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Full Length Research Paper

Effect of yeast extract and chitosan on shoot proliferation, morphology and antioxidant activity of *Curcuma mangga* in *vitro* plantlets

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This paper reported the effect of yeast extract and chitosan with combination of yeast extract on the growth and morphological changes and production of phenolics in the *in vitro* plantlets of C*urcuma mangga*. Yeast extract did not show any effect on the biomass and shoot proliferation of *in vitro* plantlets. However, the plantlets showed morphological abnormality when exposed to higher concentration of yeast extract (3.5 mgL^{-1} and above) supplemented into the culture medium. Plantlets cultured in media supplemented with 3.5 and 5.0 mgL^{-1} of yeast extract showed higher radical scavenging activity (RSA) which also indicated that stress induced by yeast extract might elicit the production of secondary metabolites which acted as free radical scavenger in 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The plantlets treated with different concentration of chitosan combined with 3.5 mgL^{-1} of yeast extract affected the biomass of *C. mangga*. The plantlets that were cultured in media supplemented with 150 mgL^{-1} of chitosan combined and 3.5 mgL^{-1} of yeast extract showed higher RSA towards DPPH as compared to the other treatments. Kinetic of DPPH free RSA from *C. mangga* extract was considered slow as compared to quercetin and the correlation between total phenolic content and RSA was poor (R^2 = 0.2293) for yeast extract and (R^2 = 0.0373) for chitosan combination with yeast extract. This indicated that the presence of phenolic compounds in the extracts were not the major factor contributing to the anti-oxidative activity of *C. mangga*.

Key words: *Curcuma mangga*, i*n-vitro*, elicitor, phenolics, anti-oxidative activities.

INTRODUCTION

Family Zingiberaceae is widely known for its multiple uses ranging from culinary to medicinal preparations. *Curcuma mangga* Val., one of the important species of Zingiberaceae family, is native to Southern Asia (Larsen et al., 1999). It is known as Temu Pauh in Malaysia, Temu Mangga in Indonesia and Mango Turmeric in India. It has the name of mango because the fresh rhizomes possess the aroma of a raw mango. Reports revealed that the rhizome of *C. mangga* possessed anticancer and antioxidant properties (Abas et al., 2005; Chan et al., 2008). It was reported that the leaves of *C. mangga* possessed higher total phenolic content and free radical scavenging activity as compared to its rhizomes (Chan et al., 2008). Therefore further study is required in order to obtain more secondary metabolites from the leaves. High anti-antioxidant activities are mainly found in the mature plants of *C. mangga* and normally, it takes a long time to reach maturity. Moreover, this plant which propagates via conventional method is season dependent. This has

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Abbreviation: MS, Murashige and Skoog; **BA,** 6-benzylaminopurine; **NAA,** 1-naphtalene acetic acid; **RSA,** radical scavenging activity; **TPC,** total phenolics content; **DPPH,** 1,1-diphenyl-2-picrylhydrazil.

created a situation of lack of plant materials for the production of bio-actives compounds with anti-oxidant activities. Generally, most plants produce a very small quantity of secondary metabolites and its production may only occur under certain circumstances. Hence, *in vitro* culture techniques can be the alternatives for the mass production of plant materials which is not season dependent. In plant tissue culture, accumulation of secondary metabolites can be enhanced by the treatment of various kinds of elicitors, which can be biotic and abiotic. Previous studies showed that the accumulation of different secondary metabolites can be efficiently induced by using elicitor (Ekiert and Gomolka, 2000; Ekiert, 2001). Yeast extract and chitosan has been used in plant tissue culture due to their ability to stimulate the defence mechanism, which leads to increase secondary metabolite production. Therefore, the study reports the effect of yeast and chitosan on (i) Biomass and morphology of *C. mangga in vitro* plantlets, (ii) anti-oxidant activity and the total phenolic content of the *in vitro* plantlets of *C. mangga*.

MATERIALS AND METHODS

Establishment of *in vitro* culture

The young rhizome buds were used as explants and washed thoroughly under running tap water for 40 min to remove the soil contaminants. The buds explants (1.5 cm) were immersed in 70% ethanol for a few minutes followed by surfaced sterilization with 20% clorox®, a commercial bleach solution which contain 5.3% of sodium hypochlorite for 20 min. The sterilized explants were rinsed three times with sterile distilled water. The sterilized bud explants were inoculated on Murashige and Skoog (1962) (MS) basal medium for three weeks and the aseptic plantlets derived from these bud explants were subsequently used for shoot proliferation. The *in vitro* shoot explants were then subcultured onto MS medium supplemented with 6-benzylaminopurine (BA) 2 mgL^{-1} and 1-naphtalene acetic acid (NAA) 0.5 mgL^{-1}, a shoot proliferation medium for *Curcuma amada* formulated by Prakash et al. (2004). The *in vitro* shoot cultures of *C. mangga* were used for subsequent studies.

