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Localization of bioactive compounds in *Zingiber officinale*: a histochemical analysis of ginger rhizome

Localização dos compostos bioativos em Zingiber officinale: uma análise histoquímica do rizoma de gengibre

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Abstract

Zingiber officinale Roscoe is a rhizomatous plant widely used as an ethnomedicinal agent and food. Although its pharmacological properties are well-studied, histochemical studies are scarce. This study aimed to perform a histochemical analysis of the *Z. officinale* rhizome to characterize the histological localization of its nutritionally and pharmacologically important metabolites. Fresh rhizome sections were prepared for light microscope analysis using histochemical methods. The results revealed anatomical features of the rhizome and identified components of its primary metabolism, such as lipids, proteins, and starch, as well as secondary metabolites like phenolic compounds, terpenoids, tannins, sesquiterpenlactones, and alkaloids. These compounds are active principles responsible for the species' pharmacological properties. The analysis defined the anatomical location and distribution of ginger's bioactive compounds, which are essential for identifying products derived from the species.

Resumo

Zingiber officinale Roscoe é uma planta rizomatosa amplamente utilizada como agente etnomedicinal e alimento. Embora suas propriedades farmacológicas sejam bem estudadas, estudos histoquímicos são escassos. Este estudo teve como objetivo realizar uma análise histoquímica do rizoma de Z. officinale para caracterizar a localização histológica de seus metabólitos de importância nutricional e farmacológica. Seções frescas de rizoma foram preparadas para análise em microscópio óptico utilizando métodos histoquímicos. Os resultados revelaram características anatômicas do rizoma e identificaram componentes de seu metabolismo primário, como lipídios, proteínas e amido, e metabólitos secundários como compostos fenólicos, terpenoides, taninos, lactonas sesquiterpênicas e alcaloides. Esses compostos são princípios ativos responsáveis pelas propriedades farmacológicas da espécie. A análise definiu a localização anatômica e a distribuição dos compostos bioativos do gengibre, essenciais para identificar produtos derivados da espécie.

Introduction

The species *Zingiber officinale* Roscoe (Zingiberaceae) has Asian origin and is used worldwide as an ethnomedicinal agent, as well as food and flavoring (Lima et al., 2014). Popularly known as ginger, it is a rhizomatous, herbaceous, and perennial species, reaching up to 90 cm in height in cultivation (Dhanik et al., 2017). Rhizomes are horizontal, aromatic, branched, thick, and pale yellowish (Kapadia, 2006). Leaves are

linear, sessile, and glabrous, and flowers are yellowish green, arranged in oblong, cylindric spikes and ensheathed in a few scarious, glabrous bracts (Joy et al., 1998).

Ginger is used as a medicinal plant in Traditional Chinese Medicine (TCM) for the treatment and prevention of diseases. Pharmacologically, it has potential effects on the cardiovascular system, including antimicrobial activity, neuroprotective activity, gastroprotective, hepatoprotective, antidiarrheal, antiemetic and anticancer (Gupta; Sharma, 2014; Mukjerjee; Karati, 2022). The phytotherapeutic application of this species is recognized in the List of Herbal Medicines by the Brazilian Health Regulatory Agency – ANVISA (Brazil, 2014a). In addition to its pharmacological uses, this species is also culturally used worldwide as a culinary spice (Mukjerjee; Karati, 2022).

The ethnobotanical value of ginger is directly related to its bioactive compounds, which are found in the rhizome. A rhizome is a type of horizontal underground stem that grows horizontally below or just above the soil surface (Taiz et al., 2017). This structure is formed by different types of specialized tissues. Therefore, microscopic study of these tissues can contribute to the understanding of the histolocalization of these compounds.

Histochemical characterization helps to improve the knowledge about the species, meeting the requirements of regulatory bodies such as ANVISA (Brazil, 2014b), which mandates morphological, microscopic, and chemical characterization for the quality control of plants used as a basis for herbal products (Santos et al., 2021). Furthermore, quality control is also mandated for the use of the species as a spice. Additionally, ginger is also marketed in powder form, which makes it susceptible to fraud, where the original product is mixed with a cheaper adulterant, causing financial loss and health risks (WHO, 1999; ANVISA, 2019; WAHO, 2020). Furthermore, knowing the localization of chemical compounds in the tissues distributed in the ginger rhizome can contribute to guiding deeper studies of such chemical groups in specific histological structures. For example, extraction methods for spectrophotometric, chromatographic, and pharmacology analyses (Yadav et al., 2021).

