6.5 Summary of results

i) Saponin was detected in both *in vitro* and *in vivo* leaf and stem samples.

ii) Terpenoids were detected in *in vivo* sample of leaf and stem, peel, pulp and seed sample. Terpenoid is absent in *in vitro* samples.

iii) Tannins were detected in all *in vitro* and *in vivo* leaf and stem samples. Tannins is present in peel sample. Absence of tannins in pulp and seed sample.

iv) Glycoside was not detected in any of the samples.

v) Flavonoids were detected in all *in vitro* and *in vivo* leaf and stem samples.

vi) Reducing sugar was detected in leaf sample of both *in vitro* and *in vivo* and peel sample. Absence of reducing sugar in both *in vitro* and *in vivo* stem, pulp and seed samples.

CHAPTER 7

7.0 DISCUSSION

Regeneration path for micropropagation including regeneration from plant cell, regeneration from *de novo* meristem and somatic embryogenesis. Shoot regeneration can be made using tissue culture technique, straight from plant cell by using axillary shoot, shoot tip or bud (George *et al*., 2008). The medium supplemented with plant hormones, cytokinins, will eventually induce multiple shoot because cytokinins trigger the apex dominant in the plant tissue. New shoot that emerges can be cut and use for subculturing onto a new media for further growth (George & Sherington, 1984).

There are 4 general steps of micropropagation. The very first crucial step is to choose the type of explant which will be propagated. This step is important as it will determine the success rate of the research. Some of the factors that should be taken into consideration when doing this research:

(i) Contamination rate of starting material is crucial. It is best to choose a plant that is healthy and vigorously growing.

(ii) Contamination rate can be further reduced by germinating the plant *in vitro*, and use aseptic seddlings as the explant source.

(iii) Some of the plant needs special treatment in order to stop dormancy of the plant. Temperature can induce or hinder the growth of the explant with relation to the storage period, hormone auxin and cytokinin concentration and the temperature of culture. The next step is explant initiation by choosing the right source as the explant.

The common explant sources for medicinal plants are nodal stem, shoot tip and axillary bud. The growth of tissue that was cultured is usually influence by the season during the explant cutting was done. Spring season is the most suitable time to do the explant cutting to get a healthy shoot. For some species, phenolic accumulation, also known as the browning problem, from the explant will produce a yellowish orange colour to the tissue. This can be prevented or reduced by using an additional of ascorbic acid, submerging the explant inside water for at least 24 hours before culture or by adding activated charcoal to the culture medium (Bhojwani & Razdan, 1997).

The third step is the multiplication of tissue. Tissue multiplication is an important step in propagation for any species for commercial exploitation to get a faster growth rate. Additional hormone added into the culture media to increase the growth rate is cytokinin. The higher the concentration of cytokinin, the higher the chances of the media to be toxic as the concentration is too high. The usage of appropriate amount of agar will control the vitrification rate to the final of propagation stage. Subculture period and explant orientation on the inducing medium can influence the growth and multiple shoots.

The fourth step is the overall plant regeneration. The success of plant regenerations for most species propagated by tissue culture are the success growth of multiple shoot and also root induction. Root induction can be induced by reducing cytokinin concentration with or without the addition of auxin. This behavior can also be induced by reducing the power of the media, for example by using half MS medium (Bhojwani & Razdan 1997; George & Sherington, 1984; George *et al*., 2008).

At the early stage of this investigation, intact plant was used as explants for tissue culture studies, but the contamination rate was very high. Leaf and stem produced lower

contamination rate compared to shoot tip and nodal stem (Jain & Ishi, 2003). Higher concentration of sodium hypochloride was used but ended up the explants died because of over-sterilized. To solve this contamination and over-sterilized problem, aseptic seedlings were used in the early stage of culture to obtain the required explants for these *in vitro* studies. Aseptic seedlings have been proven to be advantagoues as source of sterile explants.

