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In vitro Callus Induction of *Catunaregam spinosa* Using Leaves as Explant

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PKL wrote the first draft of the manuscript. Author WTPSKS and author MLAMSM proof read the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *C. spinosa* (Family Rubiaceae) is a valuable medicinal since years ago. A protocol was developed including best sterilization and best medium for callus induction of *C. spinosa* using leaf discs as explants.
Methodology: Sterilization protocol optimized using different concentrations of Carbendazim® (0.2, 0.3%) and Clorox (10, 15%) exposing to different time intervals (10, 15 min). Percentage survival and contaminations were calculated. Best medium was optimized using different concentrations of 6-Benzyl Amino Purine (BAP) and Naphthalene Acetic Acid (NAA) (1.0-6.0 mg L^{-1}). Growth regulators free Murashige and Skoog (MS) medium was used as control. Completely randomized design was followed with ten replicates in each concentration. Days taken to initiate calli, morphological characteristics and mean dry weights of calli were evaluated after 3 months of incubation.
Results: Leaf discs sterilization with 0.3% Carbendazim for 10 min, 10 % Clorox for 10 min and 70% ethanol for 30 sec followed by two washings in sterile distilled water was found to be best sterilization protocol. It recorded lowest percentage contamination (13.34%) and highest percentage survival (86.66%) after 8 weeks. No observable changes were found in calli grown in growth regulators free MS medium. Calli growth and morphologies were significantly affected by the type of growth regulator and their concentrations in MS medium. Color of the calli varied from white opaque to yellow brown to green and the texture from foamy, loose, and friable to compact. Best medium

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with 1.0 mg L^{-1} BAP and 3.0 mg L^{-1} NAA produced green friable calli with 0.0969±0.01 g mean dry weight after 3 months. Some traits were found to be depended on synergetic effect of growth regulators and genotypic characteristics of explant.
Conclusion: The study provides a better sterilization protocol and medium for *in vitro* calli induction and growth of *C. spinosa*.

Keywords: Catunaregam spinosa; tissue culture; sterilization; plant growth regulators; callus.

1. INTRODUCTION

Catunaregam spinosa (Thunb.) Tirveng is well recognized medicinal plant grows in tropical and sub-tropical countries. *C. spinosa* contains diverse range of phytochemicals including alkaloids, saponins, triterpenoids, flavonoids, cardiac and cyanogenic glycosides, tannins and volatile oils. These compounds employ in different pharmacological activities such as cytotoxicity, piscicidal, anti- bacterial, anti-inflammatory, hepatoprotective, insecticidal and anthelmintic activities. Alkaloids are important in treatments of cancer, malaria and diabetes [1]. Further it is used 25-75% in drug development [2]. Saponins have hypolipidemic, cytotoxic and anti-diabatic properties [3,4,5]. Jangwan and Singh, [6] studied *in vitro* cytotoxicity of stem bark of *C. spinosa* on Breast cancer cell line (MDA-MB-231), Human Neuronale Glioblastoma (Astrozytom) cell-Line (U87MG), Human Skin Melanoma cell line (SK-MEL-2) and Neuroblastoma cell line derived from human bone marrow (BE(2)C). It revealed significant effect of triterpene isolated from stem bark on SK-MEL-2 cell line as 93.33 µg mL^{-1} (IC50) [6]. Ragib et al. [7] studied cytotoxicity of methanolic and aqueous leaf extracts *of C. spinosa* using brine shrimp lethality assay and reported 0.94 and 0.23 µg mL^{-1} as LC50 compared to Dimethyl sulfoxide (LC50, 1.07 µg mL^{-1}) [7]. Traditional people used crushed parts of *C. spinosa* for fish harvesting and several literatures mentioned about its piscicidal activity [8-11]. Anthelmintic activity of fruit pulp of *C. spinosa* has reported against tapeworms and earthworms as folklore remedy [12]. Ethanol extract of fruits of *C. spinosa* paralyzed and killed *Pheretima posthuma* in a short period of time (8.47± 0.42 min.) compared to ethyl acetate and petroleum ether extracts (14.26 ± 0.61 min. and 26.20 ± 0.67 min. respectively) [13]. Numerous studies mentioned the antioxidant activity of different parts of *C. spinosa.* Satpute et al. [14] found high nitric oxide scavenging activity of chloroform (350.0 µg mL^{-1}) and ethyl acetate (480.0 µg mL^{-1}) fractions of fruits of *C. spinosa* [14]. Superior antioxidant activity of leaves was recorded as 85% and 2.5 µg mL^{-1} using DPPH and Fluorescence recovery after photobleaching (FRAP) assays respectively [15]. Hepatoprotective activity of *C. spinosa* was revealed by Kandimalla et al. [16] using carbon tetrachloride (CCl_4) induced hepatically damaged wistar rats. It found reversal of abnormal parameters related to hepatic damages after diagnosed with ethanol leaf and bark extracts of *C. spinosa* [16]. High anti- inflammatory activity was revealed in both methanol and ethyl acetate extracts of fruit of *C. spinosa* on induced rat paw inflammation at 200.0 mg/kg [17]. Emesis activity is mostly reputed pharmacological activity of *C. spinosa* where its extreme bitterness induces the vomiting sensation [18]. Anoopkumar, [19] studied insecticidal activity of seeds of *C. spinosa* on 4th instar larvae of *Aedes agypti* and found LC50 as 210.212 mg L^{-1} [19].

