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Seasonal changes in azulenes in leaf cells of allelopathic species *Eucalyptus cinerea* studied by spectral methods

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ABSTRACT

Using spectral methods, we studied the condition of leaf surface of allelopathically active silver dollar eucalyptus (*Eucalyptus cinerea)* F. Muell Ex. Benth (fam. Myrtaceae) in various seasons. We evaluated the presence of blue azulene pigments on the surface and inside the cells. Using microspectrophotometry techniques, the dynamics of the number of azulenes on the surface of intact cells according to their absorbance spectra (maxima in the region of 570-630 nm) was seen, confirmed by spectral analysis of ethanol and acetone extracts of these pigments during short 10-min extraction of intact leaves. Prolonged extraction for 24-48 h also indicated the presence of azulenes inside the cells as well. The largest amount of these pigments was found in all extraction samples in the spring season, characteristic of the highest ultraviolet insolation. It is assumed that this was due to the antioxidant protection of azulenes from the damaging effects of formed reactive oxygen species.

Keywords: Absorbance spectra, allelopathy, azulenes, cell surface, *Eucalyptus cinerea*, extracts, microspectrophotometry, pharmacy

INTRODUCTION

Allelopathic plants have also medicinal uses. Among perspective allelopathic and simultaneously medicinal woody species genus *Eucalyptus* is known (2,8). The active matter of the species in biocenosis and medicine is considered as complex of essential oils (28), recently the attention has been paid to azulenes, chemicals found earlier among the oils' content (3). Some natural azulenes were isolated and identified by mass-spectroscopy (7,27). Recently it has been proposed that blue or blueish image of leaves in *Eucalyptus cinerea* may be related to the azulene presence on the surface of the species that made the plant more tolerant to tropospheric ozone and ultra-violet irradiation (18). The blue pigments also can regulate some processes in plants and animals: (i). Growth reactions of plants (2,5,8,14,15), (ii). Plant and animal cell protection against ionizing radiation for pollen (11) and mouse cells (22) or (iii) Restoration of the rabbit skin from burns through anti-inflammatory mechanisms in veterinary and medicine practice (4). However, nobody analyzed seasonal contents of azulenes in plants to determine the possible maximum activity of the pigments that are important both for the maximal allelopathic activity and for the pharmacological collections of the azulene-containing plants. First attempt was been done for herbs grown in temperate climate (25). This study aimed to determine seasonal changes in azulenes in allelopathic plant *E. cinerea* inhabited in subtropic regions.

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MATERIALS AND METHODS

The leaves of *Eucalyptus cinerea* F. Muell Ex. Benth (Family. Myrtaceae) were collected from National Park Dendrarium Sochi, Caucasus from February to August 2024.

Spectral measurements. Absorption (absorbance) of the intact leaves was measured directly on slides using the microspectrophotometer/microspectrofluorimeter MSF-15 (LOMO, St. Petersburg, Russia) (20,21). The position of the maxima in the absorption spectra of intact cell surfaces was determined using Zolotarev method by the reflection spectra differentiation (29). The position of the maxima in the absorption spectra of intact cell surfaces was determined (29). Figure 1 shows a sample of the spectrum. To identify the maxima in 550-670 nm region, it was enough to provide information with positive values (top of graph) and then we used only upside images for the analysis of all other samples.