Treatments

Yeast extract

Each shoot explant was cut longitudinally into two halves and each half shoot was used as an explant. Three half shoots were inoculated into each 250 mL Erlenmeyer flask containing 60 mL liquid MS medium supplemented with BA (2 mgL^{-1}) and NAA (0.5 mgL^{-1}) and various concentration of yeast extract (0, 0.5, 2.0, 3.5 and 5.0 mgL^{-1}). The *in vitro* plantlets that were treated with 3.5 mgL^{-1} yeast extract produced favourable results and this treatment was incorporated into subsequent experiment.

Chitosan combination with yeast extract

Three half shoot explants were inoculated into each 250 mL Erlenmeyer flask containing liquid MS medium supplemented with BA (2 mgL^{-1}) and NAA (0.5 mgL^{-1}), the shoot proliferation medium. Stock solution (1 gL^{-1} chitosan) was used to prepare final concentrations 50, 100, 150, 200 mg L^{-1} in experimental medium. The optimum concentration (3.5 mgL^{-1}) of yeast extract was added to each of the medium.

Culture condition

All the cultures (yeast extract and chitosan combination with yeast extract) were agitated on the orbital shaker (SK 600 Lab. Companion, Korea) at 80 rpm placed in the culture room maintained at 25 ± 2°C. All the cultures were exposed to continuous lighting with intensity of 35 µmol m^{-2} s^{-1}.

Measurements

The fresh mass (weight) and number of shoots produced from each explant were determined after four weeks of culture. The dried biomass was determined after constant weight was obtained. The morphological features of the *in vitro* plantlets were also observed.

Experimental design and statistical analysis

Seven and ten experimental units were used for yeast extract and chitosan combination with yeast extract, respectively. Three explants were used for each experimental unit. The experiment was conducted in a Randomized Complete Block Design (RCBD). The data were analyzed using Two-Way Analysis of Variance (ANOVA) and the means were compared using Tukey test at $p \leq 0.05$ using the Statistical Package for the Social Sciences (SPSS) ver. 12.0.

Determination of anti-oxidant activities

Preparation of plant extracts

Dried samples were made into powder using blender and four grams of each sample was placed into 250 ml conical flask containing 100 ml methanol (99%- AR grade, Q-rec) and incubated in waterbath for 2 h at 40°C with continuous agitation at 90 rpm. The aliquot was then filtered using Whatman filter paper No. 1. The residue was re-extracted three times with 100 ml methanol each time. The aliquots from the same sample were combined and concentrated using rotary evaporator (Eyela N-N Series, Japan). The concentrated samples were kept at 4°C until further used.

Free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazil (DPPH)

The free radical scavenging activity was determined using modified method of Brand-Brand-William et al., (1995). DPPH 0.3 mM solution was prepared in methanol and 150 µl aliquot of this solution was added to 50 µl of each of the plant extract (16 $mgml^{-1}$, DMSO) placed in 96-wells plate incubated at 37°C. The absorbance of the samples was determined after 30 min of incubation followed by every ten minutes intervals until a constant value was reached using spectrophotometer (Multiskan Findland) at 515 nm. Quercetin was used as a positive control while DMSO served as negative control. The EC_{50} value, the concentration of the plant extract that could decrease the concentration of DPPH by 50% was then determined. We determined the EC_{50} value only for the samples with high radical scavenging activity (RSA).

Determination of total phenolic contents

The total phenolic contents in the *C. Mangga* extracts was

Table 1. Effect of yeast extract on shoot proliferation of *C. mangga in vitro* plantlets.

Yeast extract (mgL-1)	No. of shoots/ half explant ± SD
0	4.7 ± 0.6a
0.5	4.4 ± 0.7a
2.0	3.9 ± 0.6a
3.5	4.2 ± 0.5a
5.0	4.4 ± 0.4a

Mean values within the same column followed by same superscription letter are not significantly different (Tukey test, $p \leq 0.05$).

determined using Folin-Ciocalteu reagent assay according to Singleton et al. (1999) with gallic acid (Sigma, St Louis USA) as a reference standard. Folin-ciocalteu reagent (1 mL) was added to 0.5 mL extract solution (1000 mgL^{-1}) and mixed thoroughly. After 4 min, 1% sodium carbonate (3 mL) was added, and the mixture was incubated for 2 h at room temperature. The absorbance was measured at 760 nm against the blank (DMSO) using spectrophotometer (Ultrospec, Malaysia). A standard curve of gallic acid (ranging from 0.01 to 0.25 μgmL^{-1}) was used to determine the total phenolic contents expressed as gallic acid equivalent (µg GAE/mg extract).

