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CHEMICAL, BIOCHEMICAL, GENETICS, AND PHYSIOLOGICAL ROLE OF SECONDARY METABOLITES OF MEDICINAL PLANTS *VIA* UTILIZATION OF PLANT HISTOCHEMICAL TECHNIQUES

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ABSTRACT

The medicinal properties of plants are due to the presence of secondary metabolites, including flavonoids, alkaloids, tannins, and saponins, which are of great importance because they possess significant biological activities and the particular active constituents of many crude drugs are still unknown. Histochemical investigation are commonly used to verify the identity of many components either in cellular and/or in tissues. Histochemical methods are employed in the identification, density of accumulation and distribution of chemical compounds within biological cells and tissues in different organs under microscopes using the color-stain reaction technique and photographic recording. These include the preparation of fixed variably stained specimens and then the examination under the microscopic devices. It is successfully applied in detection and localization of cellular components of active cell constituents such as proteins, carbohydrates, lipids, nucleic acids, and a range of ionic elements occurring in the cell solutions, in addition to identifying the characterization of secretory structures and the chemical nature of the secreted compounds. The histochemical methods played a role in describing and tracing the ultra-structure development during different plant growth stages so as the genetic bases of plant physiological and biochemical processes could be further elucidated.

KEYWORDS

Histochemical localization, Histochemistry, Color-stain and Secondary metabolites.

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INTRODUCTION

Histochemistry is the branch of histology dealing with the identification of chemical components of cells and tissues. Starch deposition occurs widely in the plant body, but the particularly common places of its accumulation are seeds, the parenchyma of the secondary vascular tissues in the stem and root, tubers, rhizomes and corn[1]. The main ergastic compounds in protoplast are Starch and

proteinsare[2]. Tannin is the heterogeneous group of phenol derivatives, usually related to glucosides. Tannins are particularly abundant in the leaves (xylem) of many plants[3]. Saponins are the rare occurrence. Fats and lipids are vastly found in the body of plant and found in minute amount in each plant cell[4]. Fats are main reserve matter in spores and embryos in meristematic cells. Glucosides are the degradation product of the carbohydrates. Alkaloids are the degradation product of protein. Many plants contain medicinally important secondary product[5].

Histochemistry is devoted to study the identification and distribution of chemical compounds within and between biological cells, using stains, indicators and light and electron microscopy[6]. Histochemical analysis is essential for the study of plant secretory structures whose classification is based, at least partially, on the composition of their secretion. As each gland may produce one or more types of substances, a correct analysis of its secretion should be done using various histochemical tests to detect metabolites of different chemical classes[7].

Histochemistry is a methodological approach that allows the chemical analysis of cells and tissues in relation to their structural organization[8], but to achieve this objective for plant secretory structures, a wide histochemical analysis is necessary because the same gland and even the same glandular cell can produce several different metabolites simultaneously[9,10,11].

MATERIAL AND METHODS

Certain precautions must be taken to correctly interpret the results of histochemical analysis of plant secretory structures since most used reagents and dyes that are not specific,:

1. The natural color of the secretion should be observed in vivo before applying the test (avoiding the use of reagents with the same color as the secretion).
2. Attention should be paid to the color obtained in the staining since different colors can be generated in each test but the positive staining is specific.
3. The control procedure should be carefully set up, which usually consists of removing the substance to be detected prior to the test application. The color of the test and that of the control are compared visually to interpret the results[7].

Detection of polyphenolic terpenes aldehyde was detected in the tissue of Gossypium seeds. If it is necessary to use fixed material, the best fixative for hydrophilic substances is formalin-ace to-alcohol (FAA). For fixation, the material should be immersed in the FAA under vacuum for 24 h, then washed in 50 % ethanol overnight and stored in 70 % ethanol[12].

Some stains that are commonly used in histochemical localization and their methodology are illustrated in Table No.1 for hydrophilic, lipophilic substances, phenolic compounds and alkaloids.

Applications of Histochemistry in Plant Research

Localization of secondary metabolites in certain medicinal plants

Histochemical study was carried out to localize polyphenolic terpenoid aldehydes and fixed oil in healthy seeds, stems, leaves and roots of *Gossypium Barbadense* L. var. Giza 86. Polyphenolic terpenoid aldehydes and fixed oil were mainly detected inside lysigenous glands. In young leaves and roots, polyphenolic aldehydes were also observed as fine particles inside the cytoplasm of some parenchymatous cells around glands. Lysigenous terpenoid-containing glands were noticed in all tap root regions except the apical 3cm. The number of glands increased with increasing distance from the root tip. This may explain why the antimitotic activity of gossypol does not affect the growing tip of the plant[33].

Three species used in folk medicine were choosen to determine their histochemical investigation: *Adhatoda zeylanica*, *Ruta graveolence* and *Vitex negundo*. In general, these plants are used in folk medicine in the treatment of gonorrhoea, anti-periodic, bronchitis, infected wounds, scrotal swelling, synovitis, arthritis pain and rheumatic

arthritis. For histochemical studies the free hand sections of leaves and stem were taken and treated with the respective reagent to localize components, viz. starch, protein, tannin, saponin, fat, glucosides and alkaloids in the tissues[34].

A histochemical analysis of leaf and rhizome of *Curcuma neilgherrensis* was done. The study showed the identification and sites of the phytochemicals like alkaloids, saponins, tannins, oils, starch grains etc in various regions of leaf and rhizome of *C. neilgherrensis*. Free hand sections were taken and treated with respective reagents to localise the various cellular components. The observations could be of great use in chemotaxonomy and checking the drug adulteration[35].

