage, adipocyte growth, and differentiation (Yajima et al., 2004). *Plin1* has an important role in adipocyte differentiation; it is localized on the lipid droplet surface for regulating the hydrolysis of triglycerides storage into fat cells. Mice with a defect in *Plin*1 expression have been shown to downregulate the adipogenic pathway. (Lyu et al., 2015). *Fabp4* is predominantly expressed in adipose tissue, where it regulates fatty acid storage and lipolysis; for this reason, it is strongly related to obesity's metabolic disorder and vascular morbidity (Floresta et al., 2017; Iso et al., 2017). *G. lutea* extract's ability to inhibit the expression of PPARγ was also confirmed by Rau and colleagues (Rau et al., 2006). In addition to the downregulation of adipogenic gene expression, it was also demonstrated that the *G. lutea* root extract could inhibit human recombinant aldose reductase (ALR2) *in vitro*. ALR2 is an enzyme belonging to the aldo-keto reductase superfamily. It is involved in the polyol pathway, where it is responsible for reducing glucose to sorbitol using NADPH as a cofactor. Generally, sorbitol accumulation determines osmotic swelling, membrane permeability changes, and oxidative stress leading to tissue injury. It was seen that inhibition of ALR2 could prevent several complications like diabetic-related disorder; however, inhibitors identified to date have limited efficacy and several side effects. This is why the search is on for undescribed active ingredients of natural origin that combine safety and efficacy. *In vitro* studies have demonstrated that methanol or ether extract of *G. lutea* root inhibited human recombinant ALR2 whit an IC_{50} value of 23 μg and 36 μg, respectively. Thus, molecular docking studies were carried out to identify constituents responsible for this action. Among the 13 compounds tested, amarogentin represented the molecule with the highest dock score. It seems that it interacted with active site residues, namely Trp-111, His-110, Leu-300, and Leu-301, forming hydrogen interaction with Leu-300, His-110, and Trp-20, and hydrophobic bonds with Trp-219. It was even demonstrated that amarogentin is able to bind the open-type conformation of ALR2 by forming a hydrogen bond with Leu-300. In particular, compared with fidarestat, a synthetic inhibitor that binds the active site of ALR2 by forming a limited number of contacts, it was seen that amarogentin also created interaction with a hydrophobic cleft called specificity pocket (Fig. 4). This result confirms the potential inhibition of ALR2 by *G. lutea*. In addition to amarogentin also gentiopicroside, known as one of the major compounds found in *G. lutea* root, can bind ALR2. However, it bonded the specificity pocket in the closed state, where it formed a hydrophobic bond with Trp-219 but did not form hydrogen interaction (Akileshwari et al., 2012). The role of *G. lutea* extract on intracellular sorbitol accumulation was also investigated. It was seen that when red blood cells collected from healthy male volunteers were incubated with 55 mM of glucose, they led to sorbitol accumulation. However, in the presence of *G. lutea* extract, there was a reduction of intracellular sorbitol accumulation in a dose-dependent manner (Akileshwari et al., 2012). Thus, these results confirm *G. lutea*'s ability to inhibit ALR2 and its role in preventing intracellular sorbitol accumulation, indicating the promising use of this natural product for treating or preventing diabetic complications.

3.3.2. *In vivo studies*

The anti-obesogenic effect of *G. lutea* in animal models was also tested. It was seen that the administration of *G. lutea* extract (100 mg/kg/day or 200 mg/kg/day) plus a 60% fat diet to C57BL/6 J mice did not reduce total food intake but had effects on total body weight. In particular, the administration of 200 mg/kg/day of *G. lutea* extract significantly inhibited total fat and body weight induced by the high-fat diet compared with the untreated control group. Further, a prevented

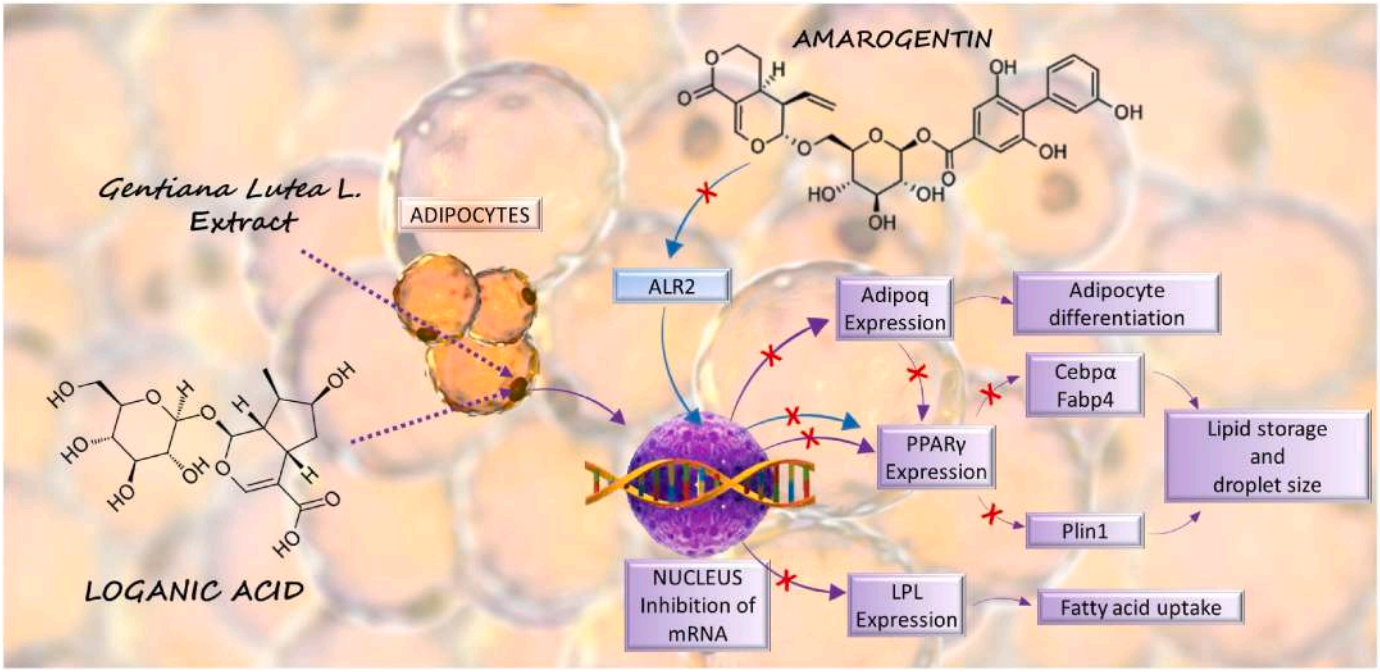


Fig. 4. Down-regulation genes implied in adipocyte differentiation. G. lutea extract and its constituent loganic acid are implied in the inhibition of the messenger ribonucleic acid (mRNA) involved in the regulation of genes, including CCAAT/enhancer-binding protein α (Cebpα), adiponectin (Adipoq), glucose transporter type 4 (GLUT4), lipoprotein lipase (Lpl), proliferator-activated receptor γ (PPARγ), perilipin1 (Plin1), and fatty acid-binding protein 4 (Fabp4). On the other hand, amarogentin indirectly down-regulating PPARγ expression throughout the inhibition of recombinant aldose reductase (ALR2).

increase in adipocyte diameter and size and reduced hepatocytes lipid deposition were also observed (Park et al., 2020). These data confirm the anti-adipogenic effects observed *in vitro*. The effect of *G. lutea* extract was also investigated on obesity-associated hormones such as leptin and insulin. Leptin concentrations in adipose tissue and plasma depend on the amount of energy stored as fat and energy balance status. The regulation of leptin is mediated, at least in part, by insulin, as leptin decreases in response to low insulin levels and increases with feeding or response to insulin stimulation (Laclaustra et al., 2007). It was seen that administering 200 mg/kg/day of *G. lutea* extract to mice fed with a high-fat diet reduces leptin and insulin serum concentration (Park et al., 2020). Thus, a reduction in weight gain was complemented by a decrease in leptin and insulin serum levels. It agrees with the knowledge that these two adipocyte-derived hormones are positively associated with fat mass and body weight (Benoit et al., 2004). Considering these good results, compounds isolated by *G. lutea* extract, like loganic acid and amarogentin, were even tasted. Loganic acid (2–10–50 mg/kg/die) was investigated on Ovariectomy (OVX)-induced Mice for 12 weeks. As in the previous *in vivo* study, if no significant differences were seen in the average daily food intake between the group treated with loganic acid and the control group, the administration of 10 and 50 mg/kg/die of loganic acid results in a reduction of body weight and total fat percentage. Besides, according to previous *in vitro* studies, the administration of 50 mg/kg/die of loganic acid to OVX mice determine the mRNA expression reduction of PPARγ in the liver and GLUT4, and *Lpl* in abdominal visceral adipose tissue (Park et al., 2018). On the other hand, the intravenous injection of amarogentin (0.1–0.3–0.5 mg/kg) to streptozocin-induced type 1 diabetic (T1DM) rats induced a marked decrease in blood sugar within 30 min. This reduction increased over time and joined a plateau from 60 to 90 min, while after 120 min, this effect was progressively reversed. Besides, 90 min later amarogentin injection, a dose-dependent reduction of hyperglycemia in diabetic rats was characterized by a lack of insulin. Moreover, after amarogentin elimination animals returned to the normal condition and no irreversible effect was observed (Niu et al., 2016). These observed effects were similar to those evidenced after metformin administration to T1DM rats (Cheng et al., 2006). The reduction of hyperglicemia was also confirmed by the OGTT test, which is widely used to assess glucose homeostasis. To investigate the mechanism underlying the hypoglycemic activity of amarogentin, the effect on the expression of skeletal GLUT4 and hepatic phosphoenolpyruvate carboxykinase (PEPCK) was also investigated. The administration of amarogentin for 1 week enhanced the expression of GLUT4 in the skeletal muscle, known for being the major site of glucose disposal, in soleus muscles isolated from T1DM rats (Niu et al., 2016). This is a good result since a decreased insulin-mediated glucose uptake due to reduced GLUT4 expression in skeletal muscle has been documented (Berger et al., 1989). Similarly, either PEPCK mRNA or protein levels have undergone a marked decrease in liver isolated from T1DM rats (Niu et al., 2016). Thus, amarogentin may influence hepatic gluconeogenesis by lowering PEPCK expression, which is highly expressed in diabetes. In another *in vivo* study, amarogentin was orally administered to streptozocin-inducing diabetes in mice in two dosages, 0.3–0.5 mg/kg body weight, once a week till the end of the study. At the end of the 8 weeks of treatment, contrarily to previous studies, no differences were observed in body weight between amarogentin-treated group and the control group; however, there was an improvement in lipid levels in the treated group, reaching values close to those of the control group. Additionally, amarogentin lowered the circulating levels of glucose, LDL-, VLDL-cholesterol, and triglycerides while improving HDL levels (Potunuru et al., 2019). It was hypnotized that this effect on cholesterol levels could be due to liver metabolism modulation through AMP-activated protein kinase (AMPK) activation. It was indeed known that AMPK activation led to the inhibition of HMG-CoA reductase, a key regulatory enzyme implied in cholesterol biosynthesis. Total serum cholesterol levels were also significantly reduced in streptozocin-induced diabetes mice fed with a regular diet supplemented with 2% *G. lutea* root powder. However, in this study, the circulating levels of HDL-, LDL-, and VLDL-cholesterol were not measured (Kesavan et al., 2016).

3.3.3. Clinical trial

Only one study has been carried out to date regarding the evaluation of *G. lutea*'s antiobesogenic effect on humans. In particular, considering that bitter-tasting compounds may modulate eating behavior by activating gastrointestinal bitter taste receptors, microencapsulated bitter *G. lutea* root extract was administrated in vanilla pudding during a randomized cross-over study. 20 healthy volunteers were treated with microencapsulated bitter ingredient-enriched pudding (EBIP), providing 100 mg of secoiridoids or control pudding (CP), on two different occasions. It was seen that EBIP administration reduced food intake by approximately 30% during the post-lunch period compared with CP. Further, a trend towards a greater glucagon-like peptide-1 response was observed after EBIP compared to CP (Mennella et al., 2016). These results aligned with releasing bitter compounds from EBIP into the gastrointestinal tract. Several studies have demonstrated that bitter-tasting compounds can modulate energy intake by inducing gastric emptying via gastric contractility inhibition and inducing the secretion of incretins (Kurpad and Swaminathan, 2011; Mani et al., 2012). Regulation of energy intake is part not only of the short-term signals that control food intake, hunger, and satiety but also of the long-term signals through which it is implicated in the conversion of energy stores (Kurpad and Swaminathan, 2011).