Shoot tips explants and nodal stem explants of *Punica granatum* L. responded well to most hormone and hormone combination in producing shoot and multiple shoots. Incorporation of cytokinin to the medium was essential to induce axillary shoot proliferation (Naik *et al*., 1999). Leaf and stem also produced shoot and multiple shoots directly and indirectly in certain hormone concentration but at very low percentage and rate (Kantharajah *et al*., 1998). Root explants only produced callus in most hormone combinations and selected hormone combinations produced callus producing shoot to stem and leaf (Omura *et al*., 1990). In certain BAP and NAA combinations on shoot tips and nodal stem explants, callus was formed at the lower tip of the explants. Shoot tip and nodal stem explants could regenerate shoots within 2-3 weeks of culture. Leaf, stem and root responded at a slower rate, usually within 3-8 weeks of culture. Shoots developed from the surface of some of the explants, which was in contact with the medium. Also, shoot bud differentiation was accompanied by the formation of a small amount of green, compact and nodular callus in most of the explants. Differentiation of isolated leaves were also a common feature along with shoot bud differentiation (Jaidka & Mehra, 1986).

MS media supplemented with BAP at (0.5-2.0 mg/L) induced shoot proliferation growth rate and capable of producing 1-5 shoots in average (Plate 2.2, Plate 2.5, Plate 2.6, Plate 2.7, Plate 2.9). Table 2.3 and Table 2.4 show MS media with NAA produced

a lower amount of shoot compared to MS media with BAP or BAP with NAA, with the average of 1-2 shoot per explant (Plate 2.16). Higher NAA concentration (1.5-2.0 mg/L) can induce root directly from shoot tips and nodal stem explants (Plate 2.26).

Combinations of BAP and NAA used with MS media resulted in shoot tips explants, nodal stem explants, leaf explants and stem explants produced shoots, with and without callus at different rates of growth (Plate 2.8, Plate 2.10, Plate 2.11, Plate 2.12, Plate 2.15). Average period for new shoots to emerge was from 12-20 days from the initial day culture. The best media for shoot growth for shoot tips was MS medium added with 1.5 mg/L BAP with 3.233 of mean shoot number, while the best media for nodal stem explants was MS medium added with 1.5 mg/L BAP with mean number of 3.833, for leaves explants was MS medium added with 2.0 mg/L BAP and 1.0 mg/L NAA with 1.533 mean shoot number and for stem explants was 2.5 mg/L BAP and 1.0 mg/L NAA with 1.767 mean shoot number.

The best medium for the highest shoot height used for shoot tip explants was MS medium supplemented with 2.0 mg/L BAP with mean of 1.947 cm per explant, while the highest shoot height for nodal stem explants was obtained from MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA. Shoot height for leaf and stem explants were not taken as the results for shoot numbers were poor.

The lowest number of shoots produced from shoot tip explants came from MS medium with 2.5 mg/L BAP and 2 mg/L NAA with 1.033 mean shoot number and the lowest obtained from nodal stem explants came from MS medium supplemented with 0.5 mg/L NAA with 1.167 mean shoot number. According to Table 2.3, shoot numbers were low using shoot tips as explants with medium combined with 2.0 mg/L NAA. Shoot heights

of shoot tips explants also low when cultured on MS medium contains NAA alone at every concentration. Addition of BAP hormone increased shoot proliferation in shoot tip explants. MS medium with only BAP treatment increased the number of shoots and also the shoot heights in shoot tip explants. The effect of BAP on the capacity to induce plant regeneration in members of Punicaceae has been reported previously (Alicja, 1974; Amin *et al*., 1999; Bhansali, 1990; Deepika and Kanwar, 2010; Mahishni *et al*., 1991) and the combination and presence of auxin proved to be unsuccessful for increasing shoot capacity and length was found in work of Murkute *et al*., (2004). The results obtained in the present work are in agreement with the above work.

In previous work by Naik and Chand (2006), 23% of the hypocotyl segments exhibited shoot bud initiation on a medium containing 8.9 μmol/L BA, 5.4 mol/L NAA and 10% coconut water within 8–9 weeks and from each explant, 2.2 shoots developed in 90 days averaging of 2.4 cm in length. In case of cotyledon, 33% of the explants exhibited adventitious shoot bud initiation within 7–8 weeks of culture in the above medium. The average number of shoots per explant was 2.6, and they had an average length of 2.5cm (Naik and Chand, 2003). According to Deepika and Kanwar (2010), the highest rate of shoot regeneration was observed in cotyledons (81.97%) followed by hypocotyls (70.57%), internodes (68.43%) and leaf-derived callus (65.32%). The data shows that 24.76%, 18.86%, 20.41%, 16.53% calli differentiated into shoots from cotyledon, hypocotyl, internode and leaf derived callus, respectively. Cotyledon derived callus yielded the best response compared to all the other explants.

