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In vitro seed germination, callus induction and naphthodianthrones production in Hypricumm triquetrifolium Turra

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Abstract

The present study was designed to investigate seed germination and callus induction *in vitro* and to produce (naphthodianthrones) hypericin and psudohypericin in *Hypericum triquetrifolium* Turra tissue cultures. Seeds were germinated aseptically on MS medium and water agar medium. Seeds germinated on water agar were better than those germinated on ½ MS medium. Germination percentage increased after treatment with GA_3 at 100 mg L^{-1}. Leaf, stem and root explants were excised from *in vitro* grown seedlings. Callus was initiated on the leaf discs which were previously cultured on MS medium supplemented with Thidiazuron (TDZ) at different concentrations included 1.0, 1.25, 1.5, 2.0, or 2.5 mg L^{-1} and indole-3-acetic acid (IAA) at 0.5 mgL^{-1}. It was observed that the combination of TDZ at 2 mg L^{-1} with 0.5 mg L^{-1} IAA supplemented to MS medium, yielded the highest fresh and dry weights of callus initiated on leaf explants. The same combined mixture was used for callus maintenance, The same components described for callus maintenance medium devoid agar, was used for initiating cell suspension cultures. To define the type and quantity of the secondary metabolites, HPLC was depended. Hypericin and pseudo-hypericin were not detected in calli, and cell suspension cultures initiated on leaf explants, while they were detected in calli and cell suspension cultures initiated on stem and root explants.

Introduction

Medically important plants are a significant source of life-saving drugs worldwide. Plant secondary metabolites are the economically important as drugs, pigments, food additives and pesticides [1]. The genus Hypericum includes nearly 460 different species of annuals, perennials, shrubs, and small trees, spread all over the world [2]. Only sixteen species wee reported in Iraq with the most abundant herbs (Hypericum perforatum L), and (H. triquetrifolium Turra) [3]. Some species were used since ancient times as folk remedies; medicinal uses included antimicrobial, antifungal, antitumor, antiviral, sedative and also for the treatment of neurological disordered and depression [4]. It was observed that the aqueous extract of H. perforatum can reduce the serum lipid profile among rats in the terms of (LDL-CH) and triglyceride (TG), also the enzymes and markers of oxidative stress which are commonly caused by hyperlipidemia [5].

Various phytochemicals were extracted from H. perforatum and other related species, included different phenolic compounds like chlorogenic acid, tannic acid, and caffeic acid. Moreover, flavonoids like rutin, hypersoid, isoquercitrin, quercitrin, quercitin, and catechin, naphthodianthrones like hypericin and psudohypericin, the phloroglucinols like hyperforin and adhypeforin as well as essential oils were extracted [6,7,8 and 9]

H. triquetrifolium Turra belongs to the Hypericaceae family and is a wild growing weed in the northern part of Iraq. The local Arabic name for this species is Roja, and the Kurdish name is Zwrnatik [10].

Plan biotechnology can provide an important alternative way for the production of a massive scale of plant secondary metabolites. Biotechnological approaches for the above purpose, particularly for plant cell and tissue culture, showed limited success due to the small and unreliable yields of the secondary products [11]. Significant improvements in product yields have been achieved through conventional biochemical approaches and the manipulation of the culture and process factors. Most applications of the plant cell cultures in biotechnology were designed for the production of bioactive secondary metabolites. There is a noticeable shortage in scientific investigations on the medical use of H. triquetrifolium in Iraq.

The current study is aimed to:

- Design a protocol for efficient seed germination for this medicinally important plant.
- Initiate callus and cell suspension culture on excised leaf, stem and root explant.
- Production of hypericin and psudohypericin from callus and cell suspension culture.

Materials and Methods

Plant material

From the intact plants, seeds of H. triquetrifolium Turra were collected during December 2010 to January 2011. The plants were from a wild population in the locality of Tasluja, in the dry rocky soil of Sulaimani city, altitude 1000 m.a.s.l. North of Iraq. Room temperature for seven days depended on for drying the collected samples, then kept in a dark, dry and cool place after putting in paper bags.