Furthermore, the histochemical studies of leaves and wood of *Sesbania grandi flora*, *Sesbania bispinosa* and *Sesbania cannabina* are medicinally important plants of Marathwada region in Maharashtra. Histochemical studies needed the free thin hand sections of leaves and wood were taken and treated with the respective reagent in localize components, viz. starch, protein, tannin, saponin, fat, glucosides and alkaloids in the tissues[36].

Solid ago chilensis Meyen (*Asteraceae*) is native plant to South America and the only example of the genus in Brazil. This species is popularly known as "arnica" and is used to treat bruises, muscle pain and inflammation. Cross-sections were made for microscopic examination of root, stem and leaf; for these parts of the plant maceration was also performed according to the method of Jeffrey. For the leaf were still made par a dermal sections, scanning electron microscopy analysis, phytochemical and histochemical tests. Therefore, it was examined the features of useful anatomy for diagnosis of the both varieties and species which, together with identification of the chemical compounds and its histolocalization, provides support to their quality control[37].

Carbohydrate storage in the form of starch grains has been examined in stems and roots of *Jatropha curcas*. The predominant starch-storing tissues were identified, and the cellular localization of the starch grains within these tissues was determined. In stem sections, starch was seen predominantly in parenchymatous cortex, medullary rays, pith while in the root sections, starch was seen highly concentrated only in cortical tissues and showed brownish black spots in the medullary rays[38].

Thymus species, is a type of wild medicinal plants. Exploitation and utilization this plant and studying the species and sites of alkaloids in its leaves. A histochemical investigation of leaves at variable developing stages was examined to determine the site of alkaloids. On the other hand, the types and amount of alkaloids in leaves were examined using GC-MS. It was found that there were two kinds of glandular trichomes, namely, peltate trichomes and capitate trichomes, on the surface of leaves, and their secretory cells could secrete alkaloids. The obtained data disclosed that trichomes could secrete alkaloids as soon as the first pair of leaves formed, and there were altogether 18 kinds of alkaloids identified by GC-MS. Nearly all of these alkaloids of leaves at different developing stages were distinct from each other, except one, 3-methoxy-a-methyl-benzeneethanamine, persists at different developing stages with high concentration[39].

Studies on the phytochemical profiling and histochemical localization in leaf and stem of *Trichosanthes cucumerina* (L) var. *cucumerina* with referring to the effect of plant age and geographical diversity were carried out using Wagner's reagent concluded that alkaloids are mostly located in the parenchyma cells bordering the vascular bundles of stem and petiole. Flavonoids, detecd with sodium hydroxide showed that they were marked as a distinct yellow band in the sub hypodermal layer of stem. Tannin localization with FeCl3 also suggested their storage in parenchyma cells[40].

The lipid composition was showed for the chemical compounds of *B. verbascifolia* and *C. adamantium*, which proved to be part of the essential oils or resins oils in *C. Adamantium* idioblasts. The chemical compounds of *B. verbascifolia, C. adamantium* and *R. montana* are present mainly in idioblasts in the parenchyma and epidermal cells. *C.*

Adamantium contains fats or lipids in secretory structures[41].

An examination mainly emphasized on the histochemical detection of secondary like metabolites; e.g., tannins, alkaloids, sugars, proteins, flavonoids, amino acids and coumarins. The active constituents were detected in different sites of the stem, petioles of leaves and roots of *B. lupulina*. It was found that presence of number of phytochemicals in xylem is higher than other tissues[42].

Detection of lignin, fats, and coumarins in the undifferentiated cells and among plants in field or *in vitro* grown plants

Bitter broom or kallurukki. (*Scrophulariaceae*), is much important in folk medicine to treat kidney, liver, and, and inflammations. Histochemical detection of protein, polysaccharided, fats and coumarins in the callus and regenerated plants. The study reveals the potential of utilizing calli in herbal formulations of the species, as this may yield better results including improved nutraceutical value[43].

Showing supposed defence mechanism actions (Parasitic plant host - root interaction)

Roots of different hosts of the holophrastic weed known as broomrape (Orobanche spp.) were examined histochemicaly for the occurrence of structural cellular barrier formation following wounding / penetration. Such barrier might function to impede the successful development of parasite haustorium interaction, i.e. as a self-defense mechanism. In faba bean and white bean, brown deposits occurred in walls adjacent to the damaged cells of the epidermis, cortex and stele. Via stain reactions and colorations these deposits were detected as melanin. Additionally, walls bordering damaged site at the level of the endodermis and within the stele become suberized and lignified. In peas, which possesses a lignified hypodermis, the response was similar but lignin was also deposited in the walls of the endodermis and hypodermis adjacent to the wound. In sunflower, which possesses a suberized hypodermis, melanin was deposited in the hypodermis and lignin and suberin occurred within the stele. In all these broomrape host species melanization conferred the modified cell wall many of the properties associated with lignified and suberized structures such as impermeability and resistance to chemical degradation[44].

Detection of active compounds in lemongrass

The location of citral terpene in lemongrass (*Cymbopogon flexuosus*) Steud) wats (cultivar OD-19) were examined by Schiff's reagent, whereas aldehydes (citral) produces a purple-red color. In this respect, however, the citral lacking cultivar GRL-1 (geraniol rich) leaf sections, which also was subjected to Schiff's reagent could be compared to the cultivar OD-19 leaf sections. In lemongrass mutant GRL-1, those specialized cells, however, are not being stained due to lack of citral. Hence, it could be confirmed that the observed schiff's staining reaction is associated with the accumulation of citral substance in a given cell[45].

New applications

Localization of monoterpene phenols accumulation in plant secretory structures

A new procedure was cited for the histochemical detection of phenolic monoterpene in essential oil secretory structures. The method was used from a spot test which was devised for *in vitro* detection of phenolic compounds in organic analyses. A positive test for phenol was indicated by the production of coloured indophenols. Monoterpene phenols were detected in the hairs of *T. vulgaris* (thymol) and *O. vulgare* (carvacrol), shown by change of color to red and green respectively[46].

