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Full Length Research Paper

Evaluation of free radical scavenging activity and total phenolic content in the petiole-derived callus cultures of *Zingiber zerumbet* Smith

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This is the first report on the antioxidant activity of callus cultures of *Zingiber zerumbet.* Callus cultures were initiated from petiole explants of *in vitro* plantlets of *Z. zerumbet* on MS medium supplemented with 0.5 mg/L 2,4-D. Bright yellow and friable callus cultured on MS medium supplemented with 0.5 mg/L 2,4-D in combination with kinetin, picloram and 1-naphthaleneacetic acid (NAA) did not show any significant difference in biomass as compared to those cultured on MS medium supplemented with 0.5 mg/L 2,4-D. 30 g/L sucrose was found the best for callus growth and proliferation. Callus of *Z. zerumbet* proliferated better in the presence of light. Subculture frequency of *Z. zerumbet* callus culture showed consistency of callus biomass production. The anti oxidant activity of Z. *zerumbet* callus extract was evaluated by 2,2 diphenyl -1- picrylhydrazyl (DPPH) free Radical Scavenging Activity (RSA) and Total Phenolic Content (TPC) assay. The RSA of callus cultures of *Z. zerumbet* was concentration dependent with an EC 50 value of 254.55 µg. At a concentration of 750 µg/ml the callus extract was able to quench 93% of the DPPH free radical in 30 min. The TPC of the callus extract was estimated to be 9 mg GAE / g extract. There was a good correlation between DPPH free radical scavenging activity and TPC.

Key words: *Zingiber zerumbet, in-vitro*, phenolics, anti-oxidative activities, Zingiberaceae.

INTRODUCTION

Zingiber zerumbet is a perennial medicinal herb belonging to the family Zingiberaceae, locally known as lempoyang or wild ginger in Malaysia (Holttum, 1950). It is also called as “shampoo ginger” because of the mucilaginous substance present in the inflorescence and is used as shampoo and natural hair conditioner (Nalawade et al., 2003; Sabu, 2003). Traditionally, *Z. zerumbet* is used in the treatment of swelling, sores and loss of appetite. The juice of the boiled rhizomes has also been used as a medicine for worm infestation in children (Faizah et al., 2002). Decoction prepared from the rhizomes or juice obtained from the fresh rhizomes is used to treat jaundice (Ong and Norzalina, 1999). The crushed leaves and rhizomes of *Z. zerumbet* produce an aromatic fragrance, indicating the presence of essential oils. According to Chane-Ming et al. (2003), the essential oils from the rhizomes were rich in zerumbone, α-pinene and camphene. The essential oils obtained from the leaves and flowers contained large amounts of (E)-neridiol, β-caryophyllene, linalool and zingiberene. Zerumbone is the major chemical compound present in *Z. zerumbet*. Other chemical constituents include zerumbone epoxide, diferuoylmethane, feuloyl-p-coumaroylmethane, di-p-coumaroylmethane, humulene, humulene derivatives, α-pinene, camphene, caryophyllene, caryophyllene epoxide and kaempferol (Matthes et al., 1980). *Z. zerumbet* was reported to have analgesic and antipyretic activities (Somchit et al., 2005), anti-giardial activity (Sawangjaroen et al., 2005), anti-amoebic activity (Sawangjaroen et al., 2006), high

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platelet-activating factor receptor binding inhibitory effects (Jantan et al., 2005), anti-inflammatory (Chien et al., 2008) and leukemia inhibitory activity (Huang et al., 2005). Zerumbone, the important sesquiterpenoid showed inhibitory effect of Epstein-Barr virus activation (Murakami et al., 1999), inhibitory effect of dextran sodium sulfate-induced colitis (Murakami et al., 2003) and anti-HIV activity (Dai et al., 1997). *Z. zerumbet* also showed significant anti-proliferative activity against various cancer cells (Shafirah-Sakina et al., 2007). The principle method of vegetative propagation of *Z. zerumbet* is through rhizomes and efficiency of vegetative propagation is low. Therefore, plant tissue culture techniques can be used as alternative methods for mass propagation and conservation of *Z. zerumbet*. Also through this technique, uniform plant materials can be obtained without the influence of environmental factors. Previous reports on *in vitro* culture of *Z. zerumbet* include micropropagation (Hsu et al., 1999; Stanly and Chan, 2007) and somatic embryogenesis (Idris et al., 2009). Some studies on the antioxidant activities of *Z. zerumbet* rhizomes had been reported (Lako et al., 2007; Ruslay et al., 2007). According to Lako et al. (2007), *Z. zerumbet* grown in Fiji contain highest levels of kaempferol compared to other fruits and vegetables and is traditionally eaten before meals.