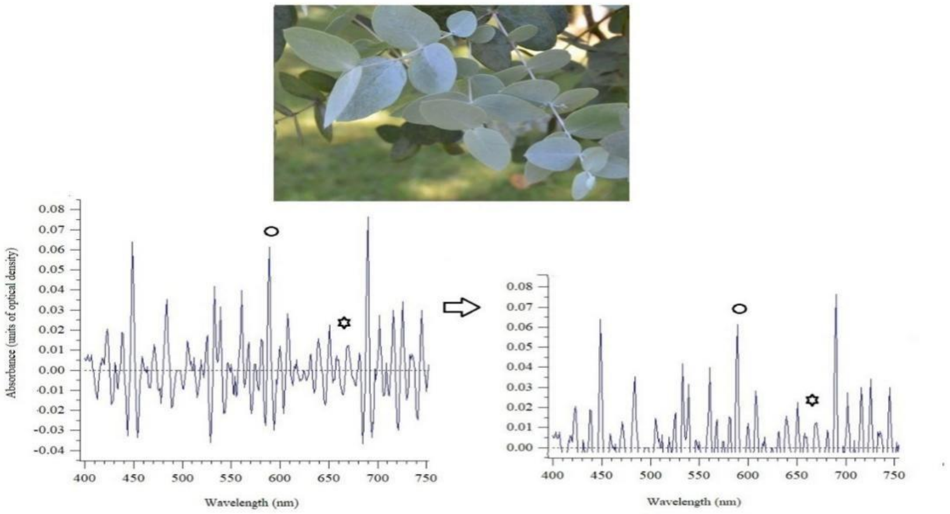

Figure 1. View of the leaf surface with a blue or silver colour of the ash *Eucalyptus cinerea* (Upper photo). At the bottom left is a view of the differential absorbance spectrum for small optical density values (first derivative) using the Zolotarev method (29) on the surface of a eucalyptus leaf. On the right is its upper part, used for comparative studies. The circle marks the maximum of azulene, the asterisk - of chlorophyll.

Extractions of azulenes. Azulenes in sample extract were determined with ethanol or acetone for 10-30 min of exposure by spectrophotometrically at 580 nm, as described earlier (18). The absorption and fluorescence spectra of extracts with 100 % acetone or 95 % ethanol from cells (1:10 w/v for 10 min to 1 h or more) in 1-0.5 cm cuvettes or on paper chromatograms were recorded using the Helios-epsilon spectrophotometer (USA), spectrophotometer Specord M-40 (Germany) and Perkin Elmer 350 MPF-44 B spectrofluorometer (Great Britain) (18).

Detection of azulenes. Azulenes in sample extracts were determined with ethanol and acetone for 10 min or 24 h of exposure by spectrophotometrically at 580 nm, as described earlier (18-23). The extracts were also chromatographed on Whatman Paper No. 1 without or after the impregnation with Vaseline oil or on thin-layer plates of Silufol silica gel, as previously described for pollens of seed plants and horsetail microspores (9,10,13). Their absorbance spectra were then recorded to compare them with data obtained on intact cells. The concentration of azulenes (A) was estimated in ethanol or acetone extracts, according to the formula:

$$A = D_{580} / \varepsilon \times l,$$

Where D_{580}: Optical density at 580 nm, ε: Coefficient of molar extinction of 328 $M^{-1} cm^{-1}$, l: Thickness of the liquid layer in the cuvette, cm.

Relative standard deviation was 5-6 % (n:3-4 samples per species), P:0.95.

The purification of extracts from chlorophyll. The ethanol extracts were purified from chlorophyll by adding sulphuric acid at 50 % concentration, filtered on glass filters, where blue crystals were formed, they were then washed off the filter, dissolved in 96 % ethanol. The resulting solution was passed through columns with silica gel LS 5/40 Chemapol (Chekhia) 2 x 30 cm and the fraction containing maxima in the region of 570-640 nm in the absorption spectra was purified by paper chromatography on Whatman 1 or FM-8. The concentration of azulenes was estimated by the blue band chromatograms with Rf 0.8-0.85 after extraction with 96 % ethanol, as described above for the initial extracts.

RESULTS AND DISCUSSION

Spectral analysis of intact leaves. Microspectrophotometry is the most suitable method for rapid analysis of azulene content in intact plants, which makes it possible to study azulenes based on the absorption spectra of microscopic objects, including single cells and individual tissue cells (20, 21). We used microspectrophotometer to obtain data absorption spectra from the surface of objects. The position of the maxima in the absorption spectra of intact cell surfaces was recorded using the MSF-15 microspectrophotometer was determined using the Zolotarev method (29) by differentiating the reflection spectra, example shown in Fig.1.The first derivative of the external reflection spectrum for low-intensity bands made it possible to directly determine the azulenes' present in the spectra after differentiation -at the maxima 580-620 nm and at 660-666 nm characteristic of azulenes and chlorophyll, relatively. The data of various months of leaves collection was shown in Figures 2-4 and summarized in Table 1.

Figure 2 showed data for eucalyptus leaves in February and March. The optical density at 580-620 nm is often different for the upper (no peak) and lower (one maximum) sides of the leaf in February. Moreover, we noted not only one peak in March, but also several that showed the presence of various forms of blue pigments. If we obtain the absorption spectra in secretory cells, then the glands in February still do not have maxima in the azulene region. They appear later in March and mainly for the glands of the lower side of the leaf (Figure 3). Here and further, data on maxima in the absorption spectra of leaves and optical density are summarized in Table 1.