RESULTS AND DISCUSSION

Yeast extract

Yeast extract used as supplement in proliferation medium did not affect the shoot proliferation of *in vitro* plantlets of *C. mangga* (Table 1). However, the *in vitro* plantlets that were cultured in medium supplemented with yeast extract that was equivalent or more than 3.5 mgL^{-1} showed sign of morphological abnormalities. The abnormalities detected was retardation of shoot growth, chlorosis and inhibition of leaf-development in the *in vitro* plantlets of *C. mangga* (Figure 1). These types of abnormalities were also detected in *Glehnia littoralis in vitro* plantlets after they were exposed to yeast extract. But the addition of yeast extract into the culture medium increased the secondary metabolites production in *G. littoralis* (Ishikawa et al., 2007)

Formerly, yeast extracts were used as growth nutrients such as crown-gall tissue cultures and callus cultures (Jonard, 1960; Vasil and Hildebrandt, 1966). Currently, yeast extract is commonly employed as a biotic elicitor for the induction and enhancement of secondary metabolites production. Reports suggest that yeast extract is used as a supplement in order to promote plant growth, due to its high amino acid content (George et al., 2008). However, different species respond in different ways to the presence of yeast extract that is, addition of higher concentration of yeast extract to MS medium, inhibit the growth whereas, lower concentration of yeast extract was found beneficial (Vasil and Hilderbrandt, 1966).

Studies done by Guo and Ohta (1994) indicated that, yeast extract enhanced the accumulation of 6-methoxymellein in carrot cells while Zhao et al. (2004) reported that, yeast elicitor-treated *Cupressus lusitanica* cell cultures also enhanced the accumulation and metabolism of polyphosphoinositol. The results indicated that the culture medium supplemented with yeast extract did not act as nutrient because addition of yeast extract did not enhance the growth of the *C. mangga* plantlets. However, culture medium supplemented with yeast extract caused morphological abnormalities. This might be the result of stress response of the plantlets to the accumulation of secondary metabolites due to the yeast extract that acted as elicitor. The results showed that addition of yeast extract into the culture medium increased the total phenolic content but only up to the certain level (3.5 $mgL^{-}1$) (Figure 2). The higher amount of yeast extract (5.0 mgL^{-1}) did not act as elicitor for phenolics production but instead acted as growth retardant as could be seen in the retarded growth of *C. mangga* plantlets that were cultured in proliferation medium supplemented with 5.0 mgL^{-1} yeast extract (Figure 1). However, the yeast extract did not affect the fresh and dried biomass of the plantlets (Figure 2). Therefore, our results indicated that yeast extract did not perform as nutrient supplement for the growth of *in vitro* plantlets of *C. mangga* but with optimum amount it could be used for the enhancement of phenolics production. It was reported that yeast extract did not affect the biosynthesis pathway of plants. However, it triggered the production of endogenous jasmonic acid and/ or methyl jasmonate, which influence the production of secondary metabolites (Sanchez-Sampedro et al., 2005). The increment of RSA and total phenolics content (TPC) of *C. mangga* plantlets indicate that yeast extract might trigger the production of endogenous jasmonic acid and/ or methyl jasmonate, which increased the production of phenolics content in *C. mangga*.

Chitosan combination with yeast extract

Addition of different concentrations of chitosan together with 3.5 mgL^{-1} of yeast extract in the proliferation medium showed significant effect on biomass. The shoot biomass decreased as the amount of chitosan added into culture medium increased (Figure 3). The addition of chitosan with 3.5 mgL^{-1} of yeast extract did not affect the *in vitro* shoot proliferation of *C. mangga* (Table 2). Morphological abnormalities of the plantlets could be detected in culture medium supplemented with 100 mgL^{-1} or more of chitosan with the presence of 3.5 mgL^{-1} yeast extract (Figure 4). Similar trends were observed in *Vitis vulgaris* where higher concentration of chitogel (chitosan solution) caused growth retardation (Barka et al., 2004). Jeong and Park (2005) also reported that higher concentration of chitosan and its oligomers decreased the biomass of *Panax ginseng* but enhanced the production of ginseng saponin. Similarly, higher concentration of chitosan showed inhibitory effect on the growth of hairy root

