Cytotoxic Activity of Taxodium Calli Extracts on Rat Liver Cells

1Abou Dahab M.A., 2El-Bahr M.K., 3Taha H.S., 1Habib, A.M.,
3Bekheet S.A., 2Gabr A.M.M. and 3Refaat, A.

1Department of Ornamental Plant, Faculty of Agriculture, Cairo University, Giza, Egypt.
2Department of Plant Biotechnology, National Research Center, Cairo, Egypt.
3Department of Phytochemistry, National Research Center, Cairo, Egypt.

Abstract: This study aimed to establish a promising protocol for callus production from T. distichum and T. distichum var. 'distichum' tree. Nodel explants were used as staring material for callic production. Calli cultures were induced from adventitious shootlet segments growing on Taxodium tree. The effect of MS or LS nutrient media supplemented with different concentrations of 2,4-D, BA and Kin on callus production were studied. LS medium supplemented with 3.0 mg/l 2,4-D + 1.0 mg/l BA showed high significant of fresh, dry weight and dry matter content (%) of mass callus production during the 3rd week of cultivation. The cytotoxic activity of calli cultures extract on rat liver cells was evaluated. Extracted Calli of T. distichum and T. distichum var. 'distichum' were not found prominent cytotoxicity activity in the range of 100 – 1000 µg on rat liver cells. Moreover, the comparative study showed that callic extracts have lower cytotoxicity activity than in vivo extracted plants.

Keywords: Taxodium distichum, Taxodium distichum var. distichum, callus, growth regulators, growth dynamic, liver toxicity.

INTRODUCTION

Taxodium distichum L. (Taxodiaceae), commonly referred to as bald cypress, is an unusual and interesting tree, often growing over 25 m in height and over 300 cm d.b.h. (diameter at breast height). Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have a remarkable role in the traditional medicine in different countries[1]. A number of reports concerning isolation and characterization of bioactive compounds from various parts of the plant have appeared in the literature. Such compounds include cytotoxic diterpenoid quinine methides, 2-furaldehyde, tannins, flavone and its derivatives, sterols and fatty acids, and proanthocyanidin[2,3,4,5]. Characterization fruit oil of Taxodium distichum growing in Egypt to be composed mainly of a-pinene (87.3%) has been reported[6]. They reported that antimicrobial, antispasmodic, and anti-inflammatory effects of the oil were attributed to its high a-pinene content. Also showed that the feminine cones, leaves, and branches of the plant are rich in a-pinene (53.7–79.7%) and limonene (3.7–18.7%)[7], while the sample analyzed from China contained caryophyllene oxide (41.67%) as the singly abundant constituent with sizeable proportion of bornyl acetate (6.24%), perilla ketone (5.45%), and a-asarone (5.39%)[8]. Furthermore, Numerous investigators[9,10,11] pointed out to the analyses leaves and fruit of Taxodium distichum, essential oils by gas chromatography-mass spectrometry (GC-MS). They reported that the main compounds were a-pinene (60.5%) and thujiopsene (17.6%) from fruits and thujiopsene (27.7%), pimara-8(14), 15-diene (13.1%), widdrol (12.8%), and p-caryophyllene (11.4%) from leaves, exhibited oils pronounced cytotoxic activities against PC-3, Hep G2, and Hs 578T human tumor cell lines at tested concentrations. Only the fruit oil displayed a promising antifungal effect (MIC 19.5 ug/mL) against Aspergillus niger. Isolation and characterization of taxodiene and taxodone from Taxodium distichum Rich (Taxodiaceae), as a novel tumor-inhibitory diterpenoid quinine methides have been reported[12]. Callus culture from young leaf explants of Khat Catha edulis Forsk. Family Celastraceous was induced. The highest value of callus induction was best achieved on MS or B5 basal media supplemented with 3.0 mg/l of either IBA or NAA. Also, they reported that the production of dark pigments was observed at the start of callus induction and continued with subcultures as a typical characteristic of Khat[13]. Moreover, isolation and identification of some antitumor compounds from Impomoea cairica (L.) sweet cell cultures[14].

