Review Article

Biological Activities and Analytical Methods for Detecting Aucubin and Catalpol Iridoid Glycosides in *Plantago* Species: A Review Study

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ABSTRACT

Background: *Plantago* species contain aucubin and catalpol iridoid glycosides used in traditional medicine for many purposes.

Objectives: To accelerate the utilization of aucubin and catalpol in *Plantago* species, research should be focused on introducing advanced purification and detection methods. In this regard, the therapeutic activities of aucubin and catalpol compounds are mentioned to confirm their effectiveness in medicinal uses.

Methods: An extensive literature search was conducted using the keywords "Aucubin and Catalpol + *Plantago*" in the public domains of Google scholar.

Results: The iridoid patterns exhibited a significant correlation with morphological and other chemical specifications of the representatives of the genus *Plantago*. Commonly, iridoid glycosides are detected with gas chromatography, liquid chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and capillary electrophoresis techniques. The most common methods are HPLC and HPTLC. Aucubin and catalpol are active compounds possessing biological activities, including anti-cancer, anti-aging, anti-inflammatory, anti-oxidant, hepatoprotective, osteoprotective, and neuroprotective properties.

Conclusion: This review article comprehensively summarizes cytotoxic activities and detection methods of aucubin and catalpol in *Plantago* species. The results suggest that Plantago species and their metabolites may benefit human health beyond their traditional uses.

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Introduction

Iridoid was first isolated at the end of the 19th century. But, the principal structure of the iridoid was identified in 1958 by Halpern and Schmid. Then, several scientific studies were conducted on iridoids relating to agriculture, biosynthesis, botany, and medicinal uses. Up to now, hundreds of iridoids have been recognized in diverse sources [1]. Iridoids are categorized into iridoid glycosides, non-glycosidic iridoids or aglycone, bisiridoids, and secoiridoids groups [2]. Iridoid glycosides are monoterpenes in at least 57 plant families [3]. Their function in plants is mostly protection [4]. Iridoids can be found in the following families: Lamiaceae, Acanthaceae, Plantaginaceae, Gentianales, Cornales Scrophulariaceae, and Rubiaceae [5-8]. Especially, aucubin and catalpol are found in plant subclasses of Asteridae, including Loganiaceae, Lamiaceae, Ericaceae, Gentianaceae, Verbenaceae, Rubiaceae, Oleaceae, Scrophulariaceae, Valerianaceae, Plantaginaceae, and Menyanthaceae [9].

Consequently, studies on *Plantago* species have just established growing attention owing to valuable components in these plants, such as aucubin and catalpol. So, the present study reviewed the cytotoxic properties and detection methods of aucubin and catalpol to create a comprehensive reference for utilizing these compounds.

Aucubin and Catalpol Names

Aucubin is known with CAS: 479-98-1 and chemical formula: (2S,3R,4S,5S,6R)-2-[[(1S,4aR,5S,7aS-5-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[c]pyran-1-yl]oxy]-6-(hydroxymethyl)oxane-3,4,5-triol [10].

Compound summary

Catalpol is introduced with CAS: 2415-24-9 and chemical formula: (2S,3R,4S,5S,6R)-2-{[(1aS,1bS,2S,5aR,6S,6aS)-6-Hydroxy-1a-(hydroxymethyl)-1a,1b,2,5a,6,6ahexahydrooxireno[20,30:4,5]cyclopenta[1,2-c]pyran-2-yl]oxy}-6-(hydroxymethyl)oxane-3,4,5-triol [10].

Aucubin and catalpol are iridoid glycosides used in herbal medicine (Figure 1).

Biosynthesis of Aucubin and Catalpol

Iridoids are a group of cyclopentanone monoterpenes found in plants as glycosides and regularly linked to glucose at C-1. The backbone of carbocyclic iridoids (Figure 2) is generally a cyclopentane unit attached to a dihydropyran ring. On the other hand, secoiridoids are formed due to C-7/C-8 cleavage. These compounds have been classified as chemotaxonomic markers, and their presence provides evidence to explain many species whose taxonomic boundaries are unclear [11, 12].

Iridoids are often found as glucosides, featuring a b-D-glucopyranosyl unit attached at C-l via a b-hemiacetalic bond (R = glucose) [13].

There are two main biosynthetic pathways for producing iridoids (Figure 3). The first pathway makes compounds that generally originate in Gentianales and Cornales orders. In this pathway, deoxyloganic acid is synthesized, the precursor of many iridoids with 8β stereochemistry, such as secologanin and loganin. The latter is caused by the oxidative cleavage of the C-7/C-8 linkage of the cyclopentane ring. After an intricate synthesis including tryptamine, secologanin gives rise to indole alkaloids; vinblastine, vincristine, and reserpine, among others, are regularly discovered in the families Rubiaceae, Apocynaceae, and Loganiaceae, order Gentianales. Another group of iridoids is biosynthetically created by the second pathway. This pathway produces 8-epi-deoxyloganic acid, a precursor to iridoids with 8-a carbon substituent, and both C-4 carboxylated and C-4 decarboxylated carbocyclic iridoids, for example, ipolamiide and aucubin, respectively. These compounds are almost particularly established in families of Lamiales [14].

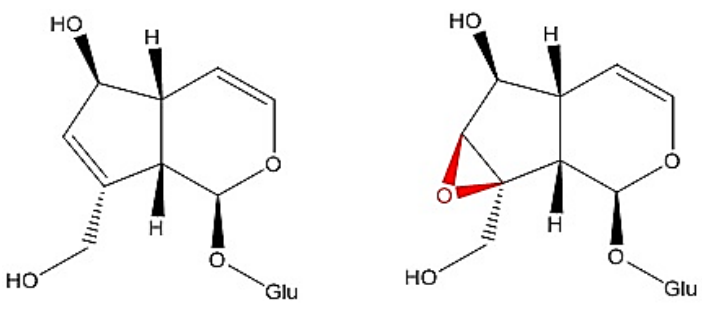

Figure 1. Aucubin and catalpol structures

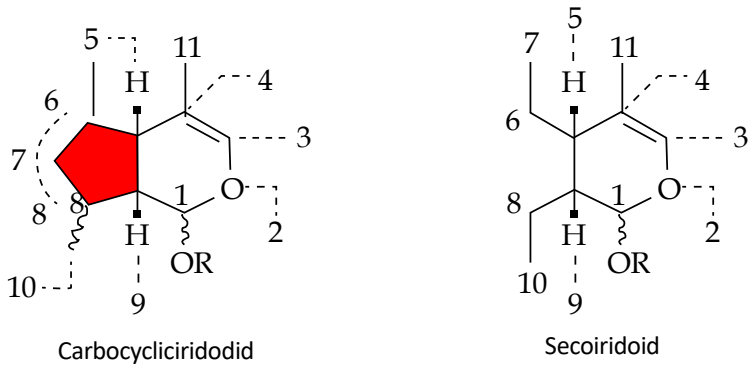

Figure 2. Basic structure of iridoids

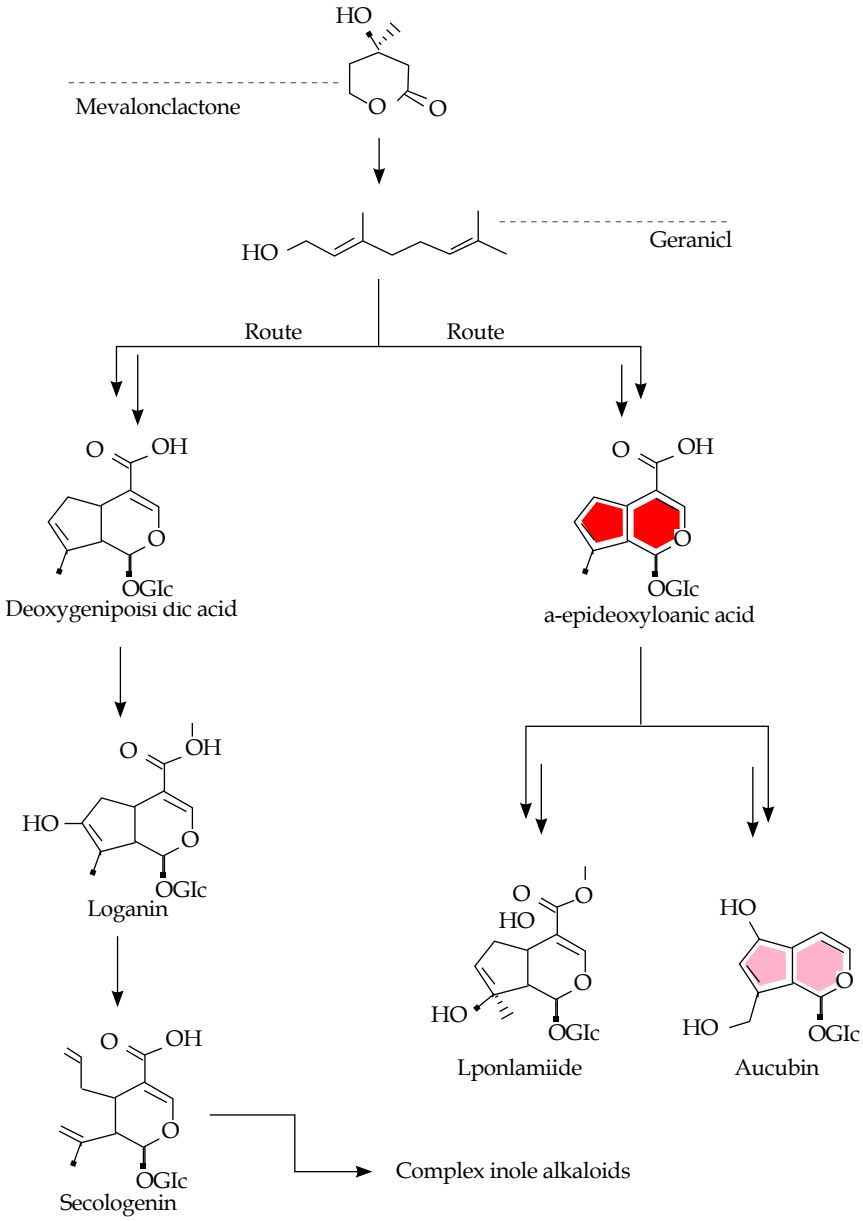

Figure 3. Iridoid biosynthesis from the pathway I and II, adapted from Jensen (1992) [14]

Rønsted et al. have revealed the biosynthesis of aucubin and catalpol, mainly in *Plantago major* (Figure 4) [15].