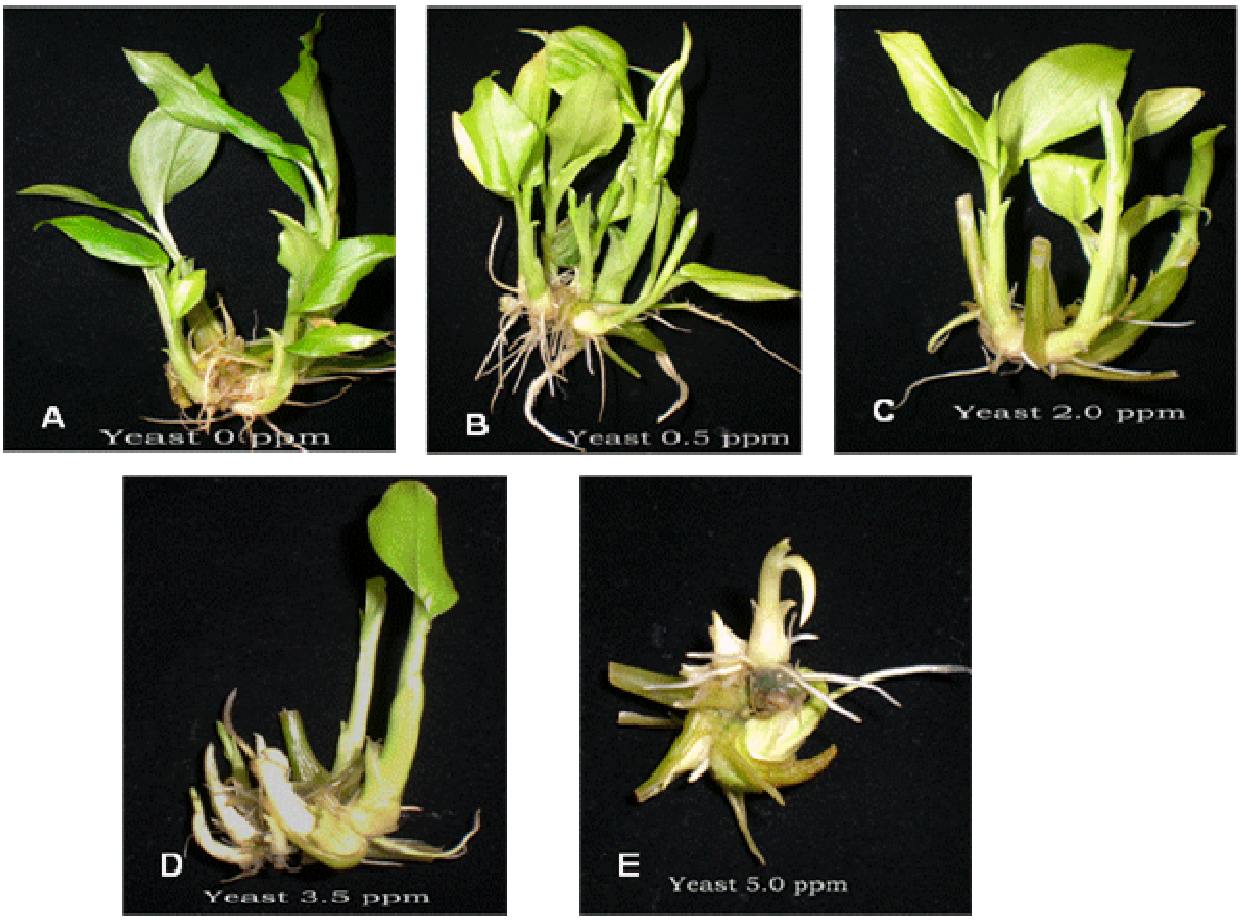


Figure 1. Effect of yeast extract on morphological characteristics of *C. mangga in vitro* plantlets. (A) 0 mgL^{-1}; (B) 0.5 mgL^{-1}; (C) 2.0 mgL^{-1}; (D) 3.5 mgL^{-1} and (E) 5.0 mgL^{-1} yeast extract in proliferation medium.

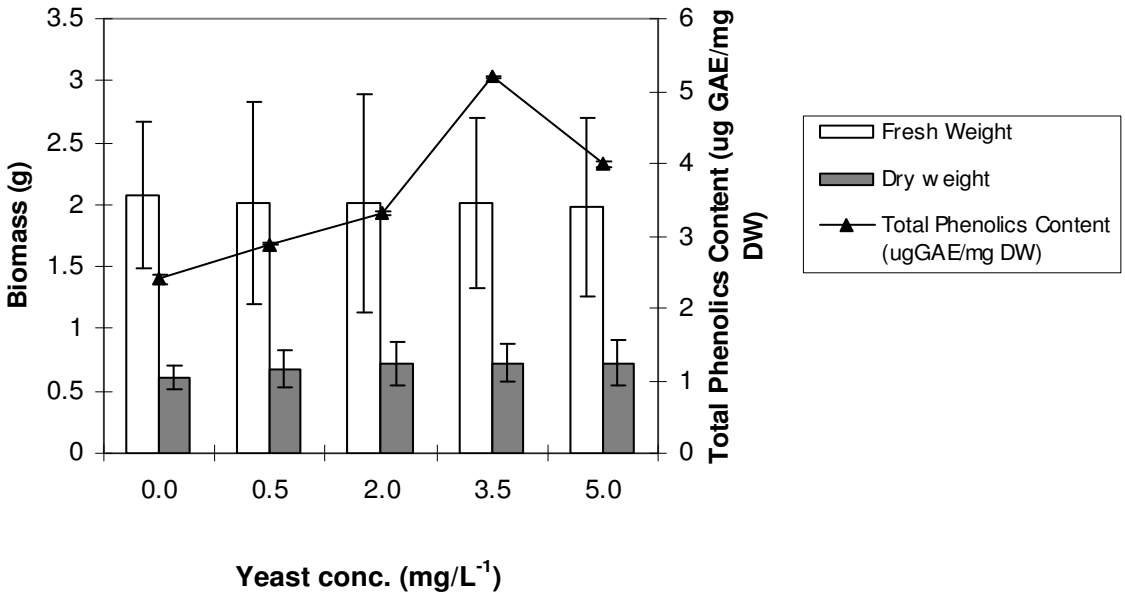


Figure 2 Effect of yeast extract on biomass (fresh weight and dry weight) and the total phenolics content of *C. mangga* plantlets.