Corresponding Author: Taha H.S., Department of Plant Biotechnology, National Research Center, Cairo, Egypt.
E-mail: Hussein03@yahoo.com
This work is important for being a primary tool for further biological investigations of both in vitro and in vivo isolated crude extracts from Taxodium tree and studies their toxicity effects. And we have two points of view: The first is supporting the idea of searching for the compounds of this plant to find the compound giving the cytotoxic effect which had minor effect. The second point of view is supporting the use of the plant therapeutically without fearing toxic effects in the given concentrations. This in vitro work is a distinct tool for biological investigation that saves money, time, and effort. Moreover, it matches with world ethical proclaims toward using intact animals in biological experiments. This part of work is considered as phase I study for evaluating a drug without the need for much time or money.

So, the present study has been undertaken with an objective to develop a protocol of callus production in vitro from adventitious shootlet explants of Taxodium distichum and Taxodium distichum var. ‘distichum’, as well as study their toxicity effect on rate liver cells.

**MATERIALS AND METHODS**

This study was carried out in Plant Biotechnology Department, and Phytochemistry Department, National Research Center, Cairo, Egypt. during the period from 2005-2007.

**Sterilization of Explants:** The nodal explants (as starting materials) were taken from Taxodium distichum and Taxodium distichum var. ‘distichum’ unique trees at Orman Garden, Giza. Egypt. Explants were washed in soapy water using septol soap, then agitated in disinfectant solution of savlon (3%) for 20 min and rinsed with running tap water for one hour. Explants were then soaked for 1 min in 70% ethanol under aseptic condition in laminar air-flow cabinet. Then, they immersed for 5 min in different concentrations (0, 20, 40, and 80% (v/v)) of commercial Clorox (NaOCl 5.25% free chlorine) with a few drops of Tween-20, alone and or with different concentrations (0.0, 0.1 and 0.2%, w/v) of mercuric chloride (MC). Three rinses with sterile distilled water were adopted after each disinfection. Each treatment contains 10 jars and each jar contains two nodal explants.

The following disinfectant treatments were used:-

- 1 - Clorox 20%.
- 2 - Clorox 40%.
- 3 - Clorox 80%.
- 4 - MC 0.1.
- 5 - MC 0.2.
- 6 - Clorox 20% + MC 0.1.
- 7 - Clorox 20% + MC 0.2.
- 8 - Clorox 40% + MC 0.1.
- 9 - Clorox 40% + MC 0.2.
- 10 - Clorox 80% + MC 0.1.
- 11 - Clorox 80% + MC 0.2.

**Starting Medium:** In this experiment, basal MS medium[15] at half salt strength was used to study the effect of disinfectant responses, and for obtaining sterilized materials.

Cultures media were solidified using 0.7 % agar which was added prior autoclaving at 1.2 Kg/cm 2 for 15 min. pH of the medium was adjusted to 5.8 by addition of 0.1 N HCL or 0.1 N KOH. The cultivation was done in 300 ml glass jars containing 50 ml of basal MS-medium, i.e., hormones free. After one month of culturing the different nodal stem segments, the obtained shootlets were used as a source material for callus production experiments.

The normal incubation conditions were: temperature of 25 ± 2°C photoperiod 16 hr, irradiance of 45 µmol m⁻² s⁻¹ (Philips white fluorecent tubes).

**Callus Production and Growth:** Segments of adventitious shootlets of each variety, induced previously from in vitro starting medium, were cultured on LS[16] and MS medium supplemented with 3% sucrose, 0.7% agar and two concentrations (1 and 3 mg/l) of 2,4-

dichlorophenoxyacetic acid (2,4-D), alone or in combination with 1 mg l⁻¹ of either 6-benzyladenine (BA) or kinetin (Kin) as follow:-

- LS medium + 2,4-D 1.0 mg/l.
- LS medium + 2,4-D 1.0 mg/l + BA 1 mg/l.
- LS medium + 2,4-D 1.0 mg/l + Kin 1 mg/l.
- LS medium + 2,4-D 3.0 mg/l.
- LS medium + 2,4-D 3.0 mg/l + BA 1 mg/l.
- LS medium + 2,4-D 3.0 mg/l + Kin 1 mg/l.
- MS medium + 2,4-D 1.0 mg/l.
- MS medium + 2,4-D 1.0 mg/l + BA 1 mg/l.
- MS medium + 2,4-D 1.0 mg/l + Kin 1 mg/l.
- MS medium + 2,4-D 3.0 mg/l.
- MS medium + 2,4-D 3.0 mg/l + BA 1 mg/l.
- MS medium + 2,4-D 3.0 mg/l + Kin 1 mg/l.