Localization of auxins and cytokinis in via histo-immunochemistry method

A new method for histochemical localization of cytokinins (CKs) in plant tissues based on bromophenol blue/silver nitrate staining was reported. The method was validated by immune histo chemistry using anti-*trans*-zeatin riboside antibody. Auxin (Indole-3-acetic acid) was detected byanti-IAA antibody in plant tissues to confirm the presence of IAA histolocalization. Root sections were used, because they are major sites of CKs synthesis, and insect galls of *Piptadenia gonoacantha* that accumulate IAA. Immunostaining

confirm the zeatin presence and sites of accumulation of IAA indicated by histochemistry. The colors shown by histochemical reactions of plant tissues were similar to those obtained by thin layer chromatography (TLC), which reinforced the reactive sites of zeatin. The histochemical procedure for examining CKs was helpful for galls and roots but IAA detection is more powerful for gall tissues. Galls presents a useful example for proving the histochemical techniques due to their fast cell cycles and relatively high augumentation of plant hormones[47].

Locating enzymes in plant tissue using nitrocellulose blotting

Nitrocellulose blotting of fresh tissue sections and the detection of enzyme activities on the blots forpolyphenol oxidase, peroxidase, glycosidases, dehydrogenase and phosphatase activity has been shown successfully utilization a simple histochemical method. Two cm squares of nitrocellulose membrane filters B A 28, (Schleicher and Schüll, 0.45µm pore size) were soaked in distilledH_2O, placed on microscope slides and blotted dry with a tissue. A2-3mm section through the plant tissue was placed on the membrane and then lightly pressed on it using an additional microscope slide. The section was removed carefully and the membrane thoroughly rinsed with distilled H_2O to remove non-proteinaceous material or soluble compounds and lightly pressed with a tissue to remove excess water. About 0.2 ml of the appropriate substrate or reagent was next spread over the surface of the membrane. The slides were incubated in a moist atmosphere for the reaction to proceed, the substrate was then rinsed off, and if necessary a developing reagent added. For fast reactions with immediate colour production the substrate was added and rinsed off as soon as colour development was optimal e.g. polyphenol oxidase or peroxidase. Alternatively the nitrocellulose blot can be placed (blot side upwards) on to a piece of filter paper moistened with substrate. This gives very good resolution and localization of enzyme activity[48].

In gene expression

The detection of plant transformation (using *β*-glucuronidase; GUS assay in *Lilium* are carried out via the application of histochemical methods. *β*-glucuronidase (GUS) assay is used to assess transient expression of the GUS gene using 5-bromo-4-chloro-3-indolyl *β* D-glucuronide (X-Gluc) as the substrate. Six days after co-cultivation, samples of 0.1g of callus collected from each treatment are subjected to transient histochemical GUS assay. The transformation efficiency of calli are evaluated by counting the number of blue spots, using stereomicroscope, showing GUS enzyme activity on each callus sample[49]. The methods /technique are described by Azadi *et al.*[50].

The use of the histochemical methods in iron and ferritin gene expression in transgenic indica rice (*Oryza sativa* L. cv Pusa Basmati) proved as efficient in such an investigation. Perl's Prussian blue staining of transgenic rice grain sections show distribution of iron accumulation (blue compound of ferric ferrocyanide) throughout the allure one and subaleurone layers and in the central region of the starchy endosperm. Whereas, in the non-transgenic grains, blue colour formation indicating iron accumulation was restricted to the aleurone layer and the intensity of color was also very low. Transverse section of the transgenic rice grains indicated the high iron accumulation in embryo as well as in the endosperm, in comparison to the non-transgenic ones. In the latter, iron appeared restricted to the embryo and aleurone layer in which the intensity of color detected in the embryo was very low. This histochemical analysis of iron in rice specifically showed temporal and spatial deposition of storage iron[51].

Advances in histochemistry and cytochemistry made are possible to retrieve quantitative data from 2D and 3D microscopic images. In this way, valid quantitative results can be regenerated (e.g. gene expression data at the mRNA, protein and activity levels) from microscopic images in relation to structures in cells, tissues and organs in 2D and 3D. Volumes, areas, lengths and numbers of cells and tissues can be calculated and related to these gene

expression data while preserving the 2D and 3D morphology[52].

Detection of heavy metals, i.e. pollution and contamination

Heavy metals accumulation

Histochemical methods were employed in the detection of the heavy metals (Cd, Pb, Ni, and Zn) and strontium, their distribution, accumulation, and translocation within the tissues of higher plants. In this respect, detailed protocols of metal detection with metallochrome indicators dithizone (Cd, Pb), dimethylglyoxime (Ni), sodium rhodizonate (Sr), zincon (Zn), and fluorescent indicator Zinpyr_1(Zn) by light and fluorescence microscopy were described[53].

The occurrence of heavy metals and their accumulation in water hyacinth [*Eichhornia crassipes* (Mart.) Solms] was investigated. The histochemical staining examinations indicated the accumulation in the epidermis and vascular bundles of the roots and petiole. In the leaf sections the palisade tissues were deeply stained, showing the high accumulation of the metals within the leaves[54].