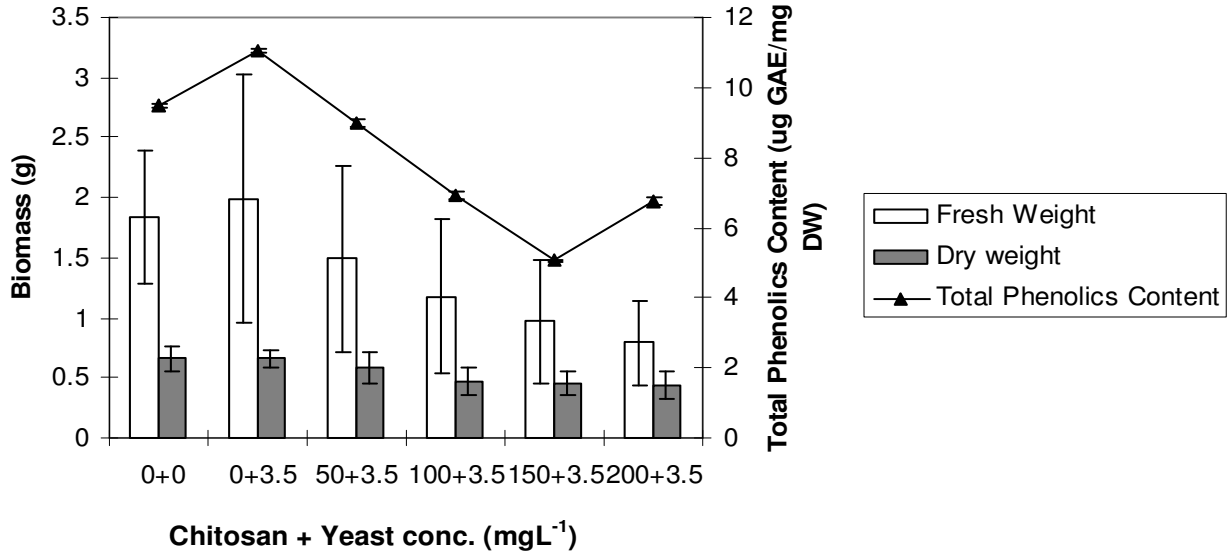


Figure 3. Effect of chitosan with 3.5 mgL^{-1} on biomass (fresh weight and dry weight) and the total phenolics content of *C. mangga* plantlets.

Table 2. Effect of chitosan combined with yeast extract on shoot proliferation of *C. mangga in vitro* plantlets.

Yeast extract (mgL-1)	Chitosan (mgL-1)	No. of shoots/ half explant ± SD
0	0	4.4 ± 1.0a
3.5	0	3.9 ± 1.1ab
3.5	50	3.8 ± 0.9ab
3.5	100	3.6 ± 0.8ab
3.5	150	3.1 ± 0.9b
3.5	200	3.2 ± 0.8ab

Mean values within the same column followed by same superscription letter are not significantly different (Tukey test, $p \leq 0.05$).

cultures of *Artemisia annua* (Putalun et al., 2007) and *Brugmansia candida* (Pitta-Alvarez and Giulietti, 1999). Our results showed that with the addition of chitosan combined with 3.5 mgL^{-1} yeast extract into the culture medium caused the reduction of phenolic contents in the *C. mangga* plantlets also up to a certain chitosan level (150 mgL^{-1}) (Figure 3). It is reported that, no specific elicitor showed a general effect on different plant species. Also, it is difficult to predict that an elicitor will be effective in a specific cell system on metabolite accumulation. The response to elicitation is dependent on many factors, such as growth stage of the culture at the time of elicitation and the contact time of elicitation (Bhagwath and Hjorts, 2000).

Anti-oxidative activity of *C. mangga in vitro* plantlets

The DPPH free radicals exhibit different sensitivity to various antioxidants. Depending on the time taken to reach the steady state of scavenging free DPPH radicals, the antioxidants can be presented as fast (1 min.), intermediate (30 min.), or slow (1 to 6 h) kinetic reactions to the DPPH free radicals (Brand-William et al., 1995). *C. mangga* exhibited slow kinetic reaction to DPPH free radicals as it took 80 min to reach the steady state of RSA as compared to quercetine which reached steady state within 30 min. Extracts obtained from yeast-extract treated sample exhibited stronger RSA as compared to the control (without yeast extract) at all incubation time (Figure 5). It indicated that the yeast extract-treated samples contained more antioxidant agents. Only 3.5 mgL^{-1}yeast extract treated samples showed the lowest EC_{50} value of 2028.50 µg. Result revealed that addition of different concentration of chitosan with 3.5 mgL^{-1} of yeast extract significantly increased the RSA at 30 min of incubation time as compared to the control (chitosan 0;0) and the other chitosan treatment. Addition of 150 mgL^{-1}

