

Full Length Research Paper

An efficient protocol for *in vitro* organogenesis and antioxidant studies in *Melia dubia* Cav.

Syed Naseer Shah^{1*}, Tareq Ahmad Wani^{1*}, Bhimi Ram², Monika Koul², Praveen Awasthi¹, Deependra Singh Rajput³ and Gillela Ravi Shanker Reddy²

¹Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu, India.

²Institute of Forest Biodiversity Hyderabad, India.

³Institute of Wood Science and Technology, Bangalore, India.

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***Melia dubia* Cav. (Meliaceae)** is a multipurpose tree of tropical and subtropical regions mainly cultivated for its medicinal and industrial importance. Due to its versatile properties, it has been depleted in its natural environment. Moreover due to sluggish and poor seed germination, there is a threat of its gene pool exclusion from the natural habitat. The alternative method for conservation and efficient mass propagation is thus need of the hour. As per the extensive literature survey there is no report on efficient protocol for mass propagation of *M. dubia* through callus organogenesis. Therefore, the present work was aimed to develop *in vitro* organogenesis protocol for rapid and large scale production of planting material. From our results, maximum callus percentage, callus weight and fragile callus was observed on 1.0 mg/l benzylaminopurine (BAP) in combination with 0.5 mg/l naphthalene acetic acid (NAA). The callus differentiation was achieved at different concentrations of BAP and indole acetic acid (IAA). Multiple Shoot number per callus propagule 5.30 was observed on 0.5 mg/l BAP and 1 mg/l IAA concentration. The maximum rooting percentage (78.5%), root number per explant (4.33) and root length per explant (4.41 cm) was observed at 0.5 mg/l indol butyric acid (IBA) after 30 days of inoculation. Further the total flavonoid content, phenolic content and antioxidant properties of leaves of *in-vitro* regenerated plants were studied. Total flavonoids and phenolic content in leaves of *in vitro* *Melia dubia* was 0.56 ± 0.8 mg quercetin equivalent (QE) and 2.97 ± 0.17 mg gallic acid equivalent (GAE) respectively. The antioxidant property was further assed through measurement of DPPH radical scavenging activity. The *in-vitro* regeneration protocol can be exploited for commercial cultivation and fulfilling the growing demand for fresh explant material through mass propagation of *M. dubia* an economically important plant species.

Key words: *Melia dubia*, antioxidant, indole-3-butyric acid, flavonoids and phenolics.

INTRODUCTION

Melia dubia, a dicotyledonous multipurpose tree belonging to family Meliaceae, has huge commercial and industrial potential. The species is native to southern Asia (India-

Pakistan-Iran) and has been introduced to South Africa, Middle East, America (Bermuda, Brazil and Argentina), Australia, Southeast (SE) Asia-Pacific Islands and

*Corresponding author. E-mail: wanitariq.bio.iim@gmail.com; shahapsu@yahoo.com. Tel: +91-9086482319.

southern Europe (Ram et al., 2014). Due to its multi-purpose uses like bioenergy production, paper and pulp manufacturing, furniture making, building constructions, making musical instruments etc., it has gained a great deal of attractiveness and elevated demand (Mandang and Aristien, 2003; Suprapti and Hudiansyah, 2004; Parthiban et al., 2009; Chinnaraj et al., 2011). Other than its industrial and commercial importance, the plant has proficient medicinal properties. The various extracts from different parts of the plant are known to have pharmacological importance which includes, antiviral, antibacterial, antifungal, antidiabetic, antineoplastic, antihelminthic and antileprosy properties (Kiritkar and Basu, 1999; Pettit, 2002; Nagalakshmi et al., 2003; Vijayan et al., 2004; Gerige and Ramjaneyulu, 2007; Susheela et al., 2008; Sukumaram and Raj, 2010; Sharma and Arya, 2011). Due to its elevated demand for commercial, industrial and therapeutic basis, *M. dubia* trees growing naturally have been indiscriminately logged which resulted in significant decline in its population. Conventionally, *M. dubia* is propagated through seeds, which have very poor (14-34.3%) germination rates because of hard stony seed coat, which makes it difficult to germinate without any treatment (Nair et al., 2005; Manjunatha, 2007; Anand et al., 2012). Therefore, it is imperative to use an efficient plant regeneration system under *in vitro* conditions for large scale production of planting material of the species from superior genotypes for quick rejuvenation. For large scale production, efficiency of propagation method is imperative. The purpose of micropropagation is to develop physiologically stable plantlets which can be acclimatized in a reduced time period and at a lower cost. In this backdrop, we have established a well developed *in vitro* regeneration system for *M. dubia* for rapid and large scale production of planting material. There are many reports of *in vitro* regeneration of the plant through axially bud proliferation (Ram et al., 2014), but it is the first report of regeneration through *in vitro* callus organogenesis. The ethanolic extract of field grown plants has promising antioxidant activity with IC₅₀ (16.89 µg/ml) value (Valentina et al., 2013). In this direction, the present study was envisaged to evaluate the antioxidant activities of leaf tissue from *in vitro* regenerated plants of *M. dubia*. This activity can be ascribed to the phenolic and flavonoid compounds present in the species. Consequently the study was extended to determine total flavonoid and phenolic content of leaf tissue. The present protocol can serve as an important tool for commercial and industrial supply of bulk plant material of *M. dubia*. The tissue culture raised plants were further evaluated for their total phenolic and flavonoid content viz-a-viz its antioxidant property.