However, there is no report on determining the antioxidant activities of *in vitro* cultures of *Z. zerumbet*. The objectives of the present study are (i) to establish callus cultures of *Z. zerumbet* and (ii) to determine the anti-oxidant activity and phenolic contents of the callus cultures.

MATERIALS AND METHODS

Establishment of callus cultures

In vitro plantlets of *Z. zerumbet* established by Stanly and Chan (2008) were used for callus induction. Different plant parts, petioles (1 cm), roots (1 cm), rhizomes (small thin strips) and leaves (1 x 1 cm) were cut from the four weeks old *in vitro* plantlets and inoculated onto MS (Murashige and Skoog, 1962) medium supplemented with different concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram. All the media were dispersed in boiling tubes (2.5 x 1.5 cm) and the culture media were supplemented with 30 g/L sucrose and solidified with 8 g/L agar (Algas, Chile). The pH of the culture media was adjusted to 5.75 prior to autoclaving at 121°C for 11 min under a pressure of 1.05 kg cm^2 (Tommy Autoclave SS-325). All the cultures were maintained at 23 ± 2°C in a culture room under continuous lighting provided with cool white fluorescent lamps at 35 µmol m^{-2} s^{-1} for a period of six weeks. The experiment was replicated six times.

Effect of different plant growth regulators, illumination and sucrose on the callus proliferation of *Z. zerumbet*

To determine the optimum concentration of 2,4-D to be supplemented into the culture medium, friable callus (0.5 g) were cultured onto MS medium supplemented with different concentrations of 2,4-D (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) dispersed in 250 ml glass jar for a period of four weeks.

To study whether there is a synergetic effect of 2,4-D with other plant growth regulators, friable callus (0.5 g) of *Z. zerumbet* were cultured onto MS medium supplemented with 0.5 mg/L 2,4-D in combination with different concentration of kinetin, 1-naphthaleneacetic acid (NAA) and picloram (4-amino-3,5,6-trichloro picolinic acid) (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) for a period of four weeks.

Friable callus (0.5 g) of *Z. zerumbet* were cultured onto MS medium supplemented with 0.5 mg/L 2,4-D dispersed in 250 ml glass jar for a period of four weeks to evaluate the effect of illumination on the callus proliferation. The callus cultures were incubated under continuous illumination with cool white fluorescent lamps at 35 µmol m^{-2} s^{-1} and total darkness.

To determine the effect of sucrose presence in the culture medium on the proliferation of callus, friable callus (0.5 g) of *Z. zerumbet* were cultured onto MS medium supplemented with 0.5 mg/L 2,4-D supplemented with different concentrations of sucrose (15, 30, 45, 60 and 75, mg/L) over a period of four weeks.

Friable callus (0.5 g) of *Z. zerumbet* were cultured onto MS medium supplemented with 0.5 mg/L 2,4-D dispersed in 250 ml glass jar every four weeks for seven subculture cycles to evaluate the effect of subculture frequency on callus proliferation.

All the experiments were replicated six times. After four weeks of culture, fresh biomass was recorded using a digital balance (Acculab VI-3mg) and the dried biomass was determined after the callus was air-dried until constant weight was attained. Data were analyzed using Analysis of Variance (ANOVA) or Student t-test at $p \leq 0.05$ with the aid of software SPSS ver. 13.