**The Following Data Were Recorded:**

Callus Production (%): The percentage of callus production was recorded after 30 days of incubation. Calli colorness were recorded as follow: W: white, Cr: creamy, B: brown. However, the morphological characters were S: spongy and F: friable.

Fresh Weight (g/jar): Fresh weight of different calli cultures, were recorded at the end of five week of cultivation. Three replicates of each explants were used.
Fresh weight was recorded weekly through five weeks of cultivation. Three replicates of each explant were used.

**Dry Weight (g/jar):** Fresh weight of different calli cultures were dried at 40 °C/ 72 hrs. Three replicates of each explant were used.

**Dry Matter Content (%):** Fresh weight of different calli cultures were dried at 40 °C/ 72 hrs. and dry matter contents were estimated as the following equation:

Three replicates of each explant were used.

**Growth Rate (G):** Growth rate of calli cultures were determined (as mg/day) at the end of every week during the five weeks of cultivation, according to the method described by[1-3] using the following equation:

\[ (P_t - P_0) / 7 \]

Where: \( P_t \) is the mass of callus fresh weight (mg) at the end of every week, during the five weeks of cultivation and \( P_0 \) is the fresh weight of callus cultures (mg) at the beginning of every week during 5 weeks of cultivation.

Three replicates of each explant were used.

**Extraction of Callus Culture and Leaves of Intact Plants:** The dried powdered leaves and collected calli cultivares of *T. distichum* and *T. distichum* var. ‘distichum’ were extracted with 80% MeOH. The dried MeOH extract was dissolved in water and left in the refrigerator over night. The samples were filtered and the filtrate were successfully extracted with pet. ether (40-60 °C) and ethyl acetate. The dried ethyl acetate extract was dissolved in water and then partitioned between chloroform and ethyl acetate. The method was carried out according to[19].

**In vitro Bioassay on Primary Culture of Rat Hepatocytes Monolayer (Hepatotoxicity):**

**Isolation and Culturing of Rat Hepatocytes Monolayer:** Primary culture of rat hepatocytes was prepared according to[19] method and modified by[20], using Wistar male rats (250-300 g), obtained from the animal house of the National Research Center, Cairo (NRC). At time of surgery, the rat was anesthetized by intraperitoneal injection of 3.4ml/kg of sodium thiopental solution (0.1g/ml). A midline incision was made, the liver was exposed and the portal vein was cannulated with a needle fitted with a teflon catheter. After the teflon catheter was tied in place and the needle removed, the inferior *vena cava* was cut below the renal vein to allow blood drainage. Perfusion of the liver was started with Ca\(^{2+}\)-free buffer (buffer 1), which contained HEPES (10 mmole/l), NaCl (137 mmole/l), KCl (2.68 mmole/l), Na\(_2\)HPO\(_4\) (0.7 mmole/l), D(+) glucose (10 mmole/l) and EGTA (0.5 mmole/l), and adjusted to pH 7.45 at 37°C. The flow-rate was 11.6 ml/min. The thoracic portion of the superior *vena cava* was cannulated, and the inferior *vena cava* was tied-off above the renal vein. After perfusing the liver for 15min., recirculation of Ca\(^{2+}\)-containing buffer (buffer 2), containing additionally to buffer 1, CaCl\(_2\) (5mmole/l) and Collagenase type IV (10g/ml) (pH 7.45 at 37°C), was started, adjusted at a flow-rate of 7.5 ml/min, for 10 min. After perfusion with buffer 2, the liver was dissected out of the body, placed in a beaker containing buffer 2 and transferred under sterile conditions to laminar flow and gently dispersed with two forceps into small pieces (2-3 mm). The cell suspension was then filtered through the cotton gauze into centrifuge tubes. Finally, the preparation was centrifuged at 600 rpm for 5 min. The supernatant was aspirated off and the loosely packed pellet of cells was gently resuspended in phosphate buffer (PB) (0.1 mole/l), which contained Na\(_2\)HPO\(_4\) (0.2 mole/l) and Na\(_2\)HPO\(_4\) (0.2 mole/l), adjusted at pH 7.6 at 37°C, then diluted two-folds with distilled water. This washing procedure was repeated twice. Viability of the cells to exclude the dye, trypan blue, was determined by incubating cell suspension (0.1 ml) with trypan blue (0.9 ml) and then counting the number of the cells that excluded the dye and the number of cells whose nuclei were stained blue, using haemocytometer under light microscope (Nikon). Complete culture medium was added over the total counted cells to reach a concentration of 2 x 10\(^5\) cells/ml.