Thus, histochemistry is a relatively low-cost and straightforward analytical tool, ideal for providing an initial assessment of plant structures. These investigations are valuable for revealing the anatomical location of pharmacological components, allowing for obtaining a histochemical profile of the medicinal plant (Yadav et al., 2021). Although *Z. officinale* is widely used, studies focused on histochemistry of the rhizome are still scarce. The existing studies have focused on other aspects of ginger structure and composition, such as tissue organization (Eltahir et al., 2018; Liu et al., 2020); vascularization pattern (Santos; Silva 1998); microscopic characterization of powdered ginger (Gavrilova et al., 2022), or the application of few histochemical reagents (Indriyani, 2017).

Therefore, our approach aimed to conduct a detailed histochemical examination of the rhizome. The correlation between the identification of metabolites and their location within the rhizome tissues allowed to provide a comprehensive description of *Z. officinale* anatomical organization in terms of bioactive compounds storage, which provides the characterization for pharmacobotanical application.

Materials and Methods

Plant Material

Ginger rhizomes (*Zingiber officinale* Roscoe) were purchased at a supermarket in Leblon, Rio de Janeiro (RJ), Brazil, since the study is part of an ethnopharmacobotany project focused on investigating plants that the population has access through commerce.

The ginger supplier for the supermarket was Grupo MNS, from which the planting data were obtained. Planting was carried out in the municipality of Tapiraí - SP, in a subtropical climate with a mean temperature of 24.8 °C and mean annual rainfall of 1429 mm. The cultivation was carried out in an open area, exposed to the sun, with irrigation twice a week and NPK fertilization in the proportions of 4:14:8 in the preparation of the planting; 12:6:12 during plant development and 14:7:28 in the pre-harvest stage.

The reference material was deposited at the Herbarium of Universidade do Estado do Rio de Janeiro (HRJ), under the registration number HRJ:13402. The project containing the research with *Z. officinale* is registered in the National Genetic Heritage and Associated Traditional Knowledge Management System (SisGen-MMA) under the registration number ABE0E83. The analyzes were carried out in the Biology Laboratory of the Instituto Federal de Educação, Ciência e Tecnologia Rio de Janeiro, Campus Maracanã.

Histochemical Analysis

The samples of *Z. officinale* fresh rhizome were cut by hand in transverse and longitudinal sections, with the aid of a razor blade. Then, the selected sections were submitted to histochemical tests, mounted on slides, analyzed under Nikon Eclipse E200 light microscope and photographed. The following reagents were used in the tests: safrablau (Kraus and Arduin, 1997) stains cellulosic structures in blue and lignified cell walls in red; lugol (Johansen, 1940) stains starch granules in purple; Ruthenium red stains pectins and mucilages in pink (Johansen, 1940); Sudan III (Pearse, 1972) stains total lipids red; Nile blue sulfate stains acid lipids blue and neutral lipids red (Cain, 1947); Coomassie brilliant blue stains proteins in blue (Fisher, 1968); Total phenolic compounds are stained by iron (III) chloride in brown to black (Gabe, 1968) and by potassium dichromate in reddish brown potassium dichromate(Johansen, 1940). Sesquiterpene lactones were stained reddish-brown using concentrated sulfuric acid, according to the method of Geissman; Griffin (1971), as modified by Higuchi (2007) with the addition of 50% glycerin to slow down tissue degradation. Ellram's reagent stains total alkaloids in orange to reddish brown (Furr; Mahlberg, 1981); hydrochloric vanillin stains tannins in red to brown (Mace; Howell, 1974), and 2,4-dinitrophenylhydrazine stains carbonyl group terpenoids in orange red (Ganter; Jollés, 1970).

Rhizome fragments were also macerated using a mortar and pestle to obtain an extract (Soares et al., 2017). After grinding the rhizome fragments, the obtained extract was analyzed through microscopic observation, and the size of the starch granules was measured by a micrometric ruler, using Neubauer Chamber. Ginger extract was tested with 2,4-dinitrophenylhydrazine and then mounted on histological slides for light microscope analysis, to observe the chemical reaction of this reagent both in the tissues and in the ginger extract.

Unstained mounted sections (blank sections) were also analyzed to perform a control test for histochemical investigations. The criteria set by Higuchi (2007) were used to indicate the presence of marked compounds: (+) for presence in isolated cells; (++) for presence in groups of cells; (+++) for presence in the entire structure and (-) for the absence of the respective metabolites.

Results and Discussion

Ginger rhizome consists of the epidermis (ep); cortex (c) and stele (s) or central cylinder (Fig. 1a). The epidermis consists of rectangular cells with suberized walls (Fig. 1b). The cortex is formed by hypodermis (hy), parenchymatous ground tissue and endodermis (en) (Fig. 1a). The hypodermis (hy) consists of multiple layers of thin-walled rectangular cells (Fig. 1b). Cortex ground tissue consists of oval-shaped parenchyma cells, among which there are scattered vascular bundles and oil cells (Figs. 1a, c, e). The endodermis corresponds to the cortex inner layer, which is formed by compactly arranged cells (Fig. 1c). The pericycle (pe) is situated internally to and contiguous with the endodermis. It corresponds to the stele outer layer, and is composed of small and compactly arranged cells (Fig. 1c). These observations agree with the anatomical analysis of Eltahir et al. (2018) and Liu et al. (2020).