MATERIALS AND METHODS

Explants collection

Experiments were carried out in plant tissue culture laboratory of

the Institute of Wood Science and Technology (IWST), Bangalore. Four Simple Randomized experiments were carried out. Explants of *M. dubia* were collected from established cultures grown under aseptic *in vitro* conditions. The leaf explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with different doses of N⁶-Benzyladenine (BA; 0.25, 0.5 and 1.0, 2 mg/l) and α -Naphthalene acetic acid (NAA; 0.1, 0.25, 0.5 and 1.0 mg/l) for callus initiation in first experiment. In second experiment callus multiplication was observed on MS medium supplemented with BA and NAA at their best combination. In the third experiment, healthy shoots were developed on benzylaminopurine (BAP; 1.0, 1.25, 0.5 and 0.25 mg/l) and Indole-3-acetic acid IAA (0, 1.0 2.0 mg/l). In fourth experiment *in vitro* raised micro-shoots were transferred to MS/2 strength rooting medium supplemented with different doses of Indole-3-butyric acid (0.1, 0.25, 0.5 and 1.0 mg/l).

Culture conditions

The inorganic salts used for preparation of culture medium were obtained from Qualigens Pvt. Ltd., India and phytohormones and B vitamins from Sigma Chemicals Pvt. Ltd., India. The medium contained 3% (w/v) sucrose, 0.6% (w/v) agar (Hi-Media chemical Ltd., India). The pH of the medium was adjusted to 6.0 before autoclaving for 15 min at 1.06 kg cm⁻² (121°C). Explants were cultured in a 150 ml Boatel Borosil[®] Boatels containing 40 ml semi-solid medium. For *in vitro* shoot multiplication and rooting experiment, the cultures were incubated at 25 ± 2°C under 16 h illuminations with fluorescent light (50 µmol Em⁻² s⁻¹).

Hardening and transplantation

Tissue culture raised microshoots of 3 to 4 cm in length with 2 to 3 nodes were tested with various concentrations of IBA, indol butyric acid (IAA) and NAA either alone or in combinations for *in vitro* rooting. The rooted plantlets of *M. dubia* were removed from agar-agar and washed with distilled water to remove the traces of agar. Plantlets were transferred to root trainers containing autoclaved potting mixture of vermiculite, sand and soil (1 : 2 : 1 v/v/v) and placed under 4 weeks in mist chamber at 30 ± 5°C temperature and > 60% relative humidity (RH). Later on the plants were kept in polythene bags containing autoclaved soil and then shifted to a shade net (50% shade) for another 2 weeks before placing it in open nursery. After successful acclimatization plants were finally transferred to natural condition.

Quantification of total flavonoid content

Total flavonoid content was quantified using spectrophotometer. Dried crude extract (prepared from 100 mg of dried plant material) dissolved in 500 µl of distilled water was mixed with 30 µl of a 5% NaNO₂ solution, followed by 5 min of incubation at room temperature. After the incubation, 300 µl of 10% AlCl₃.H₂O solution was added and the sample was further incubated for 6 min incubation. Finally 200 µl of 1 M NaOH and 200 µl of distilled water were added to the sample and absorbance was read at 510 nm. Total flavonoids were calculated using quercetin as standard (10 to 100 µg; R₂ = 0.998). The results were expressed as mg quercetin equivalent (mg QE) per gram dry weight of the plant material. The experiment was repetitively performed in triplicates.