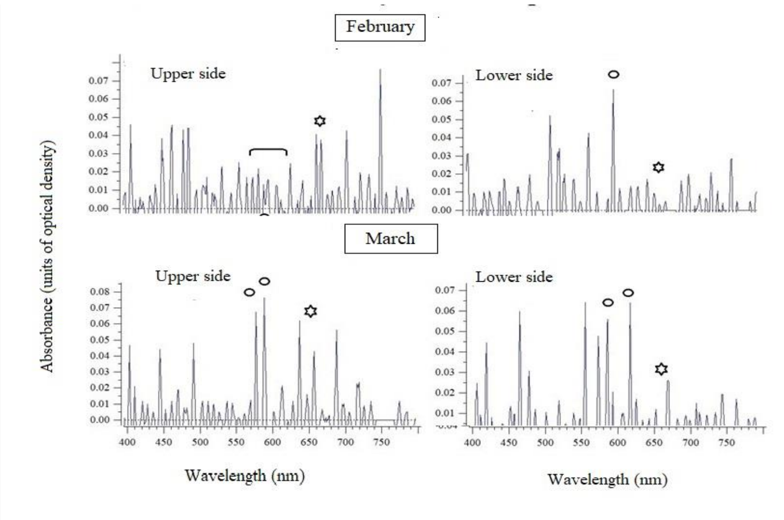

Figure 2. The absorbance spectra of intact leaves of ash eucalyptus in February - March 2024. The parenthesis indicates the areas of the spectrum characteristic of azulenes, the circles indicate the maxima of azulenes and the asterisk indicates the areas of the spectrum characteristic of chlorophyll.

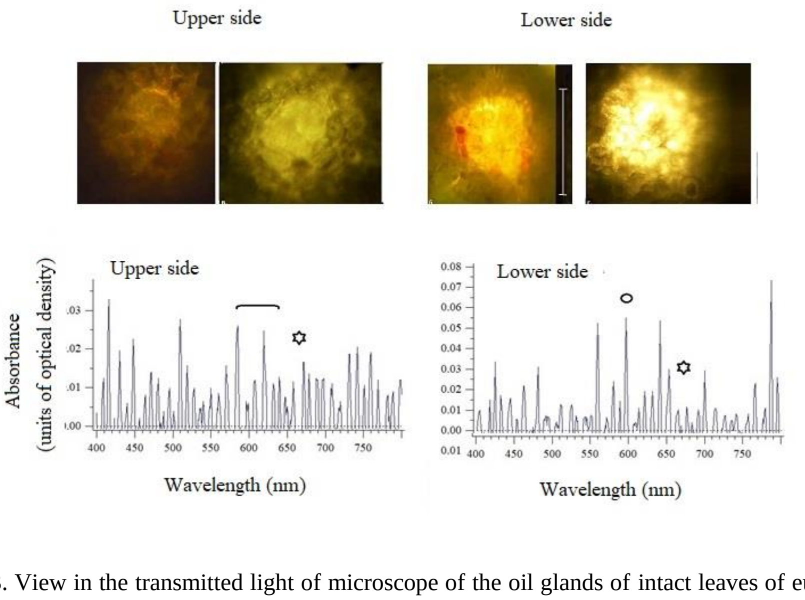

Figure 3. View in the transmitted light of microscope of the oil glands of intact leaves of eucalyptus ash and their absorption spectra in March 2024. The parenthesis indicates the areas of the spectrum characteristic of azulenes, the circles indicate the maxima of azulenes, and the asterisk indicates the areas of the spectrum characteristic of chlorophyll. the glands and at the top and bottom of the leaf.

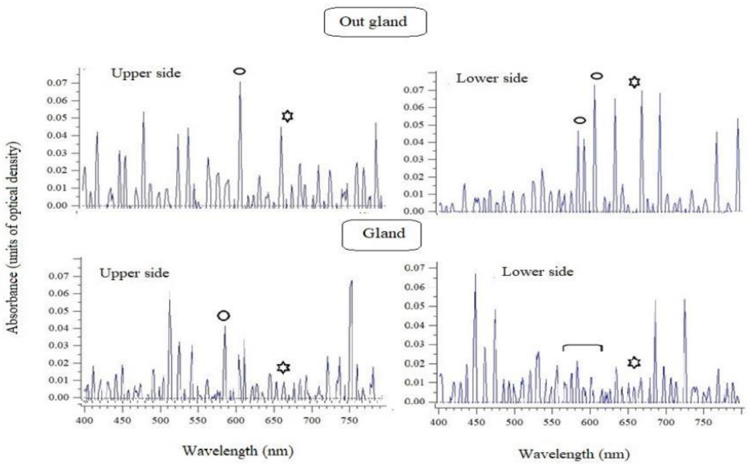

Figure 4. The absorbance spectra of eucalyptus leaves in May 2024.