Seed sterilization

For surface sterilization of the H. triquetrifolium Turra seeds, 70% (v/v) ethanol was used by immersing the seeds for about half minute. Then 20% (v/v) commercial bleach (5% NaOCl) plus 0.1 Tween 20 (polyoxyethylene sorbitan monolaurate) was used for about 25 minutes. Subsequently, they were washed four times with sterilized distilled water [12].

Seed germination

Two methods depended on for seeds germination:

1. Applying the pre-soaking treatments with different GA_3, H_2SO_4 doses, and distilled water before putting in Petri dishes. The treatments were soaked in 50, 100, and 150 mgL^{-1} GA_3, 1.5% H_2SO_4 and distilled water for 30 minutes. Petri dishes containing moisture-retaining paper liners were used by placing the treated seeds individually, then the paper liners were kept moist throughout the germination period. The seedlings were inoculated to test tubes (5×9.5cm) containing 25 ml of the ½ strength MS medium solidified with eight gL^{-1} agar.The cultures were subjected to a photoperiod of 16 hrs light/8 hrs darkness in a growth chamber at 25°C. Twenty days later, germination was measured, seeds showed radical emergence which was recorded as germination [13].

2. Sterilized seeds were cultured on eight gL^{-1} agar (water-agar medium), after pre-soaking treatments. A seed was placed in each test tube (5×9.5cm) which contains 25 ml of water-agar. The cultures were retained at 25°C under a photoperiod of 16/8 hrs (light/dark) and checked every day (20 days) [14].

MS medium

By dissolving 4.405 gL^{-1} from MS ready-made mixture medium (Duchefa-Netherland), Murashige and Skoog's medium was prepared, which contains macronutrients, micronutrients, vitamins and Myo-inositol (15), supplemented with 30 gL^{-1} sucrose. To the medium eight gL^{-1} agar was added. The total volume of the mixture was completed to one litter by DDH_2O, and the pH was adjusted to 5.8. The Hotplate magnetic stirrer was used to dissolve the medium and then dispensed into (15×2.5 cm) test tubes (10 ml/tube). They autoclaved at 1.04 Kg/cm^2, 121°C for 15 min, then allowed to cool before seeds or explants were inoculated [16].

Induction of the Callus

1. The seedlings were collected from two to three weeks germinated seeds.
2. Using surgical blade and forceps under aseptic conditions, they were fragmented into one cm long explants -leaf, stem, and roots.
3. The leaf explants were inoculated into MS medium, supplemented with IAA (0.0 or 0.5) mgL^{-1} and TDZ (0.0, 1.0, 1.25, 1.5, 2.0 or 2.5) mgL^{-1}.
4. Auxin IAA and cytokinin BAP were added to the same medium to be ready for inoculation of stem and root explants. The cultures then incubated at 25°C (16 hours in light and 8 hours in dark). A month later the callus fresh and dry weight were determined according to [12].

Cell Suspension Cultures

Nearly five to ten grams of callus fresh weight was cultured in 50 ml MS liquid medium with the same ingredients as the maintenance medium without agar. H. triquetrifolum cell suspension cultures were prepared from callus cultures of 21-28 days old. Flasks containing cell suspensions were placed on a rotary-shaker (100-120 rpm) at 27°C under certain conditions (16/8 hrs light/dark with a light intensity of 1000 lux in a growth room for 14 days) [17].

Preparation of plant extracts

Plant material was dried at room temperature (25 ± 2 °C).To obtain a homogenous powder, 0.5 g of the dried plant material (leaf, stem, and root) that dissected from seedlings in vitro; LC, SC, RC, LCs, SCs, and RCs was ground using a laboratory mill. Using ultrasonic cleaning bath (frequency=35 KHz), extraction process was carried out with 20 ml of methanol (HPLC grade) for 30 min (to remove chlorophyll contents) according to [18]. The water bath was adjusted at 30°C for two hours using a magnetic stirrer (protected from light using aluminum foil and glass covers).