Distribution of Aucubin and Catalpol in the Plant Kingdom

Aucubin was first identified in *Aucuba japonica* in 1905. However, it is also found in many other natural plants such as *Plantago asiatica* L, *Eucommia ulmoides* Oliv., and *Aucuba japonica* Thunb [16]. Aucubin is the most common compound in the iridoid glycoside class [8]. Aucubin is also an intermediate of catalpol (Figure 3) [17]. Catalpol iridoid glucoside is broadly dispensed in many plant families and is mainly acquired from *Rehmannia glutinosa* Libosch root [18]. The selection for catalpol may have resulted in a reduction in aucubin concentration [19]. Aucubin and catalpol can be applied as potential chemotaxonomic markers to regulate the quality of different plant extracts, such as *Plantago* species [20].

Plantain is known to have two important aucubin and catalpol compounds [21]. Aucubin is present in almost all *Plantago* species, whereas catalpol or its derivatives have been reported in some species [22]. Iridoid glycoside concentration also changes with the plant's developmental parts, age, genetic, and environmental factors such as weather, time of day, soil status, and arbuscular mycorrhizal fungi [3, 23-35]. Temperature, UV light, and soil nutrient conditions can alter the content of the secondary metabolites of plantain [36]. In some studies, the mean content of catalpol in the leaves of *P. lanceolata* was lower than the aucubin content [33, 37, 38], although, in one study, the opposite state was reported [3]. The contents of catalpol in *P. lanceolata* interrelated adversely with leaf age and the total number of leaves [35]. In Bowers and Stamp's study, in genotypes of *P. lanceolata*, the amount of aucubin was more in intermediate leaves compared to mature leaves, while catalpol content was higher in intermediate and young leaves [25]. Increasing leaf age leads to an increase in aucubin content relative to total iridoid glycosides [25]. On the other hand, genotype significantly affects iridoid glycoside content, although an individual plant is quite heterogeneous in terms of iridoid glycoside content. New leaves have twice as many iridoid glycosides as mature leaves [25]. Lastly, Bowers and Stamp concluded that as leaves age, less catalpol is produced, breaks down faster, and translocation occurs in the leaf [25]. Lampert and Bowers indicated that aucubin is most in old leaves, while catalpol is high in young ones [39]. The amounts of aucubin and catalpol and the ratio of catalpol to total iridoid glycosides are impassioned by interactions between the time of harvest and leaf age [40]. The amounts of catalpol in new leaves increase between the harvests, although, in intermediate and mature leaves, harvest date showed no significant influence on catalpol content [40].

De Deyn et al. showed that among the full sibling families of *P. lanceolata*, plants contained high constitutive levels of defensive iridoid glycosides in leaves and roots. They reported that the concentration of aucubin was higher than that of catalpol and was found more in the root than in the shoot tissue [41]. The difference in constitutive iridoid glycoside values in *P. lanceolata* is genetically regulated to some extent [25-27, 32].

Furthermore, environmental and ontogeny strongly affect iridoid glycoside amounts in *P. lanceolata*. Sea-

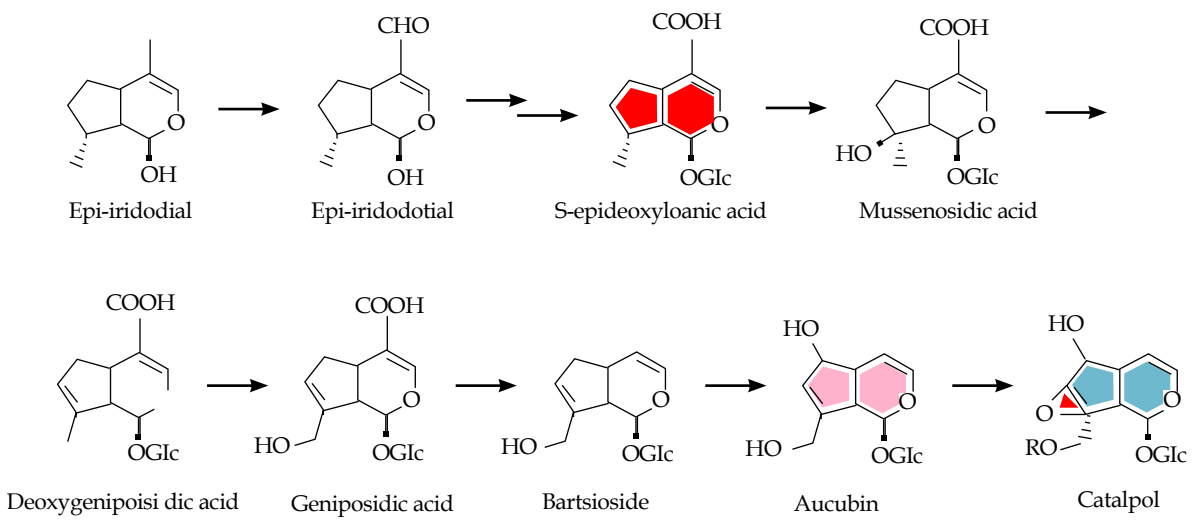

Figure 4. Biosynthetic pathway to aucubin and catalpol [15]

sonal changes probably affect the content of bioactive compounds in plantain leaves in different ways. Solar emission, nutrient disposal, and air temperature are major issues. Average levels differ among habitats and populations [36] and are mostly higher in plants grown under high light and low nutrient or water situations [28, 37]. Tamura reported that nitrogen source and low light intensity forcefully suppressed the accumulation of aucubin in plantain leaves but had no effect on catalpol concentration [36]. Tamura also explained that relatively high air temperatures (20°C/18°C, day/night) enhanced aucubin and catalpol contents. Therefore, plants grown under high temperatures can accumulate higher aucubin than those produced in low air temperatures (15°C/10°C, day/night) [36]. In total, Tamura and Nishibe assumed that the content of aucubin is more when the air temperature is optimal for growth [34].

Tamura and Nishibe investigated the effect of seasonal changes in the content of bioactive compounds in plantain leaves [34]. The amount of aucubin increased from late spring to midfall, but in midsummer, aucubin levels were relatively constant. In late fall, aucubin levels gradually decreased in Grasslands Lancelot (0.13% $°C^{-1}$) and Ceres Tonic (0.20% $°C^{-1}$), relative to the decline in air temperature from 14.7°C to 10.7°C. However, in midfall, when the temperature was still around 20°C, the levels of aucubin in both cultivars' leaves were the highest. On other hand, catalpol content was very low relative to the contents of aucubin. The seasonal changes in the amount of catalpol were less visible than in aucubin, so the concentration of catalpol increased slightly during the growing season, but its amount was low in the middle of summer. The less clear-cut changes in catalpol can be due to the low base level of its synthesis, which does not respond to environmental changes. However, in Nieminen et al. study, catalpol contents were more than aucubin in mid- and late-summer [42].

Bowers et al. also studied seasonal variations in aucubin and catalpol amounts in plantain leaves [43]. Plants were harvested at 2-week intervals from late spring to early fall (4 times). They showed a significant increase in aucubin and catalpol content during the growing season, but the levels of the compounds decreased in plants harvested in mid-summer compared to those harvested on the other three sampling dates. Since the air temperature in midsummer was very high (around 25-30°C), this result agrees with the previous explanation [34]. Furthermore, *P. lanceolata* stores more catalpol when grown in soils conditioned by grass species [44].

It is usually presumed that medicinal plants, for their pharmacological uses, should be dried at less than 60°C to minimize the loss of bioactive compounds. Therefore, the concentration of bioactive compounds gradually degenerated in the early drying stages. The reason for the decay in the amounts of catalpol and aucubin could be the presence of enzymes in charge of their degradation. The degradation of iridoid glycosides is because of β-glucosidase action. The activities of these putative enzymes can be neutralized after about 3 and 24 h after the start of drying under natural climatic status and at 60°C, respectively since the compounds' levels are fixed after these times [34]. Finally, it is suggested that midfall is the best time to harvest plantain for medicinal purposes because the amounts of the active compounds progressively decrease in the early stages of drying both under natural climatic conditions and at a temperature of 60°C [34].

Aucubin and Catalpol Content in Different Plant Parts and *In Vitro* Cultures

Plantain is applied in traditional medicines and for pasture. As that aucubin is a precursor in catalpol biosynthesis, several studies assessed the content of iridoid glycosides in plantain under different conditions, including the following studies. Aucubin is formed in plantain at very high levels, up to 3% of dry weight, depending on various aspects of the genotype, soil fertility, and so on [28, 32, 45]. The content of iridoid glycosides, including aucubin and catalpol, in a natural population of *P. lanceolata*, can attain even 9% of dry matter [3]. With increasing leaf age and dry summer conditions, the levels of these compounds increase. Also, cutting the surfaces in detached leaves can significantly increase them [25, 30, 46].