Quantification of total phenolic content

Total phenolic content in leaves of *M. dubia* was measured using the Folin-Ciocalteu reagent method as described by Pinelo et al. (2004). The total phenolic content was expressed as mg of Gallic

Table 1. Effect of of BA, NAA and their interaction on callus induction (%), callus weight and texture of callus induction in *M. dubia* at 30 days after inoculation. Values in the parentheses are arc sine transformation.

Treatment numbers	30 Days after inoculation			
	BAP +NAA (mg/l)	Callus formation (%)	Fresh weight (g)	Callus texture
T ₁	BAP (0.25) + NAA (0.1)	69(56.5)	1.84±0.20 [*]	Compact
T ₂	BAP (0.5) + NAA (0.25)	83(70.08)	2.13±0.32	Compact
T ₃	BAP (1.0) + NAA (0.5)	100(85.84)	2.44±0.36	Fragile
T ₄	BAP (2.0) + NAA (1.0)	66(54.33)	1.72±0.19	Compact
LSD (0.05)		0.60	0.08±0.01	

*S.E; (LSD= Least Significant Difference).

Table 2. Effect of BAP and NAA on in vitro callus multiplication of *M. dubia* at 30 days after inoculation.

Treatments	30 Days after inoculation	
	BA and NAA (mg/l)	Fresh weight (g)
T ₁	BAP	3.47±1.03 [*]
T ₂	BAP (0.25) + NAA (0.25)	4.60±1.65
T ₃	BAP (1.5) + NAA (0.5)	5.62±1.80
T ₄	BAP (2.5) + NAA (1.0)	4.23±1.55
LSD _(0.05)		0.19±0.05

*SE (LSD= Least Significant Difference).

acid equivalent (GAE) per gram of dry weight of the sample. The experiment was repetitively performed in triplicates.

DPPH radical-scavenging activity

Measurement of radical scavenging property of *M. dubia* was carried out according to the method described by Blois (1958). Ascorbic acid was used as positive control and % inhibition was determined according to the following equation:

$$\% \text{Inhibition} = [(A_{\text{DPPH}} - A_s) / A_{\text{DPPH}}] \times 100$$

Where, A_s is the absorption of the solution when the sample extract was added at a particular concentration and A_{DPPH} is the absorbance of the DPPH solution. Three experimental replicates were taken for the assay. The IC_{50} values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical; a lower IC_{50} value corresponds to a higher antioxidant activity of sample.

Statistical analysis

Each experiment had three replicates for *in vitro* shoot multiplication and rooting. Each replicate had 10 propagules. The data were subjected to one way analysis of variance (ANOVA). All the experiments ascertained with "F" test for level of significance. The significance at $p \leq 0.05$, $LSD_{0.05}$ was computed for comparison of treatment means.

RESULTS

Callus induction

The experiment was aimed to investigate the different

doses of BA and NAA (0.25, 0.5, 1.0, 2, 0.1, 0.25, 0.5, and 1.0 mg/l) on callus induction weight and callus texture at 30 days after incubation (Table 1). The effect of different doses of BA and NAA and their interaction on callus induction (%), weight and callus texture was found to be significant at the stage of sampling. Maximum callus induction, callus fresh weight and the best callus texture was observed on 1.0 mg/l BA and 0.5 mg/l NAA which was 57.99 and 41.86% in comparison to BAP 2.0 mg/l + NAA 1.0 mg/l. Fragile callus was observed on 1.0 mg/l BA and 0.5 mg/l NAA and compact callus was observed on other treatments.

Callus multiplication

The experiment was carried out to investigate the different doses of BA and NAA (0, 0.25, 1.5, 2.5, 0, 0.25, 0.5, and 1.0 mg/l) on callus multiplication at 30 days after incubation (Table 2). Various doses of BA and NAA significantly influenced callus multiplication at the stages of sampling Figure 1a. Fresh weight of callus was found to be maximum on 1.5 mg/l BA and 0.5 mg/l NAA which was 51.6% more in comparison to 2.5 mg/l BAP + 2.5 mg/l NAA at the stage of sampling.