The extract was separated, and the residual sample was re-extracted using methanol (10 ml) for about 1 hour (this process was repeated twice). The mixture was centrifuged at 3000rpm, using rotary vacuum evaporator the process of concentration was followed. Millipore filter paper (22 µm) were used for extract filtration. The prepared extract was kept in a dark bottle in a refrigerator until use. Using HPLC, three readings were achieved for each sample; the mean value was calculated according to [6, 19].

Separation and quantification of hypericin and psudohypericin

The extracted samples, Hypericin and psudohypericin compounds were separated for leaf, stem, and roots of H. triquetrifolium Turrra depending on a method described by [16].

A Shimadzu liquid chromatograph (Shimadzu Corp, Kyoto, Japan) were used which consist of an LC-20 AT quaternary pump, a DGU-20 A3 degasser, an SPD-M20A diode array detector and a manual rheodyne injector with a 20µl loop. Through comparing the retention times with those of the reference standard and UV spectra in the range of 200-800nm, the peaks were identified (Figure.1).

Identification of hypericin derivatives (pseudohypericin) was carried out after 30 min. Exposure of the HPLC vials to light source immediately before analysis [20]. The standard curves were obtained by plotting the peak areas of standard concentrations for hypericin and psudohypericin (0.025, 0.05, 0.08, or 0.1 $mgml^{-1}$). Standard solutions were kept in dark at -20°C to prevent oxidation of phloroglucinol and the light conversion protohypericin and protopsudohypericin to hypericin and psudohypericin respectively [21].

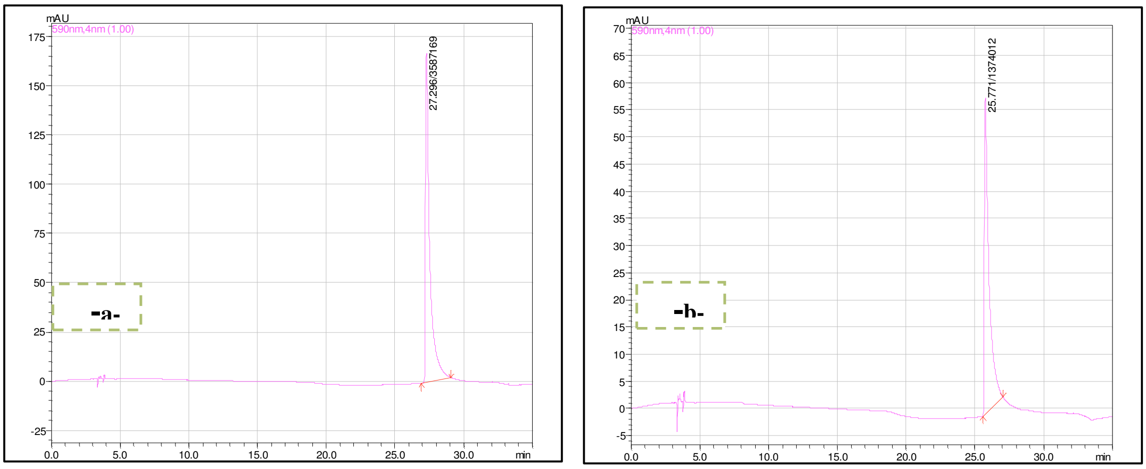

Figure- 1: Original chromatogram of standard: (a) Hypericin 0.05 mg/ml, (b) Pseudohypericin 0.05 mg/ml

Determination of total hypericin

The total hypericin contents were determined in the methanol extract depending on a method described by [22].The extracted samples were prepared as mentioned before. The absorbance was measured at the wavelength 590 nm using a spectrophotometer. For each sample, three replicates were analyzed, and the means were computed. The analysis was performed by using five-point calibration curve generated with pure hypericin using a spectrophotometer.

Standard curve preparation for total hypericin determination

Different concentrations of pure hypericin (0.00062, 0.00125, 0.0025, 0,005, and 0.01 mgml^{-1}) were prepared to generate calibration curve. Red color intensity was measured at an absorbance of 590 nm using spectrophotometer (Figure. 2).