In May (Figure 4), according to the maxima in the absorption spectra, the concentration of azulenes remained high out glands on both leaf sides. In the glands, high peaks were on the upper side of leaf, whereas, on the lower side of the leaf there was no significant absorption maxima characteristic for blue pigments.

Table 1 presents the summary of results of experiments of absorption spectra of eucalyptus plants on both surfaces. It showed the appearance of azulene maxima in the absorption spectra of the intact surface. The height of the peaks showed the values of the optical density of the pigments on the leaf surface. At the beginning of observations (February 20), the maxima of the eucalyptus absorbance spectra recorded from non-secretory and secretory cells were in the area characteristic for azulenes. However, the

Table 1. Maxima, nm and their height values in units of optical density (in parentheses) in the absorption spectra of the leaves of the studied eucalyptus plants.

Month	Upper side of leaf	Glands	Lower side of leaf	Glands
February	586 (0.0218)	597 (0.0222)	**602 (0.0666)**	580 (0.0168)
March	**580 (0.0674)** **592 (0.0764)**	596 (0.0478)	580 (0.0478) **590 (0.0576)**	(0)
April	591(0.0410)	(0)	600 (0.0358)	**590 (0,066)** **617 (0.0717)**
May	**580(0.055)**	590(0.0384)	613 (0. 0358)	580 (0.0495)
June	**612 (0.0726)**	**589 (0.0563)**	614 (0.0482)	(0)
July	**588 (0.0750)** **618 (0.0683)**	615(0.0436)	**580 (0.0597)**	(0)

The values, equal or higher 0.050 ± 0.0005 that reliable for this microspectrophotometer are marked in bold.

absorption had minimal values (if any) in upper side of leaf unlike lower surface of the leaf out glands. But the lower side of leaf out glands, showed a maximum of 602 nm with a quantifiable absorption of azulenes. There were no significant values for blue pigments in the secretory cells on upper and lower sides of the leaf. In any case, the light bluish colour overlaps the green colour of chlorophyll visually. A month later, in March, the values of maxima in area of azulene absorption increased sharply (2-3 times) on both sides of the leaves out glands, and there was already marked amount of blue pigments in the glands in the upper side of leaf. Note that the position of the maxima in the absorption spectra also changed and even new peaks appeared, unlike the February samples. Here, in the oil glands there were no azulene-related peaks with significant values. The secretions containing azulenes accumulated later were synthesized. In April the height of maxima out glands decreased, whereas there was no maxima in glands of leaf upper side. On the contrary, in gland of lower side of leaf, the values in azulene area sharply increased up to highest values of optical density, but decreased again in May. At last maximal values of optical density in out glands were seen in June-July unlike glands of both leaf sides, where they decreased, in particular up to 0 and in August, also decreased (non- illustrated).

Spectral analysis of extracts. To confirm data received on intact leaves, we studied the extracts from intact leaves with ethanol or acetone, which can dissolve azulenes. Two fractions of extracts were analyzed:1. Leachate from leaves for 10 min, where the outer layer of azulenes was washed off the surface (18,20) and prolonged infusion for 24 or 48 h, when intracellular azulenes can be extracted (18-22).