Thus, considering the *in vitro* and *in vivo* studies examined, *G. lutea* may represent a promising candidate for treating obesity, possibly thanks to the presence of bitter molecules. The anti-obesity effect of bitter molecules was also evidenced for other species like *Humulus lupulus* Linn. where it might be attributed to the presence of the bitter acids and their derivatives (Ponticelli et al., 2021). In the case of *G. lutea,* this beneficial effect seems to be mainly related to amarogentin, which, as mentioned above, is the bitterest molecule known, and loganic acid. Hence, it should be interesting to observe the effect these molecules alone may have on humans starting by studying their absorption, distribution within the tissues, metabolism, and excretion (pharmacokinetics), aspects that at the moment have not already been treated.

3.4. Gentiana lutea L. and atherosclerosis

Atherosclerosis is one of the major causes of mortality in industrialized countries. It results from hyperglycemia and lipid oxidation and is considered a disease of the vascular intima since the entire vascular system, from the aorta to coronary arteries, may be involved (Rafieian-Kopaei et al., 2014). Atherosclerosis is a chronic inflammatory disease that leads to several vascular events, including stroke, coronary artery diseases, and peripheral artery diseases (Taleb, 2016). The atherosclerotic process is characterized by several steps, including inflammation, lipids deposition, a proliferation of smooth muscle cells, and plaque formation. As a result, it is possible to observe an abnormal blood vessel narrowing due to arterial wall thickening, which causes insufficient blood flow (Rader and Daugherty, 2008). In this condition, to avoid total arterial obstruction, surgery is performed. However, one of the adverse events of this intervention is the recurrence of blood vessel constriction due to excessive vascular smooth muscle cells (VSMC) proliferation (Dzau et al., 2002). To counteract the overcome of restenosis end to contrast the excessive growth of VSMC, anti-proliferative compounds, e.g., rapamycin or paclitaxel (Taxol®), are generally administrated. Nevertheless, these drugs are characterized by several side effects; thus, alternative compounds from natural resources are demanded. Several studies have demonstrated that *G. lutea* extract and its compounds possess anti-atherosclerotic and anti-proliferative effects, making it a candidate as a potential drug for treating this condition.

3.4.1. In vitro studies

At the basis of accelerated VSMC proliferation and migration were increased levels of pro-inflammatory cytokines, like TNF-α, which are involved in the adhesion of leukocytes to endothelial cells, enhancing arterial inflammation. This led to a reduction of nitric oxide (NO) bioavailability with the consequent increased expression, on the surface of endothelial cells, of cellular adhesion molecules (CAM), such as intracellular CAM-1 (ICAM-1), E-selectin, platelet endothelial CAM-1 (PECAM-1), and vascular CAM-1 (VCAM-1) (Szmitko et al., 2003). *In vitro* studies on human umbilical vein endothelial cells (HUVECs) have demonstrated that either *G. lutea* root extract (1 mg/mL) or isovitexin (5 μmol/L), a compound isolated from the extract, blocked TNF-α enhanced expression of VCAM-1 and ICAM-1. In the same way, both extract and isovitexin also abrogated the migration of rat aortic smooth muscle cells (RASMCs) and phospholipase C-γ activation induced by platelet-derived growth factor-BB (PDGF-BB) (Kesavan et al., 2016). This is a good result since the migration of VSMCs induced by PDGF-BB requires phospholipase C-γ dependent intracellular signaling activation and the consequent intracellular calcium increase. Hence, *G. lutea* root extract or isovitexin may act upstream to activate phospholipase C-γ leading to the block of calcium release and the inhibition of VSMCs migration. Given these results, researchers have studied the activity of other compounds extracted from *G. lutea* root extract. As previously stated, increased levels of TNF-α resulted in the gene expression of adhesion molecules via NF-kB Ser^{536} phosphorylation, so treatment for 24 h of HUVECs with 20 ng/mL of TNF-α induced a 2.5-fold monocyte adhesion increase. The co-treatment with amarogentin (25 or 250 nM) blunted the inflammatory effect of TNF-α. This activity seems to be related to AMPK activation since treating HUVECs with amarogentin for 30 min has demonstrated increasing eNOS phosphorylation at the level of Ser^{1177} residue. eNOS is, indeed, a substrate for AMPK, which, after activation, can increase NO production, preventing endothelial inflammation. Furthermore, the treatment of HUVECs with amarogentin also attenuated NF-kB phosphorylation and reduced the surface expression of VCAM-1, which was increased after the TNF-α treatment. Hence, the activation of eNOS and, consequently, of AMPK mediated by amarogentin blocks the expression of VCAM-1 induced by TNF-α and so endothelial inflammation (Potunuru et al., 2019). Moreover, *in silico* docking simulation demonstrated that amarogentin could bind to the autoinhibitory domain (AID) of AMPK. The holoenzyme AMPK is a heterotrimeric protein formed by a catalytic subunit α which forms a complex with 2 regulatory subunits (β and γ). The α-subunit kinase domain (KD) in its activation loop hosts the Thr^{172} residue located between the N- and C-terminal lobes. The N-terminal KD is succeeded by an AID consisting of three α-helices. The AID, in turn, is linked to a flexible regulatory motif called α-subunit-interacting regulator (α-RIM). To prevent Thr^{172} residue phosphorylation and, thus, AMPK activation, AID interacts with the KD domain's back end. The linkage of AMP molecules to the γ-subunit enables α-RIM to pull AID away, thus relieving inhibition and facilitating long-distance allosteric AMPK activation. It was demonstrated that amarogentin could bond the conserved core of AID's α_3 helix by forming hydrophobic and hydrophilic interactions. Hence it was proposed that amarogentin could directly activate AMPK through the disruption of AID-KD interaction. This assumption was confirmed in cell culture-based experiments in which it was demonstrated that amarogentin promoted the phosphorylation of Thr^{172} in nanomolar concentration leading to AMPK phosphorylation (Potunuru et al., 2019). Based on these data, it is possible to assert that amarogentin reduced the expression of adhesion molecules induced by TNF-α by activating AMPK directly and indirectly.

These cited studies referred principally to the migration of VSMC and the expression of adhesion molecules; however, *G. lutea*'s ability to reduce VSMC proliferation was also investigated. Cells' entry and progression through the cell cycle's different stages are characterized by sequential activation of regulatory proteins like cyclins, cyclin-dependent kinase (CDKs), CDK inhibitors, retinoblastoma protein (pRB), and p53 (Golias et al., 2004; Sánchez and Dynlacht, 2005). CDK2 and CDK4 activation in complex with cyclin D1 determine cell progression from G_0/G_1 to S phase. CDK2 is, in turn, involved in pRb phosphorylation and proliferating cell nuclear antigen (PCNA) accumulation (Akiyama et al., 1992). The Extracellular signal-regulated kinase (ERK) 1/2 (ERK1/2) also directly phosphorylates pRb at the levels of Ser^{780} and Ser^{795} residues (Guo et al., 2005). This event precedes the expression of cyclin D1 and is necessary for transcription factors release, leading to DNA synthesis promotion. In *in vitro* study carried out on primary cultures of rat aortic smooth muscle cells (RASMCs), the increase in cell proliferation of 2-fold induced by PDGF-BB (20 ng/mL) was noticeably blocked after the treatment with *G. lutea* roots extract (1 mg/mL). The root extract also prevented the entry of synchronized cells into the S-phase in response to PDGF-BB. Furthermore, PDGF-BB treatment increased ERK1/2 activation, measured for ERK1 with an increase in Thr^{202} and Tyr^{204} phosphorylation and ERK2 with the dual phosphorylation of Thr^{185} and Tyr^{187}. An increase in intracellular NO levels has also been shown to coincide with Ser^{1177} phosphorylation of eNOS mediated by Akt. Treatment of RASMCs with *G. lutea* root extract completely inhibited the activation of ERK1/2 and the increase in NO intracellular levels induced by PDGF-BB. Consequently, the extract also blocked the ERK1/2 downstream target, IKKα (Kesavan et al., 2013). Based on the knowledge that the IKK-NFkB axis is directly involved in iNOS transcription activation (Jiang et al., 2001), the extract effects on iNOS expression induced by PDGF were also determined. As expected, the extract significantly blocked the expression of iNOS increased by PDGF-BB. This is a good result since, in diabetic rat VSMCs models, an increase in iNOS expression and activity was evidenced (Di Pietro et al., 2013). The iNOS is indeed known to be involved in atherosclerosis pathology, as demonstrated by the evidence that a deficit of its expression reduces atherosclerotic plaques and neo-intimal thickening in rodent models (Chyu et al., 1999; Miyoshi et al., 2006). These observed reductions are both due to LDL oxidation decrease and G_0/G_1 cell-cycle arrest (Chyu et al., 2004; Miyoshi et al., 2006); it was seen that, in the presentence of *G. lutea* root extract, there was an arrest of cell-cycle in G_0/G_1 phase in VSMCs (Kesavan et al., 2013). On the other hand, amid cell cycle regulators, *G. lutea* root decreased the expression of PCNA and cyclin D1 elicited by PDGF-BB. However, the extract did not inhibit the tyrosine phosphorylation of the PDGF-receptor induced by PDGF-BB, indicating that the extract act downstream of this receptor (Kesavan et al., 2013). The only kinase able to activate upstream ERK1/2 is MEK1/2 (Shaul et al., 2009); thus, *G. lutea* extract constituents' ability to inhibit this kinase was investigated through the use of docking analysis on the crystal structure of human MEK1. The analysis was carried out using two MEK1 crystal structures as ternary complexes with the allosteric inhibitor U1026 and the competitive inhibitor K252a. It was seen that between the *G. lutea* extract constituents, the isovitexin was able to bind the same site as U1026 and K252a in MEK1 and sowed a propensity to form hydrogen bonds with catalytic residue as Val^{211}, Asp^{208}, Met^{146}, or Lys^{97}. Corroborating these results are those obtained in cell cultures where isovitexin (0.5–10 μmol/L) showed to block PDGF-induced RASMCs proliferation (Kesavan et al., 2013). Similarly, gentisin, another molecule found in *G. lutea* extract, was also shown to inhibit VSMC proliferation with an IC_{50} value of 7.85 μM (Waltenberger et al., 2015). Hence it is possible to speculate that *G. lutea* extract and its constituents could inhibit vascular smooth muscle cells proliferation acting downstream of PDGF-receptor but upstream ERK1/2 leading to the slackening of the atherosclerotic process (Fig. 5). Nonetheless, in contrast with these results are those derived from another study in which the aqueous extract of *G. lutea* root exerted only a weak anti-proliferative activity of VSMCs (Waltenberger et al., 2015). However, it is necessary to consider the concentration of the extract used since in the latter study where only 30 μg/mL was used (Waltenberger et al., 2015), whereas, in the previous one, the concentration was much higher, 1 mg/mL (Kesavan et al., 2013). Underling ERK1/2-NFkB signaling attenuation leading to cell cycle arrest at the phase G_1, there