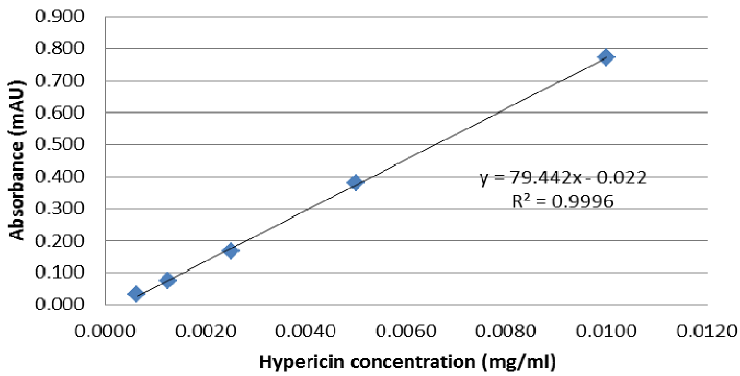

Figure-2: Calibration curve for standard total hypericin

Experimental design and statistical analysis

Completely randomized design –one way- depended for statistical analysis,15 replicates were used for tissue culture experiments. The same model depended for sample analysis with three replicates using Duncan's multiple range tests. SPSS, V. 17 software was used for data analysis [23].

Results and Discussion

Effect of Gibberellic and Sulphuric acids on seed germination

Figure. 3 Showed that the pre-soaking treatments had a significant ($p \leq 0.05$) effect on germination (%) depending on the treatment and type of media. Seeds germinated successfully within seven days (Fig. 4B), and the germination rate on water-agar was better than ½ MS medium.

Results showed that seeds were sown in ½ MS medium and pre-treated with 150 mgL^{-1} GA_3 achieved the highest % of germination (34.66%), whereas the lowest (10.33%) occurred in those pre-soaked with D.W. Germination on water-agar medium exhibited a maximum germination % in GA_3 treated seeds at 100 mgL^{-1} (41%), followed by 1.5% H_2SO_4 (37.33%), GA_3 150 mgL^{-1} (33%), and GA_3 50 mg L^{-1} (30.66%). On the other hand, germination reduced by D.W. treatment (10.66%) [13] found that the highest germination rates obtained from H. origanifolium and H. pruinatum were obtained in seeds treated with GA_3 at 100 mgL^{-1} and 150 mgL^{-1} respectively. [14]stated that water- agar supported better germination (89.9%) than ½ MS medium (67.8%) for H. triquetrifolium seeds.

The present study was performed under 16/8 hrs light/dark photoperiod; light has been recognized as germination- controlling factor and it is frequently found to be a requirement in some plant species native to arid land [24].The absence of light has an adverse effect on germination in several Hypericum species such as H. perfratum [25], H. gramineum [26], H. brasiliense [27], H. aviculariifolium [28] and H. leptophyllum [29]. [30] found that 16/8h. light/dark cycle was noticed to be more efficient to meet the light requirement for germination in H. perforatum seeds.

In the current study, H_2SO_4 treatment (Fig. 4A) improved seed germination. Results indicated the presence of physical dormancy related to internal inhibitors or hard seed coat and can be overcome by acid treatment [31].

In this study, GA_3 increased germination % significantly; when seeds were germinated on ½ MS medium, depending on its concentration combating physiological dormancy related to the partially dormant embryo. Similar results were obtained from studies carried out on other species, such as Sesamum indicium (32), Rumex dentatus [33], Zea mays and Glycine max [34], Opuntia tomentosa [35]and Physoplexis comosa [36].

Chemicals that accumulate in the fruit and seed -coat during the development remain in the seed after harvest they can act as germination inhibitors. Some of the substances associated with inhibition are various phenols, coumarins and abscisic acid which can be leached out by soaking in water [37].In the case of H. perforatum and H. aviculariifolium, soaking seeds in D.W. resulted in a significant increase in germination [38]. In the present study, similarly, D.W. treatment slightly increased germination rates in both media, but this increase was found to be non-significant when compared with the control.

