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Research article

Histochemical localization of secondary metabolites *in vivo, in vitro* leaf and leaf derived callus of *Salacia macrosperma* Wight.

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Abstract: *Salacia macrosperma* (Celastraceae) a potent anti-diabetic medicinal plant of Western Ghat, India. The present study was undertaken to evaluate the comparative histochemical characterization of *in vivo, in vitro* leaf and leaf derived callus. Histochemistry is a primary set of pharmacognestic techniques to assess the quality of herbal drugs through anatomical studies of medicinal plants. Leaf and nodal explants were used for the induction of callus and multiple shoot regeneration. 93%, 76% and 69% callus were achieved in 2, 4-dichlorophenoxyacetic acids (1.5 mg l^{-1}), Benzylaminopurine (2 mg l^{-1}) and Kinetin (1.5 mg l^{-1}) on MS medium. The histochemical investigation of fresh free hand sections of *in vivo, in vitro* leaf and callus was employed by light microscopy. The analysis showed that the presence and absence of diverse classes of secondary metabolites in various cellular localization. In the histochemical test, carbohydrates and triterpenes were present in all the three tested samples while alkaloids, lignin, tannin and flavonoids were present only *in vivo, in vitro* leaf. Most of the biochemical tests showed negative results towards callus material except starch, triterpenes, carbohydrates, oils and fats. This observation reveals field grown plant possess a maximum number of chemical constituents than *in vitro* regenerated plant and the callus. The outcome of this study concluded that the potential use of this plant species and also standardization of herbal drug formulations can be useful in the identifications of new bioactive compounds from this valuable medicinal plant species.
Keywords: Callogenesis - Histochemical - Hormones - *Salacia macrosperma*.

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INTRODUCTION

Plants are the basis for many modern pharmaceutical drugs using today for curing various diseases of human beings as well as animals (WHO 2013). The medicinal value of plants lies mainly based on the presence of various classes of secondary metabolites such as alkaloids, flavonoids, sterols and phenols, carbohydrates, proteins, saponins, triterpenes, etc. that triggered some definite physiological pathways (Nurit-Silva *et al.* 2011, Adams *et al.* 2013). The chemical characterization of the compounds in medicinal plant species is mainly based on ethnobotanical importance; it is the major strategies for the validation of its traditional use to obtaining new products. (Castro *et al.* 2011). The distinctive morpho-anatomical analysis of the medicinal plants for their authenticity and identification of secondary metabolites through specific organs where the highest concentration of bioactive substances located in herbal drug preparation (Pacheco-Silva *et al.* 2016). Some of the plants have their own ecological features, related to the environment where the plants are grown (Salvagini *et al.* 2008). The understanding of these special features may help to cultivate required plants in an optimized condition (Kuster *et al.* 2019). In search of new pharmaceuticals in plants, histochemical techniques play a major role in a quick and easy way for preliminary evaluation of phytochemicals in plants by using chemical reagents (Nurit-Silva *et al.* 2012). This technique can reduce the cost-effectiveness in herbal drug formulation as well as in search of new bioactive compounds without usage awareness of the medicinal plants (Matias *et al.* 2016).

Salacia macrosperma Wight. is an antidiabetic medicinal plant belongs to the family Celastraceae. It is a

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woody climber, under shrub, branchlets, densely lenticellate, leaves are acute at apex and base, crenulate, and flowers are many on large auxiliary or extra-axillary (Fig. 1) (Gamble 1984). The genus *Salacia* has more than 20 species, distributed in some peninsular regions of Sri Lanka, China, Brazil, Indonesia, Malaysia, Thailand, and the Philippines (Saldanha 1998). In India, it is distributed in Western Ghats regions of Kerala, Karnataka and few are found in the southern coastal region (Hooker & Hooker 1875). Some commonly used most important species of this genus are *Salacia oblonga* Wall., *Salacia chinensis* L., *Salacia reticulate* Wight., *Salacia prinoide* Willd. and *Salacia macrosperma* Wight. have high medicinal values, used for curing urinary disorders, skin diseases, leprosy, anti-inflammatory, and leaf and bark extracts used as tonic for liver disorders and stomachic (Venkatesarulu *et al*. 1992, Paarakh *et al*. 2008). *Salacia macrosperma* is the most important species among the genus *Salacia* and roots, leaves and stems of this plant have been used in Ayurvedic medicine since ancient times for curing diabetes mellitus (Nadakarni 1914, Chopra & Nayar 1956). Nowadays this plant facing the verge of rare and endemic status in the Western Ghats (Bisht *et al*. 2010). Therefore, histochemical analysis in *Salacia macrosperma* is yet to be done hence the present work is undertaken to evaluate the localization of secondary metabolites in *vivo, in vitro* leaf and leaf derived callus comparatively.