Another study reported that the level of aucubin and catalpol in seven *Plantago* species was up to 0.27% and 1.81% of dry leaf weight, respectively [47]. Furthermore, aucubin and catalpol production increased over time from 0.003% to 8.86% dry weight from seeing seedling pre-reproductive plants [48]. A significant interaction between plant age and tissue indicated differences in iridoid glycoside content between shoots and roots during plant growth. The results are presented in three cases. First, the average of iridoid glycosides was three times higher in the shoots compared to the roots. Second, the mean group differences in aucubin and catalpol among all 7 age classes were similar for the shoot and root tissues. Third, the concentration of aucubin in the shoot compared to the total iridoid glycosides caused more changes during plant ontogeny [48]. Pellissier et al. noted that total iridoid glycosides content, particularly

Table 1. The content of aucubin (AU) and catalpol (CA) in different parts of *Plantago* species

Plant Species	Plant Part	Content in Dry Weight (AU)	Content in Dry Weight (CA)	Content in Dry Weight (IG)	Method	Reference
P. lanceolata	Leaf	1.47%-4.11%	0.70%-5.04%	2.25%-7.74%	GC	[25]
P. lanceolata	Leaf	0.6%-4.4%	0.4%-3%	1.1%-7.3%	GC	[30]
P. lanceolata	Dried plant	0.02%-0.26%	0.23-0.36%	0.39-0.49%	GC-FID	[53]
P. lanceolata	Leaf	0.46%	0.32%	0.90%	GC-MS	[54]
P. major	Leaf	0.07%	n.d	0.12%	GC-MS	[54]
P. lanceolata	Leaf	0.92%	0.44%	1.39%	GC-FID	[55]
P. lanceolata	Root	0.98%	0.48%	1.50%	GC-FID	[55]
P. lanceolata	Stalk	1.01%	0.85%	1.91%	GC-FID	[55]
P. lanceolata	Spike	0.67%	1.15%	2.23%	GC-FID	[55]
P. lanceolata	Leaf	0.30%-%1.00	0.24%-0.74%	(n.d)	HPLC	[56]
P. altissima	Leaf	0.092%	0.131%	n.d	MEKC	[47]
P. argentea	Leaf	0.273%	1.809%	n.d	MEKC	[47]
P. holosteum subsp. *Depauperata*	Leaf	0.077%	n.d	n.d	MEKC	[47]
P. lagopus	Leaf	0.072%	0.082%	n.d	MEKC	[47]
P. maritima	Leaf	0.027%	n.d	n.d	MEKC	[47]
P. lanceolata	Leaf	0.115%	0.159%	n.d	MEKC	[47]
P. lanceolata	Leaf	0.33%-1.73%	0.22%-1.25%	n.d	HPLC	[57]
P. lanceolata	Leaf	0.55%-4.93%	1.57%-6.20%	2.15%-8.60%	HPLC	[58]
P. lanceolata	Leaf	0.65%-4.18%	0.0%-0.121%	n.d	HPLC	[59]
P. lanceolata	Shoot	0.1%-2.2%	0.0%-1.50%	n.d	HPLC	[41]
P. lanceolata	Root	0.5%-2.9%	0.0%-1.00%	n.d	HPLC	[41]
P. asiatica	Leaf	9358µg/g	1346 µg/g	n.d	LC	[60]
P. asiatica	Seed	104.2 µg/g	179.6 µg/g	n.d	LC	[60]
P. asiatica	Roasted Seeds	0.00	171.2 µg/g	n.d	LC	[60]
P. asiatica	Seed	160.7µg/g	105.1 µg/g	211.8 µg/g	HPLC	[60]
P. lanceolata	Leaf	3.5%	0.6%	4.1%	GC-FID	[39]
P. major	Leaf	0.77%	n.d	0.77%	GC-FID	[39]
P. lanceolata	Leaf	0.68%	0.89%	n.d	CE-MEKC	[61]
P. altissima	Leaf	0.55%	0.66%	n.d	CE-MEKC	[61]
P. atrata	Air-dried	9.28%	n.d	n.d	HPLC	[62]

Plant Species	Plant Part	Content in Dry Weight (AU)	Content in Dry Weight (CA)	Content in Dry Weight (IG)	Method	Reference
P. bellardii	Air-dried	0.54%	n.d	n.d	HPLC	[62]
P. coronopus	Air-dried	0.29%	n.d	n.d	HPLC	[62]
P. holosteum	Air-dried	5.44%	n.d	n.d	HPLC	[62]
P. lanceolata	Air-dried	7.50%	n.d	n.d	HPLC	[62]
P. reniformis	Air-dried	1.03%	n.d	n.d	HPLC	[62]
P. schwarzenbergiana	Air-dried	2.08%	n.d	n.d	HPLC	[62]
P. atrata	Air-dried	8.76%	n.d	n.d	HPTLC	[62]
P. bellardii	Air-dried	0.59%	n.d	n.d	HPTLC	[62]
P. coronopus	Air-dried	0.22%	n.d	n.d	HPTLC	[62]
P. holosteum	Air-dried	5.63%	n.d	n.d	HPTLC	[62]
P. lanceolata	Air-dried	7.13%	n.d	n.d	HPTLC	[62]
P. reniformis	Air-dried	0.84%	n.d	n.d	HPTLC	[62]
P. schwarzenbergiana	Air-dried	1.76%	n.d	n.d	HPTLC	[62]
P. atrata	Air-dried	9.33%	n.d	n.d	LC-ESI- MS	[62]
P. bellardii	Air-dried	0.68%	n.d	n.d	LC-ESI- MS	[62]
P. coronopus	Air-dried	0.30%	n.d	n.d	LC-ESI- MS	[62]
P. holosteum	Air-dried	5.33%	n.d	n.d	LC-ESI- MS	[62]
P. lanceolata	Air-dried	8.00%	n.d	n.d	LC-ESI- MS	[62]
P. reniformis	Air-dried	1.05%	n.d	n.d	LC-ESI- MS	[62]
P. schwarzenbergiana	Air-dried	2.68%	n.d	n.d	LC-ESI- MS	[62]
P. lanceolata	Leaf	n.d	n.d	1.28%-7.35%	HPLC	[51]
p. major	Leaf	0.44% to 1.72%,	n.d	n.d	RP-HPLC	[63]
P. lanceolata	Leaf	1.36%-1.72%	0.79%-1.37%	n.d	HPLC	[64]
P. lanceolata	Leaf	0.60 mg/mL	0.47 mg/mL	n.d	CE-MEKC	[65]
P. asiatica	Leaf	1.40 mg/mL	n.d	n.d	CE-MEKC	[65]
P. major	Leaf	n.d	n.d	n.d	CE-MEKC	[65]
P. lanceolata	Callus	n.d	n.d	n.d	CE-MEKC	[65]
P. lanceolata	Foliar	n.d	n.d	19.2 mg/g	HPLC	[66]
P. lanceolata	Root	n.d	n.d	15.8 mg/g	HPLC	[66]
P. lanceolata	Herbage	0.4-6.9 mg/g	0.01-0.09 mg/g	n.d	HPLC	[52]
P. lanceolata L. cv. 'Tonic'	Herbage	0.35-6.9 mg/g	0.01-0.09 mg/g	n.d	HPLC	[52]
P. lanceolata	Seed	0.02-0.23 mg/g	n.d	n.d	HPLC	[67]

Plant Species	Plant Part	Content in Dry Weight (AU)	Content in Dry Weight (CA)	Content in Dry Weight (IG)	Method	Reference
P. lanceolata	Leaf	2.34%	1.21%	n.d	LC-MS	[68]
P. lanceolata	Leaf	10.6 mg/g	1.7 mg/g	n.d	HPLC	[69]
P. lanceolata	Leaf	0.24%	n.d	n.d	TLC	[70]
P. lanceolata	Herbage	0.56-5.85 mg/g	0.003-0.39 mg/g	n.d	HPLC	[69]
P. altissima	Leaf	24.81mg/g	37.14 mg/g	n.d	HPLC	[71]
P. argentea	Leaf	39.92 mg/g	7.68 mg/g	n.d	HPLC	[71]
P. holosteum	Leaf	20.70 mg/g	n.d	n.d	HPLC	[71]
P. lanceolata	Leaf	14.36 mg/g	13.45 mg/g	n.d	HPLC	[71]
P. major	Leaf	8.99 mg/g	n.d	n.d	HPLC	[71]
P. media	Leaf	44.27 mg/g	n.d	n.d	HPLC	[71]

PBR

Mean herbage aucubin and catalpol concentration of plantain harvested in different years, different growing seasons (spring, summer, or autumn), or after establishment at different inputs harvested and various parts of the plant.

Abbreviations: n.d: not determined; AU: aucubin; CA: catalpol; IG: iridoid glycoside; GC: gas chromatography; GC-FID: gas chromatography-flame ionization detector; GC-MS: gas chromatography-mass spectrometer; MEKC: micellar electrokinetic chromatography; HPLC: high-performance liquid chromatography; LC: liquid chromatography; CE-MEKC: capillary electrophoresis- micellar electrokinetic chromatography; LC-ESI- MS: liquid chromatography-electrospray ionization tandem mass spectrometric; RP-HPLC: reverse-phase- high-performance liquid chromatography; TLC: thin-layer chromatography

for catalpol, was low (1%–7%) compared to Bowers et al., Stamp and Bowers, and Marak et al. studies [43, 46, 49, 50]. Remarkably, in the two works in which laboratory-reared plants were applied, catalpol contents were exceptionally low (0%–0.6%) [37, 38]. Consequently, Iridoid glycosides contents were lower in the greenhouse-grown plants (0.83±0.09 to 6.41±1.02 mg/g) than in field-grown plants (0.83±0.09% to 6.41±1.02%) [51]. The aucubin content in plants harvested from the field was more than 1.6%–2.7% in Bowers, 0.5%–5% in Darrow and Bowers, and 0.6%–2.2% in Nieminen et al. [3, 33, 42]. Catalpol concentrations were comparable in these studies 0.4%–3.6% in Bowers [3], 0.2%–2.2% in Darrow and Bowers [33], and 0.7%–2.0% in Nieminen et al. [42].