The vascular tissue system is formed by the stele and the vascular bundles located in the cortex. The vascular bundles are dispersed in the cortex and medulla (atactostele). The distribution of vascular bundles shows a complex organization, where collateral vascular bundles are highlighted by safrablau (Figs. 1d-f). Vascular bundles occur in distinct orientations in the transition zone from the stele to the cortex (Fig 1e). Santos and Silva (1998) associated the change in vessel orientation with the existence of direct contact between the central vascular cylinder and the cortical vascular bundles.

Figure 1. Transverse sections showing *Zingiber officinale* rhizome anatomy. A – Epidermis, cortex, and stele; B – Epidermis followed by the hypodermis; C – Inner cortex showing the endodermis and part of the stele showing the pericycle and vascular bundles. Note the high concentration of starch granules near the pericycle; D – Parenchyma cells and vascular structure from the inner cortex to the inner stele, showing the pith. E – Transition zone from the cortex to the stele where vascular bundles occur in distinct orientations. The curved arrow indicates vessel elements leaning from transverse to longitudinal orientation. F – Collateral vascular bundle. Ep = Epidermis; Hy = hypodermis; C = Cortex; en = endodermis; S = Stele; pe = pericycle; oi = oil cells; vb = vascular bundles; st = starch; xy = xylem; ph = phloem; fi = fiber. Scale bars: a, d – 500 µm; e – 200 µm; c – 100 µm; b, f – 50 µm.