The culture medium was composed of RPMI-1640 medium, supplemented with 10% inactivated (56°C for 30 min) fetal calf serum (FCS) (0.05/ml), penicillin-streptomycin (PS) (0.01/ml), insulin (0.7 (g/ml) and dexamethasone (4g/ml). Inocula of 150 µl were seeded into plastic 96-well plates and preincubated in CO\(_2\)-Incubator at 37°C, under 5% CO\(_2\) in air for 22-24 hrs. All buffers were freshly prepared and sterilized at 121°C for 30 min. before use.

**Determination of Hepatotoxicity Activity:** After pre-incubation of primary culture of rat hepatocytes for 22-24 hrs, the monolayer was checked under inverted microscope (Olympus) for attachment. Then, the monolayer was washed twice with (Phosphate Buffer Saline (PBS), which contained KCl (2.68 mmole/l), Na\(_2\)HPO\(_4\) (8.45 mmole/l), KH\(_2\)PO\(_4\) (1.5 mmole/l) and NaCl (137 mmole/l).

In order to determine LD\(_{50}\), different concentrations were prepared for each extracts. The range of used concentrations started from 100 µg/ml followed by increasing concentrations in ascending order up to the
concentration 1000 mg/ml to determine the concentration that induced death of half the number of cells. Extracts were dissolved in dimethylsulfoxide (DMSO) (1% maximum concentration). For each concentration, three replicates were carried out. The plate was incubated for 2 hrs, in CO₂ incubator.

After two hours incubation of cells with the extracts, cell viability was determined using MTT assay, that was performed according to the method of[21] modified by[22]. The MTT colourimetric assay for living cells [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] is based on utilizing a colourless substance that is modified to a coloured product by any living cell, but not dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measured the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria and so the reaction occurs only in the living cells. At the end of the incubation period, the supernatant was carefully removed by inverting flicking, blotting the plate and washing the culture with (PBS).

One hundred and fifty µL of culture medium containing all except (PS), was added to each well together with 50 µL of a solution of 2 mg MTT/ml PBS. After incubation for 4 hrs, supernatant was removed as previously described and the blue formazan product obtained was dissolved in 200 µl dimethyl sulfoxide. The plate was then shaken for 5 min. on a microtitre plate shaker and the plate on Micro-elisa reader was read at 540 and 630 nm dual wave-length using automatic kinetic microplate reader (Labsystems Multiskan RC reader).

Evaluation of the effect of the different extracts on cultured hepatocytes was obtained by calculating the absorption of the cell viability with respect to control cells (cells only without addition of extracts).

A graph was plotted with x-axis showing different concentrations of extracts used, y-axis showing absorbance percentage of viable cells.

**Statistical Analysis:** According to[23], all experiments were completely randomized designed and the obtained data were subjected to analysis of variance and treatment means were compared by an approximate Student's t-test (P<0.05). All treatments in experiments described consisted of three replicates.

**RESULTS AND DISCUSSIONS**

**Sterilization of Explants:** Viability of the explant is an important target to obtain a successful explant in the establishment stage and to be subjected in the following stage i.e., as starting materials for callus production from adventitious shootlets segments. Data in Table (1) showed the percentage viability of explants due to the use of clorox at the concentrations 0.0, 20, 40 and 80 % alone or in combination with M.C. at the concentrations 0.0, 0.1 and 0.2 %. The obtained data revealed that using clorox at 20% for 5 min followed by M. C. at 0.2% for 5 min gave the highest value of explants viability as compared with other treatments. This treatment gave 100% of viability in case of Taxodium distichum and 95% in case of Taxodium distichum var. ‘distichum’. Furthermore, it was observed that Taxodium distichum var. ‘distichum’ was generally more sensitive for sterilization condition than Taxodium distichum.