Generally, the aucubin amounts of leaves increased throughout the growing season, ranging from 0.5% to 4%-5% dry matter from July to September and October, respectively. Likewise, the catalpol content of leaves was calculated to be lower in July and increase throughout the season until October. However, it revealed that the catalpol content in the leaves was about half that of aucubin, ranging from about 0.25% dry matter to about 2.5% dry matter. In the reproductive stalks, the aucubin and catalpol levels tended to increase gradually during the early part of the season and decrease sharply between September and October. The total iridoid glycoside content in the reproductive stalks was slightly lower than in the leaves: 1%-5% and 1%-7%, respectively. While the leaves had higher levels of aucubin than catalpol, the reproductive tissues had more contents of catalpol than aucubin [33].

In another investigation, two cultivars of *P. lanceolata* L., i.e., Ceres Tonic and Grasslands Lancelot, were seeded in spring. The difference in aucubin and catalpol levels in the leaves during the growing season and by drying post-harvesting were quantitatively evaluated using high-performance liquid chromatography (HPLC). The content of catalpol was relatively low (between 1% and 2% of dry matter) throughout the growing season, and there was no obvious seasonal change. From spring to midfall, the aucubin content gradually increased from 1.0% to 2.7% in Ceres Tonic and from 2.1% to 4.8% in Grasslands Lancelot [34].

Navarrete et al. reported the level of catalpol and aucubin in plantain (cv. Ceres Tonic) during two successive growing seasons (2011–2012 and 2012–2013) [52]. There was almost no concentration of catalpol in plantain (cv. Ceres Tonic), but aucubin concentrations increased during the growing season. The content of aucubin in-

Table 2. The iridoid glycoside (IG) contents (aucubin [AU] and catalpol [CA]) by gas-chromatography (GC)

Species / Extract	Method	IG	IG (min)	Column (mm, mm, µm)	Carrier Gas (mL/min)	Oven Conditions / Rate °C/min)	Split Mode / Split Ratio	GC analyses / Reagent	Internal Standard	Reference
P. lanceolata leaf / 95% MeOH- aqueous layer	GC, GC-FID	AU, CA	7.90 8.92	DB-1 column (30, 0.32, 0.1)	Helium (1.44)	260-325 / 20	275°C / 14:1	Trimethylsilyl derivatives	Phenyl-/β-D-glucose	[25, 30, 53]
P. lanceolata: leaf / MeOH- aqueous layer	GC-FID	AU, CA	7.50 8.49	DB-l capillary (30, 0.32, 0.10)	Helium (1.44)	260-325 / 20	275°C / 14:1	Trimethylsilyl derivatives	Phenyl-β-D-glucose	[79]
P. lanceolata *P. major*: leaf / MeOH	GC-MS (HP6890-HP5973 MSD)	AU, CA		DB-1 column (30, 0.25, 0.25)	Helium (0.8)	260-290 /6.5	275°C	Trimethylsilyl reagent (TMS; Supelco)	Phenyl-β-D-glucopyranoside	[54]
P. lanceolata: Root, leaf, stalk, spike / 95% MeOH -aqueous layer	GC-FID	AU, CA	7.28 8.29	WCCT Fused Silica capillary	Helium (1.44)	260-325 / 20	275°C / 30:1	Silylation reagent	Phenyl-β-D-glucose	[55]
P. lanceolata *P. major* leaf / MeOH	Agilent 7890 GC-FID	AU, CA	9.50 11.25	DB-1 column	Helium	260-320	50:1	Silylation reagent	phenyl-β-glucopyranoside, aucubin, and catalpol (5.02,9.50,11.25)	[39]
P. lanceolata: leaf 80% MeOH	GC-MS	AU, CA	n.d	VF-5 ms column (30, 0.25, 10)	Helium (1.2)	205 / 32 250 / 2.5 300 / 9	225°C/	Silylation agent (1: 4 N-trimethylsilyl imidazole: pyridine;	Phenyl-β-D-glucopyranoside	[80]
P. lanceolata; Shoot and root / 95 % MeOH	7890 GC	AU, CA	n.d	Agilent DB-1 column (30, 0.32, 0.25)				Tri-Sil-Z™	Phenyl-β-D-glucose	[48]
P. lanceolata; Shoot and root / MeOH	Agilent 7890 GC	AU, CA	n.d	A Restek Rxi-1ms (30, 0.25, 0.25)	Helium (1.5)	260-320 / 20	275°C / 30:1	N,O-bis(trimethylsilyl) trifluoroacetamide and pyridine	Phenyl-β-D-glucopyranoside	[81]
P. lanceolata / MeOH	GC-MS	AU, CA	n.d	Zebron ZB-5ms (30, 0.25, 0.25)	Helium (1.2)	280-300 / 6	Split flow 24 mL min 21, splitless time 1 min	TMSI + PYRIDINE, 1:4	Phenyl-β-D-glucopyranoside	[49]
P. lanceolata: leaf / 80% MeOH	GC-MS	AU, CA	n.d	VF-5ms (30, 0.25, 10)	Helium (1)	220-325 / 5	1:20	Methoxyamine hydrochloride dissolved in pyridine / Silylation with N-methyl-N-trimethylsilyl-trifluoroacetamide	Phenyl-β-D-glucose	[82]

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Abbreviations: n.d: not determined; AU: aucubin; CA: catalpol; IG: iridoid glycoside; GC: gas chromatography; GC-FID: gas chromatography-flame ionization detector; GC-MS: gas chromatography-mass spectrometer

creased from 1.78 to 3.80 mg/g dry matter in the first and from 0.44 to 6.87 mg/g dry matter in the second growing season. In late fall, aucubin amounts steadily decreased in Grasslands Lancelot and Ceres Tonic. In this regard, the content of aucubin and catalpol in different parts of *Plantago* species has been assessed by researchers with various methods (Table 1).

Aucubin and Catalpol Preparation for Analysis

Aucubin is soluble in water. However, it spontaneously undergoes oxidation and forms insoluble components in aqueous solutions. It is also soluble in methanol and ethanol but is insoluble in organic solvents, such as benzene, chloroform, ether, and petroleum ether [72]. Iridoid glycosides usually tend to be hydrolyzed and undergo rearrangement under slightly acidic conditions [73]. Therefore, they must be treated and analyzed under strictly alkaline conditions. However, some structures are more unstable and may hydrolyze even under alkaline conditions or upon heating [74]. As aucubin is extracted from plants, it is suggested that aucubin be prepared at a low temperature in a weak acidic condition and dark status to increase its output and stability [75]. In Nieminen et al. study, hot water extraction was applied to separate the compounds, which was reproducible and easy. As well, it avoided the application of organic solvents, which burden the environment [42].

As a result, the dry methanolic extract was dissolved in water and partitioned with ETAC (ethyl acetate); for aucubin, only the aqueous layer gave a positive result in the Trim-Hill test [70]. The test (blue color) indicates the presence of iridoid glycosides.

Various methods for the extraction of aucubin have been developed according to its different chemical and physical properties, including cold maceration and reflux extraction. Special enzymes and ultrasound techniques to destroy the plant cell wall have been recommended to increase the permeability of active substances through the cell wall. Extraction by ultrasonic and enzymolysis help isolate aucubin. Also, the microwave extraction method has been used to extract aucubin from *Eucommia ulmoides*. Li et al. investigated the efficiency of the supercritical CO_2 and Soxhlet extraction methods for aucubin from *Eucommia ulmoides* Oliv seeds. The findings showed that supercritical CO_2 extraction provides a higher yield and lower extraction cost [75].

Analytical Methods and Techniques for Determination of Aucubin and Catalpol

Bioactive components are determined by expensive analytical methods that require chemicals, time, high competence, and know-how [76].

Commonly, iridoid glycosides (IG) are detected with chromatographic techniques, i.e., gas chromatography (GC), liquid chromatography (LC), or thin-layer chromatography (TLC) [42]. The IG contents (aucubin and catalpol) were analyzed by GC using previously described methods [26, 31, 37, 40, 77-79]. Some of the studies performed by GC analysis are listed in Table 2.

Flash chromatography was applied to separate catalpol from aucubin [83]. However, the TLC method was the first option for isolating aucubin and catalpol from the plant extracts [84-86]. Taskova et al. [87] used TLC to investigate iridoids from 44 Bulgarian collections selected from 14 species of *Plantago*, of which 14 compounds were determined using spectral methods [47]. The aqueous fraction was found to be rich in iridoids by the TLC technique [88]. In this regard, several studies applied analytical methods of high-performance thin-layer chromatography (HPTLC) and TLC for detecting aucubin and catalpol, summarized in Table 3.

Derivatization with other compounds is necessary for the visualization of the spot. So, reagents were used for post-derivatization of aucubin and catalpol, such as 10% alcoholic H_2SO_4, which burns the glucose molecule in the aucubin giving colored spots, and 10% anisaldehyde which gives a colored spot for aucubin. These reagents are applied by dipping TLC plates in the reagent solutions or by spraying the solutions on the TLC plates [70]. An ethanolic acidic solution of vanillin (1 mL H_2SO_4 conc., 3 g vanillin, 100 mL EtOH) is used to visualize iridoid glycosides as colored spots.