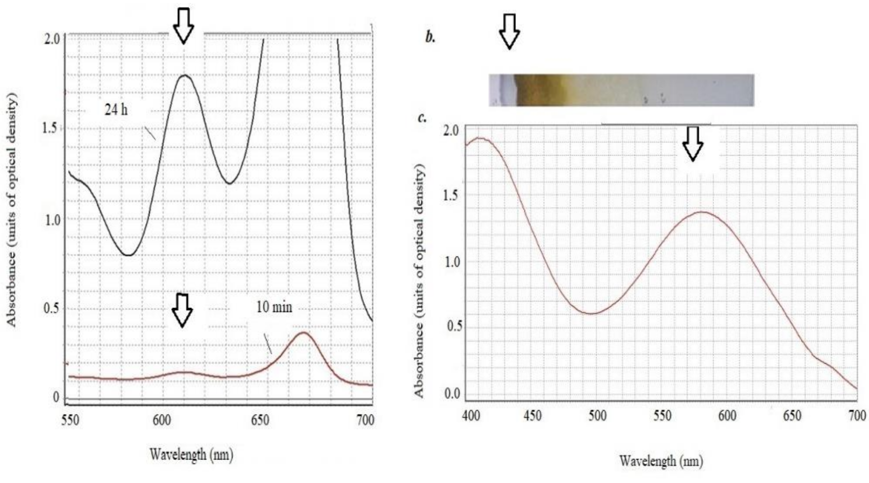

Figure 5. The absorbance spectra of 10 min and 24 h eucalyptus extracts of the azulene-containing plants. (a) Primary extracts in ethanol, (b) Chromatography of 24 h extract on chromatographic paper Whatman1, blue band with Rf 0.8-0.85 has maximum 580 nm, peculiar to azulenes. (c) Azulenes purified from chlorophyll after precipitation with sulfuric acid to its 50 % concentration and chromatography on silica gel.

Figure 5 showed the absorption spectra of alcohol extracts, where the 610 nm maximum typical for azulenes were visible as small in 10 min and as large in 24 h extracts (Fig.5a). On the Whatman chromatographic paper last extract derived and blue band Rf 0.80-0.85 was seen (Fig.5 b). After purification from chlorophyll with sulfuric acid, the blue crystals on glass filter dissolved in ethanol have a maximum of 580 nm, basic for all types of natural azulenes (Figure 5 c). In the fluorescence spectrum of 10-min and 24-h extracts, maxima of 440 nm and 460-475 nm were visible, respectively and after purification - 410 nm, characteristic of azulenes was observed (Figure 6).

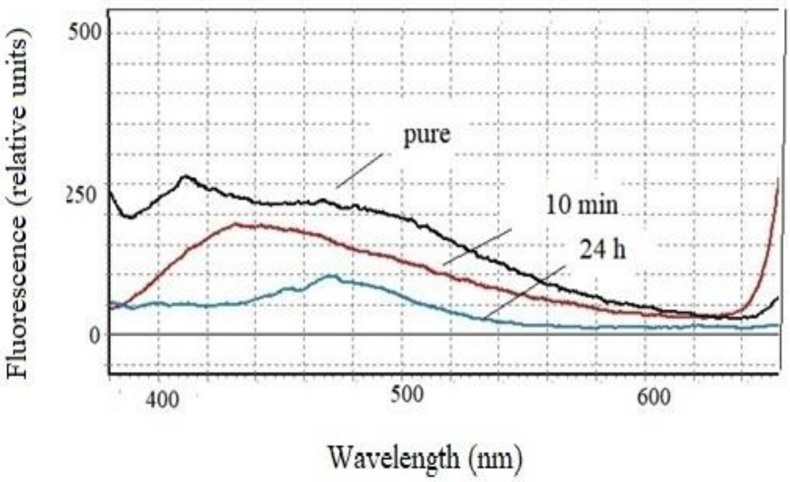

Figure 6. The fluorescence spectra of extracts during 10 min or 24 h and purified eucalyptus preparation.

Content of azulenes in extracts. The amount of azulene in 10 min and 24 h extracts was determined from the azulene maxima in their absorbance spectra (Table 2). In February, concentration of the pigments in 10 min extract was half of those in 24 h extracts.

Table 2. Amount of azulenes in ethanol extracts from leaves.

Month	**Concentration of azulenes in**	
	10 min extracts, (mg/g of fresh weight)	**24-h extracts, (mg/g of fresh weight)**
February	52.2±4	108,0±8
March	51.6±2	174,2±11
April	8.2±2	120±9
May	119±10	309±15
June	479±19	846± 22
July	158±9	390±18

Concentration of azulenes in 10-min leaf extracts in March remained approximately the same as one month ago (azulenes were on the surface of leaf cells), whereas, in 24 h fractions it increased twice. In April, the amount of azulenes decreased 5-folds on the leaf surface in 10 min extracts and on 30 % within leaf cells (24 h extract), like that we saw from the absorbance spectra of intact leaves (Table 1) in the upper and lower sides of the leaf. Sun's ultraviolet radiation is most intense in May and the concentration of ozone oxidizer was high, that correlates with the sharp burst in the azulenes' content both on the surface and within the leaves. In June, the content of blue pigments increased more in both extracts and decreased again from July (2-3folds) and decreased at the beginning of August (to 10-15 % compared with July).