Plant tissue culture is a rapid method of producing plantlets under controlled conditions. It overcomes the limitations incurred in conventional propagation such as long term seed dormancy, lack of seed viability, delayed rooting of seedlings, constraints due to seasonal changes while expanding the potential of mass propagation. Conventional propagation methods such as seed germination, rooting of cuttings and grafting show low growth rate. This can be expected due to extended time duration taken by plants to produce high content of secondary metabolites. Root suckers are also natural asexual reproduction method reported in *C. spinosa* [20]. There are many types of cultures such as protoplast, embryo, anther, ovule, ovary cell and callus cultures [21]. In present study callus cultures were used due to its common use in tissue culture and high possibility of working on most of the plant species. Thus it will be advantageous to get an idea about *In vitro* growth of *C. spinosa* using leaf discs as explants which is not previously studied. Moreover, callus culture is an easy method with basic steps compared to protoplast cultures where it undergoes enzymatic cell wall degradation, callus formation and later production of plantlets [22,23]. Diploid plantlets are produced through

callus cultures unlike ovary or anther cultures which produce haploid plantlets. Leaves, nodes and internodes are readily available for callus cultures where maternal tissues of ovules, seeds or capsules isolated in aseptic conditions are used for embryo cultures. [24]. Calli are the prerequisite element for cell cultures that can be obtained from callus cultures. Calli are potent sources of valuable secondary metabolites which are produced in a significantly short period of time [25,26]. Thus callus culture is a better alternative method compared to other types of cultures in direct production of calli for isolation of important secondary metabolites. Ramos et al. [27] used callus culture of *Ageratina pichinchensis* to produce secondary metabolites with anti-inflammatory activity [27]. Jain et al. [28] reported callus culture of *Alternanthera brasiliana* in producing antinociceptive compounds [28]. Based on these factors callus culture was used as the type of culture in producing calli of *C. spinosa* using leaf explants. Pharmaceutical industry shows raised demand over secondary metabolites. It is not sufficiently addressed due to low production rate of metabolites in plants up to the desired level [29,30]. However, literatures on *in vitro* growth of *C. spinosa* were fairly low. Further no reports were found regarding *in vitro* callus induction of *C. spinosa* using leaf explants. Begum et al. [31] studied *in vitro* seed germination of *C. spinosa*. Results mentioned maximum number of shoots (12.7) obtained from seedlings grown in MS medium supplemented with 1.0 mg L^{-1} each of BAP and NAA [31].

Nevertheless, each species demands unique plant growth regulators and concentrations for an optimum *in vitro* growth [32]. Thus, it is prerequisite to optimize particular level of concentrations of growth regulators in callus induction of *C. spinosa* whereas a sterilization protocol needs to be optimized beforehand. Present study intended to develop a successful sterilization protocol and to determine the best medium for *in vitro* callus induction of *C. spinosa* using leaf explants.

2. MATERIALS AND METHODS

2.1 Optimization of Surface Sterilization Protocol

Immature leave were collected from plants grown in an open area of University of Sri Jayewardenepura, Sri Lanka. Leaves were washed with disinfectants (Dettol), 5% teepol (v/v, liquid detergent) under running tap water for 1 h. After that leaves were washed with few drops of Tween 20 for 5 mins. Under aseptic conditions sterilization protocol was tested with 0.2 and 0.3% of Carbendazim® for 10 and 15 min. and Sodium hypochlorite (Clorox) with 10 and 15% for 10, 15 mins. Finally the explants were washed with 70% ethanol for 30 sec. Each step was followed with two successive washings in sterile distilled water. Sterilized leaves were cut into 1.0 cm^2 pieces along midrib. Leaf discs were cultured on half MS medium [32]. Well-sealed culture media were incubated under 25°C and 60% humidity. They were provided with 50 µmol m^{-2} s^{-1} white fluorescent light in 16/8 h light/ dark photoperiod. There were 15 replicates in each treatment. Percentage contamination and percentage survival were determined after three months of incubation.