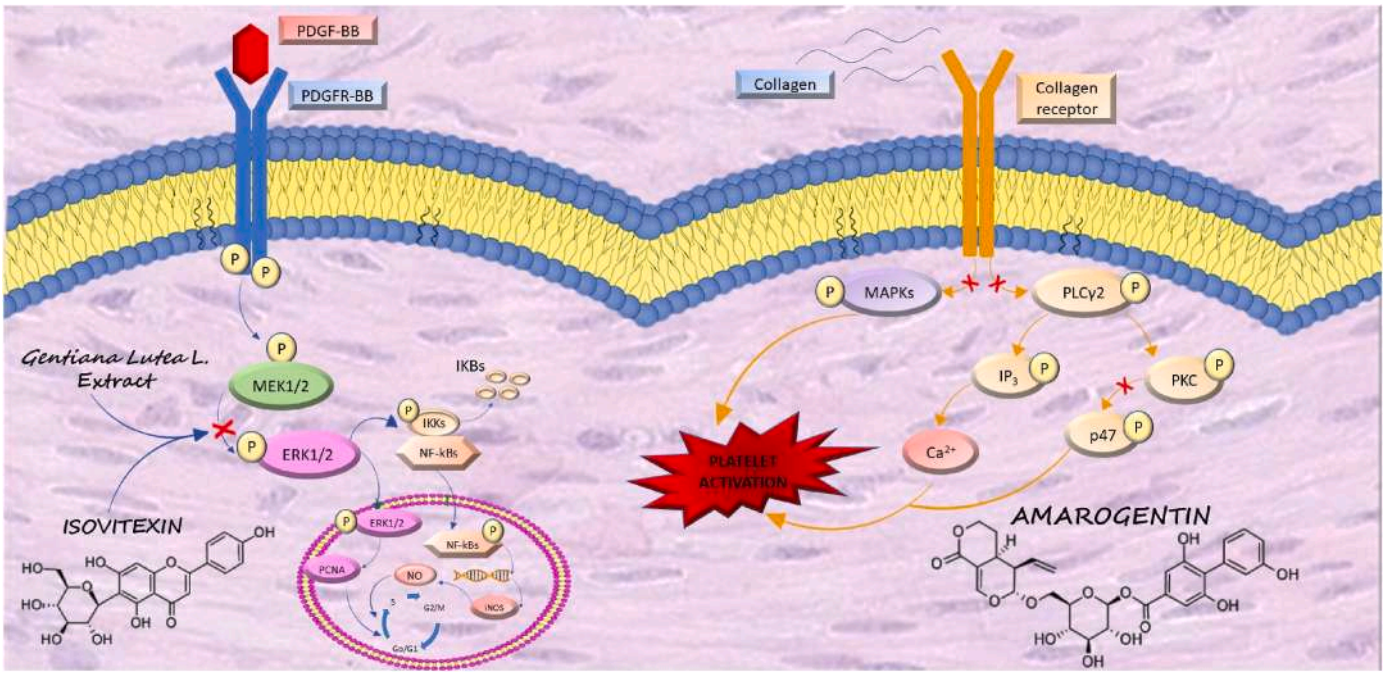


Fig. 5. G. lutea extract and its constituents could inhibit vascular smooth muscle cells proliferation acting downstream of PDGF-receptor but upstream ERK1/2 leading to the slackening of the atherosclerotic process. At the same time, amarogentin could inhibit MAPKs and PLCγ activation avoiding platelet activation.

was also the inhibition of AR (Tammali et al., 2010) whose over-expression in apoE$^{-/-}$ mice is known to accelerate atherosclerotic lesion (Srivastava et al., 2009). It is known that AR inhibition was related to blocking angiotensin II, bFGF, AGEs, and PDGF-AB induced proliferation of VSMCs (Dan et al., 2004; Ramana et al., 2002); as previously described, methanol and ether *G. lutea* root extracts showed to inhibit human and rat AR isoforms identifying amarogentin as a potential AR inhibitor (Akileshwari et al., 2012). Furthermore, myeloperoxidase (MPO) is known to be involved in the pathogenesis of atherosclerosis since it is an early marker of vascular dysfunction and plays a role in LDL oxidative modification (Schindhelm et al., 2009). MPO is a peroxidase enzyme released during neutrophils and monocyte degranulation, it is involved in the oxidation of substrates containing in their structures hydrogen peroxide (H_2O_2), leading to the production of halogenating and oxidizing agents. Several studies have demonstrated the ability of phenols in inhibiting MPO reversibly; thus also constituents of *G. lutea* root extracts were investigated. Specifically, gentiopicroside demonstrated the greatest level of inhibition (IC_{50} 0,8 ± 0.1 μg/mL) followed by isovitexin and amarogentin (IC_{50} 2.2 ± 0.1 μg/mL and 2.4 ± 0.1 μg/mL, respectively). In order to determine the contribution of each molecule on MPO inhibition, mixtures of isovitexin, amarogentin and gentiopicroside were investigated. Results highlighted the importance of gentiopicroside since mixture of the only amarogentin and isovitexin was less potent in inhibiting MPO (Nastasijević et al., 2012b).

In the pathogenesis of atherosclerosis, a crucial role was also played by the deregulation of platelet activity. Specifically, platelet exposure to collagen triggers a signaling complex that activates phospholipase C-γ2 (PLCγ2), leading to phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and the generation of either diacylglycerol (DAG) or inositol 1,4,5-trisphosphate (IP_3). DAG, in turn, determines the activation of protein kinase C (PKC), which phosphorylates p47 protein (pleckstrin) and thus platelet activation (Singer et al., 1997). Platelet treatments with amarogentin (15 ~ 60 μM) were shown to attenuate PLCγ2 and p47 protein in a dose-dependent manner. However, amarogentin did not affect platelet aggregation induced by the PKC activator phorbol-12, 13-dibutyrate (PDBu), indicating that this molecule from *G. lutea* extract may act upstream of PKC (Yen et al., 2014). The effect of amarogentin on mitogen-activated protein kinases (MAPKs) was also investigated. It is indeed well known that MAPKs, including JNKs, ERKs, and p38, have been found in platelets where, after activation by thrombin and collagen, they are involved in the process of thrombosis (Adam et al., 2008; Bugaud et al., 1999). Further, during the platelet activation, the arachidonic acid (AA) metabolism may determine a positive feedback amplifier for p38 activation, which leads to the stimulation of cytosolic phospholipase A2 and the consequent formation of thromboxane A2 (Coulon et al., 2003). On the other hand, JNK is involved in platelet aggregation, induced by collagen and thrombus formation (Kauskot et al., 2007), as demonstrated by *in vivo* studies performed on JNK1$^{-/-}$ mice where arterioles thrombus formation was significantly prolonged (Adam et al., 2010). Amarogentin was shown to inhibit MAPKs activation, suggesting that it could attenuate platelet activation and, thus, thrombus formation by inhibiting the MAPK cell-signaling pathway (Yen et al., 2014). Several studies also demonstrated the role of PI3K/Akt in regulating the aggregation of platelet and the formation of thrombus, so the ability of amarogentin to inhibit Akt was also investigated. It was seen that amarogentin was not associated with the inhibition of platelet activation induced by Akt, indicating that this secoiridoid inhibited the platelet activation induced by collagen via MAPKs, but not Akt (Yen et al., 2014). Based on these results, it is possible to assert that amarogentin could prevent platelet activation and, thus, thromboembolic disorders via inhibiting MAPK signaling pathway and the PLCγ2-PKC-p47 cascade.

3.4.2. *In vivo studies*

The anti-atherosclerotic effect of *G. lutea* and its active principles was also evaluated *in vivo*. Hyperglycemia caused by diabetes is known to exacerbate the macro-angiopathy related to atherosclerosis. For this reason, the ability of *G. lutea* root powder to prevent the formation of atheroma was evaluated in streptozotocin-induced diabetes in rats. It was demonstrated that supplementing 2% of *G. lutea* root powder to diabetic rats reduced the vessel wall media layer and collagen deposition compared to no-treated diabetic animals. Further, hyperglycemia evidenced in diabetic rats is also associated with increased expression of adhesion molecules in endothelial and smooth muscle cells leading to inflammation and vascular dysfunction. Contrarily in diabetic models supplemented with 2% of *G. lutea* root powder, the expression of iNOS,

VCAM-1, and VE-cadherin was significantly abrogated (Kesavan et al., 2016). These results confirm data obtained *in vitro* and demonstrate that *G. lutea* root may protect against macrovascular complications induced by diabetes. It is indeed known that iNOS in streptozocin-diabetic rats' aortic tissue might result from the inflammatory process characterizing the pathogenesis of atherosclerosis. Similarly, the enhanced expression of VE-cadherin and VCAM-1 is typical of ongoing inflammation and neovascularization during atherosclerotic plaque development (Kesavan et al., 2016). Based on these results, other researchers also evaluated the *in vivo* anti-atherosclerotic effect of the single compound amarogentin isolated from *G. lutea* root. The administration of amarogentin (0.3–0.5 mg/kg body weight) for 8 weeks to streptozotocin-induced diabetes in mice increased basal AMPK Thr^{172} phosphorylation in liver tissues and decreased liver fibrosis and serum Glutamic Pyruvic Transaminase (SGPT) and serum Glutamic Oxaloacetic Transaminase (SGOT) enzymes levels (Potunuru et al., 2019). Hance, amarogentin via AMPK activation may modulate liver metabolism. Further, amarogentin administration also reduced neointimal thickening, collagen deposition in aortic sections, and lipid deposition. This last result was confirmed by the presence of vacuole-like formation visible in aortic wall sections due to lipid depositions which were reduced by amarogentin treatment. The reduced neointimal thickening was closely linked to amarogentin's ability to activate eNOS and prevent endothelial inflammation mediated by TNF-α, as demonstrated *in vitro* (Potunuru et al., 2019). Considering that endothelial inflammation, collagen, and lipid deposition are related to atherosclerotic plaque formation, it is possible to identify amarogentin as a potent AMPK activator useable for occlusive microangiopathies management. This assumption is confirmed by the beneficial cardiovascular effects of other AMPK activators, like AICAR and metformin (Kalariya et al., 2012; Potunuru et al., 2019). Amarogentin's anti-atherosclerotic effects were also demonstrated by the prolongation of thrombus formation occlusion time, induced by fluorescein sodium (15 μg/kg) intravenous administration to mice. Thus 18 mg/kg of amarogentin may reduce platelet aggregation and thrombus formation in mice, confirming the data obtained *in vitro* (Yen et al., 2014).

3.4.3. Clinical trial

Only a study has been carried out to date evaluating *G. lutea*'s cardiovascular effects on humans. Specifically, the ability of bitter tastants from *G. lutea* to alter postprandial hemodynamics was investigated in normal subjects. It was demonstrated that within 5–15 min, the ingestion of Gentian flavored water (500 and 1500 mg) increased peripheral vascular resistance, which is not related to increased blood pressure. This effect was probably due to the activation of a baroceptive reflex known to be implied in maintaining blood pressure at almost constant levels. As a consequence of the peripheral vascular resistance increase, there was a reduction in cardiac workload, as also demonstrated by the lowering in cardiac activity parameters. These changes in the hemodynamic parameters were not elicited by administering gastro-resistant microcapsules containing 1000 mg of *G. lutea* bitter tastant. For this reason, it was thought that the vascular tonus increase, noted following the assumption con bitter-tasting water, could be attributed to the activation of cephalic receptors. Based on these results, the vascular response obtained after bitter tastant administration could be classified as a sympathetically mediated cephalic-phase response and may represent a way to reduce cardiac problems related to cardiac insufficiency (McMullen et al., 2014).

Overall, all these findings suggest that *G. lutea* root extract and its bitter constituents like amarogentin and isovitexin, acting on several targets, may have promising activity in preventing and treating cardiovascular diseases and, in particular, thromboembolic disorders. However, even if results from *in vivo* investigations seem to favor the antiatherosclerotic effect of *G. lutea* root, only one clinical study was done in this regard, and it is worth noting that it was done on healthy subjects. Hence, to clarify the real ability of *G. lutea* root and its active metabolites in preventing cardiovascular disorders, evaluating it in patients with different grades of cardiovascular problems is still necessary.

3.5. Gentiana lutea L. vs. inflammation and pain

Several pathological diseases are typified by pain and inflammation and thus by an increase in pro-inflammatory cytokines expression. Plant-derived compounds have been used to prevent or treat inflammatory disorders for centuries. In particular, *G. lutea* extract and its constituents have demonstrated anti-inflammatory properties *in vitro* and *in vivo*.