Rønsted et al. identified the isolated compounds by nuclear magnetic resonance spectroscopy [15, 22]. Their findings showed that the distribution pattern of the iridoids in 34 species of *Plantago* has a good correlation with the classification of Rahn [89]. The qualitative studies of *Plantago atrata*, *Plantago bellardii*, *Plantago coronopus*, *Plantago holosteum*, and *Plantago reniformis* have been reported [15, 87]. In some studies, aucubin and catalpol have been isolated from the genus *Plantago* (Table 4).

Table 3. Analytical methods by high-performance thin-layer chromatography (HPTLC) and thin-layer chromatography (TLC)

Method	Extraction / Species	Plate Type	Mobile Phase	Detection (nm) / Reagent	Rf	IG	Reference
TLC	MeOH- washed aqueous and $CHCl_3$ / *P. lanceolata*. *P. major*, leaf)	RP18, F254s,	Acetonitrile: MeOH:H_2O (6:2:17)	n.d / Ethanolic acidic solution of vanillin	n.d	AU CA	[54]
TLC- densitometry	MeOH-different fraction / 44 *Plantago* samples	Neutral alumina 60F254 Type	BuOH:MeOH:H_2O (70:5:10)	450/ Sulfuryl chloride	n.d	AU CA	[87]
Preparative TLC	MeOH-partitioning the aqueous layer with ETAC / *P. lanceolata* leaf	Silica gel GF 254 (20 * 20 cm, 0.5 mm)	n-BuOH: DW (9:1) n- -BuOH: conc. AcOH: DW 4:1:5 IsoPrOH: DW 6:4	n.d / 10% Alcoholic H_2SO_4 and 10% Anisaldehyde	0.55 0.36 0.78	AU	[70]
HPTLC	50% EtOH / *P. lanceolata* Air-dried	Silica gel 60 (20 * 10 cm, 0.25 mm)	ETAC: H_2O: formic acid-acetic acid (90:20:15:15)	520 / anisaldehyde-sulfuric acid	n.d	AU	[62]
HPTLC	MeOH - partitioning the aqueous layer with ETAC / *P. lanceolata* leaf	60 GF254 (10*10 cm, 0.2 mm)	CHCl3:MeOH:0.25M trifluoroacetic acid in ammonia (7:4:1)	n.d / 10% Alcoholic H_2SO_4	n.d	AU	[70]
HPTLC	50% EtOH / Five varieties of *P. lanceolata* leaf	Silica plate	$CHCl_3$ (35 mL):MeOH (20mL):5 mL of Ammonia (1 mL of trifluoroacetic acid in 50 mL of ammonia at 25%)	450 / 10% Sulfuric acid in Methanol	n.d	AU CA	[76]

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Note: MeOH: methanol; $CHCl_3$: chloroform; ETAC: ethyl acetate; EtOH: ethanol; BuOH, butanol

Abbreviations: n.d: not determined; AU: aucubin; CA: catalpol; IG: iridoid glycoside; HPLC: high-performance liquid chromatography; TLC: thin-layer chromatography

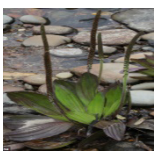
To quality control herbal samples containing iridoids, it is possible to quantify the levels of various iridoids in a mixed status by a simple method using liquid chromatography equipped with an ultraviolet detector (LC-UV) [60]. Usually, relatively large amounts of weak acids, such as phosphoric acid and acetic acid, are added to the mobile phase to prevent interference and stabilization of tautomeric rearrangements. In this way, a good separation of different iridoids can be achieved [114]. Kim et al. developed a simple LC-UV procedure to overcome such unfavorable interference by adding a small amount of trifluoroacetic acid (TFA) to the mobile phase with aqueous acetonitrile. They could simultaneously determine a small mixture of catalpol and aucubin in the aqueous extract of leaves, seeds, and roasted seeds of *P. asiatica* [60]. Aucubin and catalpol in the *P. lanceolata* extract were evaluated using liquid chromatography-mass spectrometry (LC-MS) [68, 70]. HPLC and HPTLC are frequently used to identify bioactive in samples in variety selection programs or to control new varieties [59, 115]. However, several methods can be applied to detect aucubin levels in *Plantago* extracts; the most common ones are HPLC [34, 116] and HPTLC [117, 118] methods. According to this, an attempt was also made for the quantitative analysis of aucubin in 7 *Plantago* species using HPLC and HPTLC by Janković et al. [62]. Other researchers also determined these compounds in *Plantago* species (Table 5).

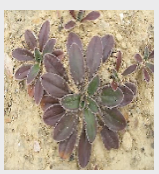
One of the conditions of HPLC is the absorption spectrum. The absorption spectra of aucubin (220, 255, 290 nm) are used for detecting aucubin extracted from plants [124].

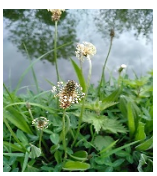
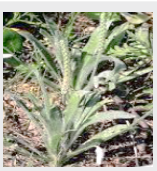
Capillary electrophoresis (CE) is a valuable tool for medicinal plant quality handling, screening, and analysis [65]. All main bioactive secondary metabolite groups can be assayed by one of the CE techniques; MEKC (capillary zone electrophoresis [CZE], Micellar electrokinetic chromatography [MEKC], microemulsion electrokinetic chromatography [MEEKC], and nonaqueous CE [NACE]) [125]. The analysis of iridoids by the CE method has already been published [2, 126, 127]. CE methods detect metabolites such as iridoid glycosides in *Plantago* species [2].

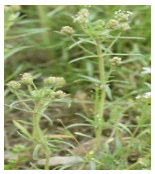
The CE and micellar electrokinetic capillary chromatography (MECC) methods were also used for evaluating neutral compounds like aucubin and catalpol [47, 128]. CE method has been confirmed to be suitable for the quantitative determination of aucubin and catalpol from aqueous extracts of leaf parts of *P. lanceolata*, *P. major*, *P. asiatica*, and *P. lanceolata* callus [65]. The content of aucubin and catalpol in *P. lanceolata* was reliably and quickly determined by MECC [42].

Table 4. The list of determination of isolated aucubin (AU) and catalpol (CA) in the genus *Plantago*

***Plantago* Species**	**Origin**	**Compound**	**References**	**Figure**
Plantago afra (*P. psyllium*)	Mediterranean	AU AU and CA	[15, 67, 85, 87]	
P. amplexicaulis	Mediterranean	AU and CA	[85]	
P. arborescens	Macaronesia	AU	[15, 85]	
P. atrata	Europe to W. Asia	AU, CA, and dihydro aucubin	[15, 62, 90]	
P. australis	Warm Americas	AU	[15]	
P. bellardii	The Mediterranean	AU	[15, 62, 85]	
P. coronopus	The Mediterranean, Europe	AU n.d	[62, 85, 87]	
P. cretica	The Mediterranean	AU	[15]	
P. lundborgii	San Ambrosio Is1	AU and CA	[15]	Not found
P. maritima	Cosmopolite	AU	[15, 85]	

Plantago Species	Origin	Compound	References	Figure
P. nivalis	Spain	CA	[15]	
P. ovata	Spain	AU and CA	[15, 67, 91]	
P. patagonica	Western USA	AU and CA	[15, 53]	
P. raoulii	New Zealand	AU	[15]	
P. reniformis	Southeast Europe	AU	[15, 62]	
P. sempervirens (*P. ynops*)	Southwest Europe	AU	[85]	
P. stauntonii	Amsterdam and St. Paul Islands	AU	[15]	Not Found
P. subspathulata	Madeira	n.d	[15]	
P. subulata (carinata)	The Mediterranean	AU	[15, 85, 92]	
P. uniflora (Littorella uniflora)	Europe	AU and CA	[15, 93]	

Plantago Species	Origin	Compound	References	Figure
P. webbii	Macaronesia	AU	[15, 85]	
P. alpina	Europe	AU	[85]	
P. altissima	Europe	AU and CA	[71, 85, 87, 90, 94]	
P. arenaria	The Mediterranean	n.d AU	[67]	
P. argentea	Southern Europe	AU and CA	[71, 85, 87, 90]	
P. asiatica	South and East Asia	AU AU and CA	[60, 95, 96]	
P. cornuti	Southern Europe	AU and CA AU	[85, 87, 97]	
P. hookeriana	Southern USA	AU and CA	[98]	
P. lagopus	The Mediterranean	AU and CA AU	[85, 87, 99]	

Plantago Species	Origin	Compound	References	Figure
P. lanceolata	Europe	AU and CA	[19, 33, 34, 44, 51, 52, 54, 58, 59, 64, 66, 67, 69, 85, 87, 90, 100-107]	
P. major	Europe	AU AU and CA	[54, 63, 67, 85, 87, 99, 100, 102, 108, 109]	
P. scabra	South Africa	AU	[87]	
P. tenuiflora	Europe	AU	[87]	
P. gentianoides	Southeast Europe, Iran., etc.	AU	[87]	
P. media	Europe	AU	[71, 87, 110, 111]	
P. myosuros	South America	AU	[112]	
P. rhodosperma	Southern USA	AU	[113]	
P. holosteum	South-eastern Europe	AU	[62, 71]	
P. schwarzenbergiana	n.d	AU	[62]	Not found

Abbreviations: n.d: not determined; AU: aucubin; CA: catalpol

Table 5. Analytical methods using high-performance liquid chromatography for quantification of aucubin and catalpol