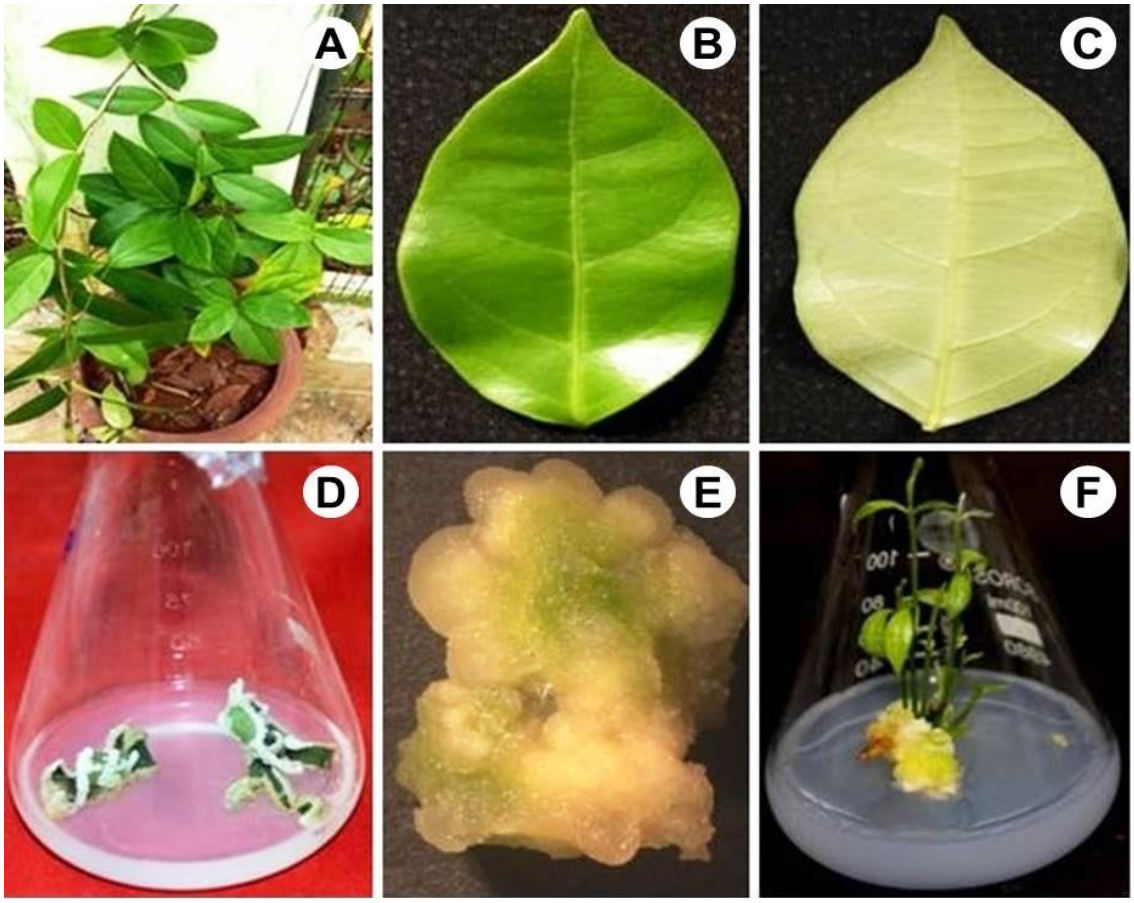

Figure 1. A, Habit of in vivo Plant; **B**, Adaxial surface of leaf; **C**, Abaxial surface of leaf; **D**, initiation of callus from leaf explants; **E**, 35 days old callus; **F**, In vitro regenerated multiple shoots.