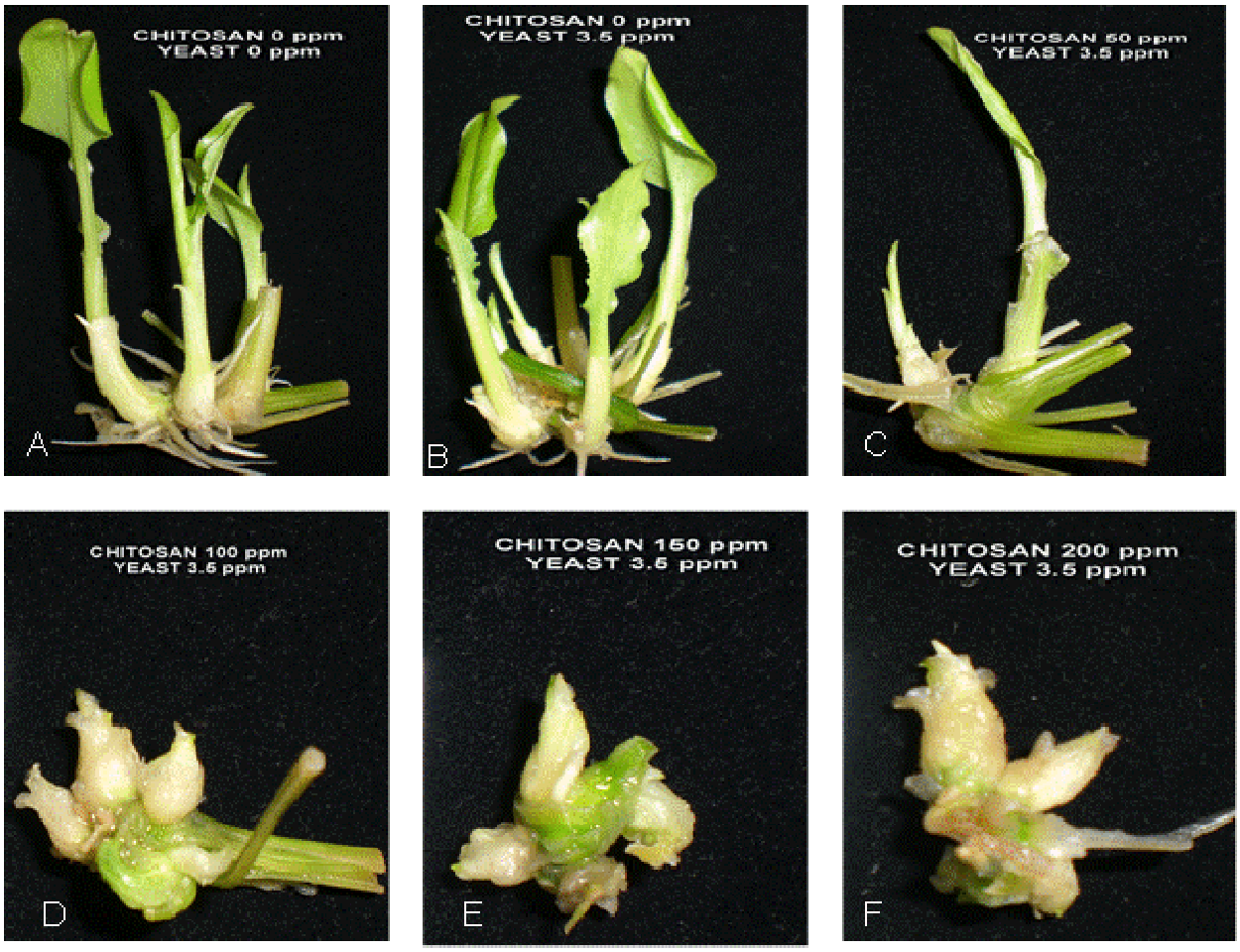


Figure 4. Effect of different concentration of chitosan with 3.5 mgL^{-1} of yeast extract on morphology of *C. mangga in vitro* plantlets.

chitosan and 3.5 mgL^{-1} yeast extract showed the highest RSA as compared to the other chitosan treatment (Figure 5) with the EC_{50} value of 1844.48 µg.

Correlation between radical-scavenging activity and total phenolics content

Plant phenolics comprise one of the major groups of compounds acting as primary antioxidants or free radical scavenger (Sanchez-Moreno et al., 1998). Theoretically, the higher phenolics content is followed by higher RSA. However, in the present study, there was no direct correlation between total phenolics content and RSA of the plant extracts of *C. mangga* derived from yeast extract treated cultures (R^2 = 0.2293) and chitosan plus yeast extract cultures (R^2 = 0.0373) (Figure 6). This might be due to the bounded phenolics in the tested *C. mangga* extracts which might not contribute to RSA in the DPPH assay, hence indicated no correlation between the phenolics content and the anti-oxidant activities.