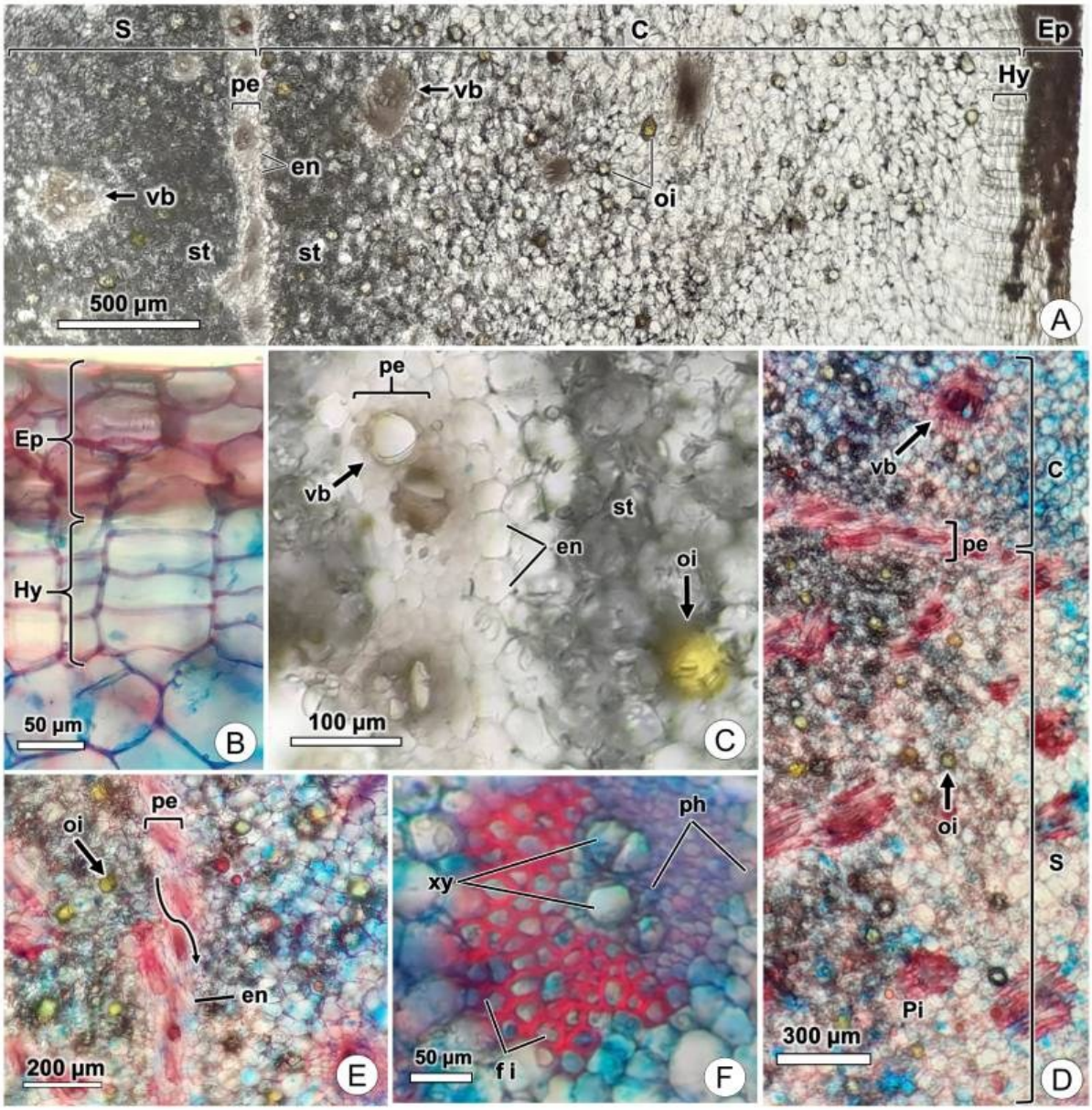


Table 1 – Results of the histochemical tests on *Zingiber officinale* rhizome. (-) Negative reaction result; (+) Positive reaction result in isolated cells; (++) Positive reaction result in groups of cells; (+++) Positive reaction result throughout the structure. * Result observed from color reaction and precipitate formation.

Reagent	Compound	Cortex		Stele		Vascular Bundles
		Outer	Inner	Outer	Inner (Pith)	
Lugol	Starch	++	+++	+++	++	-
Ruthenium red	Mucilage	-	-	-	-	-
Coomassie brilliant blue	Total protein	-	-	-	-	-
Sudan III	Total lipids	++	++	++	++	+
Nile blue sulfate	Acid lipids	+	+	+	+	-
	Neutral lipids	++	++	++	++	-
2,4-dinitrophenylhydrazine	Carbonilated terpenoids*	++	++	++	++	++
Concentrated sulfuric acid	Sesquiterpene lactone	++	++	++	++	+
Iron (III) chloride	Total phenolic compunds	++	++	++	++	-
Potassium dichromate	Total phenolic compunds					
Hydrochloric vanillin	Tannins	+	-	-	-	+
Wagner reagent	Total alkaloids	+	-	-	-	++

The stele contains starch granules, oil cells, and vascular bundles (Fig. 1d). Pith is the inner portion of the stele, which is constituted by thin-walled parenchyma cells. The oil cells are scattered throughout the cortex (except in the hypodermis) and the stele parenchyma (Fig. 1a, d, e).

The results of the histochemical tests are described in Table I, which indicates the metabolites localization and distribution in the rhizome.

The safranin in safrablau has the property of staining not only lignified cell walls but also idioblasts containing phenolic compounds (Castro; Demarco 2008; Raman et al. 2018; Novikov; Sup-Novikova, 2021). These stained structures can be observed in Figure 2.A, in the cortex tissue. The Ruthenium red reagent showed that there are no mucilage within ginger cells and marked just the middle lamella in pink (Fig. 2b), which corresponds to a region rich in pectin.

The starch production by the parenchyma cells was evidenced by Lugol (Fig. 2c). The starch granules are oval-shaped (Fig. 2d) and show an average diameter between 14 and 18 micrometers, which is following Oliveira (2019). Starch accumulation is especially observed in the first layers of the vascular cylinder, including the tissues surrounding the endodermis and pericycle (Fig. 1a). Such a pattern was also described in previous studies with *Z. officinale* and other Zingiberaceae species (Martins et al., 2010; Liu et al., 2020).

In the parenchyma tissue, lipid presence was also observed (Fig. 2e), with the differentiation of neutral and acidic lipids evidenced by Nile blue sulfate (Fig. 2f). Neutral lipids may correspond to triacylglycerols and steryl esters, whereas acidic substances may correspond to phospholipids and basophilic components of the cell (Figueiredo et al., 2007; Huang, 2018).

Coomassie Blue was used to mark cellular protein reserves, as already exemplified in research (Ventrela, 2013). However, these cellular protein reserves were not found in our study (Fig. 3a).

About secondary metabolites, phenolic compounds were detected in the parenchyma and vascular bundles (Figs. 3b-d). Gingerols have similar structures, due to the presence of the phenol function, which allows them to react with potassium dichromate and ferric chloride. The 6-gingerol is the most abundant component in fresh ginger and has been shown to have the potential as an antioxidant, anti-inflammatory, antitumor, hepatoprotective, anti-hepatoglycemic, and hypolipidemic agent (Shareef et al., 2016; Ahmed et al., 2021). When ginger rhizome undergoes a heating process, 6-gingerol is converted to 6-shogaol, and therefore, the latter is found in a smaller proportion in the rhizome of fresh ginger (Shareef et al., 2016).