2.2 Determination of Best Medium for Callus Induction from Leaf Explants

Immature leaves from *in vivo* grown plants were used as explants. Leaf cuttings were sterilized following best surface sterilization protocol determined. Half MS medium [33] containing macro and micro nutrients, vitamins, Ethylenediamine tetraacetic acid (EDTA) ferric sodium salt, sugar and agar was supplemented with different concentration of NAA and BAP (1.0 -6.0 mg L^{-1}). Nitrogen as a macro nutrient plays a vital role in production of amino acids, nucleic acids and proteins in new cells development. Micro nutrients which need in small quantities important for healthy growth of calli especially iron for chlorophyll synthesis. Proper functioning of enzymes in developing calli is assisted by vitamins such as Thiamine and Myo-inositol found in MS medium which act as coenzymes [34,35]. Sugar was used as carbohydrate and energy source and also to balance the osmotic pressure [36]. EDTA acts as chelating agent to make iron available in nutrient solution otherwise it precipitates and becomes unavailable to plantlets [37]. Different concentrations of BAP and NAA were examined which produce signals either to stimulate, inhibit or regulate the optimum growth and development of calli [38]. Medium pH was adjusted to 5.78± 0.02. Culture media were sterilized by autoclaving. Growth regulators free media were used as controls. Completely randomized design was followed with 10 replicates in each concentration. Color and texture of calli were determined by visual observations. Dry weights of calli were measured after three months of growth. Data were

statistically analyzed using ANOVA at significant level of p =0.05. Medium which produced healthy flourished calli within a minimum number of days with a significantly high mean dry weight was selected as the best medium for obtaining calli of *C. spinosa* from leaf discs explants.

3. RESULTS AND DISCUSSION

3.1 Optimization of Surface Sterilization Protocol

Protocol which exhibited lowest percentage contamination and highest percentage survival selected as the best sterilization protocol. Best sterilization protocol found to be the leaf discs washing with 0.3% carbendazim for 10 minutes, 10% Clorox for 10 minutes and 70% ethanol for 30 sec followed by successive washing twice with sterile distilled water. It exhibited lowest percentage contamination (13.34%) and highest percentage survival (86.66%) after eight weeks of incubation (Table 1). At 15% concentration of Clorox soft tissues of leaf discs tended to damage by necrosis in first week of incubation. Explants disinfected with 0.3% carbendazim for 15 mins recorded lowest contamination percentages of 13.33%. However, exposure to a high concentrations of fungicides and bleaching agents for a long period of time can lead for growing mutations, affect the regeneration capacity, cell viability and bleaching of cells causing them to die and to reduce the survival rate [39]. This trait was observed when leaf discs exposed to15% carbendazim and 15% Clorox each for 15 min. The combination significantly reduced the percentage contamination (13.33%) and concurrently decreased the percentage survival (46.66%) (T12). However, about 40.01% of explants were died following browning in this medium. Surprisingly, all replicates of best combination of carbendazim and Clorox produced calli with same percentage contamination (13.34%) with highest survival rate (86.66%). Thus 0.3% carbendazim for 10 mins and 10% Clorox for 10 min were selected as a part of best sterilization protocol for *In vitro* culturing of *C. spinosa* using leaf explants.

3.2 Determination of Best Medium for Callus Induction from Leaf Explants

Callus induction and growth rate varied among the tested concentrations (Table 2). Calli grown in plant growth regulators free MS medium (control) showed no observable changes over first 3 weeks (Plate 1a). Media supplemented with plant growth regulators took minimum 12.2±0.79 mean number of days for callus initiation. It proved the significant role of plant growth regulators in callus induction and their growth. Cells regeneration process was observed with inwardly rolled and swelled leaf discs with wavy cutting edges after two weeks of incubation (Plate 1b). After three months of growth healthy, flourished calli were observed with protuberance appearance on calli grown in media supplemented with plant growth regulators (Plate 1c).