3.5.1. In vitro studies

Myeloperoxidase (MPO) is an enzyme of the innate immune system that is principally released by activated neutrophils leading to the defense against pathogens. The activation of neutrophils determines the fusion of the lysosome with phagosomes inducing MPO release, which is implied in converting chloride and hydrogen peroxide to hypochlorite. Hypochlorous acid (HOCl) is involved in destroying microbes contained in phagolysosomes. However, MPO can also be released extracellularly, where the overproduction of HOCl promotes tissue damage and exacerbates chronic inflammation (Davies and Hawkins, 2020; Loria et al., 2008). *G. lutea* root extracts were investigated as potential MPO inhibitors at a 0.01 mg/mL concentration. The rate of enzyme inhibition was shown to increase with time, reaching a plateau after a 15-min exposure. Specifically, extracts made with 50% ethanol-water inhibited 71% MPO activity in contrast with the 96% ethanol extract, which inhibited MPO activity by only 31% after 10 min (Nastasijević et al., 2012b). Previous investigations have demonstrated that non-steroidal anti-inflammatory drugs (NSAID) like indomethacin can directly inhibit the chlorinating activity of MPO (Shacter et al., 1991). Thus, based on this knowledge, it is possible to hypothesize that *G. lutea* root extract may wield an anti-inflammatory activity. This assumption was also demonstrated *in vivo*.

3.5.2. In vivo studies

Petrol ether and alcohol extract of *G. lutea* rhizomes have demonstrated anti-inflammatory activity in different animal models. The administration of *G. lutea* extract (500 or 1000 mg/kg) to carrageen-induced paw edema, and cotton pellet-induced granuloma in rats has shown a dose-dependent inhibition of edema and reduction in granuloma weight, respectively. In particular, the anti-inflammatory activity of the ethanol extract at the doses of 1000 mg/kg was similar to that of diclofenac sodium administrated at the doses of 13.5 mg/kg. Similarly, alcohol or petrol ether extract (500 and 1000 mg/kg) exerted an anti-inflammatory activity in the case of xylol-induced mouse ear edema. Also, in this case, the anti-inflammatory activity of the ethanol extract at the doses of 1000 mg/kg was similar to that of indomethacin administrated at the doses of 25 mg/kg (Mathew et al., 2004). In another experiment, reduced doses (300 and 500 mg/kg) of *G. lutea* alcohol end petrol ether were administrated to test their potential wound healing activity. The wound healing process involves different phases, such as contraction, epithelialization, granulation, and collagenation. An indirect method used to evaluate the healing collagenation phase is the breaking strength of a resutured wound. It was seen that the administration of *G. lutea* rhizome extracts significantly increased the resistance to rupture in resutured incision wounds. These results were explained through histopathological studies, which demonstrated an increase in collagen content and the number of fibroblasts in groups treated with *G. lutea* extracts. Further, the increment in collagen content necessary for wound healing was highlighted by increasing the amount of hydroxyproline (Mathew et al., 2004). Based on these results, it is possible to exert that *G. lutea* rhizomes possess anti-inflammatory and wound-healing activity that may be ascribed to alkaloids, glycosides, and other active molecules of the rhizome. Additionally, the anti-inflammatory activity of *G. lutea* is supported by comparison with

NSAIDs, as it demonstrated activity comparable to that of diclofenac sodium and indomethacin (Mathew et al., 2004).

A single compound isolated from *G. lutea*, the gentiopicroside, was also investigated for its role in pain transmission and modulation through the down-regulation of N-methyl-D-aspartate receptors (NMDAR) into the anterior cingulate cortex (ACC) (Chen et al., 2008). It has been demonstrated that ACC plays a vital role in processing pain-related information through the modulation of NMDA receptors. Peripheral inflammation, indeed, increases the expression of NMDA NR2B receptors in ACC so that the administration of selective NR2B receptors antagonist may inhibit inflammation-related allodynia (Chen et al., 2008; Wu et al., 2005). The administration of gentiopicroside at the doses of 50–200 mg/kg (i.g. twice daily for 3 days) to inflammatory pain mice models results in a significant reduction of persistent inflammation in a dose-dependent manner. Indeed, it is found that the analgesic effect of gentiopicroside is due to its capacity to reverse the pain-induced over-expression of NR2B receptors. In particular, gentiopicroside seems to act as an adenylyl cyclases inhibitor that reduces cAMP levels with a consequent down-regulation of NR2B receptors in the ACC (Chen et al., 2008). These results are consistent with another investigation showing that gentiopicroside could induce analgesic activity in acute brain tests (Öztürk et al., 2002). However, further *in vivo* studies demonstrated that gentiopicroside failed to impair the augmented ACC presynaptic neurotransmitter release (Chen et al., 2008); thus, it is possible to postulate that this molecule may act via a postsynaptic modulation of NR2B receptors.

All this evidence supported the anti-inflammatory and analgesic activity of *G. lutea* and its major compound, gentiopicroside, also known to inhibit the release of algesic mediators like the substance P, bradykinin, TNF, and serotonin (Chen et al., 2008; Kondo et al., 1994). Hence, it should be interesting to continue investigations on this line to validate further the effect of *G. lutea* and its active molecules on pain. Furthermore, regarding the ability of this medicinal plant to induce collagen synthesis, it should be interesting to project extended-release transdermal patches or liposomal creams useable after surgery to speed up wound healing and reduce inflammation.

3.6. *Gentiana lutea L. effects on the central nervous system*

In the last year, researchers have paid particular attention to discovering undescribed active principles from plants able to modulate nervous system functions and prevent neurological disorders. Compounds like iridoids have been reported to exert several beneficial effects on the central nervous system (CNS). Several studies have indeed demonstrated that iridoids like geniposide possess neuritogenic effects on neuronal cell cultures, probably thanks to their ability to activate protein kinase leading to neuronal cell differentiation induction (Yamazaki et al., 1996). In this paragraph, the discoveries to date on the effect of *G. lutea* extract and its compounds on CNS will be treated.

3.6.1. *In vitro studies*

The stimulation of neuritogenic activity aims to prevent and treat neurodegenerative diseases like Alzheimer's disease (AD). In fact, neuritogenic agents are now considered promising molecules for neuronal injury management due to their ability to stimulate neurite outgrowth in neuronal cells (More et al., 2012). *G. lutea* extract's neuritogenic activity was evaluated on rat pheochromocytoma PC-12 cell lines since, on their surface, they express specific tyrosine kinase receptor (TrkA) known to be activated by neurotrophic factors like nerve growth factor (NGF). NFG is the best-characterized neurotrophic factor as it is essential for neuronal survival, growth, differentiation, function maintenance, and aging prevention in the peripheral and central systems (Maranesi et al., 2021; Zerani et al., 2021). TrkA phosphorylation by NFG determines the activation of signal transduction substrates, including ERK1/2, which in turn induces the phosphorylation of cAMP response element-binding protein (CREB). Once CREB is activated, it enrolls the CREB binding protein (CBP) to the cAMP-responsive genes' promoter regions responsible for dendritic spine growth, synaptic plasticity, morphology change, and long-term memory. Thus, ERK1/2 activation by NGF may determine in PC-12 cell lines the neurite outgrowth. Based on this knowledge, the neuritogenic activity of *G. lutea* extracts was investigated without the presence of NGF. It was seen that 25 μg/mL of *G. lutea* extract enhanced not only neurite outgrowth but also determined a significant increase in PC-12 lengths after an incubation of 5 days. Similarly, the incubation of PC-12 cells with either 25 μg/mL of *G. lutea* extract or 50 nM of NFG increased the number and length of neurite compared to the control cells treated with NFG alone. Hence, it is possible to hypothesize that 25 μg/mL of *G. lutea* extract may enhance the neurite outgrowth induced in PC-12 cells. These data were also confirmed by neurofilament staining evaluated through immunofluorescence dye (Mustafa et al., 2015). Neurofilament is indeed known to be a useful indicator of PC-12 cell differentiation since it is a neuron-specific protein and the major cytoskeleton component implicated in support of the axon cytoplasm (Schimmelpfeng et al., 2004). Besides its neurotrophic activity, *G. lutea* extract also significantly protected neuronal cells from apoptotic agents, as demonstrated on human neuroblastoma SH-SY5Y cells. It was seen that *G. lutea* extract (200 and 400 μg/mL) significantly enhances the viability of cells treated with vinblastine (0.1 μM). This anti-apoptotic activity seemed to be related to the Bcl-2 increased expression induced by *G. lutea* (200 μg/mL). Further, the extract also prevented Bcl-2 phosphorylation induced by the antimitotic drug vinblastine (Cafaro et al., 2020). It is a good result since Bcl-2 is an anti-apoptotic protein implicated in enhancing neuronal survival in several stressful injuries, and its phosphorylation is related to its inactivation (Ouyang and Giffard, 2014). Another crucial protein for cells' survival and protection again radiation or oxidative stress is Sirt-1 (Hisahara et al., 2005), as it plays a neuroprotective role again apoptosis induced by mechanical trauma, neurotoxins, and ischemia. It was seen that apoptosis induced by vinblastine was related to reducing Sirt-1 expression, but the co-treatment of SH-SY5Y cells with *Gentiana lutea* extract reversed this down-regulation (Cafaro et al., 2020). Hence, *G. lutea* protection against vinblastine-induced toxicity was also shown to be related to its effect on the Sirt-1 protein. These effects complemented the Gentian's ability to increase intracellular glutathione (GSH) levels when used either alone or in combination with vinblastine (Cafaro et al., 2020). Therefore, through the increase in GSH levels, *G. lutea* extract could participate in cellular antioxidant defense pathways and detoxification from chemotherapeutic agents, decreasing their effectiveness (Cafaro et al., 2020; Traverso et al., 2013).

Studies have demonstrated that inflammation is related to several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and multiple and amyotrophic lateral sclerosis (Glass et al., 2010). For this reason, *Gentiana lutea* extract was also investigated on activated RAW264.7 macrophages-like cells. It was shown that treatment with 200 μg/mL of *G. lutea* root extract decreased TNF-α levels released from RAW264.7 macrophages-like cell lines stimulated with lipopolysaccharides (LPS) from *Pseudomoas aeruginosa*. Notably, simultaneous and short-term treatment of cells with the extract only showed a weak anti-inflammatory activity indicating that Gentian did not affect LPS interaction. However, RAW264.7 long-term exposure to *G. lutea* extract exerted a high anti-inflammatory activity, leading to decreased TNF-α production (Cafaro et al., 2020). A contribution to the inflammatory process in the CNS is also made by monoamine oxidase (MAO) since this enzyme is implied in H_2O_2 production, leading to the modulation of oxidative stress. MAO, in the brain, is mainly located on the external surface of the mitochondrial membrane of neurons and astrocytes; its overexpression is related to an increased number of activated astrocytes and in the outbreaks of diseases like Alzheimer diseases (Fernandes and Özcelik, 2021). MAO exists in two different isoforms of MAO-A and MAO-B; both are implicated in monoamine neurotransmitter metabolism leading to the release of aldehydes and H_2O_2. These oxidases have attracted particular attention for their involvement in several