Azulenes, as a result of their antioxidant features the numerous double bonds in the molecule, capture the formed reactive oxygen species (1,6). It is noteworthy that the accumulation of azulenes depended on the size of the leaf. Leaves with a 1-1.5 cm dia contained fewer azulenes, and larger ones (up to 5 cm dia) had higher peaks of azulenes and chlorophyll was invisible. The glands of the lower part of the leaves were enriched with oil compared to March. In May-June, there were fluctuations in peak height and total content in extracts, but later, there were no sharp changes in both peak height and azulene content in extracts. There was stabilization in the amount.

Thus, the most productive period in the formation of azulenes in eucalyptus was the spring of May-June, when the leaves had the maximum content of blue pigments on the leaf surface and inside the cells. The internal presence of azulenes in isolated chloroplasts of some species of clover has been also demonstrated (22). We suppose that May-June was the best time to observe allelopathic effects of azulenes as well as similar time for the collection of the leaves for medicinal use in pharmacy.

CONCLUSIONS

Using spectral methods, the presence of blue azulene pigments on the surface and inside cells at various seasonal periods in the leaves of *Eucalyptus cinerea* was shown for the first time. Using techniques of microspectrophotometry, the dynamics of the azulenes' amount on the surface of intact cells according to their absorption spectra (maxima in the region of 570-630 nm) was demonstrated, confirming by spectral analysis of ethanol and acetone extracts of these pigments during short-time 10-min extraction of intact leaves. Prolonged extraction for 24 h also indicated the presence of azulenes inside the cells. The largest amount of these pigments was found in all extractions in the spring period May-June, characteristic of the highest ultraviolet insolation. It is assumed that this was due to the antioxidant protection of azulenes from the damaging effects of the formed reactive oxygen species.

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AUTHOR'S CONTRIBUTISONS

Conceptualization, Methodology, Experimentation. Writing original draft preparation V.V. Roshchina. Plant collection, Review and editing, Resources, G.A.Soltani

ETHICAL APPROVAL

The author declare that the study was carried out following scientific ethics and conduct. This study did not involve any use of animals, hence, no ethical approval has been obtained from the concerned. The search of new plant species useful for medicine and pharmacy is necessary to develop new natural drugs.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Bakun, P., Czarczynska-Goslinska, B., Goslinski, T. and Lijewski, S. (2021).*In-vitro* and *in-vivo* biological activities of azulene derivatives with potential applications in medicine. *Medical Chemistry Research* **30:** 834-846.
2. Grichi, A., Khammassi, M., Khouja, M.,Amri I. and Larbi Khouja M. (2024). Phytochemical screening and herbicidal properties of aqueous extract of *Eucalyptus cinerea* F. Muell. Ex Benth.: Source of phenolic molecules with allelopathic potential. *Vegetos* 2024: https://doi.org/10.1007/s42535-024-00815-5
3. Konovalov, D.A. (1995). Natural azulenes. *Rastitelnie Resursi* (*Plant Resources*) **31**: 101-130 (In Russian.)
4. Lysenko, L.V. (1967). Anti-inflammatory effect of azulene of eucalyptus oil. *Pharmacology and Toxicology* (Russian)**30** (3): 341-343. PMID: 5597747
5. Muir, R.M. and Hansch, C. (1961). Azulene derivatives as plant growth regulators. *Nature* **190**: 741-742
6. Murfin, L.C. and Lewis, S.E. (2021). Azulene - a bright core for sensing and imaging. *Molecules* **26** (2): 353-362
7. Nakagawa, S., Katoh, K., Kusumi, T., Komura, H., Nomoto, K., Konno, H., Huneck, S. and Takeda, R. (1992). Two azulenes produced by liverwort, *Calypogeia azurea*, during *in-vitro* culture. *Phytochemistry* **31** (5): 1667-1670.
8. Narwal S.S. (1994). Allelopathic effects of eucalyptus in agroforestry. In: *Trees and Tree Farming*. (Ed., P.K. Thampan). Peekay Tree Crops Development Foundation, Cochin, India. Pp.295-316.
9. Roshchina, V.V, Melnikova, E.V., Spiridonov, N.A. and Kovaleva, L.V. (1995). Azulenes, the blue pigments of pollen. *Doklady Biological Sciences* **340** (1): 93-96
10. Roshchina, V.V., Melnikova, E.V. and Kovaleva, L.V. (1997). The changes in the fluorescence during the development of male gametophyte. *Russian Plant Physiology* **47** (1): 45-53.
11. Roshchina, V.V., Melnikova, E.V., Gordon R.Ya., Konovalov D.A. and Kuzin A.M. (1998). The study of radio protectory effect of proazulenes on chemosensory model of pollen *Hippeastrum hybridum*. *Doklady of Russian Academy of Sciences* **358** (4): 548-551.
12. Roshchina, V.V. (1999). Mechanisms of cell-cell communication. In: *Allelopathy Update*. Vol. **2**: 3-25. (Ed. S.S. Narwal) Science Publishers, Enfield, New Hampshire.
13. Roshchina, V.V, Melnikova, E.V, Yashin, V.A. and Karnaukhov, V.N. (2002). Autofluorescence of intact spores of horsetail (*Equisetum arvense* L.) during their development. *Biophysics* (Russian) **47** (2): 318-324