Table 1. Percentage survival and percentage contaminations of different treatments used for optimizing surface sterilization protocol of *C. spinosa* leaf explants

Treatment code	Clorox		Carbendazim		% survival	% contamination
	Concentration (v/v)	Exposure time (mins)	Concentration (w/v)	Exposure time (mins)		
T1	10	10	0.2	15	40.00	60.00
T2	10	10	0.2	10	13.34	86.66
T3	10	10	0.3	15	80.00	20.00
T4	10	10	0.3	10	86.66	13.34
T5	10	15	0.2	10	73.33	26.67
T6	10	15	0.2	15	66.66	33.34
T7	15	15	0.2	10	53.33	46.67
T8	15	15	0.2	15	46.67	66.66
T9	15	10	0.3	10	73.33	26.67
T10	15	10	0.3	15	66.66	34.34
T11	15	15	0.3	10	53.33	46.67
T12	15	15	0.3	15	46.66	13.33

Table 2. Effect of different concentrations of BAP and NAA on mean no. of days and mean dry weight of *C. spinosa* leaf explants after three months of growth

Culture tag	Concentration		Mean no. of days taken to initiate callus ± SD (Days)	Mean dry weight (g)
	BAP	NAA		
C0	0	0	25.4±0.96	0.013±0.005
C1	1	0	18.5±0.52	0.014±0.005
C2	2	0	18.4±0.51	0.018±0.010
C3	3	0	17.8±0.78	0.021±0.005
C4	4	0	17.1±0.74	0.026±0.01
C5	5	0	17.2±0.78	0.030±0.006
C6	6	0	17.1±0.74	0.034±0.006
C7	0	1	15.9±0.73	0.042±0.007
C8	1	1	16.8±0.78	0.043±0.032
C9	2	1	16.0±0.82	0.057±0.009
C10	3	1	16.2±1.03	0.075±0.007
C11	4	1	16.4±0.51	0.069±0.011
C12	5	1	16.3±1.06	0.079±0.010
C13	6	1	16.0±0.94	0.074±0.014
C14	0	2	14.3±0.95	0.086±0.052
C15	1	2	16.6±0.69	0.084±0.027
C16	2	2	13.2±0.78	0.091±0.045
C17	3	2	12.6±0.69	0.094±0.045
C18	4	2	12.8±1.03	0.090±0.014
C19	5	2	12.2±0.79	0.090±0.011
C20	6	2	13.2±0.79	0.096±0.037
C21	0	3	12.8±0.78	0.086±0.020
C22	1	3	13.2±1.03	0.096±0.019
C23	2	3	13.3±1.06	0.073±0.010
C24	3	3	13.7±0.97	0.084±0.022
C25	4	3	14.7±0.67	0.074±0.018
C26	5	3	13.5±1.19	0.074±0.014
C27	6	3	13.0±0.70	0.078±0.009
C28	0	4	13.2±0.78	0.046±0.008
C29	1	4	14.2±0.78	0.074±0.007
C30	2	4	14.0±0.94	0.081±0.024
C31	3	4	14.7±0.95	0.088±0.009
C32	4	4	15.0±1.05	0.066±0.020
C33	5	4	15.4±1.33	0.051±0.019
C34	6	4	16.9±0.88	0.073±0.010
C35	0	5	15.7±0.67	0.070±0.009
C36	1	5	15.9±0.73	0.083±0.010
C37	2	5	15.8±0.79	0.074±0.007
C38	3	5	15.9±0.74	0.068±0.009
C39	4	5	16.0±0.81	0.083±0.015
C40	5	5	14.6±0.84	0.070±0.005
C41	6	5	16.2±0.63	0.064±0.014
C42	0	6	16.6±0.69	0.076±0.010
C43	1	6	15.9±0.74	0.053±0.007
C44	2	6	16.0±0.81	0.045±0.007
C45	3	6	17.0±0.82	0.032±0.009
C46	4	6	15.7±0.67	0.033±0.006
C47	5	6	17.1±0.73	0.033±0.009
C48	6	6	12.6±0.69	0.044±0.006