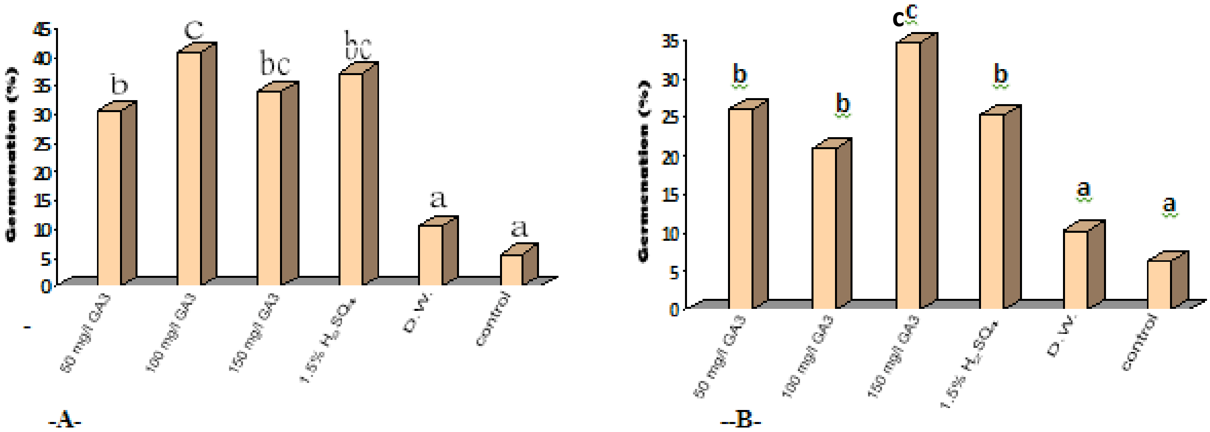

Figure-3: Germination percentages of *H. triquetrifolium* Turra seeds exposed to different pre-soaking treatments under 16/8 hrs illumination, (a) Germination on ½ MS medium, (b) Germination on water-agar medium.

Note: Different letters refer to significant differences between values ($p \leq 0.05$) according to Duncan's multiple range tests