In order to examine the correlation between total phenolic content and RSA, the RSA was observed at 120 min of incubation time considering that during that time, the reaction had reached the end of steady state. Even so, the correlation between total phenolics content and RSA was poor. It indirectly confirmed that phenolic compounds were not the major factor contributing to the anti-oxidative properties of *C. mangga*. There might be many other compounds and their complex interactions that were responsible for the anti-oxidative activity in *C. mangga*. Amakura et al. (2000) also reported that the correlation between total phenolics content and RSA varied among different species of berries. Therefore, our results revealed that two species of berries showed a significant positive correlation between RSA and total phenolics with correlation coefficient (R) of 0.95 to 0.97, while three species with R values between 0.57 and 0.72 and another four species with a wide range of R value of 0.3 to 0.78.

DPPH assay is considered as one of the simplest methods of assessing the anti-oxidative activity.

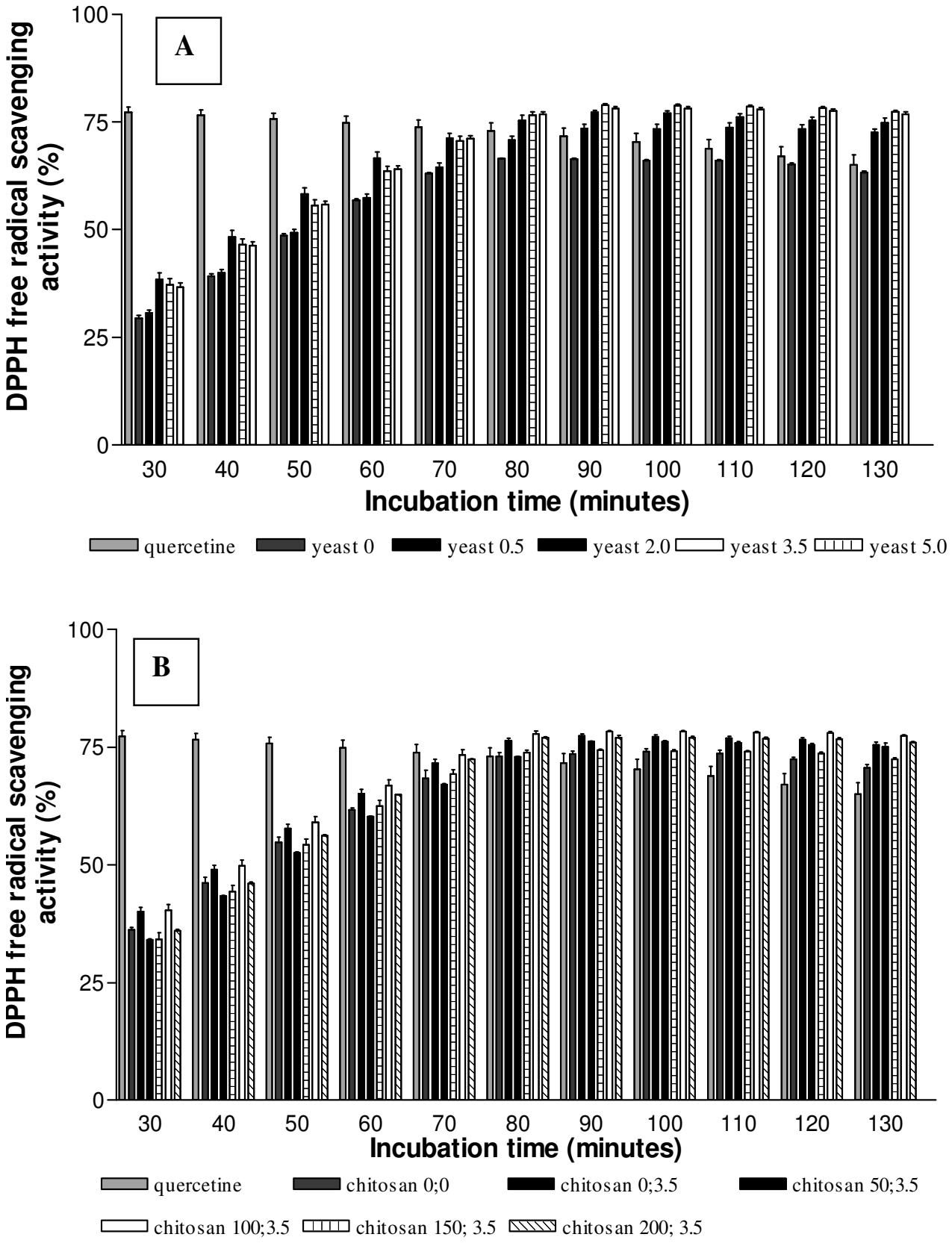
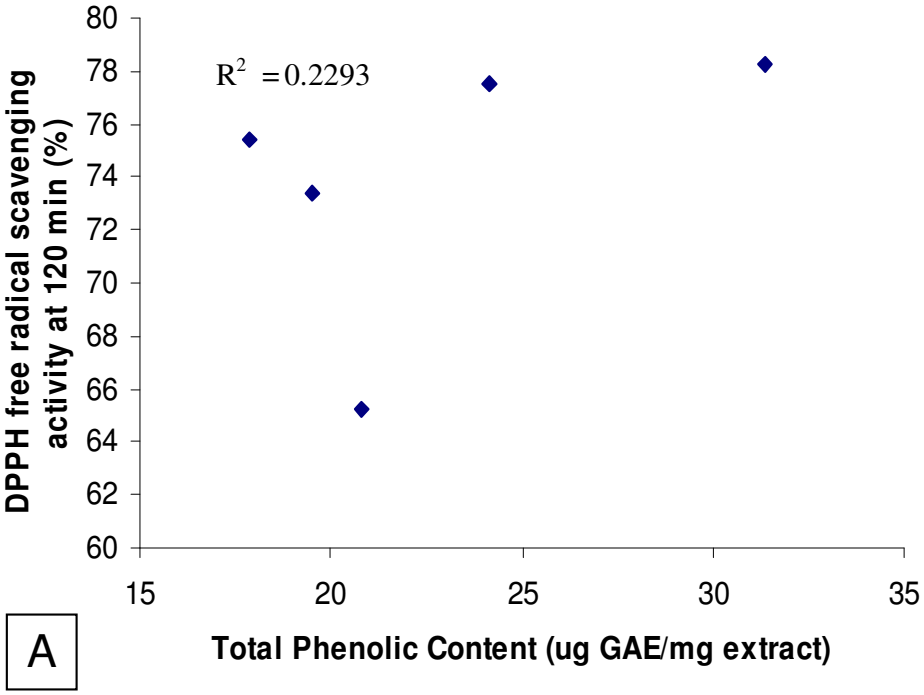