Preparation of methanolic extract

Air dried callus of *Z. zerumbet* (5 g) were agitated and extracted with 100 ml methanol three times at room temperature by placing on an orbital shaker at 120 rpm (rotations per minute) for 24 h. The methanol extract was filtered (Whatman® filter paper 90 mm) and evaporated in vacuum at 40°C using a Rotary Evaporater (Eyela Rotary Vacuum Evaporator N-N Series). The residue obtained was stored in a freezer at -20°C for further study.

Determination of free radical scavenging activity (RSA)

Free RSA of callus extract of *Z. zerumbet* was estimated using modified method described by Blois (1958). One millilitre of 2, 2 diphenyl -1- picrylhydrazyl (DPPH) solution (0.1 mM of DPPH in methanol) was mixed with 3.0 ml of various concentrations (15.6 to 750 µg/ml in methanol) of callus extract. The mixtures were shaken vigorously by covering with aluminum foil and incubated at room temperature for 30 min.

The absorbance of the resulting solution was measured at 517 nm in a UV-Visible spectrophotometer (U 2000 Hitachi Ltd, Tokyo, Japan) to measure the content of remaining DPPH free radical. The solution without any extract and with DPPH and methanol was used as control. The RSA was calculated as percentage of $DPPH^{\bullet}$ discoloration using the formula:

$$\% \text{ RSA} = 100\text{x}\left(1 - \frac{A_E}{A_D}\right)$$

(A_E is the absorbance of the solution when the extract is added at a particular concentration and A_D is the absorbance of $DPPH^{\bullet}$ without extract). The experiment was conducted in triplicates. Ascorbic acid was used as a reference antioxidant for this test.

Determination of total phenolic content (TPC)

The TPC of the callus extract was determined by a modified Folin-Ciocalteu calorimetric method (Singleton et al., 1999; Eberhardt et al., 2000; Dewanto et al., 2002), with gallic acid as a standard phenolic compound. The extracts were diluted with distilled water to a known concentration in order to obtain the readings within the standard curve ranges of 0.0 to 600 µg of gallic acid /ml. A volume of 250 µl gallic acid solution or diluted callus extract was mixed with 1.0 ml distilled water in a test tube followed by the addition of 250 µl of Folin-Ciocalteu reagent. The samples were mixed well and then allowed to stand for 6 min at room temperature in order to allow complete reaction with the Folin-Ciocalteu reagent. Then 2.5 ml of 7% sodium carbonate aqueous solution was added and the final volume was made up to 6 mL with distilled water. The absorbance of the resulting blue colour solution was measured at 760 nm using a UV-Visible spectrophotometer (U 2000, Hitachi, Tokyo, Japan) after incubating the samples for 90 min. The result was expressed as mg of gallic acid equivalents (GAE)/g callus extract by using an equation that was obtained from standard gallic acid graph. The experiment was conducted in six replicates. Correlation and regression analysis of the total phenolic content (X) versus the DPPH free radical scavenging activity (Y) was carried out using Microsoft Office 2003 Excel.

RESULTS AND DISCUSSION

Establishment of callus cultures

Leaf, roots and rhizome explants cultured on different concentrations of 2,4-D and picloram did not produce any callus at the end of six weeks of culture period. All the explants turned necrotic after six weeks of culture. However, after 3 months of incubation, petiole explants cultured on MS medium supplemented with 0.5 mg/L 2,4-D started to produce creamy white callus from its cutting edges. The compact creamy white callus along with the explant was transferred into fresh MS medium supplemented with 0.5 mg/L 2,4-D under same culture conditions. Subculture of the callus was done every four weeks interval. The compact callus proliferated into a mass of nodular and yellowish compact calli with frequent subculture cycles. Some of the nodular calli produced roots, turned green in color and eventually regenerated into plantlets indicating their embryogenic potential. 2,4-D has been reported to stimulate cell division and promote the growth of embryogenic callus in previous studies for ginger (Guo and Zhang, 2005; Lincy et al., 2009). Present study confirmed that 2,4-D served as a trigger for the initiation of callus for *Z. zerumbet*. After a period of six months of subculture, the texture of the callus changed into granular type. With increasing subculture frequencies, the granular and yellowish callus turned into friable and bright yellow callus (Figure 1). This study indicated that *Z. zerumbet*, a monocotyledon plant, required longer duration for callus induction and proliferation. This hence proved that monocotyledonous plants often require longer time for callus initiation as compared to dicotyledonous plants as stated by Geier (1986). So far to our knowledge there was only one report on the induction of embryogenic callus from *Z. zerumbet* (Idris et al., 2009) using 2,4-D, NAA, and IAA under dark conditions. Callus induction had also been reported from other Zingiberaceae species using 2,4-D such as *Zingiber officinale* (Malamug et al., 1991; Guo and Zhang, 2005; Lincy et al., 2009), cardamom (*Elettaria cardimomum*) (Parvin et al., 1999) and *Kaempferia galanga* (Rahman et al., 2004). The present study also confirms that 2,4-D was essential for the initiation of callus for zingiberaceae species.