**Callus Production and Growth:**

**Callus Production:** An equal segments of in vitro growing adventitious shootlets of T. distichum and T. distichum var. ‘distichum’, were cultured on LS or MS media supplemented with 1.0 or 3.0 mg/l 2,4-D alone or in combination with either 1.0 mg/l BA or Kin. After 5 weeks of cultivation, all treatments induced calli, but LS medium with 3.0 mg/l 2,4-D + 1.0 mg/l BA showed remarkable callus formation. Calli obtained from this medium were faster growing, delicate, and mostly spongy and white creamy in color. Whilst culturing the segments onto MS medium supplemented with 2,4-D combine with BA or Kin gave sponge and brown calli, (Table 2).

Data tabulated in Table (2) showed that the highest values of callus induction were 100% and 96.67% with T. distichum and T. distichum var. ‘distichum’, respectively. LS medium showed the best nutrient salt medium for callus induction as compared with MS nutrient salt medium. Furthermore, supplementation of LS medium with 3.0 mg/l 2,4-D + 1.0 mg/l BA gave the maximum value of callus induction on both Taxodium varities as compared with the other supplementations. Regarding the morphological characters, creamy and friable calli were observed on the same previous medium with T. distichum as compared with T. distichum var. ‘distichum’.

Concerning the statically data analysis of callus induction, LS nutrient salt medium gave significant responses as compared with MS nutrient salt medium. Supplementation of LS medium with 3.0 mg/l 2,4-D + 1.0 mg/l BA gave the highest significant value (98.33%)as compared with the other supplementations. Concerning the cultivar types T. distichum showed the highest significant of callus induction (80.56%) than T. distichum var. ‘distichum’ (75.97%).

**Callus Growth:** Data in Table (3) showed that the highest value of callus fresh weight, dry weight and dry matter content (%) were 9.300, 0.8367 (g/jar) and 9.057% for T. distichum, while they were 7.567, 0.6900 (g/jar) and 9.143% with T. distichum var. ‘distichum’, respectively. LS nutrient medium showed...
showed that the growth rate(s) of derived calli cultures callus was obviously higher than those of increased by increasing the time of cultivation. During the five weeks of cultivation. Data in Fig. (2) showed that fresh weight of callus was gradually increased by increasing the time of cultivation. Furthermore, the fresh weight values of *T. distichum* callus was obviously higher than those of *T. distichum* var. 'distichum'.

**Growth Rate (mg/day):** Data illustrated in Fig. (3) showed that the growth rate(s) of derived calli cultures were determined (as mg/day) at the end of every week during the five weeks of cultivation. Data clearly showed that the significant increase was recorded in the 3rd week of cultivation in both *T. distichum* and *T. distichum* var. 'distichium', respectively.

In conclusion the 3rd week of cultivation showed best results of callus growth dynamic as compared with other weeks, calli of *T. distichum* was better than *T. distichum* var. 'distichium'.

**In vitro Evaluation of Taxodium distichum and Taxodium distichum Var. 'Distichum' Samples, Calli Cultures and Intact Plant, for Hepatotoxic Effect on Hepatocytes Monolayer Culture:** With the given extracts, increasing absorbance values were observed with increasing concentrations from 100 µg/ml to 1000 µg/ml in monolayer culture of primary rat hepatocytes, and no cytotoxicity was observed microscopically in the cellular systems.

### Table 1: Effect of different concentrations (20, 40, and 80%) of Clorox in combination with two concentrations (0.1 and 0.2%) of mercuric chloride (M.C.) on percentage of viability of *Taxodium distichum* and *Taxodium distichum* var. 'distichum' nodal explants.

<table>
<thead>
<tr>
<th>M.C. % (w/v)</th>
<th>Clorox 0%</th>
<th>Clorox 20%</th>
<th>Clorox 40%</th>
<th>Clorox 80%</th>
<th>Clorox 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>N. D.</td>
<td>65</td>
<td>40</td>
<td>N. D.</td>
<td>65</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
<td>75</td>
<td>35</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>0.2</td>
<td>25</td>
<td>80</td>
<td>25</td>
<td>30</td>
<td>95</td>
</tr>
</tbody>
</table>

### Table 2: Effect of LS or MS media supplemented with 2,4-D, Kin and/or BA on callus induction (%) from segments of adventitious shootlets of *Taxodium distichum* and *Taxodium distichum* var. 'distichium' and their morphological characters after 5 weeks of cultivation.