Figure 5. Kinetic of RSA of *C. mangga* Yeast extract treated sample (A) and chitosan in combination with 3.5 mgL^{-1} yeast extract treated sample (B).



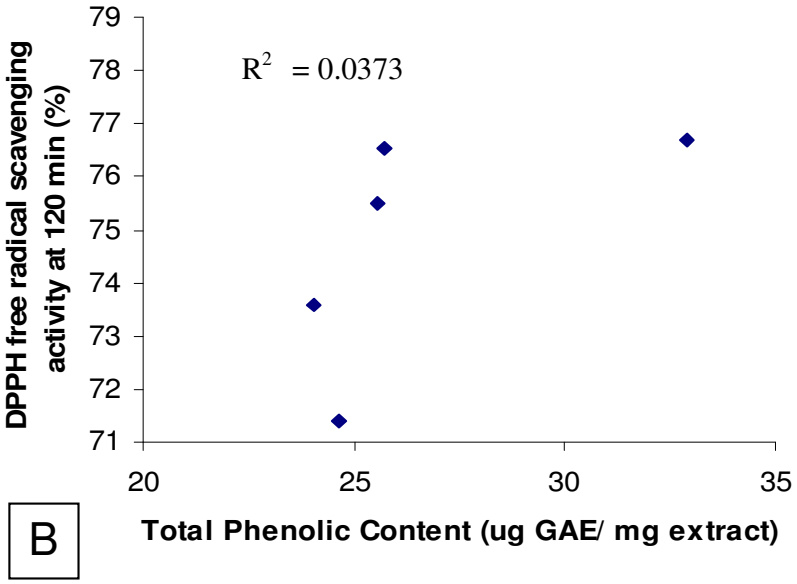


Figure 6. Scatter plot showing correlation between total phenolics content (µg GAE/ mg extract) and free radical scavenging activity (RSA) of *C. mangga in vitro* plantlets cultured in medium supplemented with yeast extracts (A) and chitosan plus 3.5 mgL^{-1} yeast extract (B).

However, different anti-oxidative assay gives different result due to their mechanism of reaction. Generally, anti-oxidative process follows the mechanism of hydrogen atom transfer. While DPPH is a nitrogen radical with a long lifespan, which is not similar to the highly reactive and transient peroxyl radicals involved in lipid peroxidation process. Many compounds that easily react with peroxyl radicals might not or slowly react to DPPH (Huang et al., 2005). Despite these limitations in this study, DPPH assay and total phenolics content indirectly illustrated the role of non phenolic compounds that might contribute to the anti-oxidative mechanism in *C. mangga*.

Conclusion

On the basis of the present study, it can be concluded that yeast extract can be used as elicitor for the production of phenolic compounds in the *C. mangga* plantlets. Media supplemented with 3.5 and 5.0 mgL^{-1} of yeast extract showed higher radical scavenging activity, indicated that stress (morphological abnormality) induced by yeast extract might elicit the production of secondary metabolites which acted as free radical scavenger in DPPH assay. Chitosan combined with yeast extract acts as growth retardant and inhibitor for phenolics production. Both caused morphological abnormalities when used in higher concentration.

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