Callus differentiation

Various doses of BA and IAA (1.0, 1.25, 0.5, 0.25, 0, 1.0 and 2.0 mg/l) significantly influenced callus differentiation

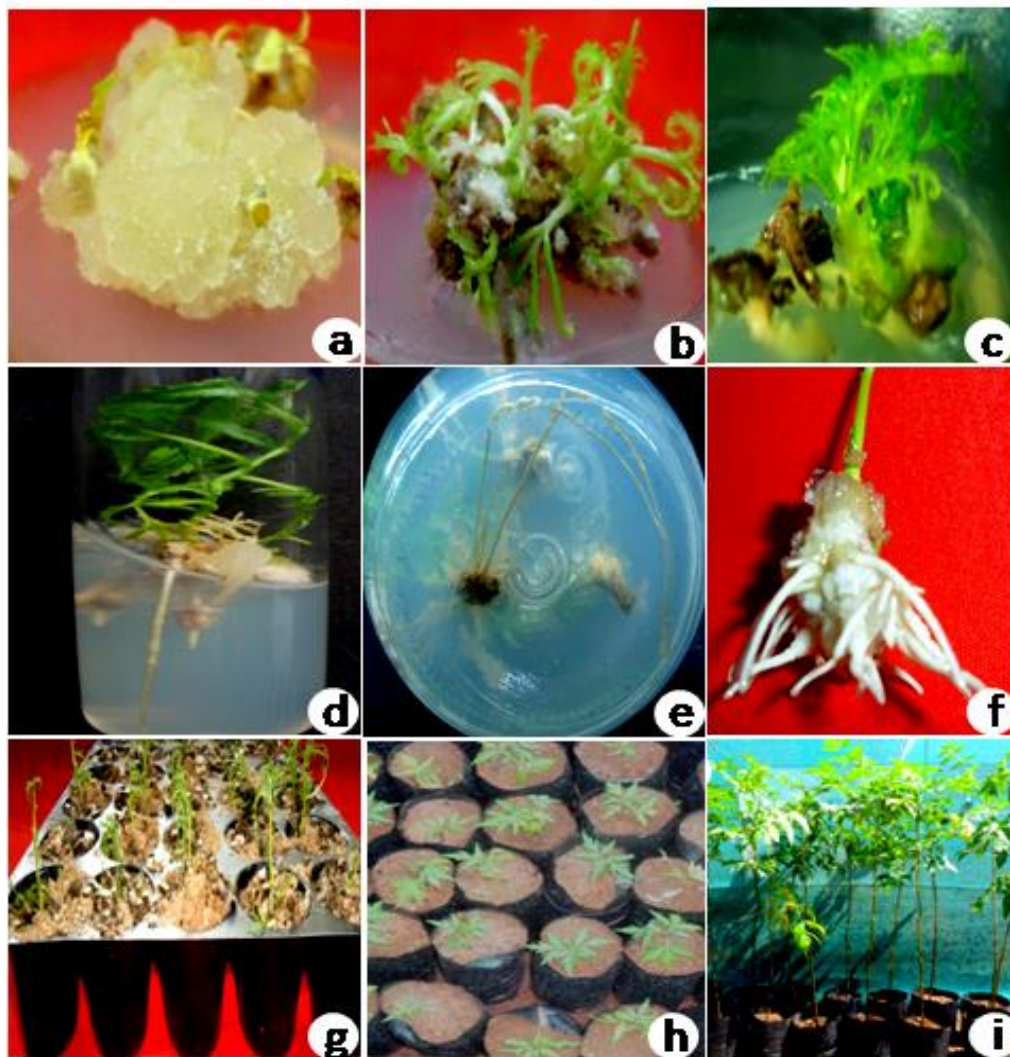


Figure 1. Effect of different phytohormones on different developmental stages: Callus multiplication (a), Callus differentiation (b), shoot induction (c), Efficient rooting (d-f), Hardening and acclimatization of *in vitro* raised plants transferred in root trainers containing potted mixture for four weeks in mist chamber (g), Plants transferred to polythene bags containing autoclaved soil (h) and full grown hardened plants in open environment (i).

at 30 days after inoculation Figure 1b. The highest shoot number per callus propagule was observed on 0.5 mg/l BA and 1mg/l IAA. Shoot number per callus propagule was 133% more in comparison to treatment 1 Figure 1c. Shoot length per callus propagule was recorded at 30 days after inoculation. Shoot length per callus propagule was found to be maximum at 0.5 mg/l BA and 1 mg/l IAA which was 175% more in comparison to treatment 1 (Table 3).

***In vitro* rooting**

The experiment was carried out to study the effect of four doses of IBA (0.1, 0.25, 0.5 and 1.0 mg/l) with MS/2

medium and their all possible interactions on *in vitro* rooting (%) and root number per explant at 30 days after inoculation (Table 4). Various doses of IBA significantly influenced rooting (%) at the stage of sampling Figure 1d-f. The effect of 0.5 mg/l IBA provide significantly maximum rooting % which was 71% more in comparison to 0.25 mg/l IBA.