<table>
<thead>
<tr>
<th>Callus induction (%)</th>
<th>Callus colour and morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Taxodium</strong></td>
<td><strong>T. var. 'distichum'</strong></td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D</td>
<td>68.33</td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D + 1 mg/l Kin</td>
<td>70.00</td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D + 1 mg/l BA</td>
<td>86.67</td>
</tr>
<tr>
<td>LS medium + 3.0 mg/l 2,4-D</td>
<td>100.00</td>
</tr>
<tr>
<td>LS medium + 3.0 mg/l 2,4-D + 1 mg/l Kin</td>
<td>90.00</td>
</tr>
<tr>
<td>LS medium + 3.0 mg/l 2,4-D + 1 mg/l BA</td>
<td>100.00</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D</td>
<td>53.33</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D + 1 mg/l Kin</td>
<td>56.67</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D + 1 mg/l BA</td>
<td>73.33</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D</td>
<td>90.00</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D + 1 mg/l Kin</td>
<td>85.00</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D + 1 mg/l BA</td>
<td>93.33</td>
</tr>
<tr>
<td>Mean (B)</td>
<td>80.56</td>
</tr>
</tbody>
</table>

**LSD**<sub>0.01</sub> = 4.888  B = 1.888  AB = 6.913

W: white, Cr: creamy, B: brown, S: spongy and F: friable. Each treatment is the average of 3 replicates.
Comparing the results of leaves of intact plant and derived calli cultures of *Taxodium distichum* indicated the following:

Results in Fig. (4) showed increased viability in the side of leaves of intact plant over derived calli. On the other hand, the data in Fig. (5) showed that although the leaves of intact plant of *T. distichum* var. ‘distichum’ showed increased viability over calli cultures in two concentrations (750 and 1000 μg/ml), the overall activity lies in the side of calli cultures.

### Table 3: Effect of LS or MS media supplemented with 2,4-D, Kin and/or BA on fresh, dry weight and dry matter content (%) of callus of *Taxodium distichum* and *Taxodium distichum* var. ‘distichum’, after 5 weeks of cultivation.

<table>
<thead>
<tr>
<th></th>
<th>Fresh weight (gm/jar)</th>
<th>Dry weight (gm/jar)</th>
<th>Dry matter content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. var. distichum</em></td>
<td>Mean (A)</td>
<td><em>T. var. distichum</em></td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D</td>
<td>5.000</td>
<td>4.947</td>
<td>0.0467</td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D + 1 mg/l Kin.</td>
<td>4.333</td>
<td>4.100</td>
<td>0.2177</td>
</tr>
<tr>
<td>LS medium + 1.0 mg/l 2,4-D + 1 mg/l BA.</td>
<td>5.267</td>
<td>5.500</td>
<td>0.4667</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D</td>
<td>6.900</td>
<td>6.567</td>
<td>0.6400</td>
</tr>
<tr>
<td>LS medium + 3.0 mg/l 2,4-D + 1 mg/l Kin.</td>
<td>5.400</td>
<td>5.400</td>
<td>0.4767</td>
</tr>
<tr>
<td>LS medium + 3.0 mg/l 2,4-D + 1 mg/l BA.</td>
<td>9.300</td>
<td>7.567</td>
<td>0.8367</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D</td>
<td>2.467</td>
<td>3.000</td>
<td>0.1767</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D + 1 mg/l Kin.</td>
<td>3.000</td>
<td>3.400</td>
<td>0.2267</td>
</tr>
<tr>
<td>MS medium + 1.0 mg/l 2,4-D + 1 mg/l BA.</td>
<td>3.133</td>
<td>3.200</td>
<td>0.2800</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D</td>
<td>3.100</td>
<td>3.000</td>
<td>0.2367</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D + 1 mg/l Kin.</td>
<td>3.500</td>
<td>3.633</td>
<td>0.2167</td>
</tr>
<tr>
<td>MS medium + 3.0 mg/l 2,4-D + 1 mg/l BA.</td>
<td>5.267</td>
<td>5.500</td>
<td>0.4667</td>
</tr>
</tbody>
</table>

| LSD<sub>0.05</sub> | A = 0.6875 | 0.06365 | 0.9625 |
| LSD<sub>0.05</sub> | B = 0.2807 | 0.02599 | 0.3930 |
| LSD<sub>0.05</sub> | AB = 0.9723 | 0.09002 | 1.361 |