In addition to phenolic compounds, terpenoids with carbonyl groups were also found in the parenchyma, evidenced by 2,4-dinitrophenylhydrazine (Figs. 3e, f). As a result of the chemical reaction of this reagent with aldehyde and ketone groups, an insoluble and crystalline precipitate is formed, resulting in strongly stained dinitrophenylhydrazone molecules (Shriner et al., 1983).

This effect was observed in the cells, a tissue microenvironment, where the formation of these precipitates occurred in several points of the tissue, showing orange and red precipitates (Fig. 3e, 3f). Saturated ketones and aldehydes are usually yellow to light orange, and conjugation of the carbonyl group with a double bond or benzene ring shifts the color to shades of red (Sachin et al., 2012). Other aromatic plants also showed terpenoids with carbonyl groups (ketones and aldehydes) marked by this reagent, as in the studies by Santos et al. (2013), Tiago et al. (2020), Szabo et al. (2022) and Parafiniuk et al (2023).

Examples of carbonylated terpenoids that could be marked by 2,4-dinitrophenylhydrazine are neral and geranial, isomers of the citral molecule that are involved in the composition of the aroma and involved in antitumor effects (Yeh et al., 2014). Gingerols and shogaols also present a carbonyl group in their structure, which also makes them detectable by this reagent, although they are not terpenoids.

The formation of crystalline precipitates was also observed in ginger extract. Thus, this reaction with 2,4-dinitrophenylhydrazine could indicate the potential of this reagent as a low-cost marker for the quality control of ginger. This method could be particularly important in powdered ginger products, where it is not possible to identify fraud macroscopically. We suggest that further analysis be carried out to improve this application. The use of histochemical reagents that could differentiate ginger from adulterants such as chickpeas or wheat flour (Mohiuddin, 2019; Jahanbakhshi et al., 2021) would make the analysis more dynamic and less costly.

Tannins were found in very few cells, in ginger parenchyma, and in the vascular bundles (Figs. 4a-b). This result is by analyses performed on the total phenolic compounds of the species, which report low concentrations of these compounds (Fortes et al., 2015).

Figure 2. Results of *Z. officinale* histochemistry. A – Safrablau: Cortex transverse section showing the shape of the parenchyma cells. The cortex anatomy is characterized by the presence of oil cells, vascular bundles and isolated idioblasts. B – Ruthenium Red: Cortex transverse section showing parenchyma cells with starch granules and oil. Pectin is highlighted in the middle lamella. Mucilage was not observed. C – Lugol: Stele transverse section showing parenchyma cells with starch granules; D - Ginger extract with starch, observed in the Neubauer chamber; E – Sudan III: Cortex longitudinal section showing total lipids scattered in the parenchyma cells; F - Nile blue sulfate: Cortex transverse section showing parenchyma cells with blue droplets, indicating acid lipids, and pink droplets, indicating neutral lipids. oi = oil cells; vb = vascular bundles; st = starch; pc = idioblasts with phenolic compounds; p = pectin; tl = total lipid; al = acid lipid; nl = neutral lipid. Scale bars: a – 200 µm; b-f – 50 µm.