Effect of different plant growth regulators, sucrose, light and subculture frequency on the callus proliferation of *Z. zerumbet*

In order to optimize the callus growth of *Z. zerumbet*, it was necessary to manipulate the plant growth regulators as well as physical conditions. The petiole-derived callus of *Z. zerumbet* that was subcultured on MS medium supplemented with different concentrations of 2,4-D (0.5, 1.0, 1.5 and 2.0 mg/L) produced fresh biomass between 1 to 2 g and a dry biomass between 0.06 to 0.126 g from the initial inoculum of 0.5 g after 4 weeks of culture. MS medium supplemented with 0.5 mg/L 2,4-D induced the highest fresh and dried biomass as compared to other concentrations. It seemed that absence of 2,4-D and higher concentration of 2,4-D showed negative effect on the growth of the callus cultures of *Z. zerumbet*. Callus cultured on MS medium supplemented with 0.5 mg/L 2,4-D in combination with kinetin, picloram, or NAA did not show any significance difference in terms of fresh and dried biomass when compared to callus cultured on MS medium supplemented with only 0.5 mg/L 2,4-D (Figure 2). This indicated that 0.5 mg/L 2,4-D supplemented into the MS medium was sufficient for proliferation of *Z. zerumbet* callus tissues.

MS medium supplemented with higher concentrations of sucrose (45, 60 and 75 g/L) reduced callus proliferation. Highest callus biomass was obtained on MS medium supplemented with 30 g/L sucrose (Figures 3A and B). Decrease of callus biomass in higher concentration of sucrose was also reported for wheat callus (Javed and Akram, 2008). Sucrose has been used in plant tissue culture as a major source of carbon and energy. Sucrose also functions as an osmotic agent. According to Cleland (1977) and Zimmermann (1978) high levels of sucrose would inhibit the formation of turgor pressure which is required for cell expansion. The stress induced by this condition inhibits the callus growth which might the reason that high sucrose concentration lead to the reduction in callus growth. We can deduce that 30 g/L sucrose supplemented into the culture medium was suitable for inducing correct osmotic stress for optimum growth of *Z. zerumbet* callus. In our present study, MS medium supplemented with 0.5 mg/L 2,4-D and 30 g/L sucrose produced the highest fresh and dry biomass in the callus cultures of *Z. zerumbet.*

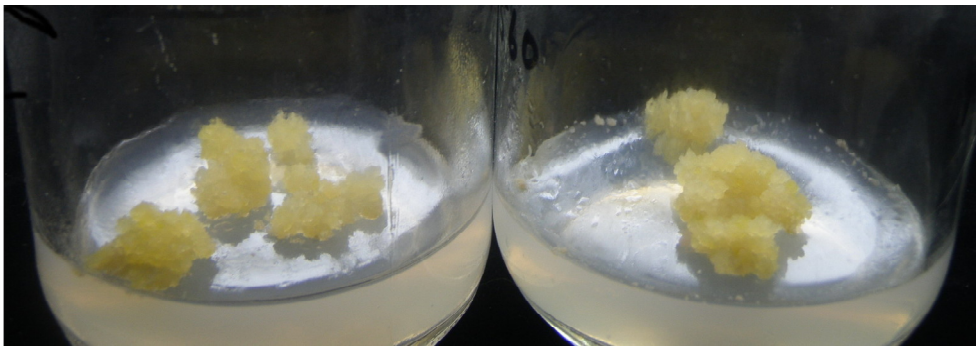

Figure 1. Bright yellow and friable petiole-derived callus of *Z. zerumbet* cultured on MS medium supplemented with 0.5 mg/L 2,4-D with frequent subcultures for 6 months.