Root number per explant

The effect of four doses of IBA significantly enhanced root number per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum on root number per explant which was 270% more in comparison to 0.25

Table 3. Effect of BAP and IAA on callus differentiation in *M. dubia* at 30 days after inoculation.

Treatments	30 Days after inoculation		
	BAP+IAA (mg/l)	Shoot number/explant	Shoot length/explant (cm)
T ₁	BAP (1.0)	2.27±0.32*	1.27±0.22*
T ₂	BAP (1.25)	3.13±0.54	1.37±0.27
T ₃	BAP (0.5) + IAA (1.0)	5.30±1.23	3.53±0.64
T ₄	BAP (0.25) + IAA (2.0)	4.27±1.10	3.27±0.61
LSD (0.05)		0.28±0.03	0.28±0.02

*SE (LSD= Least Significant Difference).

Table 4. Effect of different concentrations of IBA and MS/2 medium on *in vitro* rooting %, root number and root length in *M. dubia* at 30 days after inoculation. Values in the parentheses are arc sine transformation.

Treatment numbers	30 Days after inoculation			
	IBA (mg/l)	Rooting (%)	Root number per explant	Root length per explant (cm)
T ₁	IBA (0.1)	56 (49.2)	1.27±0.26*	1.37±0.32*
T ₂	IBA (0.25)	53 (48)	3.22±1.25	3.23±1.30
T ₃	IBA (0.5)	96 (78.5)	4.33±1.54	4.41±1.61
T ₄	IBA (1.0)	60 (51.4)	1.17±0.21	1.13±0.30
LSD (0.05)		20.21	0.19±0.02	0.13±0.02

*SE (LSD= Least Significant Difference).

Table 5. Measurement of total flavonoids, phenolics contents and antioxidant activity in extracts prepared from leaves of tissue culture raised plant of *Melia dubia*.

Material	Total flavonoid content (mg QE)	Total phenolic content (mg GAE)	IC ₅₀ (µg/ml)
Leaves	0.56 ± 0.08*	2.97 ± 0.17*	242.88 ± 12.55*

*S.E, P-value < 0.05. IC₅₀: the concentration of extracts (µg/ml) causing 50% inhibition of DPPH radical.

mg/l IBA.

Root length per explant

Administration of different doses of IBA significantly enhanced root length per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum for root length per explant which was 290% more in comparison to 0.25 mg/l IBA.

Hardening

Following hardening procedures, 6-weeks old hardened plants (Figure 1g-i) were successfully transferred to field conditions. The survival rate of *in vitro* raised plants under hardening conditions showed prominent growth with about 94% success. This gives the suitability of protocol.

Total phenolic and flavonoid content

Flavonoids significantly contribute to the total antioxidant property of the plants (Luo et al. 2002). Total phenolic content were found to be high in *M. dubia* in comparison to total flavonoids content (Table 5).

DPPH radical-scavenging activity

DPPH method is a simple, rapid and reproducible assay used for measuring the antioxidant activity of plant extracts (Mishra et al., 2012). The IC₅₀ value for methanolic extract of leaves of *M. dubia* was found to be 242.88 ± 12.55 µg/ml (Table 5). The antioxidant activity (DPPH radical-scavenging activity) of all the extracts was lower than that of ascorbic acid (IC₅₀, 6.1 ± 0.32 µg/ml), used as positive control; a lower IC₅₀ value corresponds to a higher antioxidant activity of sample.