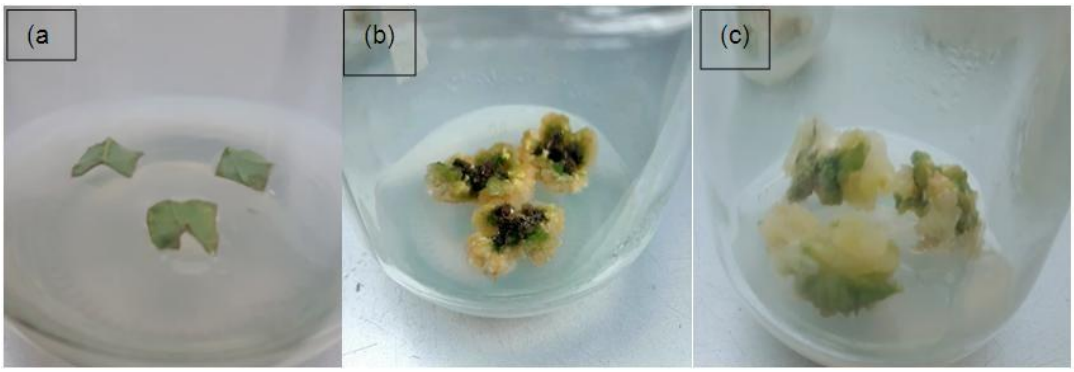

Plate 1. Calli grown in plant growth regulators (a) free MS medium after three weeks (b) added medium after two weeks (c) added medium after three months of incubation

Morphology and callus inductions have significantly influenced by both individual and combined concentrations of BAP and NAA. Texture was varied from friable, foamy, loose or compacted (Plate 2). Color varied from white opaque to yellow brown to green (Plate 3). Initiate calli. MS media supplemented with 1.0, 2.0 and 3.0 mg L^{-1} NAA concentrations alone only spent 15.9±0.73, 14.3±0.95 and 12.8±0.78 mean number of days for callus induction whereas no significant prompt callus induction was observed at high concentrations of NAA alone (4.0, 5.0 and Concentration dependent callus growth was observed (Table 2). Callus induction and growth varied with single or combined use of BAP and NAA. NAA alone initiated the callus induction. Both callus induction and growth rate were low when BAP alone used. High concentrations of NAA inhibited the callus induction where it recorded relatively high mean number of days to 6.0 mg L^{-1}). Greenish compact calli were observed at low concentrations of NAA independent of the BAP concentration. Their mean dry weights were <0.04g and mean number of days taken to initiate calli were between 18.5 - 17.1 days. Calli grown in MS media supplemented with 2.0 – 3.0 mg L^{-1} NAA (C16-C27) recorded lowest mean number of days (12.2- 14.7 days) for callus induction compared to all other combinations. They produced flourished, friable calli with high mean dry calli weights. MS media treated with 4.0 – 6.0 mgL^{-1} concentrations of NAA (C29-C48) produced more whitish and loosen calli with 14.0 -17.1 days mean number of days. There were some instances followed opposite of this scenario where treatments with high concentration of NAA recorded low mean number of days and vice versa. MS media supplemented with 5.0 mg L^{-1} (C40) and 6.0 mg L^{-1} (C48) NAA only spent 12.6±0.69 and 12.8±0.78 days respectively and MS medium treated with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} NAA recorded relative high mean number of days (15.5±0.92 days) to produce calli. These can be probably caused by effect of genotypic characteristics of explants on callus induction [40].

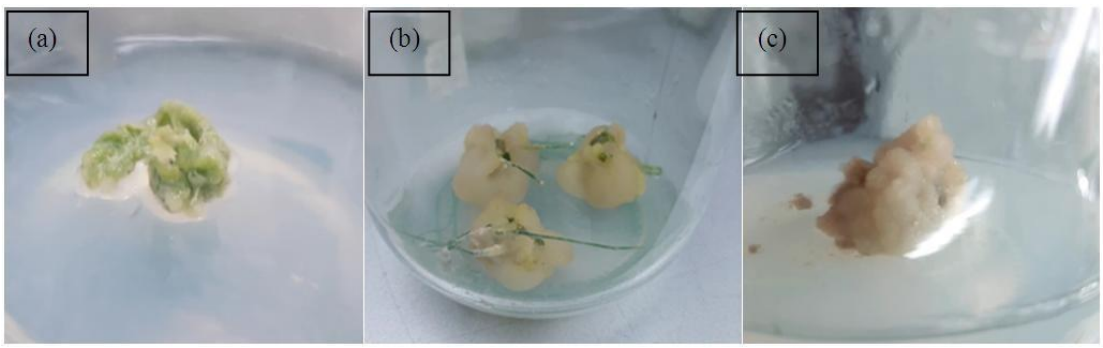

Plate 2. Texture morphology of calli grown in MS media supplemented with different concentrations of BAP and NAA (a) compact (b) foamy (c) friable