MATERIALS AND METHODS

Chemicals

All the chemicals were procured from Sigma Aldrich and Hi-Media Lab. Pvt. Ltd. Bangalore. $HgCl_2$ (98%), Macronutrients, Micronutrients, Agar and Sucrose (Bacteriological grade), Bavistin, LB reagent (98%), FC reagent (98%), Potassium ferricyanide, Ferric chloride, Iodine, Glacial acetic acid, Sudan III, Toluidine Blue, Picric acid, and Methylene blue.

Plant material collection and explant preparation

Healthy and young (leaf) plant materials of *Salacia macrosperma* samples were collected from Virajpete, Kodagu district, Western Ghats of Karnataka, India (latitude: 12° 25' 37″ N and longitude of 75° 44' 51″ E). Authenticated with the help of plant taxonomist and few plant saplings were maintained in the medicinal garden for further work. For callus induction, young leaf explants were selected and washed thrice under tap water, then treated with Bavistin (5%) for 10 min followed by 10% tween-20 for 2 min. Further, the leaf samples were

surface sterilized with 70% ethanol for 30 seconds and finally with 0.1% $HgCl_2$ for 2–3 min and washed 2–3 times with sterile double distilled water and air-dried between sterile blotter discs and then aseptically inoculated on MS medium (Bhojwani 2012).

Callus induction and direct regeneration

The MS medium (Murashige & Skoog 1962) supplemented with 2, 4-dichlorophenoxy acetic (2, 4- D), benzylaminopurine (BAP) and kinetin (KN) various concentrations. The media contains 3% sucrose and 0.9% agar as a gelling agent and the pH of 5.8 was adjusted and autoclaved at 121°C for 15 lbs pressure for 20 min. Prepared aseptic leaf explants (1–2 cm) were inoculated on MS medium for callus induction under aseptic condition. The room temperature was maintained at 25°C with 80% relative humidity and a photoperiod of 16 h light and 8 h dark light intensity of 3000 lux. Each experiment was conducted thrice with 10 replicates per treatment. The nodal explants were used for direct regeneration of multiple shoots on MS medium supplemented with 2, 4- D in all the treatments in combination with BAP, TDZ (Thidiazuron) along with activated charcoal.

Histochemical analysis

Histochemical studies of fresh material of *Salacia macrosperma in vivo, in vitro* leaf and leaf derived callus were carried out through transverse sections made with a razor blade and microtome. Thin and clear sections were selected and treated with different reagents/dyes for the detection of various classes of secondary metabolites (Table 1). The control sections (unstained) were also maintained together for comparative investigation and specific localization of secondary metabolites in various types of tissues and cells. Fresh sections were treated with Wagner's reagent (Furr & Mahlberg 1981) for alkaloids detection, Schiff's reagent test for carbohydrates (Sass 1951), Methylene blue (aq.) for resins (David & Carde 1964), Phloruglucinal for lignin (Jensen 1962), Fast green for protein (Ramasheshan *et al.* 2017), Sudan III for oils and fats (Kraus & Arduin 1997), Vanillin for flavonoids (Mamoucha *et al.* 2016), concentrated H_2SO_4 for triterpenes (de Alcantara Guimarães *et al.* 2016), and Antimony dichloride test for triterpenes containing sterols (Mamoucha & Christodoulakis 2016). All specimens were observed under QUASMO ecostarplus optical microscope with a camera attached and all the specimens micrographs were recorded digitally.

Table 1. Histochemical staining methods used for the detection of various classes of secondary metabolites in *in vivo* leaf, *in vitro* leaf and leaf derived callus sections of *Salacia macrosperma* Wight.