neuropsychiatric disorders. Inhibition of MAO and, thus, H_2O_2 production is indeed related to preventing depression and other neurological diseases (Haraguchi et al., 2004). Several plants are characterized by compounds able to inhibit MAO suggesting that vegetable extracts could be used as potential neuroprotectors. For this reason, the effect of the *G. lutea* constituent on MAO was investigated. Specifically, it was demonstrated that three compounds isolated from the methanolic extract of *G. lutea*, namely 3-3″linked-(2′-hydroxy-4-*O*-isoprenylchalcone)-(2‴-hydroxy-4″-*O*-isoprenyldihydrochalcone), 2-methoxy-3-(1,1′-dimethylallyl)-6a, 10a-dihydrobenzo(1,2-*c*)chroman-6-one and 5-hydroxyflavanone, exerted a higher competitive inhibition against MAO-B than MAO-A (K_i values of the three compounds against MAO-B were 24.2, 1,1 and1.4 μM, respectively) (Haraguchi et al., 2004). These results suggested that *G. lutea* may be a potential vegetable resource for preventing and treating Parkinson's and Alzheimer's disease thanks to MAO-B inhibition. Researches demonstrated that neuroinflammation in brain tissue is even related to increased ATP levels whose metabolism is directly correlated to the ecto-nucleotide triphosphate diphosphohydrolases (E-NTPDases) activity (Roszek and Czarnecka, 2015). E-NTPDases are membrane enzymes implied in the hydrolyzation of extracellular tri- and di-phosphatase nucleotides when Ca^{2+} and Mg^{2+} are in millimolar concentration at extracellular pH between 7 and 8 (Zimmermann et al., 2012). Alteration in E-NTPDase activity was evidenced in different neurodegenerative and neuropsychiatric diseases; therefore, molecules able to modulate E-NTPDase activity and their gene expression could be very important for neurological disorders treatments (Roszek and Czarnecka, 2015). Considering the demonstrated effects of *G. lutea* compounds on the brain, it was decided to test the extract and its constituents as possible inhibitors of E-NTPDase located in the synaptosome membrane. It was demonstrated that using 200 mg/mL of *G. lutea* extract, an enzyme inhibition of about 35–50% was achieved. Based on these results, the single compound contribution to E-NTPDase activity was investigated. Specifically, amarogentin, gentiopicroside, and isovitexin exerted an enzyme inhibition in the concentration range between 1×10^{-7} and 3×10^{-4} M and an inhibition time of 20 min. These constituents were also investigated together in four different concentrations, and it was seen that the greater inhibition degree, about 16%, was obtained with the mixture containing isovitexin, amarogentin, and gentiopicroside at the concentration of 20 μg/mL. Contrarily, the mixture formed by two constituents (final concentration 20 μg/mL) exerted lower inhibition, about 10, 5, and 3%. Nonetheless, when molecules were investigated separately, it was seen that they achieved a higher E-NTPDase inhibition than the mixture (25.30%, 28.50%, and 29.30% for amarogentin, isovitexin, and gentiopicroside, respectively). Therefore, it was thought that these three active principles acted via competitive inhibition when used together at the same concentration. In fact, molecular docking studies demonstrated that only one binding site for isovitexin, amarogentin, and gentiopicroside on the E-NTPDase2 isoform overlapped with those of ATP (Nastasijevic et al., 2016). The importance of these results is related to the location of E-NTPDase2 in astrocytes. During cerebral ischemia, ATP concentration increases, which is first deprotonated to ADP by E-NTPDase2 and then dephosphorylated by other ectonucleotidases to AMP. Since ATP can activate purinoceptor 2 (P2Y2), it might have a protective action in glial cells. Therefore, it was thought that the inhibition of E-NTPDase2 might be helpful for treating ischemic brain conditions, e.g., for stroke treatment (Brunschweiger et al., 2008). In addition, stimulation of cancer progression has been demonstrated with high expression of E-NTPDase2 (Buffon et al., 2007; Knowles and Chiang, 2003). All these findings make Gentian extract and its constituents potentially safe resources for treating CNS diseases.

3.6.2. *In vivo studies*

The effect of *G. lutea* extract on the central nervous system was also evaluated *in vivo*. The administration of methanol extract of Gentiana root at the dosage of 250 and 500 mg/kg (i.p.) to adult male albino mice showed adaptogenic and analgesic activity. This effect may be attributed to the activation of CNS by three secoiridoids compounds: swertiamarina, sweroside, and gentiopicroside (Öztürk et al., 2002). Specifically, gentiopicroside, the main secoiridoid present in *G. lutea* extract, demonstrated analgesic properties and the ability to inhibit the expression of GluN2B-containing N-methyl-D-aspartate (NMDA) in mice anterior cingulate cortex (Liu et al., 2014). NMDA is one of the ionotropic glutamate receptors known to play several physiological roles. It is indeed involved in maintaining cellular homeostasis; any variation in its activity leads to neuropsychiatric pathologies like psychosis, schizophrenia, and mood disorders (Lakhan et al., 2013). It was demonstrated that NMDA agonists like ketamine rapidly ameliorate depression symptoms (Zarate et al., 2006). For this reason, Gentiopicroside has been investigated as a potential depression modulator through the downregulation of GluN2B-containing NMDA. Specifically, gentiopicroside was administered to the reserpine-induced pain/depression mice model at different concentrations (50−100−200 mg/kg) twice daily for 3 days. Reserpine is known to induce depression-like behavior and nociceptive pain; thus, it was often used to screen undescribed promising treatments for pain/depression-centred symptoms. Tests used to investigate the dyad pain/depression related to behavior are classic forced swimming test, pain hyperalgesia test, open field test, and tail suspension test. It was seen that gentiopicroside improved the behavioral deficit bonded to the pain/depression dyad induced by reserpine in a dose-dependent manner. This effect could be related to gentiopicroside ability to restore monoamine levels, exert antioxidant activity, and downregulate the GluN2B receptor in the amygdala (Liu et al., 2014). Monoamines (serotonin, dopamine, noradrenaline) are indeed known to play an important role in depression syndrome development (Elhwuegi, 2004) and pain perception (Marks et al., 2009; Potvin et al., 2009). Besides, the amygdala is renowned for being involved in major depressive disorder (Sacher et al., 2012) and switching on/off chronic pain (Rouwette et al., 2012). Reserpine administration to mice caused a decrease in monoamines, whose levels are restored by gentiopicroside (Liu et al., 2014). An increase in monoamine levels was indeed shown in the mice's amygdala BLA region (Liu et al., 2014), which is known to be implied in the mediation of emotional disorders (Tye et al., 2011). Several studies have demonstrated that NMDA receptors play a crucial role in pain and emotional disorders development (Amaral and Roesler, 2008; Wu and Zhuo, 2009). After reserpine administration to mice, an increase in the expression of GluN2B containing NMDARs was seen. However, treatment with gentiopicroside reversed this upregulation and inhibited the dyad of pain/depression (Liu et al., 2014). The down-regulation of GluN2B was also confirmed by caspase-3 levels reduction and Bcl-2 levels increase when reserpinized mice were treated with Gentiopicroside (100 mg/kg) or the GluN2B antagonist Ro25-6981 (0.5 mg/kg). Caspase-3 is a mediator required for kay apoptotic events (e.g., fragmentation of DNA and collapse of the nucleus) and represents the link between the extrinsic and intrinsic death pathways (Widmann, 2007). On the other hand, Bcl-2 is a cellular protein that suppresses apoptosis (Cho et al., 2015). Either gentiopicroside or Ro25-6981 reversed the decrease of CAT activity and increased malondialdehyde (MDA) levels in reserpinized mice (Liu et al., 2014). These are other good results since CAT is an enzyme involved in converting hydrogen peroxide into water and oxygen molecules, protecting cells from damage by ROS (Singh and Kumar, 2019). MDA is instead an oxidative damage marker produced by the peroxide PUFAs breakdown; its toxicity is derived from the ability to facilitate protein cross-linking, form Michael adduct with thiol groups, and stimulate mutagenesis (Landau et al., 2013). It is known that the increase in caspase-3 and MDA levels and the reduction of Bcl-2 and CAT activity are typical signals of glutamate excitotoxicity induced by the NMDARs, containing NR2B subunit, selective activation. Hence, gentiopicroside and thus *G. lutea* extract could be used as NMDARs inhibitor in the same way as Ro25-6981; this is also confirmed by the evidence for which inhibitors of GluN2B containing NMDARs induce a rapid and sustained decline in depressive symptoms (Li et al., 2010). However, are still insufficient the information regarding the real mechanism of action

by which gentiopicroside may induce the enhancement of monoamine and so serotonin levels. Thus the study of the polimorfism of MAO, Serotonon trasporter protein, Tryptophan Hydroxylase 1, or hydroxytryptamine receptor-1A, -2 A, and -3 A should be elucidated to evaluate gentiopicroside like antidepressant activity.

3.7. Gentiana lutea L. cytoprotective and antitumoral activity

Treatments used today for cancer have several negative effects not only related to the cost and, therefore, difficult to access for everyone but also for the side effects that often affect the patient's quality of life. For this reason, it is necessary to find undescribed molecules, also of natural origin, able to produce beneficial effects in this field.

3.7.1. In vitro studies

The cytotoxic effect of *G. lutea* leaf extract was investigated in different cell lines like human cervix adenocarcinoma (HeLa), human breast cancer (MCF7), human prostate cancer (PC3), and human colon carcinoma (LS174) cell lines by MTT assay. After 72 h, gentian methanolic leaf extract manifested a moderate cytotoxic effect only against HeLa cells with an IC_{50} value of 41.1 ± 1.5 μg/mL compared to cisplatin used as control (IC_{50} 0.7 ± 0.14 μg/mL). Regarding compounds, isogentisin was moderately effective against MCF7 (IC_{50} 36.3 ± 4.4 μg/mL), PC3 (IC_{50} 36.2 ± 1.1 μg/mL) and LS174 (IC_{50} 39.6 ± 4.4 μg/mL) cells and together with mangiferin and gentiopicrin showed marked cytotoxic activity against HeLa cells with IC_{50} values ranging from 5.7 ± 0.4 to 8.8 ± 0.9 μg/mL (Balijagić et al., 2012). Different studies also reported the cytotoxic activity of *G. lutea* root. The aqueous extracts of *G. lutea* root induced inhibition of about 15–20% only at the highest concentration (500 μg/mL) in HeLa cells as well as in MCF-7 after 72 h. Otherwise, the ethanolic extract reduced the cell viability of Hela cells in a dose-dependent manner with a maximum effect at 500 μg/mL (100% inhibition), while it manifested only 25% of cell growth inhibition at 125 μg/mL in MCF-7 cells. Thus, the solvent and cell line influenced the behavior of the extracts (Rodrigues et al., 2019). In addition to the antiproliferative activity, chemoprotective effects were also investigated. Cafaro et al. reported neuroprotective effects of the methanolic root extract in human neuroblastoma cell lines (SH-SY5Y). In particular, the extract (200 μg/mL) combined with the apoptotic agent vinblastine (0.1 μM) reduced the percentage of apoptotic cells evaluated by acridine orange/ethidium bromide double staining assay. Gentian extract treatment reduced caspase-3 activity and antagonized the phosphorylation of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) typically induced by vinblastine, improving neuron survival. Vinblastine treatment produced a reduction in Sirtuin-1 (Sirt-1) levels. Sirt-1 regulates the expression levels of genes important for proliferation and ATP generation; the co-treatment with yellow gentian restored the basal levels of Sirt-1, determining a neuroprotective effect (Cafaro et al., 2020).

The protective effect of yellow gentian root was also investigated on the peripheral blood mononuclear (PBMC) cell line. Valenta Šobot et al. demonstrated that the incidence of chromosomal aberrations decreased after 72 h compared to 48 h of treatment with gentian; in fact, there were no chromosomal breaks at the lowest dose (0.5 mg/mL) tested, which might be due to the activation of DNA repair mechanisms in cells with aberrations that could be fixed, and the death of cells damaged beyond repair. Even the percentage of DNA in the comet tail decreased after 72 h. The comet assay tests whether a clastogenic substance is capable of generating structural damage in chromosomes, resulting in chromosomal mutations. Normally a fluorescent substance capable of binding to DNA is used, and the nucleus is observed under the fluorescence microscope. In the presence of damage, the DNA fragments migrate to the anode forming an elongated structure that looks like the tail of a comet (Olive and Banáth, 2006). Chromosomal radial figures' formation evidenced the DNA repair mechanism's activation after 72 h of treatment with the highest concentration of 2 mg/mL (Valenta Šobot et al., 2020).

In addition, regarding the protective effect of gentian root, Meschini et al. reported the application of the aqueous-alcoholic root extract in the clastogenicity induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(α) anthracene (DMBA). The first is responsible for single- and double-stranded DNA breaks and the production of free radicals. The second is converted into carcinogenic diol epoxide by cytochrome P450 producing oxidative damage due to increased production of ROS and a notable increase in chromosomal abnormalities. The anti-clastogenic activity of *G. lutea* activity was investigated in the HepG2 cell lines through a cytokinesis-block micronucleus (CBMN) assay. Post-treatment reduced the frequency of micronuclei (21.6%) induced by MNNG (25 mM) due to the reparation of damaged DNA, while the simultaneous treatment with DMBA (2 mM) and gentian extract (1.25 mg/mL) induced a significant increase in the % of cytostasis with respect to the only treatment with DMBA may be due to a physic-chemical interaction between them (Meschini et al., 2015).