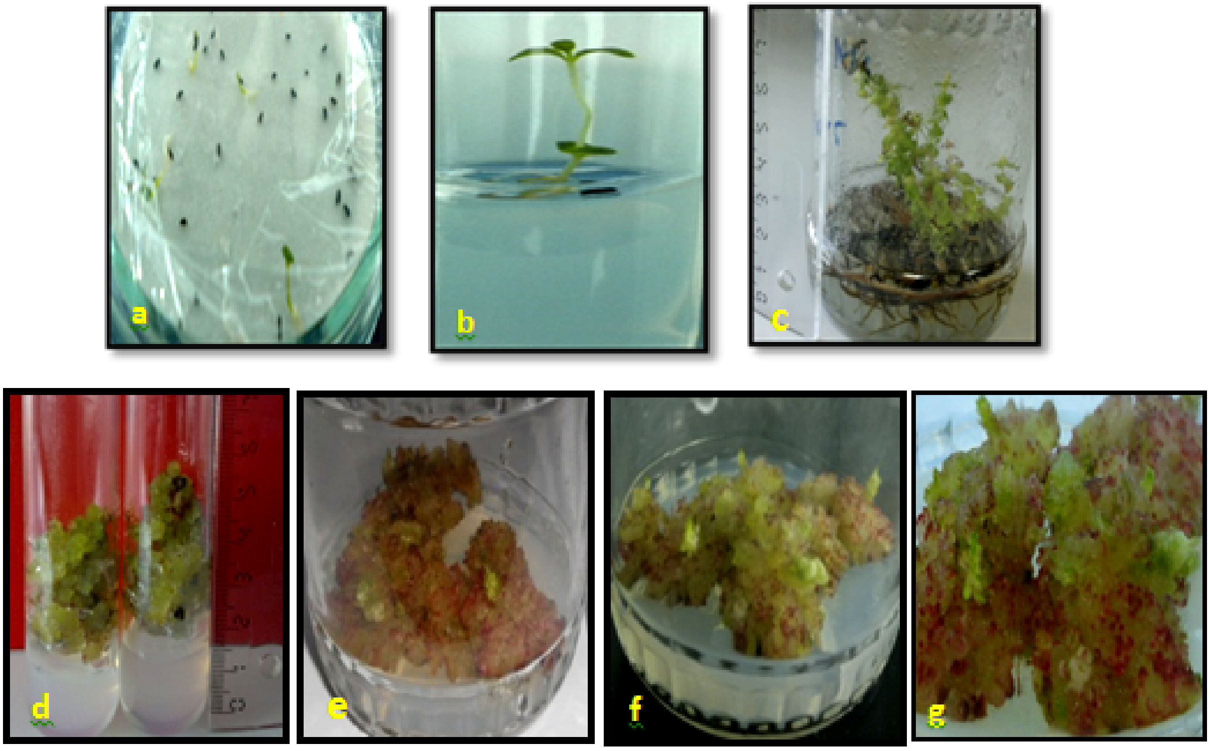

Figure-4: *In vitro* seed germination of *H. triquetrifolium* Turra. (a) H_2SO_4 pre-soaking treatments, (b) Germination of seeds after seven days, (c) Seedlings grown on ½ MS medium, 35-42 days after sowing, (d) Callus derived from leaf explants treated with a combination of 2 mgL^{-1} TDZ and 0.5 mgL^{-1} IAA, (e) Callus derived from stem explants treated with a mixture of 1.25 mgL^{-1} BAP and 0.5 mgL^{-1} IAA, (f) Callus derived from root explants treated with a mixture of 1.25 mgL^{-1} BAP and 0.5 mgL^{-1} IAA, (g) Red pigmentation in callus cultures derived from stem explants indicating the presence of hypericin *in vitro*.

Effect of TDZ in the presence of IAA on callus induction

Callus initiated on wounded ends and spread towards the middle region of explants. The effect of TDZ concentrations on callus fresh weight in the presence of 0.5 mgL^{-1} of IAA is shown in Table 1. Explants formed calli at all the concentrations tested except controls. The best response occurred on MS medium containing 2.5 mgL^{-1} TDZ which gave the highest value (%100); callus fresh and dry weight were significantly higher than other treatment when the concentration of TDZ decreased to 2 mgL^{-1} recording 77.51 g and 2.90 g respectively. Other values fluctuated depending on the concentration of TDZ (fig. 4 D).

It was reported that a few species within the genus Hypericum had been used to produce callus cultures. In H. erectum, callus induction was obtained by inoculating seedling explants in the presence of IAA and BAP in the dark [39].Using nodal explants of H. brasiliense, the combination of cytokinins and auxins did not support callus growth. However, it was obtained in the presence of 2,4-D or NAA using either MS or B5 medium under a 16 hrs photoperiod [40].

Callus induction and proliferation are known to be very useful for the study of the biosynthesis of natural products and the factors influencing it, giving some possibilities of controlling production. In several Hypericum species this approach has been used successfully [41 and 42].

TDZ is more efficient than other cytokinines for inducing callus formation in some plant species. TDZ may work as a coordinator for the induction of mRNA and sucrose transportation. Additionally, it induces the synthesis of mRNA which in turn increases the synthesis of protein and ultimately induces cell division [43].

From the current study and within one month, compact green, mostly with dense areas, and granular calli were formed on leaf explants (Fig. 4 D). The majority of calli were green and friable exhibiting dense red pigmented areas indicating the presence of hypericin (Fig. 4 E, F, and G).

Results obtained from this study agreed with observations reported by others [44] who indicated that different concentrations of TDZ tend to promote the formation of compact, green calli on leaf explants in Hypericum spp.

Table-1: Effect of different concentrations of TDZ mgL^{-1} in the presence of 0.5 IAA mgL^{-1} on the response (%) of callus initiation, the fresh and dry weight of leaf explants.