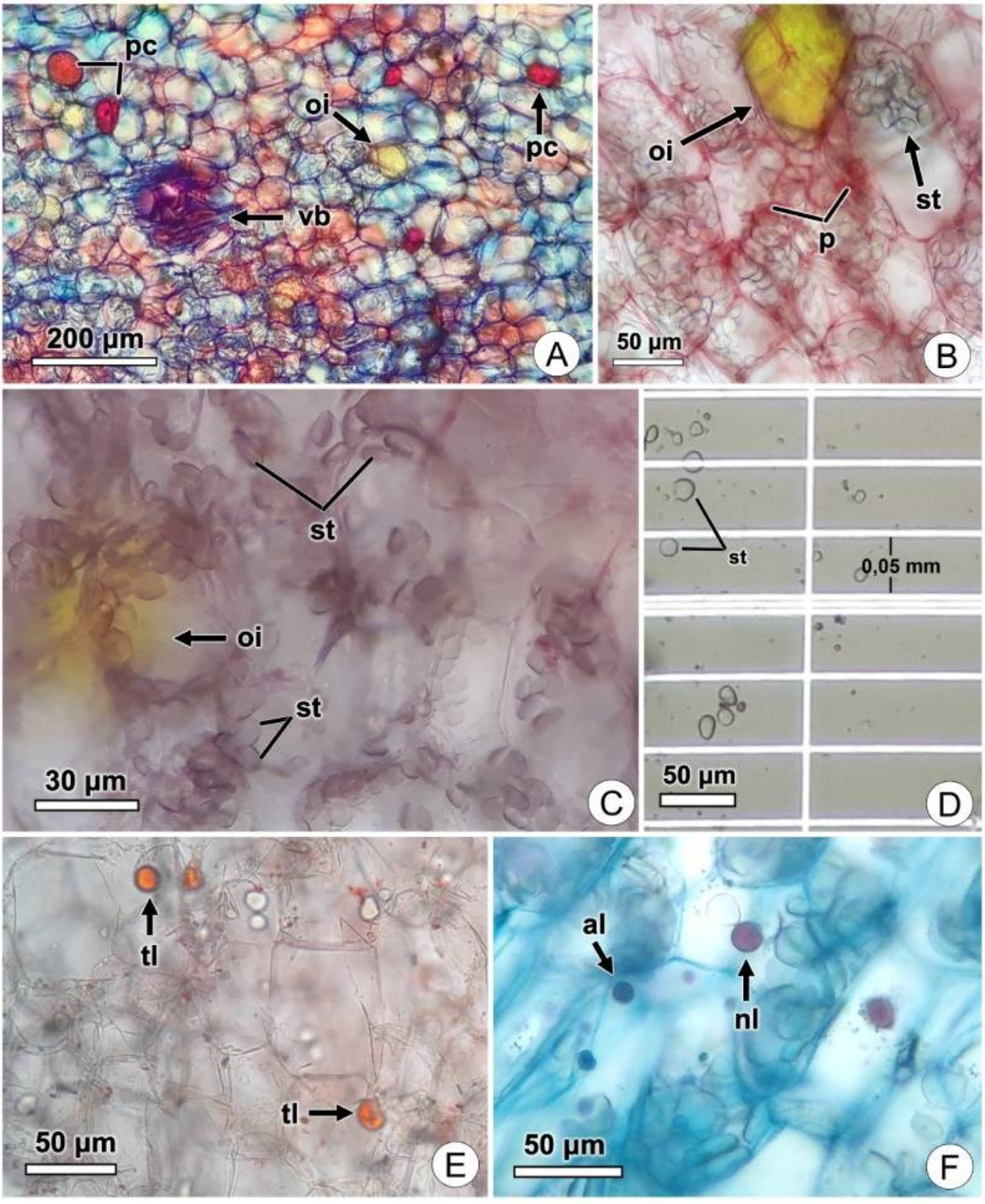


Figure 3. Results of *Z. officinale* histochemistry. A – Coomassie brilliant blue: Stele transverse section with no protein reserves inside the cells; B – Potassium dichromate: Stele longitudinal section showing vessel elements with total phenolic compounds; C – Potassium dichromate: Cortex transverse section showing parenchyma cells with total phenolic compounds; D – Iron (III) chloride: Cortex transverse section showing parenchyma cells with total phenolic compounds; E-F – 2,4-Dinitrophenylhydrazine: Stele longitudinal section with cells showing crystalline precipitates, which mark the presence of terpenoids with a carbonyl group. f - Section detail to highlight the presence of orange and red precipitates. Casparian strips can be observed in the endoderm. pc = phenolic compounds; op = orange precipitate; rp = red precipitate; Cs = Casparian strip. Scale bars: b,e – 150 µm; a, c, d, f – 50 µm.