S.N.	Secondary metabolites	Histochemical reagent	Observations	References
1	Alkaloids	Wagner's reagent	Golden color appearance	Furr & Mahlberg (1981)
2	Carbohydrates	Schiff's reagent test	Dark pink or magenta color	Sass (1951)
3	Resins	Methylene blue (aq.)	Blue color indication	David & Carde (1964)
4	Lignin	Toluidine blue test	Magenta color	Jensen (1962)
5	Oils and fats	Sudan III test	Brown color	Kraus & Arduin (1997)
6	Protein	Fast green	Appearance of bright green	Ramasheshan *et al.* (2017)
7	Starch	Potassium iodide solution	Blue or black color appearance	Johansen (1940)
8	Tannin	10% Ferric chloride test (aq.)	Blue-green appearance	Mace & Howell (1974)
9	Glycosides	Guignard's	Red or magenta color	Reshi *et al.* (2015)
10	Sterols	Antimony dichloride	Orange color	Mamoucha & Christodoulakis (2016)
11	Terpenes	Concentrated H_2SO_4	Light purple color	de Alcantara Guimarães *et al.* (2016)
12	Flavonoids	Vanillin test	Red in color	Mamoucha *et al.* (2016)

RESULTS AND DISCUSSION

Callus induction and direct regeneration

The induction of callus was achieved by using leaf explants of *Salacia macrosperma* supplemented with various plant hormones like 2, 4-D, BAP, and KN in MS medium. The effect of hormones for callus induction and the percentage of callus responses was reported in our previously published data (Mahendra *et al.* 2020) (Fig. 1D & E) and concentration of the plant growth regulators (PGR's) used for callus induction was standardized. The callusing was started 15 days after inoculation in leaf explants on MS medium supplemented with 2, 4-D, BAP, and KN with a concentration range of 0.5 to 2.5 mg l^{-1}. In the absence of PGR's *i.e.*, basal medium has shown negative results and explants became brownish. Among the different PGR's tried, the

highest percentage (93%) of callus formation was observed in 2, 4-D at 1.5 mg l^{-1} concentration. Likewise, in BAP and KN the maximum callus induction of 76.66% and 69.71% observed in leaf explants at 2.0 mg l^{-1} and 1.5 mg l^{-1} respectively. On the other hand, callus formation is greatly reduced due to recalcitrance when concentration of PGR's increases. Similar results were also reported by Manasa *et al.* (2017) in *Mussaenda frondosa* L. wherein, the maximum of 78%, 80%, and 70% callus induction was achieved in MS medium supplemented with 2, 4-D (3 mg l^{-1}), BAP (0.5 mg l^{-1}) and KN (0.5 mg l^{-1}) respectively. The nutritive composition of the medium, as well as concentration of PGR's, has greatly influenced the rate of induction and its morphology of callus formation (Johnson *et al.* 2010, Verma 2016). The direct regeneration was succeeded in nodal explants of *Salacia macrosperma* (Fig. 1F) on MS medium but failed to succeed indirect regeneration from leaf induced callus. The multiple shoot regeneration from nodal explants on MS medium supplemented with various PGR's was tried. The Nodal explants in some replicates induced slight callus and about 93% of multiple shoots were induced simultaneously on the MS medium supplemented with 2, 4-D (1.5 mg l^{-1}) + BAP (2.5 mg l^{-1}) + TDZ (1.5 mg l^{-1}) besides 1% AC as an antioxidant source. These findings are in line with earlier reports of Zhai *et al.* (2011) and Faisal *et al.* (2014) where they have achieved successful direct regeneration of *Caragana fruticosa* Pall. and *Mentha arvensis* L. by the combination of BAP and NAA. Our results point out that the 2, 4-D is the suitable hormone for maximum callus formation and also with the combination of other hormones for direct shoot regeneration.

Histochemical analysis

The free-hand sections of leaf and microtome section of leaf derived callus of *Salacia macrosperma* were treated with various reagents to examine the histochemical test for the presence or absence of secondary metabolites. The result obtained from the histochemical tests and their specific localization of secondary metabolites in various cells or tissues was presented in table 2. The histochemical analysis is the preliminary technique for the detection of phytochemicals localized in various cells or tissues that are examined by the change in color reactions upon staining the sections with specific chemical reagents (Sass 1951, Kuster & Vale

Table 2. Histochemical and localization of phytochemicals in in *in vivo* leaf, *in vitro* leaf and leaf derived callus sections of *Salacia macrosperma* Wight.