Species	Extraction	Stationary Phase Column Type	Column Size (L×i.d. mm)	Temp (°C)	Mobile Phase	Flow Rate (mL/min)	Detection (nm)	IG	Rt (min)	Reference
Aerial parts (*P. altissima*, *P. atrata*), root parts (*P. atrata*)	Different fractions	RP-18 column Whatman ODS-3	250×4.6, 10 µm	n.d	MeOH:H_2O (20:80)	3	n.d	AU CA	n.d	[90]
P. lanceolata (Leaf and stalk)	70% MeOH	Analytical column	250×4	n.d	NaOH (1 M):Milli-Q water (10:90%).	1	n.d -PAD	AU CA	2.90 4.35	[101]
P. lanceolata (Leaf and stalk)	70% MeOH	Analytical column	250×4	n.d	NaOH (1 M):Milli-Q water eluents (10: 90%).	1	n.d -PAD	AU CA	2.90 4.35	[101]
P. lanceolata	70% MeOH	Analytical column	250×4	n.d	NaOH (1M) Milli-Q water eluents (10:90%).	1	n.d -PAD	AU CA	2.90 4.35	[41, 56, 57, 119, 120]
P. lanceolata (leaf)	MeOH	YMC-pack ODS-A	100 × 6.0	40	1% ACN in water	1	204 -DAD	AU CA	11.28 5.45	[34]
P. lanceolata (leaf)	70% MeOH	Analytical column	250×4	n.d	NaOH (1M) Milli-Q water eluents (10:90%).	n.d	n.d -PAD	AU CA	3.12 4.57	[50]
P. lanceolata (leaf)	MeOH	YMC–pack ODS-A	250×4.6	40	ACN and water (2:98)	0.8	204	AU	29.8	[59]
P. lanceolata (leaf)	70% MeOH	Analytical column	250×4	n.d	NaOH (1 M): Milli-Q	1	PAD	AU CA	3.25 4.40	[58]
P. lanceolata (leaf)	70% MeOH	Analytical column	250×4	n.d	NaOH (1 M) and Milli-Q water eluents (10:90)	1	PAD	AU CA	3.25 4.40	[58]
P. asiatica (seed)	H_2O	C18	150×4.6;5 µm		3% ACN:0.1% trifluoro-acetic acid-water	n.d	205	AU CA	n.d	[121]
P. atrata, *P. bellardii*, *P. coronopus*, *P. holosteum*, *P. reniformis*, *P. schwarzenbergiana* (aerial)	50% EtOH	Zorbax SB-C18 column	150 x 4.6, 5 µm	25	1% orthophosphoric acid in water: acetonitrile	1	210-DAD	AU	5.4	[62]
P. lanceolata (leaf and root)	70% MeOH	Analytical column	250×2	n.d	NaOH (1 M): ultrapure water eluents (10:90%).	n.d	(PAD)	AU CA	3.5 5.0	[103]
P. lanceolata (leaf)	70% MeOH	analytical column	250×4	n.d	NaOH (1 M): Milli-Q	1	PAD	AU CA	3.25 4.40	[51]
P. lanceolata (Leaf)	70% MeOH	Nucleodur Sphinx RP	250×4.6, 5 µm	25	0.05% trifluoroacetic acid: ACN gradient	1	200-DAD	AU CA	n.d	[104]

Species	Extraction	Stationary Phase Column Type	Column Size (L×i.d. mm)	Temp (°C)	Mobile Phase	Flow Rate (mL/ min)	Detection (nm)	IG	Rt (min)	Reference
P. major (flowers, petiole, root, leaf)	MeOH	Eurospher 100-5 C-18	250 x 4.6	n.d	ACN: H_2O (7:93)	0.6	204	AU	4.9	[63]
P. lanceolata (leaf)	MeOH	Analytical column	125×2.0	40	Ultrapure water: pure MeOH (solved in ultra-pure water)	Gradient (0.250)	204	AU CA	20.7 13.9	[64]
P. lanceolata (leaf, root)	70% MeOH	Nucleodur Sphinx RP-column	250 x 4.6, 5 µm	25	0.05% trifluoroacetic acid: ACN gradient	1	200- DAD	AU CA	n.d	[66]
P. lanceolata	MeOH, EtOH	YMC pack ODS-A	100 × 6.0	40	1% ACN in water	1	240	AU CA	n.d	[19, 52]
P. ovata Forsk; *P. lanceolata*; *P. arenaria*, *P. major*; *P. psyllium* (seed and leaf)	MeOH	Reverse phase chromatography on water spheri-sorb ODS2	250×4.6, 10 µm	25	MeOH:H_2O 0.6(17:83) (v/v)	0.6	210-DAD	AU	6.2	[67]
P. lanceolata (leaf)	99% MeOH	GraceSmart C18	250 ×4.6,5 µm	25	MeOH:H_2O (10:90),	n.d	n.d	AU	n.d	[122]; method adapted [123]
P. lanceolata (shoot)	70% MeOH	CarboPac PA1	250×2	20	100% 0.1 M NaOH	0.25		AU CA	3 5	[44]
P. lanceolata (herb)	MeOH	n.d	n.d	40	1% ACN in water	1	204	n.d	n.d	[69]
P. lanceolata (leaf)	MeOH	Reverse phase SiC18 Biosphere packed stainless steel	250 × 4	n.d	H_2O:ACN with 0.1% acetic acid	1	210-PDA	AU CA	n.d	[105]
P. lanceolata, *P. major*, *P. altissima*, *P. argentea* Chaix, P. holosteum Scop. *P. media* L. (leaf)	80% MeOH	Zorbax SB-C18 column	150 x 4.6, 5 µm	25	1% orthophosphoric acid in water: ACN	1	210	AU CA	n.d	[71] according to [62]
P. lanceolata, *P. major* (Invitro aerial, Invitro root, hairy roots)	MeOH	C8 analytical column	150 x 4.6, 5 µm		1% ACN: 1% orthophos-phoric acid in water	1	204	CA	3.7	[106,107, 109]

Note: MeOH: methanolic; ACN: acetonitrile; EtOH: ethyl acetate

Abbreviations: n.d: not determined; AU: aucubin; CA: catalpol; PAD: Pulsed amperometric detection; DAD: Diode-array detection

Micellar electrokinetic chromatography (MEKC) was used to isolate and analyze aucubin and catalpol in hot water extraction of several *Plantago* species growing in Croatia: *P. altissima* L., *P. argentea* Chaix, *P. coronopus* L., *P. holosteum* Scop. (subsp. *depauperata*, subsp. *holosteum* and subsp. *scopulorum*), *P. lagopus* L., *P. lanceolata* L., and *P. maritima* L. Significant differences were exhibited between the iridoid contents in mentioned species using this method [47].

An authentic and simple CE-MEKC method has been validated and developed to quantitatively determine aucubin and catalpol of *Plantago* species, *P. lanceolata* calli, *P. lanceolata* matrices, *P. altissima*, *P. major*, *P. media*, and *P. maritima* [61]. However, TLC pattern analysis could recognize the species mentioned above in a single run in a system commonly applied for the quality management of *P. lanceolata* leaves. However, *P. altissima* and *P. lanceolata* did not represent enough pattern difference to be fully isolated [61]. Consequently, according to iridoid content, *P. altissima* was chemically indistinguishable from *P. lanceolata* [61].

Also, Gonda et al. evaluated the changes in aucubin and catalpol concentration in dry leaves of *P. lanceolata* subjected to the atmosphere with different relative humidity (0%, 45%, and 75%) by CE-MEKC for 24 weeks [115]. CE-MEKC method showed that it is suitable for aqueous extracts of *P. lanceolata*, *P. major*, *P. asiatica* leaves, and *P. lanceolata* callus culture [65].

In another study, aucubin from *P. lanceolata* was separated and quantified by preparative TLC and then determined by HPTLC fingerprinting. Aucubin that was isolated from the plant material was analyzed by Fourier-transform infrared spectroscopy (FTIR) and LC-MS, respectively [70]. Each TLC-isolated compound exhibited a single spot on the HPTLC plate, which confirms an idea about the purity of the isolated compound. Aucubin accompanied by catalpol were determined using LC-MS in different ionization mode. In continuing, many functional groups were recognized in the TLC-isolated aucubin by FTIR [70]. Nevertheless, aucubin and catalpol in *Plantago* can be quantified by other different methods such as LC-ESI-MS (liquid chromatography-electrospray ionization tandem-mass spectrometry) [62], MPLC [88], LC-TOF-MS (liquid chromatography-time of flight-mass spectrometry) [80], and uHPLC–TOF-MS (ultra-high performance liquid chromatography combined with time-of-flight mass spectrometry) [35]. Various selection programs require rapid and low-cost methods to analyze bioactive components in thousands of samples. FTNIR (Fourier transform near-infrared spectroscopy) supported using chemometric analysis could be a tool to reduce time and costs. A suitable method has been developed to quantify bioactive compounds in plant species [129, 130].

Understanding the mechanism of measuring tools can help us choose the correct and accurate method for measuring compounds (Table 6).

Techniques for determining aucubin and catalpol in pharmacokinetic studies

Although this study has been performed on the methods of determining aucubin and catalpol in *Plantago* species, mentioning some methods for detecting these compounds in pharmacokinetics can also be useful.