In a study by Naik *et al*., (1999), of the two cytokinins tested, BAP was more effective than Kinetin. Shoot development increased with the increment of the concentration of cytokinin (BAP or Kinetin) up to 9.0 mM. Ninety-four percent of the explants exhibited

shoot development within 7-8 days on a medium supplemented with 9.0 mM BAP. Explants took 10-12 days for shoot development on a Kinetin supplemented medium. Shoot number and shoot length were significantly affected by the concentration and type of cytokinin used. Significantly higher shoot number were developed on a medium containing 9.0 mM BAP. Each cotyledonary node produced an average of 9.8 shoots in 30 days and had an average length of 5.5 cm. On the other hand, an average of 2.4 shoots were developed from a single cotyledonary node in 30 days on a medium containing 9.0 mM Kinetin and the average shoot length was 4.7 cm.

Another previous study by Naik *et al*. (1999), MS medium containing BAP at an optimum concentration of 1.0 mg induced bud break in 93% of the explants in 12-15 days. Each explant developed 2.1 shoots averaging 3.7 cm length in 30 days. On the other hand 85% of the explants exhibited bud break within 1012 days on a medium containing 2.0 mg/L Zeatin and from each explant an average of 5.2 shoots were developed in 22-25 days. The shoots elongated within 4±6 days upon transfer to a medium containing a lower concentration of Zeatin (1.0 mg/L) and attained an average height of 3.9 cm. Concentrations of BAP or Zeatin higher than the optimum level had an inhibitory effect on shoot development.

In experiment by Murkute *et al*. (2004), TDZ at 0.01-0.5 mg/L was used to induce shoot development from the nodal explants. The optimum concentration of TDZ, at which maximum number of explants exhibited bud break, was 0.05 mg/L. The frequency of bud break was 63% and from each explant a single shoot was developed in 15-18 days. However, the shoot failed to elongate. This problem was overcome by transferring the shoot to a medium containing 0.5 mg/L BAP. Within 3-4 days of transfer the shoot

elongated and attained an average height of 3.6 cm in 10 days. The frequency of shoot development was reduced markedly at 2.0 mg/L concentration of TDZ.

Other than hormone effect, removal of shoot apex helps in breaking and weakens the apical dominance or axillary buds that can be activated to form new buds (Kanwar *et al*., 2010). This effect is due to a change in hormone balance within the plant allowing dormant buds to grow (Sharon & Sinha, 2000). Addition of a cytokinin to the medium was essential to induce bud break and multiple shoots formation in the explants.

MS medium supplemented with different concentrations of BAP did not only enhance the multiplication rate, but also favored the proliferation of a more vigorous shoots (Singh *et al*., 2007). BAP proved to be more effective compared to other cytokinins tested in shoot multiplication of this species according to previous work by Murkute *et al*. (2002 & 2004). BAP hormone is also more potent cytokinins for organogenesis, absence or low concentration of auxins but moderate to high concentration of cytokinins concentrations were required for axillary shoot production (Naik & Chand, 2003 & 2006). However, in some cases even in the presence of shoot apex (shoot tip explants), there will be multiple shoot formation attributed to the explants being from young seedlings and the axillary buds can proliferate in new shootlet giving cluster of multiple shoots (Guohua *et al*., 1999). Shoot length in comparison between BAP and NAA hormone treatment for shoot tip explants was not significantly different. Thus, the hormone combination for every concentration did not affect or influence the shoot height from shoot tip explant.

Exogenous cytokinins are required to obtain *in vitro* shoots proliferation and auxins are usually not necessary (van der Krieken *et al*., 1993). Kanwar *et al.,* (2010) reported that

the use of cytokinins for *in vitro* cultures of *Punica granatum* L. lead to better rates of axillary branching and improved quality of shoot development. The elimination or lower rate of auxin favors the micropropagation in this species. A medium with high concentration of cytokinins generally inhibit root formation and growth. It could also prevent the promotive effects of auxins on root initiation.