S.N.	Ergastic substance	Histochemical reagent	Localization of secondary metabolites		Leaf derived callus
			In vivo leaf	*In vitro* leaf	
1	Alkaloids	Wagner's reagent	Around the xylem and phloem vessels	Golden color appearance in cortical cells	-ve
2	Carbohydrates	Schiff's reagent test	Pink coloured cells at cortex region	Appearance of pink color in parenchyma cells at cortex	Pinkish color at the edges of callus cells
3	Resins	Methylene blue (aq.)	Upper and lower epidermis	-ve	-ve
4	Lignin	Toluidine blue test	Magenta color at vascular region	Magenta color at vascular region	-ve
5	Oils and fats	Sudan III, Sudan IV test	Cortex cells	-ve	Parenchyma cells
6	Protein	Fast green	Appearance of bright green at cortex regions of parenchymatous cells	parenchymatous cells	-ve
7	Starch	Potassium iodide solution	-ve	Dark blue color granules around the vascular elements	Granules at the cortical cells of callus
8	Tannin	10% Ferric chloride test (aq.)	Sclerenchyma cells appears blue green color	Sclerenchyma cells	-ve
9	Glycosides	Guignard's	Sclerenchyma and peripheral cells of vascular elements	-ve	-ve
10	Sterols	Antimony dichloride	Orange color at vascular elements	-ve	-ve
11	Triterpenes	Concentrated H_2SO_4	Light purple color cortex cells	Cortex cells	Blue color at the cortex region
12	Flavonoids	Vanillin test	Golden yellow cells around vessel elements	Cells around vessel elements	-ve