The HepG2 cell lines were also used as a model for the investigation of the gentian protective effect against heterocyclic aromatic amines. These latter compounds, generated during the high-temperature cooking of fish and meat and after the activation by cytochrome P450, can create covalent bindings with DNA (DNA-adducts) and oxidative DNA damage for ROS production, becoming dangerous for cells. As reported by Cvetkovic et al. methanolic extract of gentian root (2 mg/mL) produced the highest inhibition of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)-induced genotoxicity (80%) while the leaf extract was more effective (72%) on 2-amino-3-methylimidazo [4,5-f quinoline] (IQ) at the same concentration. The interesting activity of gentian is probably connected to its phytochemical components responsible for the antioxidant action that reduce the danger of amines involved in the etiology of human cancer, in particular colon cancer, suggesting its potential application as a food supplement (Cvetkovic et al., 2019).

3.7.2. In vivo studies

Plants are a source of molecules that possess important pharmacological properties. However, these must not be harmful to health. For this reason, the genotoxic effect of *G. lutea* was investigated by Drosophila Wing Spot Test, a somatic mutation and recombination test (SMART). This eukaryotic system shows a metabolic activation system similar to that of mammals. Patenković et al. demonstrated no genotoxicity effects in acute and chronic treatments with water infusion of gentian roots at 25 mg/mL in somatic cells of *Drosophila melanogaster*. However, the co-treatment of gentian with methyl methanesulfonate (MMS) (3 mM) showed that gentian increased the frequency of mutant clones compared to treatment with MMS alone, suggesting a synergistic action with MMS probably due to the interactions with excision repair mechanisms (Patenković et al., 2013).

3.7.3. Ex vivo studies

The protective effect of gentian root was also investigated in a clinical study. The root and rhizome hydroalcoholic extracts demonstrated radioprotective and sensitizing effects on PBMC and HeLa cell lines. Ionizing radiations are responsible for tissue damage; when they penetrate into the cells, they induce the production of reactive oxygen species by breaking chemical bonds and, consequently, cellular damage. Nine healthy volunteers (3 males and 6 females) were treated with 15 g of gentian extract, and PBMC cells were extracted from the blood before and after treatment. Gentian extract protected PBMC cells from x-ray irradiation without affecting the susceptibility of HeLa tumor cells to be destroyed by radiation. In addition, the treatment with mangiferin, a xanthone present in flowers and leaves of *G. lutea,* increased cell viability at the highest x-ray irradiation doses considered (6 Gy and 8 Gy) measured by Kenacid Blue R dye binding method, which evaluates the change in total cellular protein (Menkovic et al., 2010).

Based on the data analyzed, it is not possible attributing to *G. lutea* an

anticancer activity since there is a lack of investigation on *in vivo* models and only a trend of activity in *vitro* cancerous models. Hence the need for an intensive investigation explicating the mechanism by which gentian may have an antitumor activity remain.

3.8. *Gentiana lutea L. Activity again atopic dermatitis and psoriasis*

The *stratum corneum* is formed principally by differentiated keratinocytes surrounded by an extracellular lipid bilayer. Lipids forming this bilayer are secreted from lipid droplets, consisting of intracellular organelles specialized in lipids storage, assemblage, and supply (Feingold et al., 2007). In keratinocytes, lipid droplets are generated during their differentiation, reaching the maximum number in the *stratum granulosum* when there is the highest extracellular calcium level (Feingold, 2009). Several lipids contribute to optimizing epidermal barrier function, and ceramides are particularly important among these. Indeed, reducing ceramide levels is involved in several skin disorders, such as atopic dermatitis and psoriasis, which are also characterized by inflammation and dysregulation in keratinocytes synthesis. Active principles from *G. lutea* have been demonstrated to improve skin disorders.

3.8.1. *In vitro studies*

Amarogentin, the bitterest compound of *G. lutea*, was demonstrated to stimulate keratinocyte differentiation by up-regulating endothelial bitter taste receptors (TAS2R1 and TAS2R38) (Fig. 6). It is known that the activation of bitter taste receptors leads to the formation of inositol-trisphosphate and diacylglycerol via the G protein α-gustducin and subsequent phospholipase C-β_2 induction. This intracellular cascade determines the increase in calcium levels and the consequent activation of the transient receptor potential cation channel 5 (Lindemann, 2001). Based on this knowledge, the ability of amarogentin to increase calcium levels by activating bitter taste receptors was investigated using Diphenidol as a reference standard. Diphenidol is indeed a synthetic molecule known to be a TAS2Rs agonist (Meyerhof et al., 2010). It was seen that the incubation of HaCaT cells with different concentrations of Diphenidol (30-100-150 μM) and amarogentin (30-100-300 μM) led to a dose-dependent elevation of calcium. Specifically, a concentration of 100 μM amarogentin induced an increase in calcium influx comparable to 30 μM of Diphenidol without cytotoxic effects (Wölfle et al., 2015a). Generally, the keratinocytes differentiation process is triggered by a high extracellular calcium concentration of 2 mM leading to an up-regulation of involucrin, keratin 10, and transglutaminase 1. Keratin 10 is the earliest differentiation marker, while transglutaminase 1 and involucrin are the latest keratinocyte differentiation marker. Incubation of HaCaT cells with 100 μM of Diphenidol and amarogentin for 72 h has shown an increase in the expression of these markers (Wölfle et al., 2015a). Furthermore, near the rise in keratinocyte differentiation, there was the ability of amarogentin to show immunomodulatory effects by interacting with keratinocytes and mast cells (Wölfle et al., 2015b). Mast cells are located in the upper dermis and, after activation, are responsible for the release of histamine and pro-inflammatory cytokines [e.g., tumor necrosis factor α (TNF-α)] release. Mast cell numbers and subsequent histamine levels increase during chronic skin inflammation disorders, such as psoriasis, leading to keratinocytes activation. This latter, through the activation of histamine receptors (H1), is responsible for the increase in the expression of pro-inflammatory cytokines [e.g., interleukin 1 (IL-1)], chemokines [e.g., interleukin 8 (IL-8)], and matrix metalloproteases 1 and 9 (MMP-1 and MMP-9) (Gschwandtner et al., 2008; Kohda et al., 2002). MPP-1 is implied in the cleavage of type 1 collagen, the main dermis constituent, and in the activation of MMP-9, which have dermal elastin and fibrillin as specific substrates (Tsoureli-Nikita et al., 2006). The breaking of these constituents results in epidermal T cells invasion with the consequent enhancement of skin inflammation. Hence, mast cells, keratinocytes, and T cells play a crucial role in skin inflammatory processes. To verify if amarogentin can inhibit the release of histamine or TNF-α, human mast cells line LAD-2 were preincubated with 100 μM amarogentin or 24 μM azelastine (a known histamine receptor antagonist) as a positive control. LAD-2 treatment with amarogentin before substance P-induced inflammation (2 μM) was shown not to inhibit histamine release. However, pre-incubation with amarogentin and azelastine blocked the *ex novo* secretion of TNF-α by LAD-2 mast cells 24 h after SP stimulation. This suggests that amarogentin was not acting as a histamine receptor antagonist but as an inhibitor of TNF-α synthesis. Further, it seems that this effect of amarogentin is related to the activation of TAS2R since cells' treatment

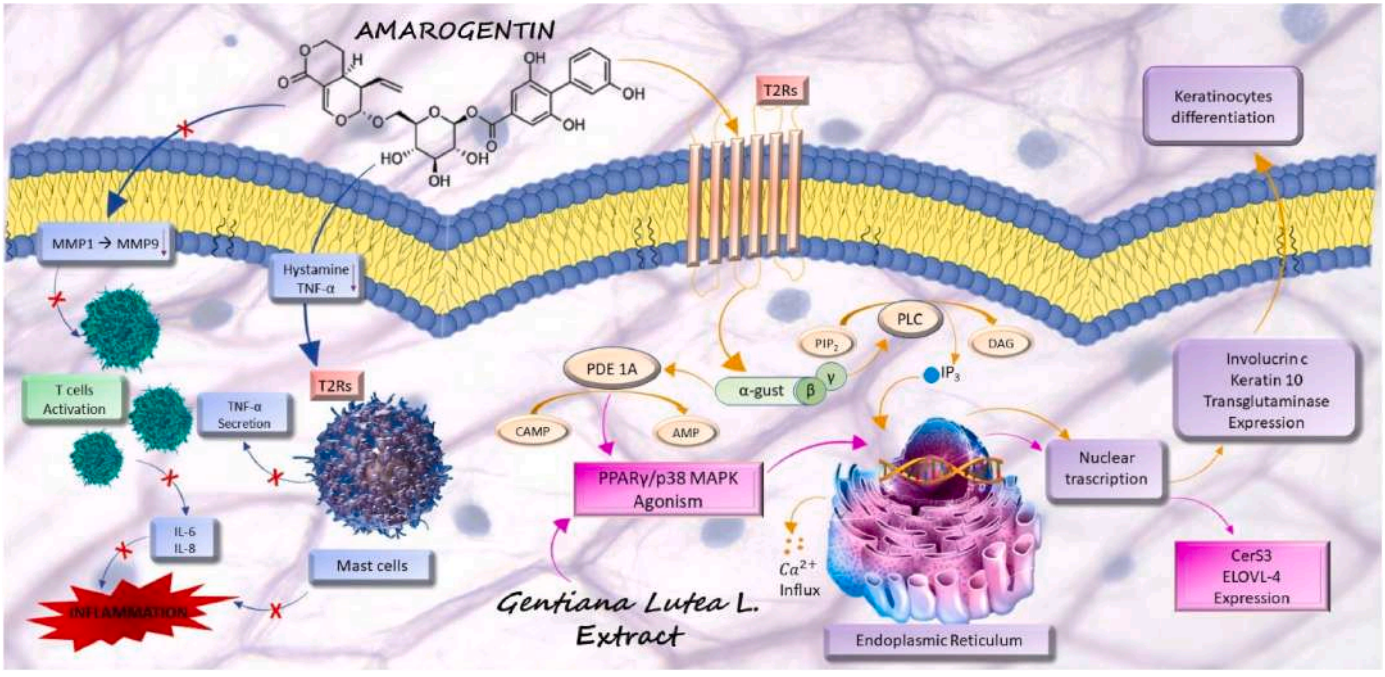


Fig. 6. Amarogentin was demonstrated to stimulate keratinocyte differentiation and reduce inflammation by up-regulating endothelial bitter taste receptors expressed on keratinocytes and mast cells.

with inhibitors of these receptors, like U73122, reversed the inhibitory effect on TNF-α newly synthesized. The same receptors also underlie the ability of amarogentin to inhibit IL-8 and MMP-1 release in human HaCaT keratinocytes costimulated with histamine and TNF-α (Wölfle et al., 2015b). Based on these results, it can be stated that TAS2R are expressed on both mast cells and keratinocytes and that their activation by bitter compounds led to amarogentin's immunomodulatory effects in the skin (Fig. 6).