Detection of H_2O_2 and O_2 −; a result of cadmium contamination

The effect of cadmium on H_2O_2 and $O_2\cdot-$ production in leaves from pea plants grown for 2 weeks with 50μm Cd, by histochemistry with diaminobenzidine (DAB) andnitroblue tetrazolium (NBT), respectively was studied. Thesubcellular localization of the reactive oxygen species (ROS) was studied by using $CeCl_3$ and Mn/ DA Bstaining for H_2O_2 and $O_2\cdot-$, respectively, followed by electron microscopy observation. In leaves from pea plants grown with 50μm $CdCl_2$, a rise of six times in the H_2O_2 content took place in comparison to control plants. The accumulation of H_2O_2 was localized mainly in the plasma membrane, mesophyll and epidermal cells, as well as in the tonoplast of bundles heath cells. In mesophyll cells, the accumulation ofH_2O_2 was observed in mitochondria and peroxisomes. Localization of $O_2\cdot-$ production was demonstrated in the tonoplast of bundle sheath cells, and plasma membrane from mesophyll cells. The Cd-induced production of the ROS, H_2O_2 and $O_2\cdot-$, could be attributed to the phytotoxic effect of Cd. In this connection, lower levels of ROS were assumed to function as signal molecules for the induction of defense genes against the injurious effects of the heavy metal[55].

However, Jin *et al.* found that exposure to cadmium resulted in significant ultra-structure changes in the root meristems and leaf mesophyll cells of *Sedum alfredii.* Hence, Damages were more pronounced in NHE (non-hyper accumulator ecotypes) even when Cd concentrations were one-tenth of those applied to HE (hyper accumulator ecotypes). In the cadmium stress damaged chloroplasts resulted in imbalanced lamellae formation which is coupled with early leaf senescence. Histochemical results revealed that glutathione (GSH) biosynthesis inhibition led to overproduction of hydrogen peroxide (H2O2) and superoxide radical (O2−) in HE but not in NHE. The GSH biosynthesis induction in root and shoot exposed to elevated Cd conditions, however, might be involved in Cd tolerance and hyper accumulation in HE of *S. alfredii* H[56].

Investigation of augumentation of AL (Aluminium), oxidized lipids, production of callose, and integrity of plasma membrane

Staining was shown in the roots of peas (*Pisum sativum* L.) and distributed on the entire surface of the root apex. Meanwhile, the loss of plasma membrane integrity was examined by using Evans blue which was concentrated around the periphery of the cracks on the surface of root apex. The enhancement of four phenomena, i.e. aluminum accumulation, lipidperoxidation, callose production and root elongation inhibition displayed similar aluminum dose dependencies which occurred at 4hs exposure. The loss of membrane integrity, however, was enhanced at lower aluminum concentrations and after a longer aluminum exposure of 8h. The addition of butylated hydroxyanisole (a lipophilic antioxidant) during aluminum treatment was found to completely prevent only the lipid per oxidation and callose production by 40%. Thus, lipid per oxidation was suggested to represent relatively early symptom induced by the accumulation of

aluminum and appear to cause, in part, callose production. Whereas, the loss of plasma membrane integrity represented a relatively late symptom caused by cracks in the root is due to the inhibition of root elongation[57].

Table No.1: Some stains that are commonly used in histochemical tests

Detection of Hydrophilic substances	
1. Mucilage	
Ruthenium red staining	This method stains acidic mucilages, pectins[12,13], and nucleic acids magenta or red (Figure No.1a). 1. Apply 0.1 % ruthenium red to sections for 5 min. 2. Wash sections twice in distilled water to remove surplus stain. 3. Mount the sections between slide and coverslip with glycerin gelatin.
Alcian Blue Staining	This test has a similar result as ruthenium red, staining acidic mucilages, pectins[14] and nucleic acids light blue (Figure No.1b). 1. Stain sections with 1 % Alcian Blue for 30 min. 2. Rinse sections twice with distilled water to remove surplus stain. 3. Mount the slide with glycerin gelatin.
Tannic Acid and Ferric Chloride	This method is based on the reaction of tannic acid with mucilages[15] and pectins, substances which are further revealed by the addition of ferric chloride, producing a grey to black color (Figure No.1c). 1. Apply 5 % tannic acid for 20 min. 2. Rinse briefly with distilled water. 3. Submerge sections in 3 % ferric chloride for 5 min. 4. Wash twice in distilled water to remove surplus ferric chloride. 5. Mount the sections using glycerin gelatin. 6. Control: Compare the staining obtained in the test with that of sections treated only with tannic acid or with ferric chloride.
2. Starch	
Lugol's Reagent	This reaction highlights the starch grains in dark blue to black (Figure No.1d)[12]. Almost all other structures stain yellow, but this color has no specific significance. 1. Submerge the sections in the Lugol's reagent for 10min. 2. Rinse briefly with distilled water. 3. Mount the slides using distilled water or Lugol's reagent itself.

Table (1): Cont.

Triple Staining for Starch Detection	This triple staining was developed to analyze structural tissue components and the starch grains concomitantly[16]. The application of safranin, astra blue and iodine–potassium iodide solution stains starch grains black, acidic substances (e.g., nucleic acids and lignin) brown, and non-lignified cell walls green (Figure No.1e). 1. Stain the sections with 1 % safranin for 1 min. 2. Rinse 3 times for few seconds in 50 % ethanol to remove surplus stain. 3. Stain with 1 % astra blue for 1 min. 4. Wash three times for few seconds in distilled water to remove surplus stain. 5. Apply the iodine–potassium iodide solution for 10 min. 6. Dip sections rapidly in distilled water. 7. Mount the slide with the smallest amount of water.
3. Carbohydrates	
PAS Reaction (Periodic Acid: Schiff's reagent)	This method is based on the reaction of periodic acid with carbohydrates, forming carbonyl groups revealed by Schiff's reagent[17]. Carbohydrates stain magenta (Figure No.1f). 1. Apply 1 % sodium tetraborate (freshly prepared) for 30 min. 2. Transfer sections to 1 % periodic acid for 10 min. 3. Rinse briefly in distilled water. 4. Apply Schiff's reagent for 15 min in dark. 5. Wash the sections with sodium metabisulfite for 10 min. 6. Rinse in tap water for 10 min. 7. Mount the slides using glycerin gelatin. 8. Control: Repeat the test excluding step 2 (periodic acid).
Aniline Blue Staining	This staining marks callose, which may be detected by a green fluorescence under UV light (Figure No.1g)[18]. 1. Apply 0.05 % aniline blue for 10 min. 2. Rinse briefly in distilled water. 3. Mount the slide in the same buffer used for staining.
Calcofluor White Staining	This test is used to detect cellulose in cell walls, which fluoresces light blue under UV light (Figure No.1h)[19]. 1. Place sections into 0.01 % calcofluor white for 10 min. 2. Rinse briefly in distilled water. 3. Mount in distilled water.