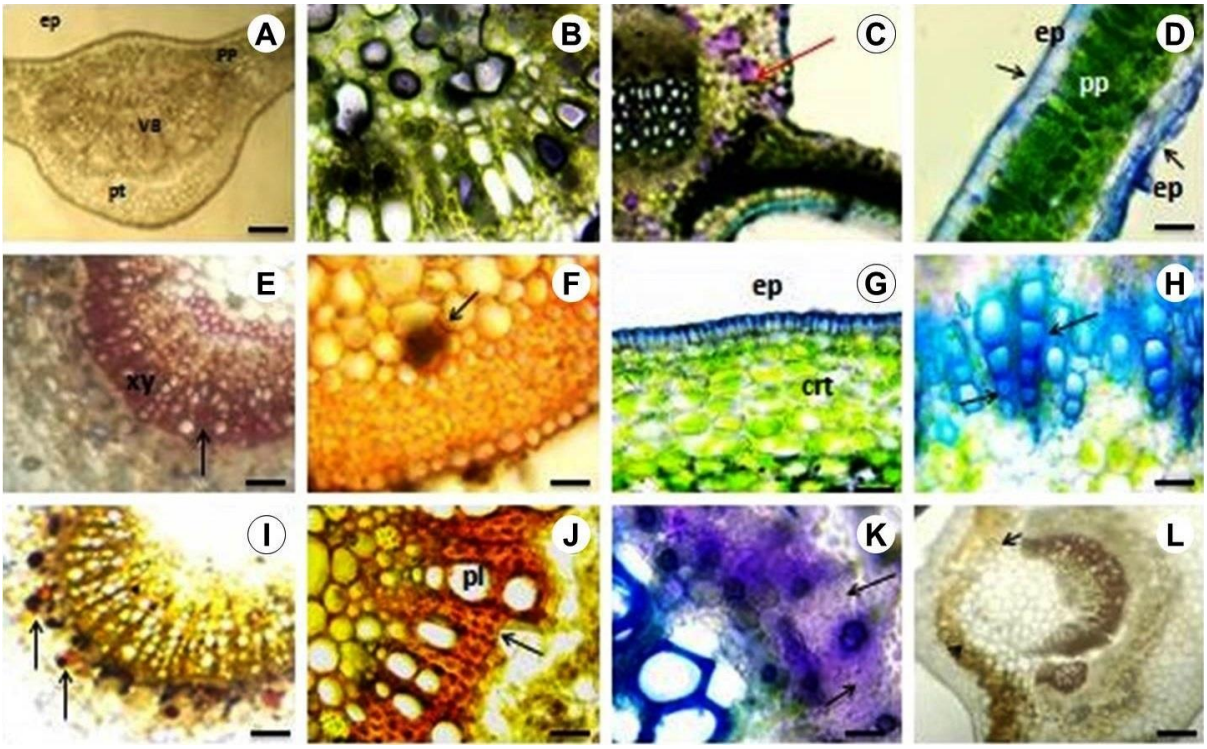

Figure 2. Leaf histochemical Studies *in vitro* plant leaf: **A**, Control section; **B**, Alkaloids (slight golden color); **C**, Carbohydrates (dark pink); **D**, Resins (bluish); **E**, Lignin (Red color cells at vascular region); **F**, Essential oils in the secretary cells (dark brownish); **G**, Protein (Bright green); **H**, Tannin (blue green appearance); **I**, Glycosides (Red color peripheral cells of vascular elements); **J**, Sterols (Orange color Xylem vessels); **K**, Triterpenes (Light purple color cortex cells); **L**, Flavonoids (Greenish golden yellow).

2016). The confirmation of alkaloids in *in vivo*, *in vitro* leaf and leaf derived callus free hand sections and microtome sections were revealed the appearance of golden yellow colored cells after treating the sections with Wagner's reagent (Furr & Mahlberg 1981). The alkaloids were located around the cortical region of xylem and phloem vessels of *in vivo* and *in vitro* leaf (Figs. 2B & 3B), but which is absent in leaf derived calli section. The intensity of the colour reaction shows that, the alkaloid content for *in vivo* and *in vitro* leaf studies was unique. Alkaloids are the major class of compounds in most of the plant species played a vital role in pharmaceutical industries like antimicrobial agents, haemoglobinizators of leukemia cells and also acts as active stimulators, inhibitors and terminators of endogenous security regulation mechanism (Waller 2012). Detection of carbohydrates was made by treating the sections with Schiff's reagent solution accordance with the report of Sass (1951), the result shows that pink color parenchymatous cells at mesophyll region of both *in vitro* and *in vivo* leaf and edge cells of leaf derived callus, which clearly indicates the presence of carbohydrates (Figs. 2C, 3C & 4E). Carbohydrates themselves have not been found to have a therapeutic effect, but may possibly increase the effectiveness of the therapeutically important ingredients. Recently it has been employed in producing polysaccharide which act as immunomodulators with therapeutic and vaccine implications (Opdenakker *et al.* 2012).

The occurrence of resins and lignin was noticed in *in vivo* and *in vitro* leaf of *Salacia macrosperma*, are in accordance with David & Carde (1964); Jensen (1962) respectively. The sections were treated with methylene blue and toluidine blue, which results that upper epidermis and mesophyll cells turns bluish-green color in *in vivo* leaf indicates the presence of resins (Fig. 2D) but lignin present only in both *in vivo* and *in vitro* leaf (Figs. 2E & 3D). Lignin and its derivatives are the most abundant complex organic polymer particularly helps in the formation of cell wall thickenings of vascular plants and also has many applications in tissue engineering (Spiridon *et al.* 2018).

Oils and fats in leaf and callus sections were confirmed by the presence of blue-colored cells located in the upper and lower epidermis of the leaf (Fig. 2F), whereas in the callus section it was observed in the uniform parenchymatous cells (Fig. 4G). Earlier reports made by Ramasheshan *et al.* (2017) staining the sections with fast green stain resulted in the formation of bright green colored cells of vascular bundles of *in vivo* leaf section and pith regions of *in vitro* leaf section confirming the presence of proteins (Figs. 2G & 3E). Proteins and amino acids are the primary metabolites help to produce secondary metabolites by their metabolic process (Gutzeit & Ludwig 2014). According to the report of Johansen (1940) around the vascular elements, cells colour changes to