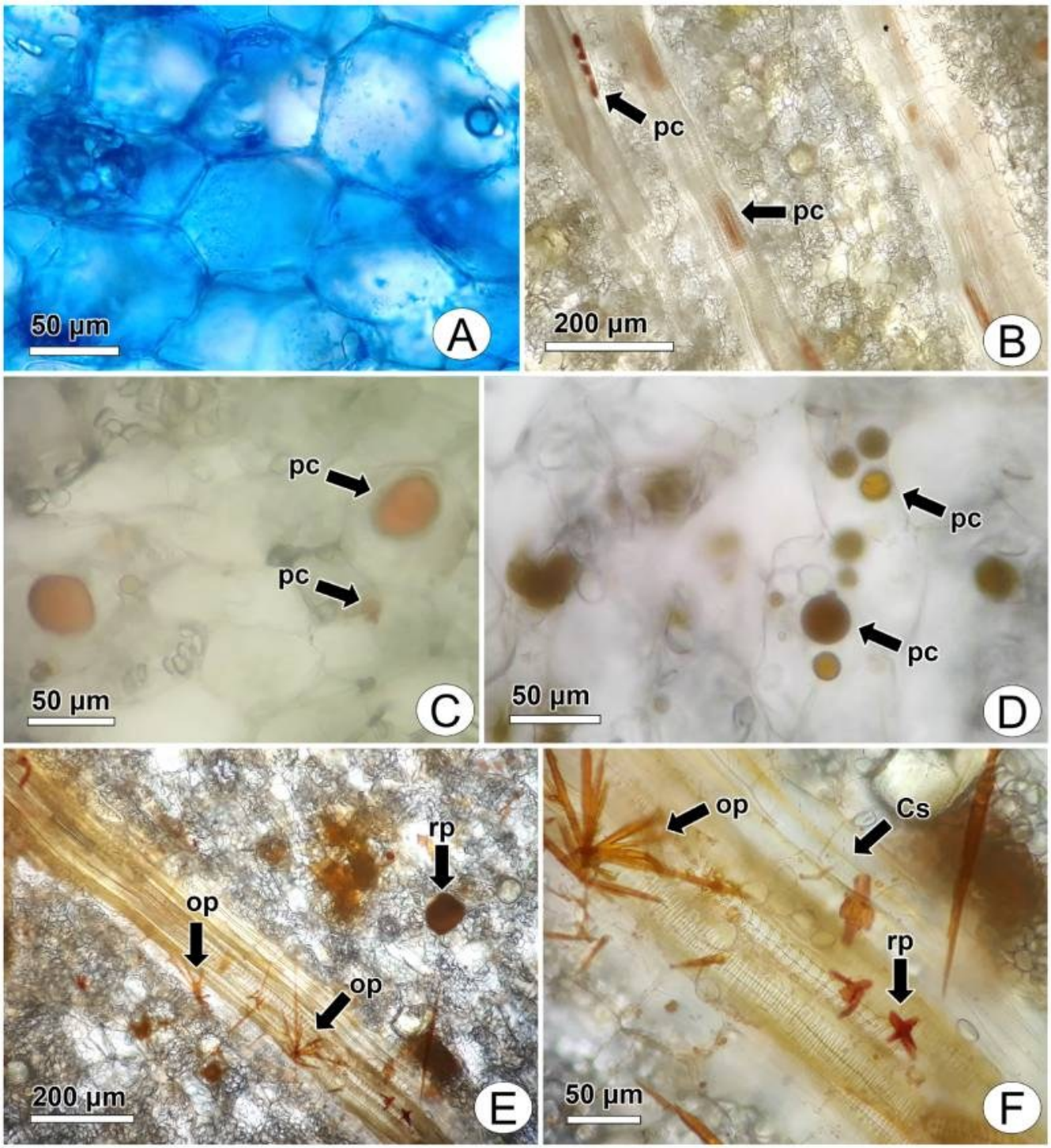
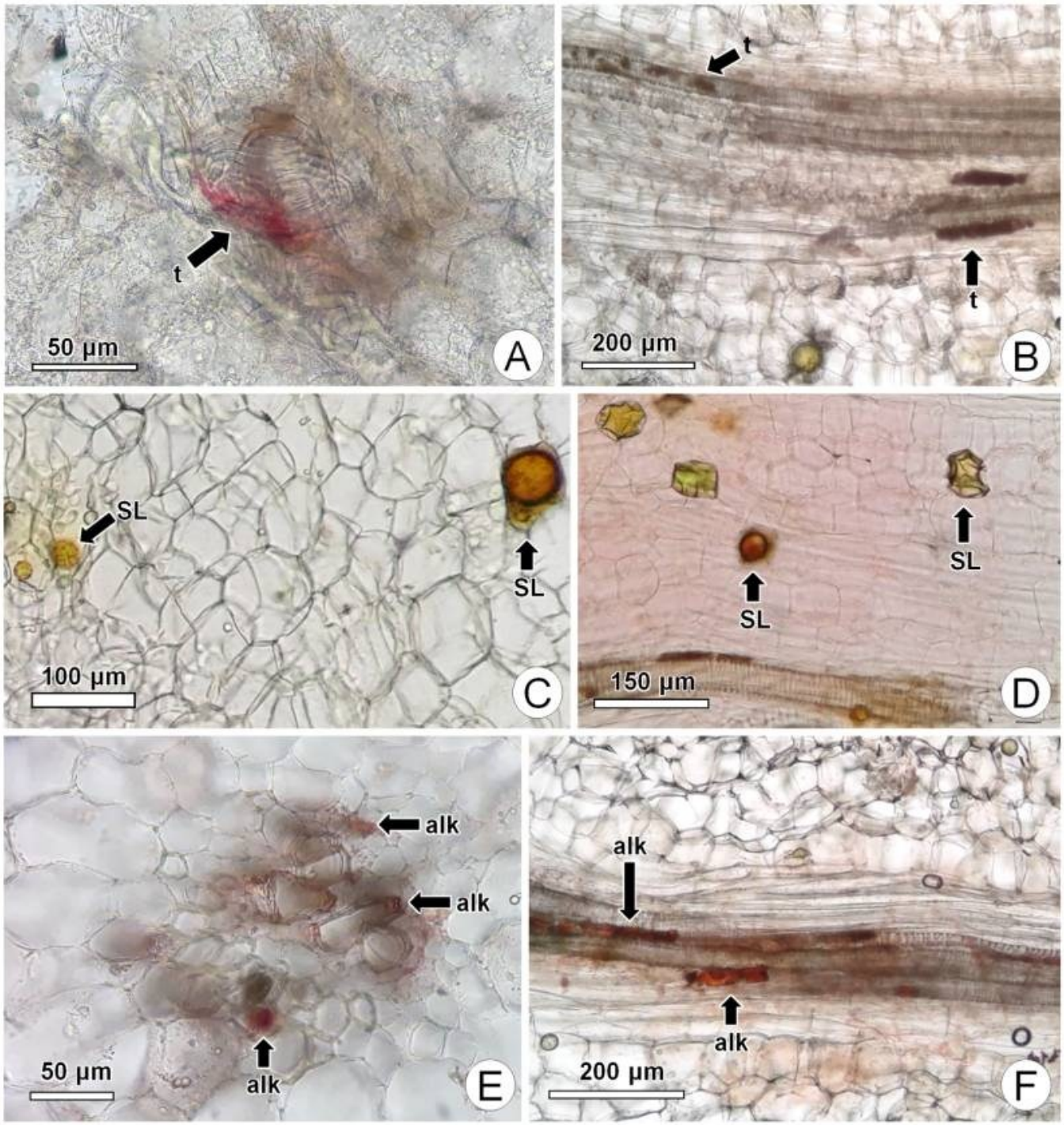