Table (1): Cont.

4. Proteins	
Aniline Blue Black Staining	This stain reveals proteins in blue (Figure No.1i)[20], whether structural or acting in the primary or secondary metabolism. 1. Dip sections into 1 % aniline blue black for 1 min. 2. Wash twice in 0.5 % acetic acid to remove excess stain. 3. Rinse briefly in distilled water. 4. Dehydrate sections passing quickly through 90 %, 100 % ethanol, then a mixture of 100 % ethanol and xylene (1:1, v/v), and finally pure xylene. 5. Mount slides using synthetic resin.

	6. Control: Put sections in a solution of acetic anhydride and pyridine (4:6, v/v) for 6 h prior to staining.
Coomassie Blue Staining	This method stains proteins blue (Figure No.1j)[20] and produces a similar result to aniline blue black. 1. Stain in 0.25% Coomassie blue for 15min. 2. Differentiate in 7 % acetic acid. 3. Rinse briefly in distilled water. 4. Mount in glycerin gelatin. 5. Control: Put sections in a solution of acetic anhydride and pyridine (4:6, v/v) for 6 h prior to staining.
5. Detection of Lipophilic substances	
Lipids	
Sudan Black Staining	This is a general method which stains lipids dark blue to black (Figure No.2a)[14]. 1. Stain with Sudan black B for 20 min. 2. Rinse briefly in 70 % ethanol. 3. Wash in distilled water. 4. Mount in glycerin gelatin. 5. Control: Sections should be kept in the extraction solution[21] for 6 h or more, depending on the composition of the secretion (determined empirically). After this time, the sections should be transferred to distilled water and washed in a period of 4 h (4 × 1 h). Then, the staining proceeds as described.

Table (1): Cont.

Sudan IV Staining	Sudan IV also stains lipids, in general[14], which become red or red-orange (Figure No.2b). 1. Apply Sudan IV for 30min. 2. Rinse briefly in 80 % ethanol. 3. Wash in distilled water. 4. Mount in glycerin gelatin. 5. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.
Neutral Red Staining	This fluorochrome emits different colors depending on the lipid composition[22]. Under blue light, the lipids of secretion fluoresce yellow or green (Figure No.2f), cuticle fluoresces yellow and lignified cell walls fluoresce red. 1. Stain with 0.1 % neutral red for 20 min. 2. Rinse briefly in distilled water. 3. Mount in distilled water. 4. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.

6. Acidic and Neutral Lipids	
Nile Blue Staining	Since lipids were detected in the material, Nile blue distinguishes acidic lipids, which stain blue, from neutral lipids, which stain pink (Figure No.2c)[23]. 1. Stain with Nile blue solution for 5min at 60°C. 2. Wash twice with 1 % acetic acid at 60°C. 3. Rinse in distilled water. 4. Mount in glycerin gelatin. 5. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.
7. Fatty Acids	
Copper Acetate and Rubeanic Acid Staining	This method for lipids is slightly more specific than the Sudan tests and identifies fatty acids through the reaction of copper acetate with these acidic lipids, which subsequently turn dark green when exposed to rubeanic acid (Figure No.2d)[24,25]. 1. Treat sections with 0.05 % copper acetate for 3 h. 2. Apply 0.1 M Na2 EDTA (EDTA acid disodium salt solution) for 5 min. 3. Wash in distilled water for 5 min. 4. Transfer sections into 0.1 % rubeanic acid (freshly prepared) for 20 min. 5. Wash in 70 % ethanol for 5 min. 6. Rinse in distilled water. 7. Mount in glycerin gelatin. 8. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.
8. Terpenes	
NADI Reaction	This reagent produces differential staining[26], with essential oils (Figure No.2e, monoterpenes and sesquiterpenes) staining blue and resins (diterpenes, triterpenes, tetraterpenes and derivatives) staining red. Mixtures of essential oils and resins produce varied shades of violet to purple, depending on the prevalence of each compound. 1. Apply NADI reagent for 1 h in the dark. 2. Wash in sodium phosphate buffer (0.1 M, pH 7.2) for 2min. 3. Mount in the same buffer. 4. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.
9. Detection of Phenolic Compounds and Alkaloids	
Phenolic compounds	
Ferric Chloride Staining	This method highlights phenolic compounds through iron precipitation, producing a dark color[12], usually black (Figure No.3a), sometimes brown. 1. Apply 10 % ferric chloride for 30min. 2. Wash twice in distilled water to remove surplus ferric chloride. 3. Mount in glycerin gelatin.
Potassium Dichromate Staining	This method also highlights phenolic compounds; in general[27], producing a brown or red-brown color (Figure No.3b).

Table (1): Cont.

	1. Apply 10 % potassium dichromate for 30min. 2. Wash twice in distilled water to remove surplus reagent. 3. Mount in glycerin gelatin.

Table (1): Cont.