bluish-black upon treating the section with potassium iodide solution indicates the presence of starch granules, in the present investigation *in vitro* leaf and leaf derived callus exhibits positive result (Figs. 3F & 4H). The appearance of bluish-green-colored cells in the vascular bundle and upper epidermis regions of *in vivo* and *in vitro* leaf confirmed the presence of tannins respectively (Figs. 2H & 3G), wherein the results are contradictory to the earlier reports of Mace & Howell (1974). Tannin compounds are widely distributed and play a role in protection from predation and in plant growth regulation (Edeoga & Ogbeboro 1999) and also it can evoke an antidiarrheal effect and these substances may precipitate proteins on the enterocytes reducing peristaltic movement and intestinal secretion (Su *et al.* 2000). Glycoside test was carried out accordance with Reshi *et al.* (2015) by using Guignard's reagent led to the appearance of reddish to magenta color in the peripheral sclerenchymatous region of vascular elements of *in vivo* leaf exhibits the presence of glycosides (Fig. 2I), but absent in *in vitro* leaf and leaf derived callus sections.

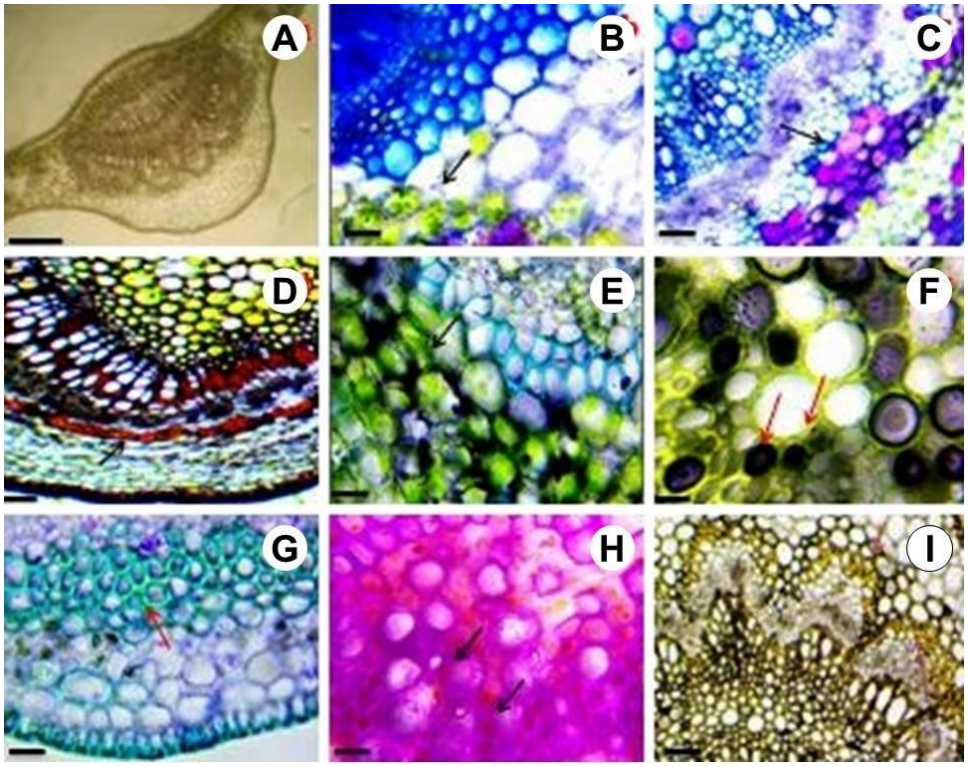

Figure 3. Leaf histochemical Studies *in vitro* plant leaf: **A**, Control section; **B**, Alkaloids (Golden color appearance cortical cells); **C**, Carbohydrates (Pink color in parenchyma cells at cortex); **D**, Lignin (Magenta color at vascular region); **E**, Proteins (Bright green at cortex region); **F**, Starch (Dark blue color granules around the vascular elements); **G**, Tannin (Blue green color schlerenchyma cells); **H**, Triterpenes (Light purple color cortex cells); **I**, Flavonoids (Greenish golden yellow).