Dicotyledon plant tissue is believed to rely on external cytokinin usage than monocotyledon plant tissue (Alicija, 1974). However, different variety of a species may give different response to every hormone and media. In previous reports on *P.granatum* by Naik *et al*. (2003), from different variety, Kinetin gave influence on shoot proliferation. Maximum shoot formed in that study was 9, by using medium of 9.0 μM BAP, the very same hormone that gave the best shoot number in this study. While medium with 0.54 μM NAA resulted in the highest percentage of shoots and significantly higher number of roots than other concentrations. The study of Naik *et al* in 2000 resulted in the highest shoot percentage (9.8 shoots per cotyledonary nodes and 5.5 cm height in) 30 days, 10 days shorter than the data taken in this study. However, in the present study the concentration of BAP used was much lower than used in the study by Naik *et al* (2000; 2006). The average shoot multiplication and the shoot length were higher compared to the results obtained in this experiment with highest number was from nodal stem explants (4.4 shoots), suggesting cotyledonary nodes were better explants in producing shoot. It was reported that MS basal medium supplemented with Zeatin on nodal segment mature pomegranate tree being the most effective medium producing shoot (Naik *et al*. 2006). For direct adventitious shoot organogenesis and complete plant regeneration from seedling-derived explants of pomegranate, MS medium enriched with 8.9μmol/L BA, 5.4μmol/L NAA and 10% coconut water (CW) induced adventitious shoot bud differentiation in axenic seedling-derived cotyledons as

well as hypocotyl segments. The cotyledons were more responsive than the hypocotyls. Addition of ethylene inhibitors such as AgNO3 (10–40 μmol/L) and aminoethoxyvinylglycine (AVG) (5–15 μmol/L) to the medium markedly enhanced regeneration frequency as well as number of shoots obtained per explant (Naik *et al*, 2003).

Same species with different variety may have differences and variations of biochemical contents. In this research, shoot elongation and shoot number from both shoot tip and nodal stem explants gave different results with other previous studies. Factors such as duration of result taken from initial culture day, different cultivar, additional of several nutrients and different hormone concentration may affect this outcome (Amin *et al*., 1999). However the cytokinin used (BAP) gave the most optimum results in shoot growth and multiple shoot growth from previous report from Naik *et al.* (2000).

Sucrose had been used as the carbohydrate source in most *in vitro* investigations. It is added to the media at various concentrations, depending on the method and media used. In a normal MS media, 30 gram per liter was the standard concentration of sucrose added (Hartman *et al*., 1990). In this study, sucrose concentration was varied to observe which sucrose concentration will exhibit the highest shoot number compare to normal sucrose concentration used (30 g/L). For shoot tip explant, the highest shoot number was obtained from 40g/L sucrose concentration with 4.100 shoots per explant (Plate 2.17, Plate 2.18), which was higher than the normal sucrose concentration used (30g/L) with only 3.233 shoots per explant. However, for nodal stem explant the highest was obtained from the normal sucrose concentration used (30g/L) with 3.833 shoots per explant. The results implied that the optimum sucrose concentration used was at the range of 20-40 g/L. The shoot proliferation process is facilitated by a high osmolarity,

but relies on adequate supply of sucrose for optimal production. A tentative suggestion would be the slight excess of sucrose triggers shoot initiation, and the high osmolarity ensures the continuous production of carbohydrate in the explants and shoots during their growth (Murkute *et al*., 2002).