14. Roshchina, V.V. (2004). Cellular models to study the allelopathic mechanisms. *Allelopathy Journal* **13** (1): 3-16
15. Roshchina, V.V., Yashin, V.A. and Vikhlyantsev, I.M. (2012). Fluorescence of plant microspores as biosensors. *Biochemistry* (Moscow), Suppl ser.A: *Membrane and Cell Biology* **6**(1): 105-112
16. Roshchina, V.V. (2022). Possible role of azulenes in plant life: Experiments with models. *Environmental Science and Technology* **1**: 1-10
17. Roshchina, V.V. and Konovalov, D.A. (2022). Single cell plant model of *Equisetum arvense* for the study antihistamine effects of azulene and sesquiterpene lactones. *Future Pharmacology* **2**: 126-134.
18. Roshchina, V.V., Kuchin, A.V., Kunyev, A.R, Soltani, G.A., Khaibulaeva, L.M. and Prizova, N.K. (2022). The presence of azulene on the surface of plant cells as a test for ozone sensitivity. *Biochemistry* (Moscow), Suppl ser. A: *Membrane and Cell Biology* **16** (2): 167-174.
19. Roshchina, V.V., Prizova, N.K. and Khaibulaeva, L.M. (2022). Azulenes of the leaf surface as a protective optical filter. *Russian Journal of Biological Physics and Chemistry* **7**(1): 36-39 (Russian.)
20. Roshchina, V.V., Yashin, A.V. and Kunyev A.R. (2023). Spectral characteristics of the plant cell surface: Occurrence of azulenes and biogenic amines. *Biochemistry* (Moscow), Suppl ser. A: *Membrane and Cell Biology* **17** (4): 276-285
21. Roshchina, V.V. (2023). Plant leaf surface as a sensory system in allelopathic relations: 1. Role of azulenes. *Allelopathy Journal* **59** (2): 109-122.
22. Roshchina, V.V. and Sergievich L.A. (2024). Effects of azulenes as protection factors on the mouse vitality. *Russian Journal of Biological Physics and Chemistry* **9** (2): 168-173
23. Roshchina, V.V. (2024). Azulenes in plant cell: Clover as their useful resource. *Annals of Agricultural and Crop Sciences* **9** (1): 02-07.
24. Roshchina, V.V. (2024). Spectral methods for the search of azulene-containing allelopathic and medicinal species. *Allelopathy Journal* **63** (1): 59-70
25. Roshchina, V.V., Shovkun, M.M. and Demidov, V.E. (2024). Spectral search of azulene-containing plants of temperate climate as possible new biological resources for pharmacy. *Journal of Medicinal Plants and Herbs* **3**(3): 1-14.
26. Rybalko, K.S. (1978). *Natural Sesquiterpene Lactones.* Medicine, Moscow. Pp. 319.
27. Siegel, U., Mues, R., Dönig, R., Eicher, Th., Blechschmidt, M. and Becker, H. (1992). Ten azulenes from *Plagiochila longispina* and *Calypogeia azurea. Phytochemistry* **31** (5): 1671-1678
28. Zhang, J.B., An, M., Wu, H., Stanton, R. and Lemerle, D. (2010). Eucalyptus essential oils: Chemistry and bioactivity. *Allelopathy Journal* **25**: 313-330
29. Zolotarev, V.M. (2012). Application of differentiation in reflection spectroscopy. *Optics and Spectroscopy* **112** (1): 150-154