A fast and accurate LC-electro spray ionization (ESI)-MS/MS method has been developed and validated to quantify catalpol in rat plasma [133]. Another group of researchers also validated LC-MS/MS method. However, APCI (atmospheric pressure chemical ionization) was replaced by ESI for the determination of catalpol (m/z of 380/165) in rat plasma and cerebrospinal fluid (CSF) [134]. Therefore, LC-MS/MS was validated as a rapid, sensitive, accurate, and robust method and applied for quantifying aucubin, a main bioactive component of *P. asiatica*, in rat plasma [135, 136]. On the other hand, Xue et al. introduced the LC-ESI-MS/MS method for simultaneously determining aucubin and catalpol in rat plasma [137]. Zhang et al. created a specific and sensitive high-performance liquid chromatography coupled with a tandem mass spectrometric (HPLC-MS/MS) method. The method was developed to simultaneously determine geniposidic acid and aucubin in rat plasma after oral administration of Du-Zhong tea extract [138]. In this regard, the simultaneous determination of catalpol, morroniside, loganin, and acteoside in the plasma of normal and chronic kidney disease rats by ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) was developed to investigate the combined medicinal extract of *R. glutinosa* and *Cornus officinalis* Sieb [139]. Hu et al. presented a selective, sensitive, and efficient ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for simultaneously determining 5 active substances, including aucubin in male and female rat plasma after oral administration with *Eucommiae cortex* extract [140]. Lian et al. developed a novel method for aucubin determination in rat serum with type 1 diabetes using UPLC-MS/MS with supramolecular solvent (SUPRAS)-based on dispersive liquid-liquid microextraction. In general, regarding instrumental analysis, UPLC-MS combines the

Table 6. Specifications of analysis methods of iridoid glycosides (aucubin and catalpol)

Method	Problems	Advantages	References
TLC	Poor resolution, aucubin, and catalpol-related compounds were poorly separated, and this could lead to erroneous spot assignment	First choice for the selection of aucubin and catalpol	[47, 84]
HPTLC	Impurities in and additives to solvents can cause irreproducible separations, enhanced UV-background, additional noise	Often applied to detect bioactive samples in the framework of variety selection plans or to control new varieties	[76, 131]
LC-UV	Adding large amounts of 1% weak acids (v/v), change of the detection wavelength to 230–240 nm, a high boiling point of acetic acid (108°C), and non-volatile properties of phosphoric acid can lead to additional disturbances in the separation of iridoids and maintaining the columns due to the retained metallic acid	Adding a small amount of trifluoroacetic acid (TFA) to the mobile phase (aqueous acetonitrile) has great practical effectiveness.	[60, 114, 126]
HPLC	Long run time, failure to resolve iridoids as a separate peak cluster	To characterize bioactive compounds in samples in the framework of variety selection schedules or to control new varieties	[59, 65, 115]
CE and MECC-CE	Poor separation between aucubin and catalpol	High speed and resolution	[47, 128]
CZE	Aucubin and catalpol have very high pKa values (> 12); these molecules are non-ionogenic and not be isolated by CZE	All main bioactive secondary metabolite groups can be evaluated by one of the CE techniques	[47]
MEKC	Separating highly hydrophobic analytes, which is difficult with MEKC	Quick analysis, satisfactory resolution between iridoids and their good isolation, specificity, resolution, acceptable accuracy, and precision. a worthwhile tool in fingerprint analysis	[47, 65, 132]
FT-NIR	Not mentioned	A rapid and low-cost method	[129, 130]

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Abbreviations: TLC: Thin-layer chromatography; LC-UV: liquid chromatography-UV; HPLC: high-performance liquid chromatography; HPTLC: high-performance thin-layer chromatography; CE: capillary electrophoresis; MECC, micellar electrokinetic capillary chromatography; CZE: capillary zone electrophoresis; MEKC: Micellar electrokinetic chromatography; FTNIR: Fourier-transform near-infrared spectroscopy

resolution capability of chromatography with the benefits of speed, specificity, and sensitivity acquired by mass spectrometry (MS). The analytical community has widely admitted this hyphenated technique as a common tool in pharmacokinetic studies [141]. The UHPLC-MS/MS method for aucubin determination exhibited low limits of quantification, good linearity, high extraction recoveries, acceptable accuracy, precision, and stability, in plasma and tissue samples of mice [142].

Biological Activities of Iridoid Glycosides

Iridoids, including secoiridoids, glucosides, esters, and aglycones derivatives, have been reported for medicinal applications [143]. Iridoids are classified as dietary supplements, medicinal foods, and drugs. Iridoids or iridoid-rich plants have established the following biological activities *in vitro*, *in vivo*, and clinical research [143]. Iridoids act as a defense for specified plant species and cause various medicinal effects in animals [144]. A large number of iridoids isolated from plants used in traditional medicine have shown various biological activities, thus validating their popular use all over the world. A broad range of biological activities has been reported for iridoids which include anti-diabetic, anti-cancer, anti-inflammatory, anti-microbial, anti-bacterial, anti-oxidant, anti-spasmodic, hepatoprotective, hypolipidemic, hypoglycemic, cardioprotective, choleretic, neuroprotective, purgative, molluscicidal, immunomodulatory, stimulation of bile acid excretion, hepatic dysfunction, anti-tumor, antidotal activities for noxious *Amanita* mushroom poisoning and anti-viral effects against hepatitis B virus [18, 145-153]. Besides, some iridoids have exhibited anti-protozoal effects against *Plasmodium* spp. [154, 155], *Trypanosoma* spp. [156], and *Leishmania* spp. Indeed, the anti-leishmania activity of iridoids has drawn the scientific community's attention for decades [157, 158].

Pharmaceutical properties of aucubin and catalpol

Most iridoids, including aucubin and catalpol, have been reported to exhibit significant medicinal properties, such as anti-inflammatory [159], anti-cancer and anti-bacterial activities *in vitro* and *in vivo* assay systems. The allure of aucubin and catalpol as cosmetic ingredients in hydrogel formulations was obvious, especially

Table 7. Anti-bacterial activities of iridoids glycoside (aucubin and catalpol)

Compound	Type of Organism/Mechanism	Result	Reference
Aucubin, Aucubigenin	*Staphylococcus aureus*	Aucubigenin, the enzymatic hydrolysis product of aucubin, was an active matter for the antimicrobial activity	[163]
Aucubin, Aucubigenin	*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853) / Disk diffusion method; MIC and MBC values by Micro-Well dilution assay method	Aucubin was not active no against all of the tested bacteria; aucubigenin exhibited considerable antibacterial activity; MIC (0.03-2 mg/mL), MBC (0.06-2 mg/mL)	[164]
Aucubin	*Candida albicans* / MIC, MFC, MBIC; Biofilm by a 2,3-bis (2- methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide reduction assay; cell surface hydrophobicity; hydrophobicity percentage of the cell surface	MICs of aucubin (61 to 244 µg/mL); MFC of aucubin (244 µg/mL), MBIC of aucubin (61 to 244 µg/mL); a potent fungicidal activity	[165]
Aucubin, Catalpol	*Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Candida krusei* (ATCC 6258), *Candida albicans* (ATCC 90028), *Candida parapsilosis* (ATCC 90018) / Broth microdilutions	MICs of aucubin and catalpol (256 to 512 µg/mL) on bacteria and (128 to 256) on fungi.	[166]

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Abbreviations: MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; MBIC: minimum biofilm inhibitory concentration.

when iridoid glycosides were used as lipid nanoparticles [10]. However, several properties have been reported for the compounds aucubin and catalpol, and understanding the mechanism of action of many of these properties requires more extensive studies.

The study on aucubin confirmed that it possesses extensive pharmacological effects, including anti-aging, anti-bacterial (Table 7), anti-cancer, anti-fibrotic, anti-oxidant, anti-osteoporosis, anti-toxic, neurotrophic, neuroprotective, hepatoprotective, osteoprotective, anti-inflammatory properties as like suppressing the inflammatory act produced by the injection of carrageenan, and healing of skin wounds as like local treatment of oral wounds [152, 160-162].

Aucubin established a significant protective effect against ramanitin intoxication in mice [167]. Aucubin also stimulates the elimination of uric acid from tissues to the blood and the repulsion of uric acid from the kidneys [168]. Aucubin acts as a particular inhibitor of NF-kB (nuclear factor NF-kappa B) in mast cells, which may effectively remedy chronic allergic conditions [169].

Aucubin, and to a greater extent catalpol, are deterrents or toxic to generalists [3, 170]. These compounds apply as oviposition and feeding stimulants for some specialist herbivores [43, 58, 171, 172].

Catalpol has been assessed widely for its biological properties *in vitro* and *in vivo* [18]. Although catalpol is more toxic to generalist herbivores than aucubin [3, 43, 170, 173], the research on its properties has shown various pharmacological effects, including sedative, anti-inflammatory, analgesic, anti-tumor, liver protective, anti-microbial (Table 7), purgative, anti-apoptosis actions, and anti-catarrhal for the upper and lower respiratory tract [174-176]. In addition to the listed activities, catalpol has been confirmed as a significant neuroprotective agent against experimental Alzheimer and Parkinson diseases. Catalpol has shown a potential glucose-lowering activity in experimental type 1 and type 2 diabetes mellitus. These activities may be due to improved glucose use in insulin-sensitive tissues and cured mitochondrial biogenesis/function. In addition, catalpol has shown potentially beneficial results in experimental diabetic complications. The significant protective effect of catalpol on cardiovascular was also confirmed. However, in experimental models, catalpol was effective against asthma, hepatotoxicity, and ovarian failure [18].