Figure 4. Results of the histochemistry of *Z. officinale* cortex. A-B – Vanillin hydrochloride: Tannins marked on the vessel elements in cross and longitudinal section, respectively; C-D – Sulfuric Acid: Sesquiterpene lactones evidenced in oil droplets of longitudinal parenchyma; E-F – Ellram reagent: Alkaloids marked on the vessel elements in cross-section and longitudinal section, respectively. t = tannin; SL = sesquiterpene lactones; alk = alkaloids. **Scale bars:** f –



The sesquiterpene lactones were evidenced by the sulfuric acid with glycerin in the ginger oil droplets, which turned reddish brown (Figs. 4c-d). Sulfuric acid is also able to detect sesquiterpenes not linked to the main constituents of essential oil (Geng et al., 2012). Sesquiterpene lactones or sesquiterpenlactones are sesquiterpene class molecules containing a lactone ring. These compounds have a great structural variety and consequently, their bioactive properties are very rich, such as antimicrobial, antifungal, antioxidant, anti-inflammatory, and antitumor, for example (Reyes; Suarez, 2015; Parafiniuk et al., 2023).

The reaction with Ellram's reagent, which indicates total alkaloids, caused colorimetric changes in vessel elements and regions close to them (Figs. 4e-f). Alkaloids are associated with therapeutic properties of this plant, for example, acting as a coagulant factor and analgesic, the latter due to the characterization of morphine-type alkaloids (Raaof et al., 2013; Umeh et al., 2013).

Thus, the histochemical characterization supports the identity and quality control of products of plant origin. The analysis performed allowed us to define the anatomical localization and distribution of ginger chemical bioactive compounds, that are related to *Z. officinale* pharmacological and alimentary properties.

Conclusion

Thus, the analysis performed allowed for the definition of the anatomical location and distribution of the bioactive chemical compounds in ginger, which are related to the pharmacological and nutritional properties of *Z. officinale*. The histochemical technique revealed the presence of total phenolic compounds, tannins, terpenoids with carbonyl groups and other carbonyl-containing molecules, sesquiterpene lactones, alkaloids, lipids and starches, located in different tissue regions. The results enhanced the understanding of the distribution of metabolites in the rhizome tissues, facilitating more targeted analyses of such compounds. Additionally, the histochemical characterization helps in identifying and ensuring the quality of ginger marketed for consumption.

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Authorship Contributions

Conceptualization: MIT, MCS. Data curation: LHOC, MIT, MCS. Formal Analysis: LHOC, MIT, MCS. Funding acquisition: MIT. Investigation: LHOC, MIT, MCS. Methodology: LHOC, MIT, MCS. Project administration: MIT, MCS. Resources: MIT. Software: LHOC. Supervision: MIT. Validation: LHOC, MIT, MCS. Visualization: LHOC, MIT, MCS. Writing – original draft: LHOC. Writing – review & editing: LHOC, MIT, MCS.

Conflict of interest

The authors declare that there are no conflicts of interest to report.

Data Availability

The complete set of data analyzed during the current study is presented in the body of the manuscript and a species is deposited in the HRJ Herbarium.

Ethical compliance

Not applicable.

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