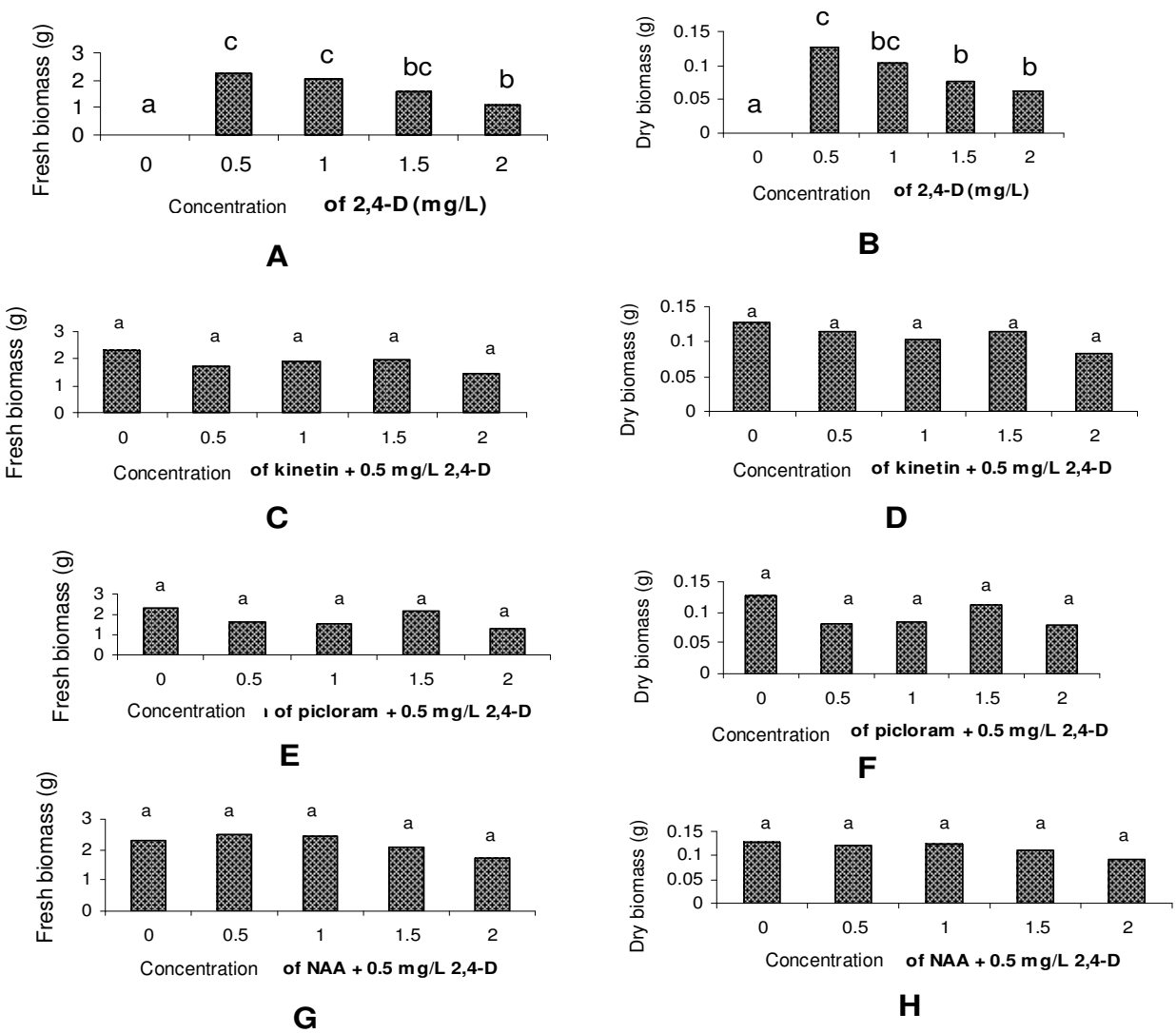

Figure 2. Effect of MS medium supplemented with 2,4-D alone and in combination with kinetin, picloram and NAA on the fresh and dry biomass of *Z. zerumbet* callus after 4 weeks of culture. (A) Fresh biomass and (B) dry biomass obtained from different concentrations of 2,4-D. (C) Fresh and (D) dry biomass from different concentrations of 2,4-D+kinetin. (E) Fresh and (F) dry biomass obtained from different concentrations of 2,4-D + picloram. (G) Fresh and (H) dry biomass obtained from different concentrations of 2,4-D + NAA.