Ferrous Sulfate–Formalin Fixation	The best method to detect phenolic compounds is to introduce iron salts into the fixative since the iron compound fixes and stains the phenolic compounds (Figure No.3c)[12]. 1. The samples should be fixed in the ferrous sulphate- formalin solution under vacuum for 48 h. 2. Wash 4 × 2 h (totaling 8 h) in distilled water. 3. Dehydrate the material in 30 %, 50 %, 70 % ethanol for 12 h each. 4. Embed the material according to the chosen technique (Paraplast, Historesin, or PEG) and then section in a microtome
Vanillin– Hydrochloric Acid Staining for Tannins	This test is more specific for some phenolic compounds, staining tannins red (Figure No.3d)[28]. Use only sections of fresh material. 1. Treat with 0.5 % vanillin for 20 min. 2. Mount the slide using 9 % hydrochloric acid.
Phloroglucinol– Hydrochloric Acid Stainingfor Lignin	Phloroglucinol in an acidic medium stains lignin in cell walls pink to red (Figure No.3e)[12]. It is possible to use either fresh or embedded material. 1. Apply 10 % phloroglucinol for 15min. 2. Mount the slides carefully with 25 % hydrochloric acid.
Acridine Orange	This fluorescent dye is useful to identify several acidic compounds under blue light, such as nucleic acids and components of the cell wall[29], distinguishing lignified cell walls (yellow-green fluorescence) from non-lignified cell walls (red fluorescence; Figure No.3g). It is possible to use fresh material as well as embedded material in this test. 1. Apply 0.01 % acridine orange for 20min. 2. Mount the slides with distilled water.
Autofluorescence	Plant tissues have several auto fluorescent components which permit their analysis under UV radiation[30]. In relation to secondary metabolites, many phenolic compounds (including lignin) emit a blue or blue-green fluorescence (Figure No.1g, 3f). However, it is necessary to be cautious in identifying compounds through autofluorescence because some alkaloids and terpenoids may also emit fluorescence in the blue band[32].

Table (1): Cont.

10. Alkaloids	
Dragendorff's Reagent	This reagent marks alkaloids in red-brown (Figure No.3h)[31]. Fresh and fixed material may be used in this method, but fixed material shows a considerably loss of the alkaloids and the staining color when compared to fresh material. 1. Treat with Dragendorff's reagent for 20min. 2. Rinse briefly in 5 % sodium nitrite. 3. Mount in distilled water.

	4. Control: Treat sections with 5 % tartaric acid in 95 % ethanol for 72 h and repeat the staining procedure.
Wagner's Reagent	This method also stains alkaloids red or red-brown (Figure No.3i)[32]. It is recommended that fresh material be used for this test. 1. Apply Wagner's reagent for 20 min. 2. Rinse briefly in distilled water. 3. Mount in distilled water. 4. Control: Treat sections with 5 % tartaric acid in 95 % ethanol for 72 h and repeat the staining procedure.