As mentioned above, lipids contribute to optimizing epidermal barrier function; for this reason, *G. lutea* extract and its constituents were investigated for their role in keratinocytes' lipid metabolism modulation. *G. lutea* extract was shown to enhance lipid accumulation in HaCaT keratinocytes in a dose-dependent manner (50–100–200–400 μg/mL), and in human primary keratinocytes (hPKs) at the dose of 200 μg/mL. Specifically, after treatment with *G. lutea* extract, a 2–4-fold increase of palmitic acid and linoleic acid in either young or old keratinocytes and an increase in ceramide levels in old keratinocytes were observed. It is known that the nuclear transcription factors PPARs and the MAPK pathway are involved in lipid formation and packaging. For this reason, to understand the *G. lutea* extract mechanism of action, hPKs cells were pre-incubated with SB203580 (p38 MAPK inhibitor) or GW9962 (PPARγ inhibitor) before extract treatment. It was seen that either p38 MAPK inhibitor or PPARγ inhibitor significantly antagonized lipid production induced by *G. lutea* extract. The same results were obtained for ceramide synthesis as ceramide synthase 3 (CerS3) expression, induced by *G. lutea* in hPKs, is reduced to initial levels after pre-incubation with both inhibitors (Wölfle et al., 2017). Thus, the natural extract's ability to enhance lipid synthesis and CerS3 expression could be related to PPARγ/p38 MAPK agonism. This is in line with a previous investigation in which it was shown that PPARγ could be activated by the extract of *G. lutea* (Rau et al., 2006). The ability to improve epidermal barrier function by increasing ceramide synthesis was also investigated in psoriasis-like keratinocytes. In this study, psoriasis-like hPKs were obtained by stimulating health hPKs with cytokines usually involved in psoriasis pathogenesis (IL-17, IL-22, TNF-α, and INF-γ), showing an increase in the levels of IL-6 and IL-8 and a decrease in the expression of elongases (ELOVL1 and 4) and CerS3 (Gendrisch et al., 2020). ELOVLs and CerS are known to be involved in the generation of long-chain ceramides, and their expression is reduced in psoriatic skin leading to the development of epidermal barrier abnormalities (Tawada et al., 2014). Treatment of psoriasis-like hPKs with 200 μM of *G*L extract significantly increased the expression of CerS3 and restored the reduced expression of ELOVL-4 (Gendrisch et al., 2020). However, ceramides are not only involved in skin barrier recovery but also inflammatory responses (Di Nardo et al., 1998). Studies on human fibroblasts have demonstrated that ceramides are implied in the modulation of prostaglandin E_2 (PGE_2) secretion (Ballou et al., 1992). For this reason, to ensure that *G. lutea* extract was not engaged in keratinocyte inflammatory response, the release of PGE_2 and IL-6 by HPKs was measured. It was demonstrated that hPKs treatment with the extract does not induce these inflammatory mediators' expression (Wölfle et al., 2017). These results confirm the immunomodulatory and anti-inflammatory activity evaluated for amarogentin, the bitterest compound of *G. lutea* roots.

3.8.2. Clinical trial

The ability of *G. lutea* extract to enhance lipid synthesis of the *stratum corneum* was also tested *in vivo* in a placebo-controlled double-blind half-side comparison study. In this trial, 33 volunteers with normal or dry skin were treated for 4 weeks, 2 times daily, with a cream containing 5% of *G. lutea* extract. As a site of cream application was chosen the volar forearms because, unlike other areas of the skin, in this area, lipids are almost exclusively produced by keratinocytes and not by sebaceous glands. The other arm of the trial was treated with a vehicle unguentum. Lipid contents were measured with a sebumeter after 2, 3, and 4 weeks; 60% of the volunteers showed a 25% increase in lipid content, and 8 of them resulted in an enhancement of more than 50% (Wölfle et al., 2017).

Hence, these studies demonstrate that *G. lutea* extract may increase lipid amounts not only *in vitro* on keratocyte cell line but also *in vivo* on healthy volunteers. These indicate that *G. lutea* could represent a source of molecules with therapeutic value in inflammatory skin diseases characterized by reduced lipid content and an impaired epidermal barrier. Hence, further investigation should be done to evaluate which active molecules may be responsible for the analyzed activity and try to formulate innovative pharmaceutical products to convey these molecules as best as possible.

3.9. Gentiana lutea L. gastroprotective and hepatoprotective activity

3.9.1. In vivo studies

Gentianae radix has been extensively studied for its choleretic and hepatoprotective properties, thus representing a good remedy for stomach and liver inflammations. As regards the gastroprotective effect, in pylorus-ligated mice treated with methanolic extract of gentian root in the duodenum, there was a decrease in gastric juice secretion and total acid output with a dose-dependent effect considerable at doses of 500 and 1000 mg/kg. The same results were achieved by EtOAc and *n*-BuOH fractions with an activity comparable to that of 60 mg/kg of cimetidine (histamine H2 receptor antagonist that blocks stomach acid secretion during ulcer treatment). These effects could be related to increased secretin levels or other mechanisms. The *radix* extract and the fractions obtained (EtOAc and *n*-BuOH) also showed a protective effect in the case of pyloric-ligation plus aspirin-induced ulcers (20 mg/mL) in a dose-dependent manner, and in particular, for the fractions, the effect was comparable to cimetidine. Furthermore, oral administration of the two fractions revealed protective effects in gastric ulcers induced by immersion stress, while EtOAc soluble fraction showed protection against ethanol-induced gastric lesions. In these two fractions, a high concentration of gentiopicroside and amarogentin was observed, and these bitter compounds are probably responsible for the gastroprotective effect by acting on the prostaglandin pathway. In fact, both secoiridoids showed a protective effect in the case of ulcers induced by immersion stress, while amarogentin was also effective in gastric lesions induced by ethanol. However, no effect was observed when indomethacin (5 mg/kg), an inhibitor of prostaglandins synthesis, was used as pre-treatment (Niiho et al., 2006).

As for hepatoprotective action, in recent years, there has been an increase in the incidence of liver problems (Xiao et al., 2019). Several plant species have shown important liver effects, such as silymarin from *Silybum marianum* induces a reduction of inflammatory factors like interleukin-10, TNF-α, interferon, and IFN-γ with a consequent hepatoprotective effect (Vargas-Mendoza et al., 2014). The beneficial effect of gentian on the liver may be due to the gentiopicroside since its protective effect in the presence of cholestasis has been reported in previous studies (Han et al., 2018).

3.9.2. Clinical studies

Regarding human investigations, *G. lutea* was studied for its effectiveness in reducing the increase in intestinal permeability, a problem that causes greater absorption of endotoxins due to a loss of integrity between epithelial small intestine cells. In Complementary and Integrative Medicine (CIM), characterized by applying multiple and personalized treatments, *G. lutea* showed a reduction in the time needed to solve the intestinal permeability alteration from more than 6 to 4–5 months (Leech et al., 2019).

The Committee on Herbal Medicinal Products (https://www.ema.europa.eu/documents/herbal-summary/gentian-root-summary-public_en.pdf) approved the use of *G. lutea* for mild stomach and gut complaints, but they fonded their approbation only on their traditional and long-standing use as there was insufficient evidence from clinical evidence trials. It was done, indeed, only an observational study involving 205 subjects with mild gut and stomach complaints and treated with *G. lutea*

root. This study suggested an improvement in symptoms, but this trial did not include a placebo group or the treatment with another treatment, so it is impossible to come to a firm conclusion. Hence the need for further randomized clinical trial remain.

3.10. Gentiana lutea L. Antimicrobial activity

Over the years, the excessive and improper use of antibiotics has generated resistance phenomena by pathogens. Therefore, it is necessary to search for undescribed molecules capable of fighting bacterial infections, obviating the problem of resistance. Several studies have reported that *G. lutea* and its bitter agents exert an antimicrobial action. In this regard, it was observed that the effect of gentian corresponded to that of the antibiotic ampicillin, which is used to treat different gram-positive and negative bacterial infections, including meningitis, endocarditis, salmonellosis, respiratory and urinary tract infections (Brogden et al., 1979; Šavikin et al., 2009b).

3.10.1. In vitro studies

Pseudomonas aeruginosa, Bacillus subtilis, Proteus mirabilis, Staphylococcus epidermidis, and *Candida albicans* were the most sensitive to gentian leaf extract with MIC values between 0.12 and 0.31 mg/mL. However, flower extract was lightly active against the tested microorganisms, and the most susceptible was *Salmonella enteritidis* (MIC 0.15 mg/mL). Both leaf and flower extracts showed an antitubercular effect against *Mycobacterium bovis.* Regarding the isolated compounds, gentiopicrin showed a broader spectrum of action with a great effect against *E. coli* (0.12 mg/mL) and a moderate effect against *S. aureus* and *S. typhimurium* (0.15 mg/mL). In contrast, the xanthone isogentisin was active against *M. bovis* and showed moderate activity against the gram-negative *E. coli* and *P. aeruginosa* (0.15 mg/mL) and the gram-positive *Micrococcus luteus* (0.15 mg/mL). The single compound does not have a greater antimicrobial effect than the extracts, probably because a synergistic action is required (Šavikin et al., 2009b). Moreover, Mahady et al. reported that *G. lutea* root methanolic extract exhibited a weakly activity (MIC 100 μg/mL) against 15 strains of *Helicobacter pylori* (Mahady et al., 2005), a slow-growing, spiral, gram-negative organism responsible for different gastrointestinal diseases like peptic ulceration, mucosa-associated lymphoid tissue lymphoma, gastritis, and gastric cancer (Makola et al., 2007). On the contrary, the aqueous *G. lutea* root extract showed no significant activity against other yeast and bacteria in the agar. In particular, it was weakly active against aerobic bacteria such as *Streptococcus pyogenes, Corynebacterium amycolatum*, and *Corynebacterium pseudodiphtericum,* and anaerobic bacteria like *Fusobacterium nucleatum* and *Porphyromonas gingivalis* with MIC values of 100 μg/mL except for *C. amycolatum* whose MIC was 10 μg/mL (Weckesser et al., 2007).

To date, there is a lack of *in vivo* investigation able to demonstrate the antimicrobial activity except for one study conducted on horses which was not taken into consideration because *G. lutea* was tested in combination with other plants making the study off-label for this systematic review. Thus in order to clarify the rial antimicrobial activity still need to test the extract *in vivo* on infected animal models. This might also be necessary to assess the mechanism by which *G. lutea* and its active molecules could exert their activity against bacteria and viruses.

4. Conclusions

To date, the pharmacological use of traditional remedies derived from plants has gained much consideration thanks to their proven safety and efficacy. Obviously, they need further careful phytochemical and biological investigation to evaluate possible pharmacological interactions and the exact mechanism of action of natural isolated compounds. Specifically, *Gentiana lutea* L. attracted the attention of many researchers for its countless activities beneficial to human health. In fact, this natural resource and its constituents have been demonstrated to exert antioxidant, anti-inflammatory, anti-microbial, anti-obesogenic, anti-atherosclerotic, gastroprotective, neurotrophic, anti-genotoxic effects, thus becoming a plant with pleiotropic properties. In this systematic review, all the current knowledge has been treated to provide a detailed overview of the potential use of Gentian for the treatment of various diseases. However, these are mainly pre-clinical results that need further confirmation by clinical trials.

5. Experimental

5.1. Search strategy

Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, the systematic search of the literature was done in May 2021 and included all items published until June 2022. It comprised all articles inherent to the review's object found on two specialized databases: PubMed and Scopus. The keywords used for the search were *Gentiana lutea* paired with the following words: obesity, antioxidant, inflammation, diabetes, atherosclerosis, cardiovascular diseases, neurological diseases, antimicrobial, antiviral, antibacterial, antifungal, cytotoxicity, apoptosis. Research papers were restricted to English-language publications.

5.2. Study Selection

The inclusion criteria for the review writing comprised pre-clinical studies (*in vivo* and *in vitro*) and clinical studies involving *Gentiana lutea* extracts and their related compounds. Only articles writing in English and with the keyword in the abstract or title were selected. Other review articles, meta-analysis, retrospective studies, abstracts, editorials, letters, and manuscripts, or articles without full text available were not considered for writing out this systematic review. Two investigators (M.P. and I.F.) selected the manuscripts by screening titles, abstracts, and finally full texts. In cases of dissensus, other independent reviewers were consulted (L.M.). All chosen articles were closely reviewed to include or exclude manuscripts that did not fit the specified criteria.

5.3. Data Extraction

All chosen articles were reviewed attentively, and information concerning the activity of Gentiana lutea L. was extracted, as well as the study design, experimental models, doses used, main results, and general mechanism of action. Articles reporting the most important results were summarized in Table 2.

5.4. Methodological Quality assessment

The authors assessed the quality of each research and the risk of bias by adapting the checklist of Cochrane Handbook for Systematic Reviews of Interventions, adjusted explicitly for animal intervention studies (SYRCLE's) (Higgins et al., 2019) and clinical trials (Higgins et al., 2019). Studies' qualities evaluation was made based on the presence or absence of the information reported in Tables 3 and 4. Articles that did not are in line with all the criteria were linked as items with a medium risk of bias, while papers lacing these criteria were included in the high risk of bias group. Finally, manuscripts respecting all parameters were assessed as having a low risk of bias.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 2
Description of the main biological activity of *G. lutea* and its specialized metabolite.