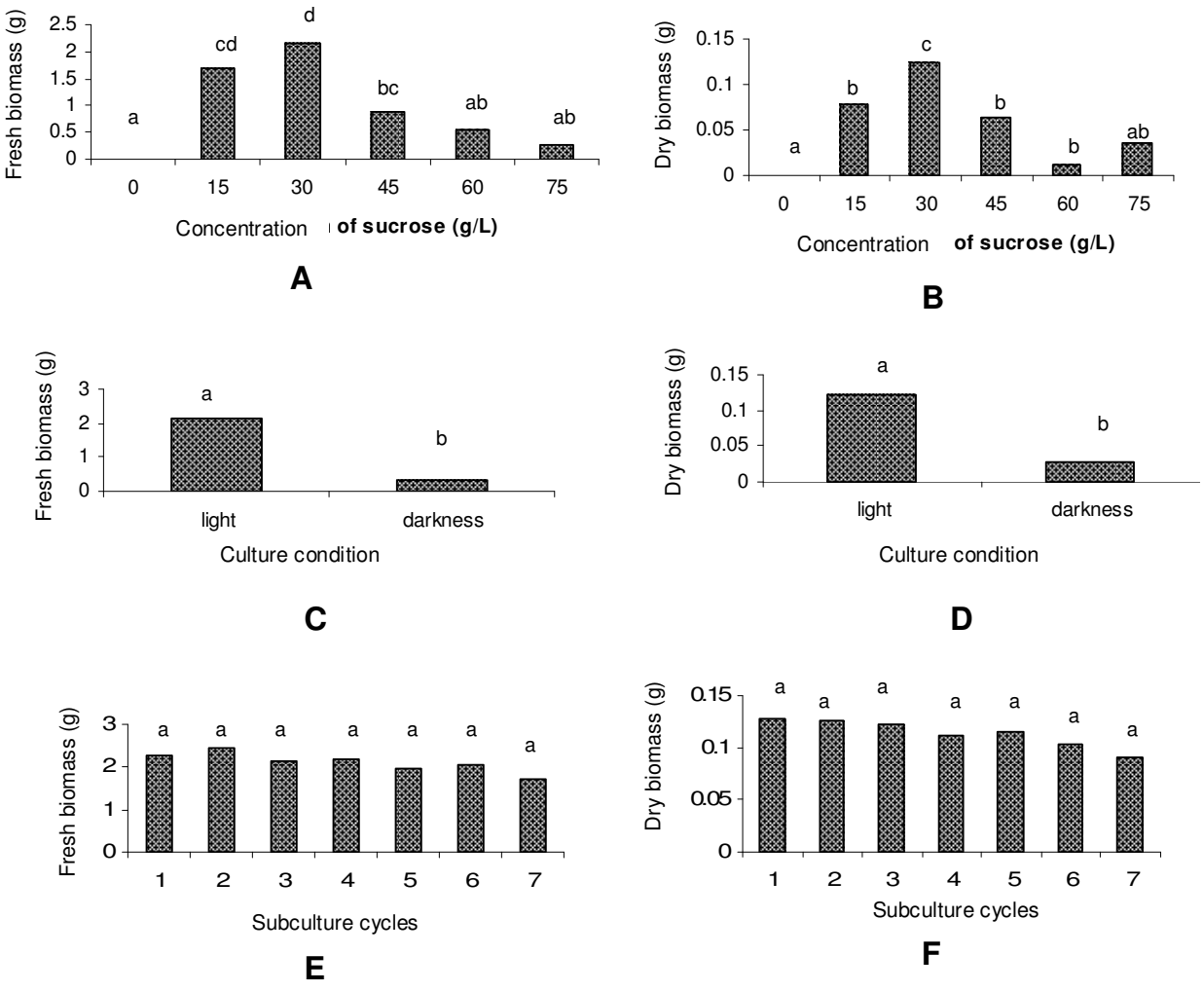

Figure 3. Factors affecting callus proliferation of *Z. zerumbet*. Effect of MS medium supplemented with different concentrations of sucrose on fresh (A) and dry biomass (B), effect of illumination on fresh (C) and dry biomass (D) and effect of subculture frequency on fresh (E) and dry biomass (F) of Z. zerumbet callus after 4 weeks of culture.

Callus cultured in the presence of light produced higher biomass (fresh and dried) than the callus cultured in total darkness (Figures 3C and D). The callus that was cultured in total darkness turned brown and necrotic. This showed that light is crucial for callus proliferation and maintenance. Light irradiation play important role in photosynthesis and is considered as an essential factor for the growth regulation, differentiation and metabolism (Wang et al., 2001). Similar findings was also observed by Tawfik and Mohamed (2007) who reported that callus proliferation rate in *Salvia officinalis* was higher under light condition. The callus of *Z. zerumbet* was found to proliferate consistently from the first until the seven subculture cycles. From