TDZ (mgL^{-1})	**Response to Callus formation (%)**	**Fresh weight (g) (Mean ± S.E.)**	**Dry weight (g) (Mean ± S.E.)**
0.0	0.0	0.0	0.0
1.0	80	61.11 ± 0.22^{b}	2.55 ± 0.05^{c}
1.25	72	62.16 ± 0.17^{c}	2.15 ± 0.03^{b}
1.5	72	61.68 ± 0.21^{bc}	2.05 ± 0.03^{b}
2.0	88	77.51 ± 0.33^{d}	2.90 ± 0.03^{d}
2.5	100	12.92 ± 0.22^{a}	0.76 ± 0.06^{a}

Note: Values followed by different letters in the columns are significantly different at ($p \leq 0.05$, bars; ± S.E.) according to Duncan's multiple range test.

Determination of Hypericin, Psudohypericin and total hypericin

Results showed that explants were grown on solid MS medium (leaves, stems, and roots) Fig. 5 A, B and C contain the highest level of hypericin, psudohypericin and total hypericin (0.0062, 0.0289 and 0.0651), (0.0039, .0157 and 0.0403), and (0.0003, 0.0019 and 0.0088 mgg^{-1}) for explants and compounds respectively when compared with calli and cell suspension derived from these parts. This fact was confirmed by researchers who stated that hypericin contents in methanol extracts of dried flowers, leaves, stems and roots of H. triqutrifolium Turra were determined [20]. However, these results disagreed with those of [6]who reported that, in the case of H. triqutrifolium, the dark glands containing hypericin are distributed along the leaf margin and stems while they are absent in the other plant organs (not detected in root).

In the current study, callus and cell suspensions derived from leaf stem and root explants contained significantly less hypericin, psudohypericin and total hypericin compared to the in vitro initiated plant parts. Hypericin and psudohypericin were not detected in calli and cell suspension cultures derived from the leaf. Observation by this study was n parallel with the results of [45 and 46], who concluded that the production of hypericin and psudohypericin in vitro plants were not detected in calli and cell suspensions, and significantly less than that found in in vivo plants. Moreover our results disagreed with conclusions noticed by [47], they analyzed Hypericin contents in vitro by a UV-VIS spectrophotometer at 589 nm and reported the level of hypericin found in callus were 0.0527 and 0.0485 mg g^{-1}, whereas for the cell suspension cultures the rates were lower (0.0018 and 0.0016 mg g^{-1}). Their observations suggested further modifications are needed for accumulation of this compound in the cell suspension.

Other investigators indicated that the absence of hypericin in cell suspension cultures and calli derived from leaves seems to be in contrast with their ability to synthesize xanthones, which partially share the same pathway as hypericin, and enzymes involved in anthranol synthesis and dimerization to hypericin are missing [48].

It was noticed from the current study, that the accumulation of hypericin and psudohypericin in calli and cell suspension cultures derived from stems and roots exhibited visible red pigments containing hypericin (Fig. 4 F and G). [49] indicated that hypericin content in calli and cell suspension cultures derived from stems and roots was attributed to the presence of BAP in the medium for initiation of callus and cell suspension. The investigation was conducted to establish the relationships between plant growth regulators (including BAP) and naphtodianthrones production [50 and 51] They reported that the concentration range of BAP from 1-2 mgL^{-1} improved the production of hypericin. Similarly, in this study supplement of 1.25 mgL^{-1} of BAP to the culture medium improved hypericin content in H. triqutrifolium tissue cultures.