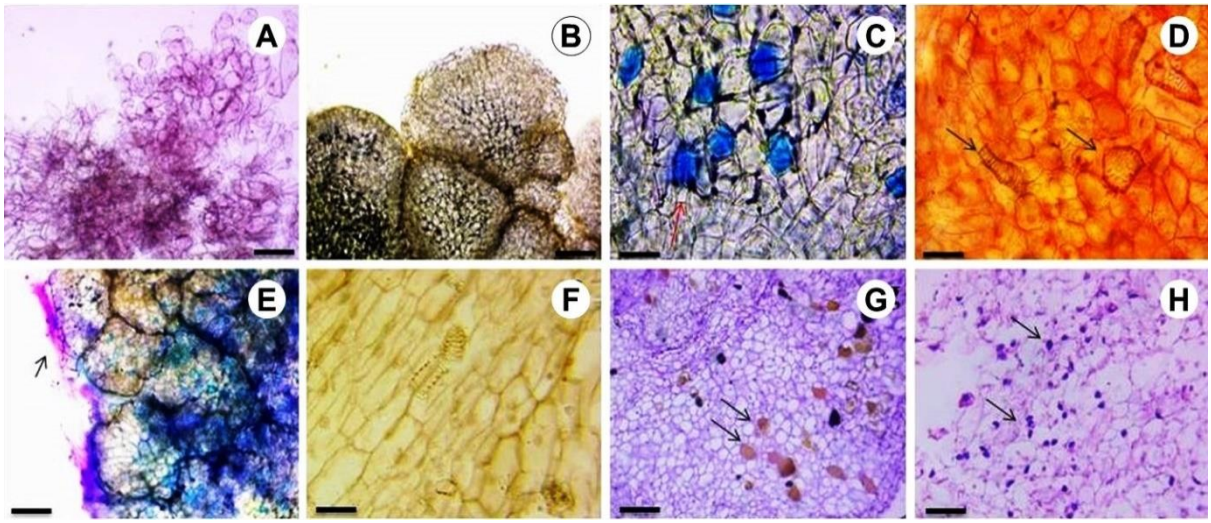

Figure 4. Callus histochemical studies: **A**, Normal callus cultured cells; **B**, Control section of callus; **C**, Terpenoids (blue color at the cortex region) **D–E**, Carbohydrate (Pink color at the peri vascular region); **F**, Vascular elements; **G**, Essential oils in the secretary cells (Brown color cells); **H**, Starch (Dark blue color granules in cortex region).

Presence of sterols was evaluated by using antimony dichloride reagent in accordance with the report of Mamoucha & Christodoulakis (2016), results shows that orange colour appearance at vascular elements of *in vivo* leaf exhibits the positive results (Fig. 2J), and the negative result was observed in callus cells and *in vitro* leaf due to the lack of sterols. Steroids have been reported to have antimicrobial properties, analgesic properties and act on central nervous activities (Neethu *et al.* 2018). Triterpenes was evaluated using concentrated H_2SO_4 in accordance with the de Alcantara Guimarães *et al.* (2016), reveals the presence of triterpenes through the appearance of light purple colour cortex cells of *in vitro* and *in vivo* leaf section (Figs. 2K & 3H), blue color at the cortex region in leaf derived callus section (Fig. 4C). According to Mamoucha *et al.* (2016) appearance of greenish golden yellow cells reveals that the presence of flavonoids. In our study, cells around the vessel elements of the *in vitro* and *in vivo* leaf section show the positive results for flavonoids (Figs. 2L & 3I) and callus shows a negative result. Flavonoids mostly impart pigmentation in plants to attract pollinators. Various *in vitro* studies have been proved that flavonoids are medicinally significant. It has already been reported that flavonoids and phenolic compounds possess a wide array of medicinal properties like hepatoprotection, anti-inflammation, immunomodulation, anti-carcinogenic, anti-infertility (Saravanakumar *et al.* 2009, Sandhar *et al.* 2011).

CONCLUSION

This study revealed the presence of secondary metabolites in callus, *in vivo* and *in vitro* leaf comparatively *Salacia macrosperma* by histochemical techniques. The histo - anatomical analysis provides more specific data and determined the actual storage site of chemicals substances in the plants. The result obtained from the study exhibited *in vivo* plant shown maximum positive results than *in vitro* regenerated leaf and callus. The outcome of this study concludes that the characterization of this valuable medicinal plant species in various aspects can be useful and it paves the way to various pharmacological aspects.

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