the initial inoculum of 0.5 g of callus, a fresh biomass of 1.70 to 2.45 g (dried weight, 0.09 to 013 g) was obtained and was found to be not significantly different within the seven subculture cycles (Figures 3E and F). This showed that the callus culture of *Z. zerumbet* was stable as shown in the consistency of callus biomass production.

Free radical scavenging activity) and total phenolic content

DPPH• occurs as a stable free radical in aqueous or methanolic solution and accepts hydrogen atoms or electrons from the antioxidant molecules to become a stable diamagnetic and yellow coloured diphenyl-picryl hydrazine. It is a commonly used reagent to evaluate the antioxidant activity of plant extracts. A freshly prepared DPPH solution shows a deep purple coloration and the purple colour changes to yellow in the presence of the antioxidants. Hence, as the absorbance decreases the more efficient the antioxidant activity of the extract in terms of hydrogen atom donating capacity. The curve for

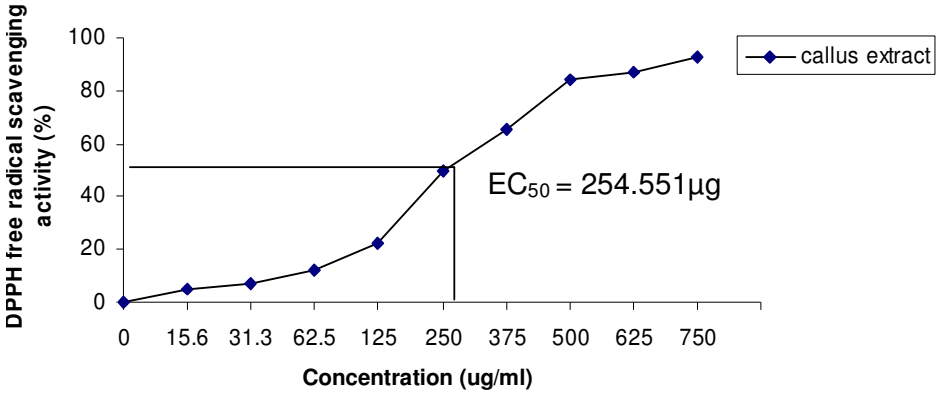

Figure 4. DPPH• free radicals scavenging activity of callus extract of *Z. zerumbet*.