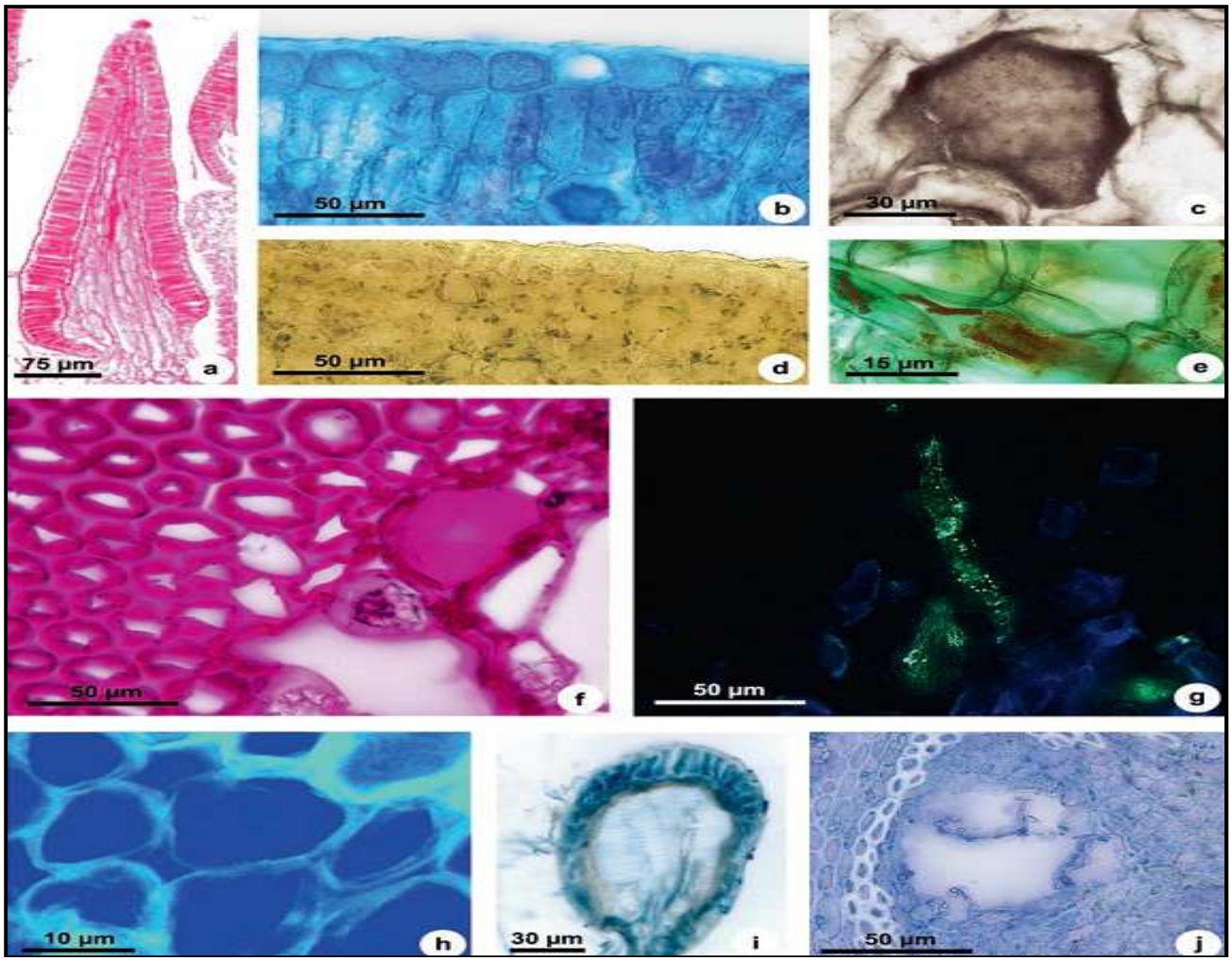

Figure No.1: Histochemical analysis of plant secretory structures. (a, c, g–i) Embedded material. (b, d–f, j) Fresh material. (a) Detection of acidic mucilage in the colleter of *Asclepias curassavica* L. (Apocynaceae) by ruthenium red. (b) Identification of acidic mucilage in the epidermis of *Rhododendron* sp. (Ericaceae) by Alcian Blue. (c) Positive result for mucilage in secretory idioblast of *Cattleya walkeriana* Gardner (Orchidaceae) using tannic acid and ferric chloride. (d) Starch grains in the nectary of *Inga edulis* Mart. (Fabaceae) detected by Lugol's reagent. (e) Observation of starch grains in laticifer of *Euphorbia milii* Des Moul. (Euphorbiaceae) and the tissue structure using the triple staining. (f) Detection of carbohydrates in the secretory idioblast of *Ceiba speciosa*[7]