Extract/ Compound	Assay/Model/Clinical trial	Treatment Time	Doses/Concentration	Biological activity	References
ANTIOXIDANT ACTIVITY					
G. lutea extract	Rats	5 days	1 g/kg	↑ CAT ↑ SOD	Amin (2008)
ANTI-OBESOGENIC ACTIVITY					
G. lutea extract	3T3-L1 preadipocyte	8 days	2–10–50 μg/mL	↓ adipocyte differentiation ↓ *Cebpα* ↓ *Adipoq* ↓ GLUT4 ↓ *Lpl* ↓ PPARγ	(Park et al., 2018, 2020)
	Cos7 cells		10–30–100 mg/mL	↓ PPARγ	Rau et al. (2006)
	Spectrophotometric assay		0.6–2.4 μg	↓ ALR2	Akileshwari et al. (2012)
	Ex vivo				
	Male C57BL/6 J mice fed a 60% fat diet	12 weeks	100–200 mg/kg/day	↓ Total fat ↓ body weight ↓ hepatocytic lipid deposition ↓ leptin ↓ insulin	Park et al. (2020)
	Male C57BL6/J mice	8 weeks	regular diet supplemented with 2% *G. lutea* root powder	↓ total cholesterol	Potunuru et al. (2019)
	Randomized cross over study on healthy volunteers	two 1-d experimental sessions	Microcapsules providing 100 mg of secoiridoids	↓ food intake ↑ GLP-1	Mennella et al. (2016)
Amarogentin	*In silico* assay			↓ ALR2	Akileshwari et al. (2012)
	T1DM rats	1 weeks	0.1–0.3–0.5 mg/kg	↓ blood sugar ↓ hyperglicaemia ↑ GLUT4 in skeletal muscle ↓ PEPCK	Niu et al. (2016)
	Male C57BL6/J mice	8 weeks	0.3–0.5 mg/kg	↓ glucose, ↓ LDL ↓ VLDL ↓ cholesterol ↓ triglycerides ↑ HDL	Potunuru et al. (2019)
Gentiopicroside	*In silico* assay			↓ ALR2	Akileshwari et al. (2012)
Loganic acids	3T3-L1 preadipocyte		2–10–50 μg/mL	↓ adipocyte differentiation ↓ *Cebpα* ↓ *Adipoq* ↓ GLUT4 ↓ *Lpl* ↓ PPARγ ↓ *Plin1* ↓ *Fabp4* ↓ TNF-*α*	Park et al. (2018)
	OVX female ddY mice	12 weeks	2–10–50 mg/kg/day	↓ body weight ↓ total fat percentage ↓ GLUT4 ↓ *Lpl* ↓ PPARγ	
ANTI-ATHEROSCLEROTIC ACTIVITY					
G. lutea extract	HUVEC cell lines	24 h	1 mg/mL	↓ TNF-*α* ↓ VCAM-1 ↓ ICAM-1 ↓ RASMCs migration ↓ phospholipase C-γ activation	Kesavan et al. (2016)
	Primary cultures of RASMCs		1 mg/mL	↓ ERK1/2 activation ↑ NO production ↓ IKK*α*	Kesavan et al. (2013)
	Aldose Reductase (ALR2) Assay		0.6–2.4 μg	↓ ALR2 activity	Akileshwari et al. (2012)
	Ex vivo on humanRed blood cells			↓ sorbitol	
	In silico				
	Streptozotocin inducing diabetic rats	12 weeks	2% *G. lutea* root powder in the animal feed	↓ vessel wall media layer ↓ collagen deposition ↓ iNOS, ↓ VCAM-1 ↓ VE-cadherin	Kesavan et al. (2016)
	Control placebo clinical study				McMullen et al. (2014)

(*continued on next page*)

Table 2 (*continued*)

Extract/ Compound	Assay/Model/Clinical trial	Treatment Time	Doses/Concentration	Biological activity	References
		1-d experimental sessions	Gentian flavored water (500 and 1500 mg)	↑ peripheral vascular resistance ↓ reduction in cardiac workload ↓ cardiac activity	
Isovitexin	Primary cultures of RASMCs *In silico*		5–10 μmol/L	↓ RASMCs proliferation ↓ PDGF-induced ERK1/2 activation ↓ MEK1 activity	Kesavan et al. (2016)
Amarogentin	HUVEC cell lines	30 min	25–250 nM	↓ TNF-α ↑ eNOS ↑ NO production ↓ NF-kB phosphorylation ↓ VCAM-1	Potunuru et al. (2019)
	In silico			↑ AMPK	
	Ex vivo	3 min	15~60 μM	↓ MAPK	Yen et al. (2014)
	Streptozotocin-induced diabetes in mice	8 weeks	0.3–0.5 mg/kg	↑ AMPK ↓ SGPT ↓ SGOT ↓ hepatic fibrosis ↓ neointimal thickening ↓ collagen deposition ↓ lipid deposition	Potunuru et al. (2019)
	mice		18 mg/kg	↓ thrombus formation	
Gentisin	Rat aortic VSMC	30 min	3–10–30 μM	↓ VSMC proliferation	Waltenberger et al. (2015)
Gentiopicroside	Spectrophotometric assay		1×10^{-5} to 1×10^{-3} mg/mL	↓ MPO	(Nastasijević et al., 2012)
ANTI-INFLAMMATORY ACTIVITY					
G. lutea extract	RAW264.7 macrophage cells	7 h	200 μg/mL	↓ TNF-α	Cafaro et al. (2020)
	rats	7 days	300–500–1000 mg/kg	↓ edema ↓ granuloma ↑ wound healing	Mathew et al. (2004)
	Male albino mice	1-d experimental sessions	250–500 mg/kg	↑ analgesia ↑ fatigue resistance	Öztürk et al. (2002)
Gentiopicroside	Inflammatory pain mice models	twice daily for 3 days	50–200 mg/kg	↓ persistent inflammation ↓ NR2B	Chen et al. (2008)
NEUROTROPHIC ACTIVITY					
G. lutea extract	PC-12 cell line	5 days	25 μg/mL	↑ neurite outgrowth	Mustafa et al. (2015)
	SH-SY5Y cells	48 h	200–400 μg/mL	↓ apoptosis ↑ Bcl-2 ↑ Sirt-1 expression ↑ GSH	Cafaro et al. (2020)
Isovitexin	Spectrophotometric	20 min	1×10^{-7} and 3×10^{-4} M	↓ E-NTPDase	Nastasijevic et al. (2016)
Amarogentin	*In silico*				
Gentiopicroside	reserpine-induced pain/depression mice	twice daily for 3 days	50–100–200 mg/kg	↓ GluN2B ↓ pain/depression ↑ Bcl-2 ↓ caspase-3 ↑ MDA ↓ CAT	Liu et al. (2014)
ANTITUMORAL EFFECT					
G. lutea extract	HeLa, MCF7, PC3 cell lines	72 h	125–500 μg/mL	↑ anti-proliferative	(Balijagić et al., 2012; Rodrigues et al., 2019)
	PBMC cell lines	72 h	1.25–2 mg/mL	↑ DNA reparation	(Cvetkovic et al., 2019;
	HepG2 cell lines			↑ cytostasis ↓ genotoxicity	Meschini et al., 2015; Valenta Šobot et al., 2020)
	Drosophila melanogaster		25 mg/mL	↓ genotoxicity	Patenković et al. (2013)
	Ex vivo on healthy volunteers' PBMC cells	1-d experimental sessions	15 g	↑ radio-resistance	Menkovic et al. (2010)
ACTIVITY AGAIN ATOPIC DERMATITIS AND PSORIASIS					
G. lutea extract	HaCaT and hPKs keratinocytes		50–100–200–400 μg/mL	↑ palmitic acid ↑ linoleic acid ↑ ceramide ↑ CerS3	Wölfle et al. (2017)
	Psoriasis-like hPKs		200 μM	↑ ELOVL-4 ↑ CerS3	Gendrisch et al. (2020)
	Placebo-controlled double-blind half-side comparison study	2 times daily for 4 weeks	cream containing 5% of *G. lutea* extract	↑ lipid content	Wölfle et al. (2017)
Amarogentin	HaCaT keratinocytes	72 h	30–100–300 μM	↑ keratinocyte differentiation	Wölfle et al. (2015a)
			100 μM	↓ histamine release	Wölfle et al. (2015b)

(*continued on next page*)

Table 2 (*continued*)

Extract/ Compound	Assay/Model/Clinical trial	Treatment Time	Doses/Concentration	Biological activity	References
	LAD-2 cell lines			↓ TNF-α	
	HaCaT keratinocytes			↓ IL-8	
				↓ MMP-1	
GASTROPROTECTIVE AND HEPATOPROTECTIVE ACTIVITY					
G. lutea extract	pylorus-ligated mice		500–1000 mg/kg	↑ protective effect against gastric ulcer ↓ gastric juice ↓ total acid output	Niiho et al. (2006)
	Complementary and integrative medicine (CIM) practitioners	Not specified	Not specified	↓ intestinal permeability	Leech et al. (2019)
ANTIMICROBIAL ACTIVITY					
G. lutea extract	*Streptococcus pyogenes Corynebacterium amycolatum Corynebacterium pseudodiphtericum Fusobacterium nucleatum Porphyromonas gingivalis*		MIC 100 μg/mL	↓ vitality	*Weckesser* et al. *(2007)*
	C. amycolatum		MIC 10 μg/mL	↓ vitality	
	Helicobacter pylori		MIC 100 μg/mL	↓ vitality	Mahady et al. (2005)
	Pseudomonas aeruginosa		MIC 0.12–0.31 mg/mL	↓ vitality	Šavikin et al. (2009b)
	Bacillus subtilis Proteus mirabilis Staphylococcus epidermidis				
	Candida albicans				
	Mycobacterium bovis				
	Salmonella enteritidis		MIC 0.15 mg/mL	↓ vitality	
Gentiopicrin	*Escherichia coli*		MIC 0.12 mg/mL	↓ vitality	
	Streptococcus aureus		MIC 0.15 mg/mL	↓ vitality	
	S. typhimurium				
Isogentisin	*Escherichia coli*		MIC 0.15 mg/mL	↓ vitality	
	Pseudomonas aeruginosa				
	Micrococcus luteus				

Table 3
Checklist for assessment of risks of bias in pre-clinical studies (Higgins et al., 2019).

Checklist for Assessment of Risks of Bias in Pre-clinical Studies
Are the hypothesis and objective of the study clearly described?
Are the main outcomes to be measured clearly described?
Are the main findings of the study clearly described?
Are the samples size calculations reported?
Are the animals randomly housed during the experiment?
Are the investigators blinded from knowledge which treatment used?
Are the outcome assessors blinded?
Is the dose/route of administration of *Gentiana lutea* L. properly reported?
Is the dose/route of administration of the drug in co-treatment properly reported?
Is the frequency of treatments adequately described?

Table 4
Checklist for assessment of risks of bias in clinical studies (Higgins et al., 2019).

Checklist for Assessment of Risks of Bias in Clinical Studies
Are the hypothesis and objective of the study clearly described?
Are the main outcomes to be measured clearly described?
Are the main findings of the study clearly described?
Are the samples size calculations reported?
Are the animals randomly housed during the experiment?
Are the investigators blinded from knowledge which treatment used?
Are the outcome assessors blinded?
Is the dose/route of administration of *Gentiana lutea* L. properly reported?
Is the dose/route of administration of the drug in co-treatment properly reported?
Is the frequency of treatments adequately described?

Data availability

No data was used for the research described in the article.

Acknowledgments

This research was supported by Regione Basilicata; Project PAPRI-KANET - Plant Active Products: Research, Innovation And Knowledge Advancements Through An International Network CUP: C39J20001740002 programme 2014IT16RFOP022; Italian Ministry of the Economic Development "Fondo per la Crescita Sostenibile—-Sportello "Agrifood" PON I&C 2014–2020", Project FULLNESS n. F/200099/01–03/X45.

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