Anti-cancer properties of aucubin and catalpol

The researchers reported that aucubin inhibited the proliferation of A549 human non-small lung cancer cells by upregulating the expression of p21 and p53 proteins to prevent cell cycle progression in the G0/G1 phase. However, hydrolyzed aucubin showed better anti-leukemia activity than aucubin [177]. Catalpol significantly affected various cancer models, including lung, breast, stomach, and colorectal cancers. One placebo-controlled clinical study confirmed the effect of catalpol

Table 8. Anti-cancer activities of iridoids glycoside (aucubin and catalpol)

Compound	Type of Organism/Mechanism	Result	References
Aucubin	Chronic myelogenous leukemia K562 cells; normal lymphocytes /XTT-based Colorimetric Assay	Inhibits cell proliferation, a weak antileukemic activity	[178]
Catalpol	Human solid tumor cell lines SW1573 (non-small cell lung), HBL-100 (breast), T47D (breast), WiDr (colon), A2780 (ovarian), HeLa (cervix) / *in vitro* antiproliferative activity; in silico calculation; cell cycle studies	Formation of analogs with one to three silyl ether groups - formation of antiproliferative compounds against a panel of six human solid tumor cell lines – the arrest in G0/G1 phase and inhibition of DNA polymerase	[179]
Aucubin	Human non-small cell lung cancer (A549) / XTT assay; ELISA; Immunoblot assay	Blocking cell cycle progression at the G0/G1 phase and inducing apoptosis	[177]
Catalpol	Human solid tumor cell lines (HeLa, HBL-100, A2780, SW1573, WiDr and T-47D) / Chemosensitivity tests; Flow cytometric; Annexin V binding; Immunoblotting,	Growth inhibition, cell cycle arrest in the G1 phase, reduction in cyclin D1 expression. inducing apoptosis	[180]
Catalpol	Breast cancer cells (T47D and MDA-MB-231) / Phytoestrogenic effects, expressions of ERá/ERâ protein and ps2 mRNA	Antiproliferation effects by specific concentrations of catalpol and increase the level of ERâ protein expression in T47D cells by catalpol	[181]
Catalpol	OVCAR-3 cells / MTT; Caspase-3 Activity Assays; Flow Cytometry; Gelatin Zymography Assays of MMP-2; Q-PCR Analysis of miR-200 Expression; Transfection of anti-miR-200and miR-200	Suppressing cellular proliferation, accelerating apoptosis in OVCAR-3 ovarian cancer cells, promoting microRNA-200 expression levels, and restraining MMP-2 signaling	[182]
Aucubin, catalpol, hydrolyzed-aucubin, hydrolyzed-catalpol	Human myeloid leukemia cell lines (K562) / MTT; Cell cycle analysis (PI); Annexin V assay; Western blotting; Immunocytochemistry for STAT3 and STAT5 localization	Catalpol and aucubin were not cytotoxic, down-regulated BCR–ABL phosphorylation, and inhibited constitutive STAT3 activation by H-catalpol and H-aucubin, enhancing the apoptosis induction by hydrolyzed-catalpol	[183]
Catalpol	Human bladder cancer cells T24 / MTT assay and flow cytometry, Western Blot Analysis	Antiproliferation of T24 cells, promoting apoptosis, arrest at G2/M phase, the modulation of PI3K/Akt pathway, inhibits the expression of B cell lymphoma-2 (Bcl-2) family proteins; up-regulated Bcl-2, Bcl-2-associated X protein, and Bcl-2 associated death promoter	[184]
Catalpol	Human breast cancer (MCF-7) / MTT assay, caspase-3 activity assays, flow cytometry, gelatin zymography assays, reverse transcription-quantitative polymerase chain reaction, miR-146a, and anti-miR-146a	Decreasing cell proliferation, promoting apoptosis in MCF-7 cells, reducing MMP-16 activity, and increasing the expression of miR-146a in MCF-7 cells	[185]
Catalpol	CT26 colon cancer cell lines/cytotoxicity assays, matrigel invasion assay; Boyden chamber assay, xenograft tumor transplant model; rat aortic ring assay, Capillary tube formation assay, enzyme-linked immunosorbent assay, Immunohistochemical assay, western blot analyses	Suppressing growth, proliferation, and invasion of colon cancer cells, inhibiting inflammation and tumor angiogenesis, reducing inflammatory factors in colon cancer tumors (IL-1β, IL-6, IL-8, COX-2, and iNOS)	[186]
Catalpol	Human colorectal cancer cells (HCT116) / MTT, caspase3 and caspase9 activities, flow cytometric assays for annexin VFITC/PI, DAPI staining assay, and Western blot analysis, RTqPCR	Antiproliferative effect, downregulation of the PI3K-Akt signaling pathway; Inducing apoptosis of HCT-116 cancer cells; increasing caspase-3 and caspase-9 activities; and upregulation of microRNA-200 expression	[187]
Catalpol	Human osteosarcoma cancer cell lines (MG63 and U2OS), a non-tumor cell line of hFOB1.19 / MTT assay, flow cytometry analysis, wound healing assays, Migration analysis, Western blot analysis, RT-qPCR, ROS assessment, Xenograft mice model, Immunohistochemistry (IHC) analysis	Suppressing migration of osteosarcoma; reducing KRAS expression; apoptosis; improving cleave of caspase-8/-9/-3 and Poly-(ADP-ribose) polymerase; Release of Cyto-c in the cytoplasm and Bax up-regulation, down-regulated of mitochondrial Cyto-c and cellular Bcl-2, reactive oxygen species (ROS) production, ROS scavenger, N-acetylcysteine, imped of catalpol-caused apoptosis, suppression of signal transducer and activator of transcription 3/Janus kinase 2 gene/Src (STAT3/JAK2/Src), *in vivo* reduction of the tumor growth	[188]

Compound	Type of Organism/Mechanism	Result	References
Catalpol	MKN-45 human gastric cancer cells, Athymic nude mice / MTT, Western blot analysis, flow cytometry analysis; Measurement of ROS generation, GSH/GSSG ratio, athymic nude model experiment, immunohistochemical analysis	Decreasing migration and proliferation of cancer cells, suppression of MMP-2, α-SMA, RhoA, ROCK1, and N-cadherin; apoptosis in cancer cells by elevation of apoptosis-associated markers, cleaved caspase-3, and PARP, prevention of tumor growth in xenograft nude mice	[189]
Catalpol	Locally advanced colon adenocarcinoma (patients with surgical resection) / serum levels of carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA); matrix metalloproteinases-2 (MMP-2), matrix metalloproteinases-9 (MMP-9); Patient overall survival (OS), cancer-free survival (CFS), adverse effects, cost of therapy	Reduction of serum levels of CA 19-9, CEA, MMP-2, MMP-9; non-fatal adverse effects; significantly increasing of OS and CFS, favorably cost	[190]
Catalpol	NSCLC human non–small-cell lung cancer cells-A549 cells/cell viability assay, cell migration, and invasion assays, quantitative real-time polymerase chain reaction, Western blot analysis, Zymography assay	Inhibition of TGF-β1-induced cell migration and invasion of A549 cells, Attenuated MMP-2, and MMP-9 expression; Significant attenuation of Smad2/3 activation and NF-κB signaling pathways induced by TGF-β1 in A549 cells	[191]
Catalpol	Hepatocellular carcinoma (HCC) cell lines (Huh7 and HCCLM3) / qRT-PCR, Western blot, MTT assay, colony formation assay, transwell invasion and migration assays, flow cytometry, a luciferase reporter assay, *in vivo* nude mice model for HCC tumor growth assay	Suppressed cell viability, and colony growth, reduced the number of migrating/invading cells, increased apoptosis with an increase in the number of cells in the G0/G1 phase of the cell cycle, up-regulation of miR-22-3p expression and down-regulation of MTA3	[192]
Catalpol	CRC cells / CCK-8 assay, flow cytometry, electron microscopy, western blotting	Reduction of miR-34a expression levels, over-expressed SIRT1 in most of the CRC tissues and all the CRC cell lines, reduction of cell viability, suppressed autophagy, promoted apoptosis, regulation of the expression of SIRT1- inducing miR-34a *in vitro* and *in vivo*	[193]
Catalpol	HCC cells / Reverse transcription-quantitative PCR, western blotting, protein expression levels of miR-140-5p, vimentin, N-Cadherin, E-Cadherin	Antiproliferative activity; invasion and migration; decreasing vimentin and N-cadherin expression; increasing E-cadherin and miR-140-5p expression, inhibition of morphological changes in the epithelial-mesenchymal transformation of cells induced by TGF-β1	[194]

on colorectal cancer, which seems to have an anti-cancer impact due to the reduction of inflammation, apoptosis, angiogenesis, and cell cycle arrest [18].

The researchers' findings show that aucubin and catalpol are known as potential cancer treatments due to their ability to prevent cancer progression and metastasis and induce the death of cancer cells. Therefore, in the present study, some of the properties of these compounds were briefly described. However, anti-cancer properties have been reported in detail (Table 8).

Conclusion

The iridoid patterns exhibited a significant correlation with morphological and other chemical specifications of the representatives of the genus *Plantago*. Aucubin has extensively been detected in wild plants and especially in *P. asiatica*. It was reported that aucubin has only been extracted from plants. However, pure products can barely be obtained due to the unstable structure of aucubin. In some studies on *Plantago* species, aucubin was also found to be more frequently exist compared to catalpol, which could be related to the fact that aucubin is a biosynthetic precursor of catalpol. However, the catalpol value was also observed to be more compared to aucubin content in some *Plantago* species (*P. lanceolata*, *P. altissima*, *P. lagopus*, and *P. argentea*) where both iridoids were existence. As a result, leaf age, plant genotype, seasonal changes, and environmental factors affected these variables and influenced the iridoid glycoside concentrations.

The iridoid glycosides, i.e., aucubin and catalpol, are active components with wide pharmacological activities. These compounds have anti-microbial, anti-oxidative, anti-inflammatory, and anti-fungal properties. There-

fore, aucubin and catalpol are compounds with abundant sources, good safety, and various biological effects, which demonstrate high value in pharmaceuticals and deserve further research and development.

Aucubin and catalpol have been identified as biologically active in the *Plantago* species. Furthermore, aucubin and catalpol play many important roles in the medicinal effects of *Plantago* species, including their hepatoprotective, spasmolytic, collagen synthesis promoting effects, pancreas-protective, neuroprotective, anti-atherogenic, and anti-arthritic. These results suggest *Plantago* species and their metabolites may apply to human health beyond their traditional uses.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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