DISCUSSION

A culture medium is defined as a formulation of inorganic salts and organic compounds (apart from major carbohydrate sources and plant growth regulators) used for the nutrition of plant cultures (George, 1993). It usually consists of a balanced mixture of macro-and-micro elements together with vitamins and other organic nutrients, including a carbon source. Nutritional requirements for optimal growth of a tissue, *in vitro*, may vary with the species and parts of a plant. When starting with a new system, it is essential to work out a medium that will fulfill the specific requirements of the tissues (Bhojwani and Razdan, 1996; Smith, 2000). During the present investigations on *M. dubia* MS nutrient medium was used. There are many reports comparing different media for their effect on *in vitro* shoot multiplication (Rugini, 1984; Mehta et al., 2000; Lu, 2005; Jain et al., 2009). Most of the workers have routinely used MS medium for shoot multiplication (Reddy et al., 1998; Komalavalli and Rao, 2000; Devi and Srinivasan, 2008). Sha Valli Khan et al. (2002), reported the multiplication of white friable callus on MS medium with NAA (1.0 μM) in combination with BAP (1.0 μM) in *Bixa orelliana* L. Nirmalakumari et al. (1993) reported 6-7 shoots from one month old callus of leaf and stem on MS medium supplemented with BAP (2.0 mg/l) and IAA (0.5 mg/l) in *Azadirachta indica*. Chaicharoen et al. (1996) observed maximum 9 shoot from callus, when medium was supplemented with 1.0 mg/l BA in *Melia azedarach*. Chaturvedi et al. (2003) revealed that 5 μM BAP alone favored shoot induction from anther derived callus on MS medium. Srivastava et al. (2009) observed maximum (78%) shoot regeneration when callus was sub cultured on MS medium containing 5 μM BAP alone in *A. indica*. Contrary to the above observation, Islam et al. (1993) reported that shoot organogenesis was the best from cotyledons origin callus on MS medium with 2.0 mg/l BA and 0.2 mg/l NAA used in *A. indica*. Vila et al. (2003) in *M. azedarach* reported successful regeneration of plantlets from leaf derived callus by using BA (4.4 μM) and NAA (0.46 μM) in MS medium. Sharry et al. (2006) reported induction of multiple shoots from callus in MS medium with NAA (0.5 mg/l) and BAP (1.0 mg/l). In the present study, IBA was found to be the most excellent auxin for rooting in terms of number of roots and root length. In accordance to our result, IBA have been used extensively for rooting in a wide range of plant species of Meliaceae family such as; *M. azedarach* (Thakur et al., 1998; Sen et al., 2010), IBA 1.0 mg/l in MS/2 medium favoured the best root induction 83 to 90% respectively. Chemically MS medium shows variation in concentration of different components as compared to other media. It has been observed that IAA, IBA and NAA enriched medium was also responsible for root induction in *B. tulda* (Saxena, 1990) and *D. strictus* (Mascarenhas and Murlidharan, 1989). In case of *M. dubia* IBA is best which

play a key role in root induction. The procedure offers an efficient and rapid method which can be adopted commercially for mass multiplication of *M. dubia*. MS medium supplemented with NAA 0.5 mg/l + BAP 1.0 mg/l on callus multiplication, BAP 0.5 mg/l + IAA 2 mg/l for shoot regeneration and MS/2 with IBA 0.5 mg/l for *in vitro* rooting and has been selected for efficient and rapid multiplication of *M. dubia* 30 days after inoculation. Also the technology developed may be used to get sustained supply of *M. dubia*, which requires raising them on a mass scale for plantations and forestation purposes. Commercial exploitation of this protocol for multiplication of this economically important species is possible as demand for fresh explant can meet easily.

Plants produce a diverse number of secondary metabolites in response to changing oxidative environment. As such plant extracts are expected to contain a diversity of molecules with different structures and functions. *M. dubia* is being previously reported for the presence of flavonoids and phenolic compound (Murugesan et al., 2013; Gopal and Manju, 2015). In the present study total flavonoids content, phenolic content and antioxidant potential were determined from the leaves of tissue culture raised plant of *M. dubia*. In this study, flavonoid content was found to be 0.56 ± 0.08 mg quercetin equivalent (mg QE) per gram dry weight of the plant material, while total phenolic content was 2.97 ± 0.17 mg gallic acid equivalent (mg GAE) per gram dry weight of the plant material. Flavonoid and phenolic content reported in other species of meliaceae family such as; *A. indica* was 0.2 to 1.07 mg/g of plant material (phenolic content) and 0.61 to 5.29 mg/g of plant material (flavonoids content). These report are comparable with the total flavonoids and phenolic content of *M. dubia* (Khamis Al-Jadidi and Hossain, 2015).

Conclusion

As such there is no report on efficient protocol for mass propagation of *M. dubia*. Therefore, the present work was aimed to develop *in vitro* propagation protocol for rapid and large scale production of planting material. The results demonstrate that the leaves may act as excellent source for isolation of potential antioxidants with significant amount of flavonoids and phenolics. They may serve as natural antioxidants in pharmaceutical preparations.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

BAP, 6-Benzylaminopurine; **NAA**, α -naphthalene acetic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **DPPH**, 2, 2-diphenyl-1-picrylhydrazyl; **GAE**, gallic acid equivalent; **QE**, quercetin equivalent.

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