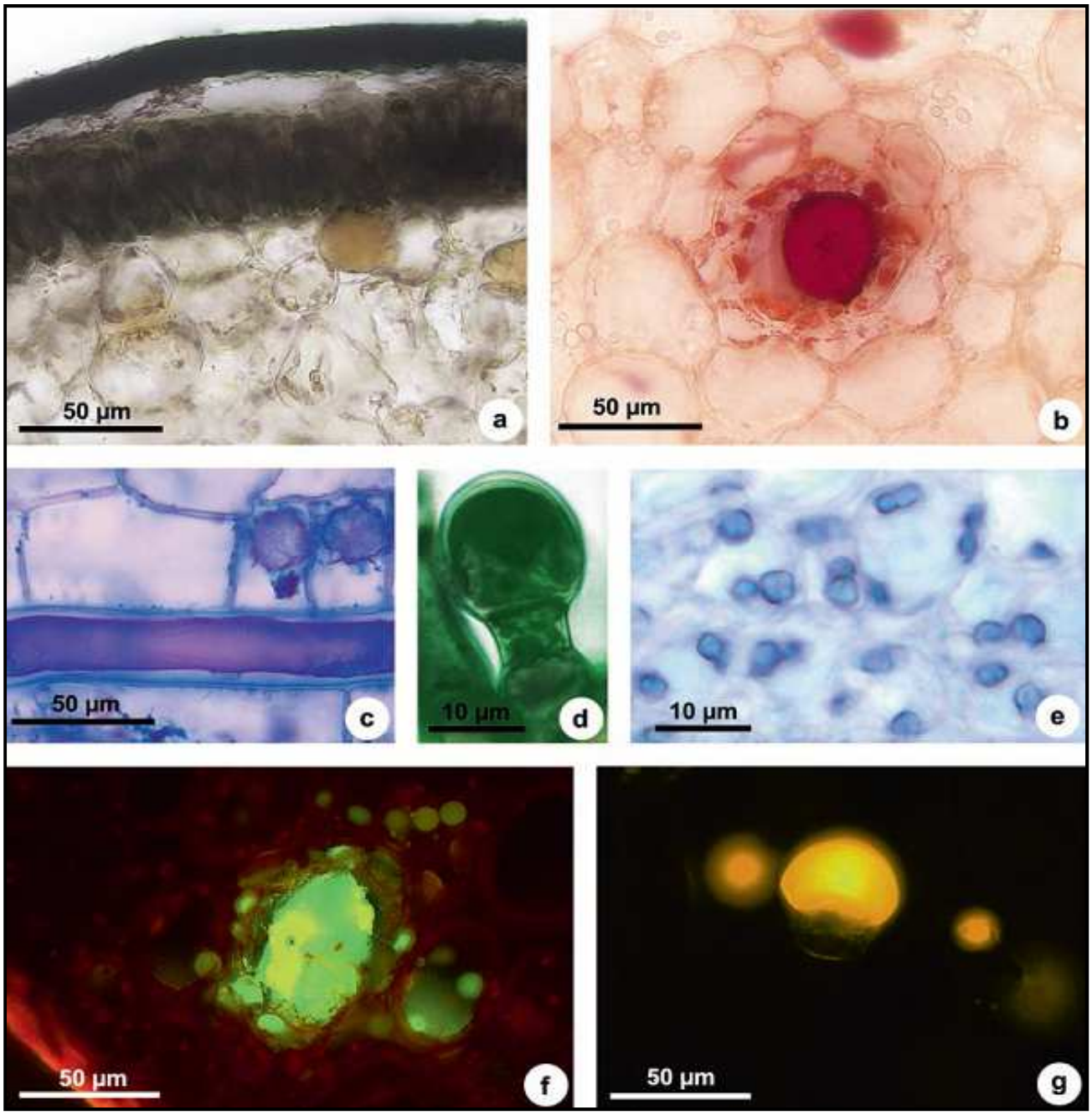

Figure No.2: Histochemical analysis of plant secretory structures. Fresh material. (a) Detection of lipids production by elaiophore of *Byrsonima intermedia* A. Juss. (Malpighiaceae) by Sudan black B. (b) Identification of lipids in the secretory duct of *Philodendron* sp. (Araceae) by Sudan IV. (c) Positive result for neutral lipids in the laticifer of *Sapium glandulatum* (Vell.) Pax (Euphorbiaceae) by Nile blue. (d) Observation of fatty acids in the glandular trichome of *Tetradenia riparia* (Hochst.) Codd (Lamiaceae) using copper acetate and rubeanic acid. (e) Detection of essential oils in the secretory idioblasts of *Peplonia axillaris* (Vell.) Fontella and Rapini (Apocynaceae) by NADI reagent. (f) Identification of lipids in secretory duct of *Kielmeyera appariciana* Saddi (Calophyllaceae) by neutral red under blue light. (g) Positive result for lipids in glandular trichome of *Tetradenia riparia* by Nile blue under blue light[7].

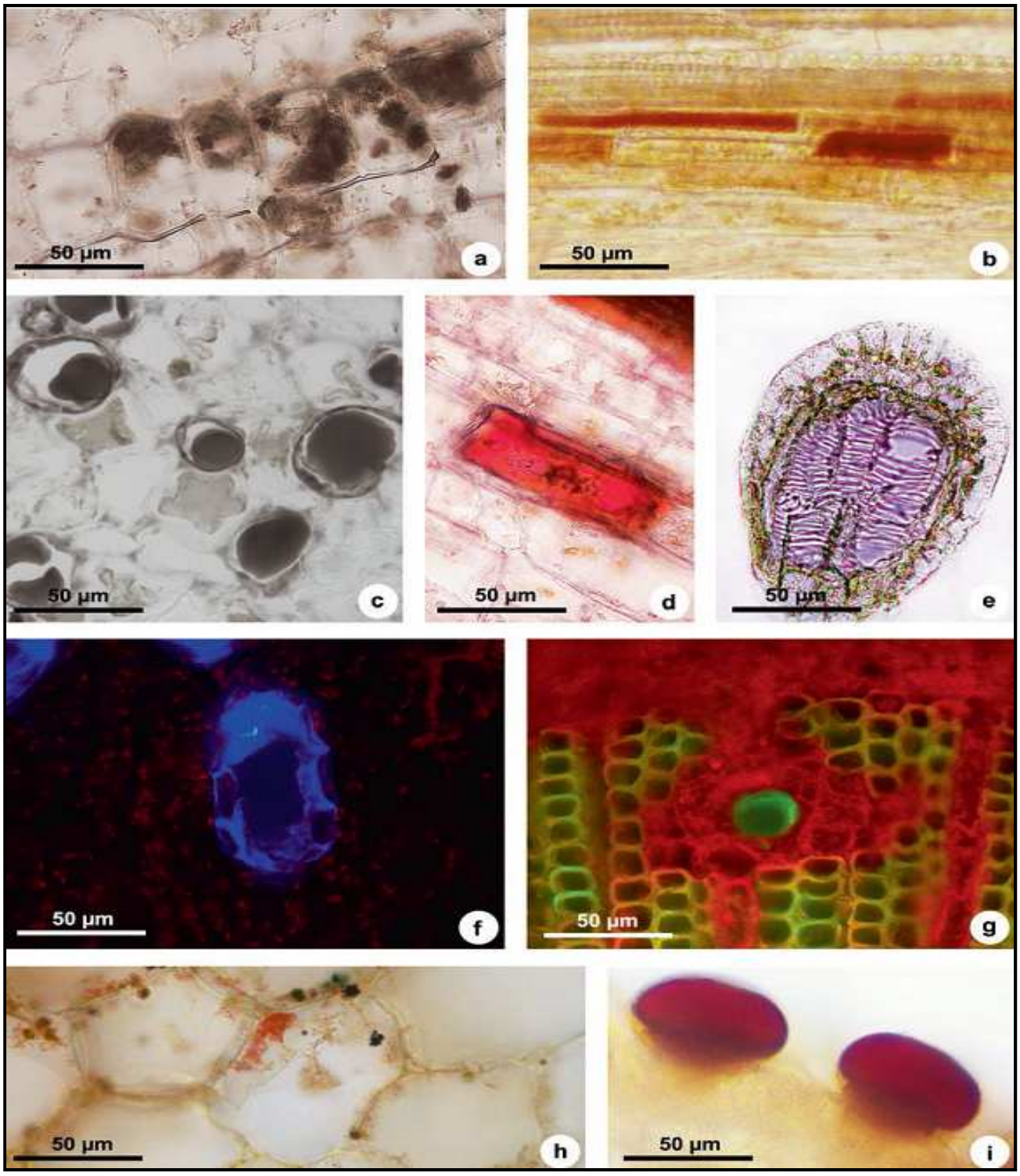

Figure No.3: Histochemical analysis of plant secretory structures. (a, b, d, f–i) Fresh material. (c, e) Embedded material. (a–c) Detection of phenolic compounds in secretory idioblasts. (a) Ferric chloride. *Acalypha amentacea* Roxb. (Euphorbiaceae). (b) Potassium dichromate. *Calliandra tweediei* Benth. (Fabaceae). (c) Ferrous sulfate in formalin[7]

CONCLUSION

Many plants contain medicinally important secondaryproducts. Therefore, histochemical investigations of different plant parts dealing with theidentification of chemical components of cells and tissues. Moreover, histological localization has many and valuable economic and pharmaceutical applications. Based on the investigation, this review concludes that histochemical localization could be used ina rapid field survey to identify the existence ofbioactive compounds in certain plants.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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