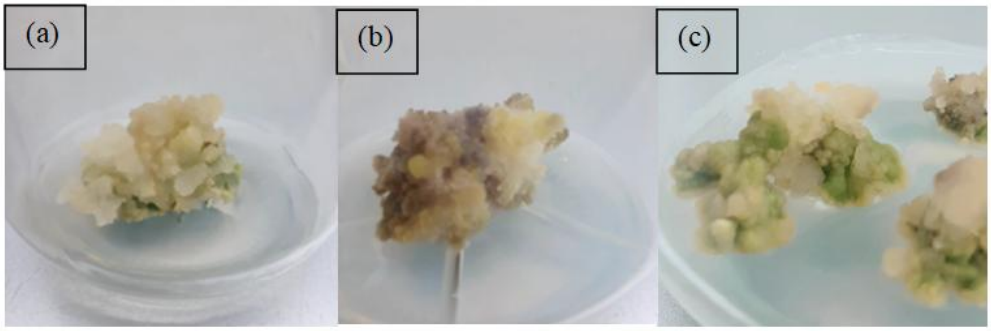

Plate 3. Color morphology of calli grown in MS media treated with different concentrations of BAP and NAA (a) white opaque (b) yellow brown (c) green

BAP started to form new cells by stimulating cell division at the presence of optimal concentration of NAA. New cell formation expanded the leaf explants to form calli with optimum weights. Based on that we can predict the possible reason compared to all other combinations. They produced flourished, friable calli with high mean dry calli weights. MS media treated with 4.0 – 6.0 mgL^{-1} concentrations of NAA (C29-C48) produced more whitish and loosen calli with 14.0 -17.1 days mean number of days. There were some instances followed opposite of this scenario where treatments with high concentration of NAA recorded low mean number of days and vice versa. MS media supplemented with 5.0 mg L^{-1} (C40) and 6.0 mg L^{-1} (C48) NAA only spent 12.6±0.69 and 12.8±0.78 days respectively and MS medium treated with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} NAA recorded relative high mean number of days (15.5±0.92 days) to produce calli. These can be probably caused by effect of genotypic characteristics of explants on callus induction [40].

BAP started to form new cells by stimulating cell division at the presence of optimal concentration of NAA. New cell formation expanded the leaf explants to form calli with optimum weights. Based on that we can predict the possible reason days (13.2±1.03 days) to produce pronounced, healthy, green and friable calli. Ikeuchi et al. [41] mentioned intermediate ratio of auxin to cytokinin promotes callus formation and high ratio of auxin to cytokinin and vice versa promote root and shoots respectively [41]. However according to Neibaur et al. [42] high concentration of auxin to low concentration of cytokinin is typical combination to promote mass cell proliferation [42]. The same scenario has proven in a study of callus induction of *Coffea arabica* L. using leaf cultures. The prominent friable calli have formed in 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP concentrations [40]. In our study also best medium was contained with high concentration of auxin (3.0 mg L^{-1}) to low concentration of cytokinin (1.0 mg L^{-1}) ratio.

4. CONCLUSION

Callus production is possible in *C. spinosa* as a plant with significant phytochemicals. Best sterilization protocol for explants grown in open environment is disinfecting with 0.3% carbendazim for 10 mins, 10% Clorox for 10 mins. and 70% ethanol for 30 sec. which recorded lowest contamination and highest survival percentages compared to other combinations. Half MS medium supplemented with 3.0 mg L^{-1} of BAP and 1.0 mg L^{-1} NAA was best medium for callus induction of *C. spinosa* from leaf discs. Callus morphology and mean dry weights of calli were depended on the type of plant growth regulator and their concentrations. Present study provides better sterilization protocol and medium for *in vitro* callus induction and growth of *C. spinosa* using leaves as explants which would assist any future studies regarding secondary metabolites isolation or other applications calli of *C. spinosa* are involved.

DISCLAIMER

Commonly and predominantly used products in Sri Lanka have been used for this research. This research was conducted solely for advancement of knowledge. Thus there is no conflict of interest between authors and companies of products supplied. Further, research is completely funded by University of Sri Jayewardenepura under the grant no: ASP/01/RE/2019/15 not by any other product producing company.

SIGNIFICANCE OF THE STUDY

This study provides a better protocol for *in vitro* callus induction of *C. spinosa* which is a reputed medicinal plant since years ago. Mass propagation of *C. spinoa* plantlets can be ensured via indirect organogenesis if the callus production protocol was developed. Further calli can be used as the source of secondary metabolites which would stamp the value of *C. spinosa* as a medicinal plant more in the pharmaceutical industry.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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