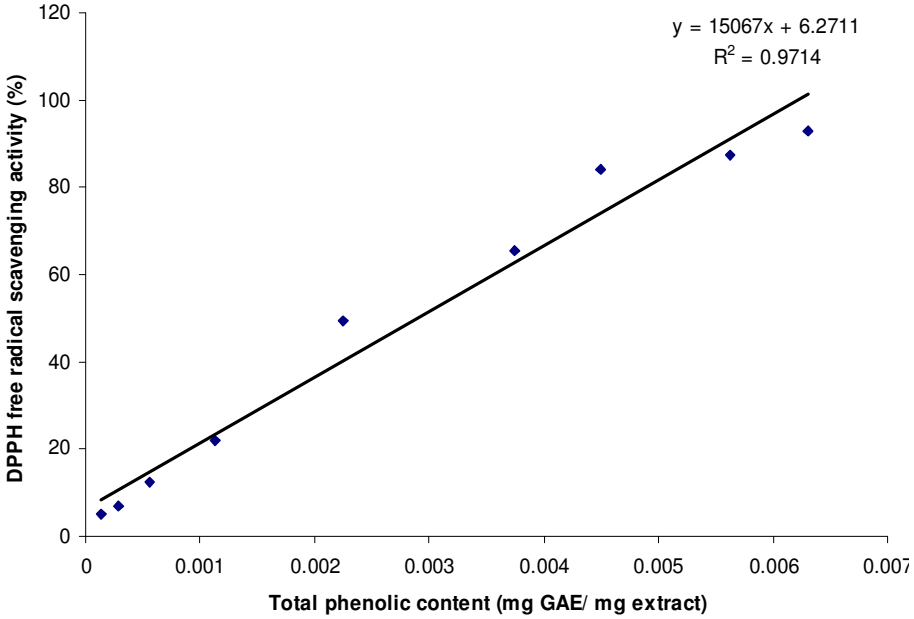

Figure 5. Linear correlation between total phenolic content and DPPH free radical scavenging activity.

the radical scavenging activity of *Z. zerumbet* callus extract was found to be dose dependent. Scavenging of DPPH radicals was found to be increasing as the concentration of the callus extract increased. Capability of removal the free radical was found to increase from 5 to 93% with the increase of the concentrations of the extract from 15.6 to 750 µg/ml. The minimum concentration of *Z. zerumbet* callus extract to scavenge 50% of DPPH [(EC_{50}) value] was 254.551 µg (Figure 4). The antioxidative property of *Z. zerumbet* callus extracts can be attributed to the presence of active compounds in them.

Phenolic compounds are important plant antioxidants which exhibited considerable scavenging activity against radicals. Thus antioxidant capacity of a sample can be attributed mainly to its phenolic compounds (Zheng et al., 2003; Chinnici et al., 2004; Huang et al., 2009). The antioxidant activity of phenolic compounds is due to their redox properties which help them to behave as reducing agents, hydrogen atom donors, and ability to quench the singlet oxygen. They also have metal chelating properties (Rice-Evans et al., 1995). TPC of the callus extract of *Z. zerumbet* was estimated by modified Folin-Ciocalteu calorimetric method. The TPC was found to be 9 mg GAE /g extract. In our present study, the correlation between TPC and DPPH scavenging activity was significantly high (r^2 = 0.9714; $p < 0.01$) (Figure 5). We could hence conclude that the anti-oxidative activities of *Z. zerumbet*

callus extract were mainly due to the phenolic compounds produced in the callus cultures.

Conclusion

It can be concluded that callus could be induced from petiole explants of *in vitro* plantlets of *Z. zerumbet* on MS medium supplemented with 0.5 mg/L 2,4-D. The friable callus cultures could be maintained by frequent subculture in the same medium. The antioxidant activity of *Z. zerumbet* callus extract was found to be increasing in a dose dependent manner. The correlation between total phenolic content and DPPH scavenging activity was significantly high. From our study it can be concluded that the antioxidant activity of the callus extracts is mainly due to the presence of phenolic compounds in the extract.

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