Macromorphology research of reproductive and vegetative plant parts gives a clearer picture of the plant external structures (Brutti *et al*., 2002). Micromorphology gives information on the ultrastructural characteristics of the plant. Scanning electron microscopic studies of *in vivo* and *in vitro* of *P.granatum* will show the difference characteristics between *in vitro* cultured *P.granatum* with the normal pomegranate grown on soil. Using scanning electron microscopy, the leaf and stem from *in vitro* and *in vivo* of *P. granatum* were taken as samples to be observed under the SEM. Structures such as stomata opening and other ultrastructures were observed under certain magnification. Stomata are small pores on the surfaces of leaves and stems, bounded by a pair of guard cells, that control the exchange of gases, most importantly water vapour and carbon dioxide between the interior of the leaf and the atmosphere. In this capacity they make major contributions to the ability of the plant to control its water relations and to gain carbon. Gas exchange is regulated by controlling the aperture of the stomatal pore and the number of stomata that form on the epidermis (Esau, 1965). Environmental signals such as light intensity, the concentration of atmospheric carbon dioxide and endogenous plant hormones control stomatal aperture and development. The acquisition of stomata and an impervious leaf cuticle are considered to be key elements in the evolution of advanced terrestrial plants.

Trichome can be classified into two types, unicellular and multicellular. Trichome is an epidermis appendage which can be in various shapes, structures and functions. It is

usually represented in hairy form to give support and protection to the cells (Esau, 1965). Depending on the plant species, trichome can sometime has a scaly structure all along its surface and various of papillae for absorption of nutrients from root (Metcalfe & Chalk, 1972)

A higher density of stomata can be observed at the abaxial surface of *in vitro* leaf sample at 500 times magnification compared to the adaxial surface of *in vitro* leaf sample at the same magnification (Figure 2.1). Stomata counts are critical for plant function. Smaller plants such as grasses do not have as much stomata as trees which have larger mass and therefore require more stomata for photosynthesis (Brigidaa *et al*., 2007). However, there was no presence of trichome on *in vitro* and *in vivo* samples of pomegranate leaf and stem. The adaxial surface of the leaf observed to have less irregularities compared to the abaxial surfaces in both *in vitro* and *in vivo* samples (Figure 2.2 and Figure 2.3). The features found on both leaf and stem *in vitro* and *in vivo* such as the abundance and shapes of the stomata could be related to its surrounding during cultivated or cultured.

Index count of stomata was higher at the abaxial surface of both *in vitro* and *in vivo* samples compared to the adaxial surface of pomegranate leaf (Figure 2.1 and Figure 2.7). As expected, there were some differences of stomata distribution between abaxial surface and adaxial surface of the leaf.

In this work, *in vitro* and *in vivo* leaf and stem samples of *P.granatum* were taken at 3 months age. Previous studies on SEM for *in vitro* samples stated that stomata had lost of it's function in *in vitro* culture, caused by immature plant (Hetherington & Woodward, 2003). Therefore, it is advisable to select a more mature and older plant to be used for

ultrastructural observation under SEM. According to observation made in this study, the occurrence of stomata density was higher on *in vitro* leaf samples compared to *in vivo* leaf samples, because of high transpiration rate occur *in vitro* culture (Brutti *et al*., 2002). The anatomy of *in vitro* leaf, such as reduced mesophyll differentiation and thin cuticle with scarce wax deposition, exhibited typical features described in other *in vitro* plants (Apóstolo & Llorente 2000). The *in vivo* plant leaf had both, thick and well developed palisade parenchyma that according to France *et al*. (2011) indicates an adaptation to greater photon flux density. Higher concentration of sugar in culture will reduced cell elongation and increase thinning of the cell wall, resulting in the formation of fewer cells. Additionally, the poor ventilation of the culture vessel results in a high relative humidity inside them, which forces the *in vitro* shoot to keep the stomata open. *In vivo* plant developed had similar proportions of open and close stomata, while in the *in vitro* cultured samples have more than 90% of the stomata were opened. These stomata were observed supported on epidermis cells protruding from the cuticle. The elliptical cell shape is frequently associated with stomata that function normally (Brutti *et al*. 2002), indicating somaclonal variation did not occur in *in vitro* and *in vivo* samples of *P.granatum* L. obtained from this study. It can be summarized that the shape of the stem and leaf *in vitro* and *in vivo* are strongly similar, and showed that both samples did not undergo any changes during tissue culture process (Negi *et al*., 2003).

Regeneration of plants by micropropagation of *in vitro* cultures can be achieved from leaf primordia existing in shoot tips and axillary bud explants. Alternatively, plants can be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous plant growth regulators. Plant regeneration from callus is possible by *de novo* organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant

regeneration from callus permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the *in vitro* applied environmental stimuli, including growth factors added to the cultured cells.