Fig. 1: Callus induction and morphological characters of *T. distichum* and *T. distichum* var. ‘distichum’ cultured on LS medium + 3.0 mg/l 2,4-D + 1.0 mg/l BA under light condition.
Fig. 2: Growth dynamic of callus fresh weight (g/jar) of *T. distichum* and *T. distichum* var. 'distichum' during five weeks of cultivation.

Fig. 3: Growth rate (mg/day) of *T. distichum* and *T. distichum* var. 'distichum', calli culture during five weeks of cultivation.

Fig. 4: Comparison between leaves of intact plant and calli cultures of *Taxodium distichum* for hepatotoxic effect on hepatocytes monolayer culture.
The viability assay was applied with a broad range of concentrations of the studied extracts (from 100-1000 mg/mL) on monolayer of rat hepatocytes. It revealed that the extracts have no toxic effect on monolayers of rat hepatocytes up to concentration of 1000 mg/mL, as shown.

**Discussion:** Concerning callus production,\(^{24}\) showed that one of the basic prerequisite for any tissue culture system is the establishment of efficient callus. They reported that callus of seedling roots and glumes of maize on LS medium supplemented with 2,4-D at the range of 0.5 – 4 (mg/l). This results are in agreement with our results which indicated that LS medium supplemented with 3.0 mg/l 2,4-D + 1.0 mg/l BA was more significant for callus production from advanced shootlets explants of either *T. distichum* or *T. distichum* var. ‘distichum’. The differential optimal requirement of auxins for callus induction from explants may be due to physiological status of the explants and that auxin sites may have a higher affinity for analogues than other auxins. Furthermore, the obtained results are agreement with \(^{23}\) they found that auxin was proved to be necessary for high percentage for callus induction from *viola tricolor*, and 2,4-D or NAA were more effective than IBA, while high concentration of BA would inhibit callus information. In other study, 2,4-d plus NAA were also used and high callus induction percentage was obtained in *Viola wittrockiana*. This results in close with our results which indicated that the use of low concentration of 1.0 mg/l BA more suitable for callus induction from either *T. distichum* and *T. distichum* var. ‘distichum’, than high concentration of BA.

Also, our results in close with\(^{26}\) they showed that *Atriplex gmelini* plants callus lines were induced from the hypocotyl explants on LS medium supplemented with 1 µM BA and 5 µM NAA in the dark. The obtained results are in lines with our results which indicated that LS medium supplemented with 3.0 mg/l 2,4-D was more suitable for callus induction from derived shootlets segments of *Taxodium distichum* and *Taxodium distichum* var. ‘distichum’.

Concerning the effect of type of nutrient media on callus production,\(^{27}\) observed that callus induction from leaf and node explants of strawberry (*Fragaria sp.*) was initiated earlier on media with high auxin concentrations as compared with lower concentrations. Some explants produced friable and white to light creamy callus while others produced soft watery callus white or light gray in color. Best callus induction was recorded on MS medium supplemented with 4.0 mg/l NAA from leaf (86.1%) and inter nodal explants (89.3%). These results in contrast with our results which clearly showed that LS medium more efficient on callus production than MS medium.

Similar combination of auxin with cytokinin for callus induction has been reported by\(^{28}\), our investigation also accordance to\(^{29}\) they reported that highest value of callus induction from leaf base as explant of *Kaempferia galanga* were induced from MS medium supplemented with 2,4-D at 1.5 mg/l with BAP at 1.0 mg/l. Concerning the callus growth dynamics,\(^{30}\) showed that the highest frequency of callus induction of *Ocimum basilicum* at the end of fourth weeks on MS medium supplemented with 0.5 mg/l 2,4-D with 1.0 mg/l BAP. These results are in contrast with our results which indicated that the significant value of callus production was recorded during the 3rd week of cultivation.
REFERENCES