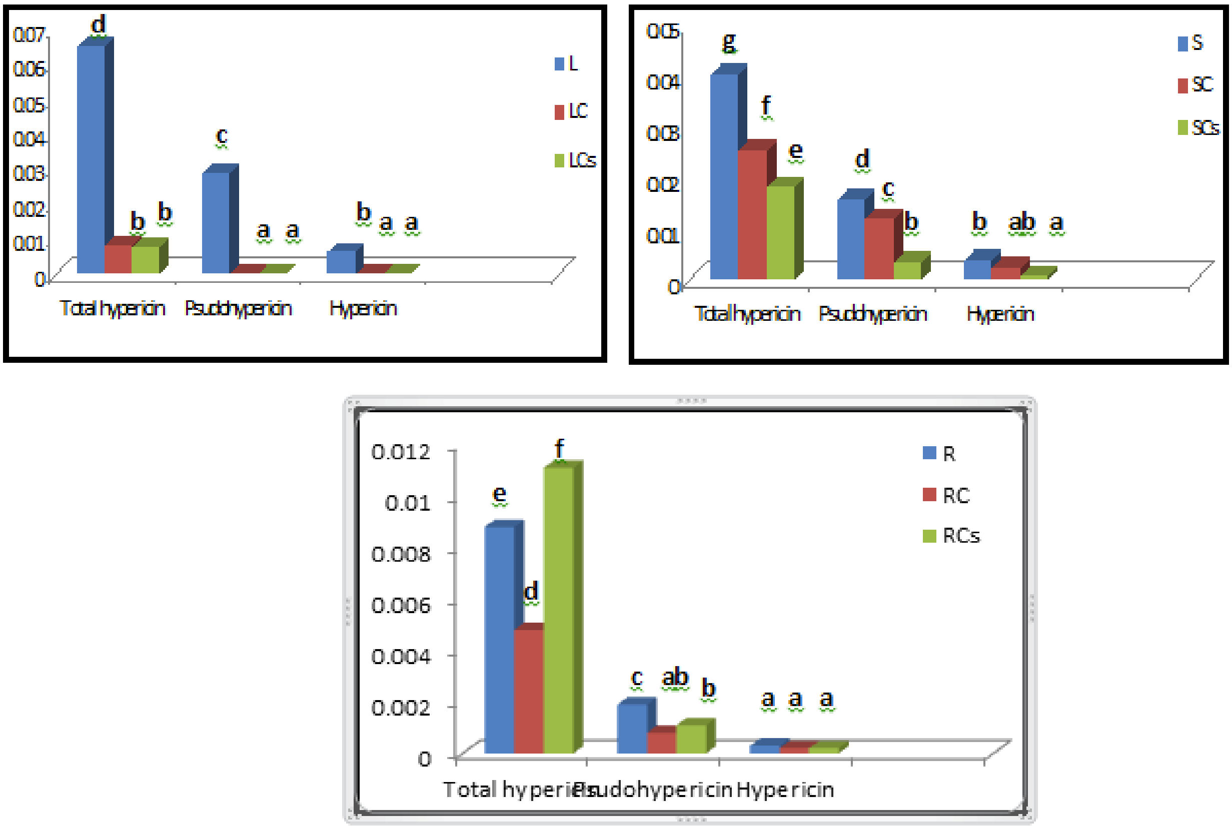

Figure-5: Concentrations (mgg^{-1} dwt.) of hypericin, psudohypericin and total hypericin in different plant parts, (a)- L: Leaves initiated *in vitro*, LC: Callus derived from leaf, LCs: Cell suspension cultures derived from leaf, (b)- S: Stem initiated *in vitro,* SC: Callus derived from stem, SCs: cell suspension cultures derived from stem, (c)- R: Root initiated *in vitro*, RC: Callus derived from root, RCs: Cell suspension cultures derived from root.

Note: Values followed by different letters significantly different at ($p \leq 0.05$) according to Duncan's multiple range test.

Additionally, leaves grown in vitro accumulated higher amounts of pseudohypericin than hypericin compared with stems and roots. Similar results were carried out on different Hypericum species which reported the yield of psudohypericin was greater than hypericin. Leaves were covered by dark glands, mainly in margins, containing hypericin [13 and 52].

Statistical analysis justified that explant type was the only factor affecting the hypericin content. Similar results were found by [53], who noticed that hypericin percentage in shoots obtained from leaf disc-originated calli was higher than that of stem segment-originated ones recording 0.048 and 0.031% respectively. Explant type is one of the main factors regulating and directing secondary metabolite synthesis.

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