From the experiments conducted, there was a significant difference in the response of different explants for callus formation. It was found that, the highest percentage of responding explant to callus induction was from leaf explant (95.44%), followed by stem explant and root explant. The highest callus weight among the media with combination of BAP and NAA hormones was obtained from root explants with the mean callus weight of 0.477 g cultured on 1.5 mg/L BAP and 1.5 mg/L NAA (Plate 3.7). For other hormones (IAA, IBA, 2,4-D and Kinetin) the highest weight obtained from leaf explants with the mean callus weight of 0.186 g cultured on 1.5 mg/L 2,4-D (Plate 3.17). Stem explants was the second best explants with the highest mean of callus weight at 0.411 g among the media enriched with BAP and NAA hormones only, with 1.5 mg/L BAP and 1.0 mg/L NAA (Plate 3.29) as the optimum medium, and MS medium supplemented with 1.5mg/L Kinetin (Plate 3.25) obtained the highest mean of callus weight for stem with 0.127g, among media supplemented with other hormones (IAA,IBA, 2,4-D and Kinetin). For leaf explants, the optimum medium among the BAP and NAA combination media was obtained from 0.5 mg/L BAP and 0.5 mg/L NAA with the mean of 0.221g. There were no significant difference in between medium of hormone concentration of 2,4-D, from 0.5mg/L to 2.0 mg/L when treated on leaf explants. However, medium with any concentration of 2,4-D obtained the optimum callus weight among all the media with 0.5 mg/L-2.0 mg/L of IAA, IBA and Kinetin. This result was similar to previous study by Deepika and Kanwar (2010), where leaf explants yielded a significantly higher response (54.06%) than the internode explants

(48.14%) where it took six weeks for optimum growth of callus on the explants in comparison with five weeks in internode explants.

Most explants treated with 2,4-D hormone gave result of cream-colored callus, and eventually turned black if left without subculture to a new medium within 40 days of initial culture for medium with 0.5 mg/L 2,4-D and medium with 2.0 mg/L 2,4-D using stem explants and medium with 0.5 mg/L 2,4-D and 1.5 mg/L 2,4-D for root explant. There was also no significant value in between all the concentrations used in 2,4-D hormone- enriched medium.

The callus initiation observed at cut ends and wounded regions of leaf and stem explants, while callus initiated at all area for root explants after two weeks of culture. The whole surface of the explants was covered with the callus within six weeks of incubation (Plate 3.1, 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7). In previous report, callus was initiated after 1 week from cut ends of both types of *P. granatum* L. explants, cotyledons and hypoctoyl (Kanwar *et al*., 2010). A very low percentage of leaf and stem explants generated indirect shoot, shoot that emerged from callus. Theoretically, auxin is generally required to be incorporated into the medium for callus induction and being a strong promoter for callus induction, 2,4-D is the most frequently used hormone to initiate callus growth (George & Sherrington, 1984).

Callus formed from root explants observed to be in creamy color. Similarly in previous study the callus from *P.granatum* root sample was compact nodular, creamy yellow in colour and showed excellent callus growth with 1.5- 2.0 cm (Chaugule *et al*., 2005; Jaidka and Mehra, 1986; Omura *et al*., 1987). While callus from leaf and stem explants will usually generate green or light green colour when cultured on BAP and NAA

fortified medium. This is strongly similar to previous report where *P.granatum* L. explants were incubated on MS medium supplemented with BAP and NAA resulted in compact, green in color, and fast-growing callus (Kanwar *et al*., 2010). Indirect root was formed at higher concentration of IAA, IBA and 2,4-D for leaf, stem and root explants. There was scattered root, identified by the presence of a small root tip and a cluster of white root hairs, appeared on the surface of the callus. Although this did not yield in a high percentage, root formation was possible for this type of explants when treated with medium with higher concentration of auxins. It was observed that there was an increase in the percentage of explants showing callus formation with increasing concentrations of BAP hormone with or without combination with NAA hormone. With this combination of hormones in media, the callus produced is green and light green in color. The callus was observed to be loose, friable and proliferated upon transferring to fresh medium. The optimal time for subculture is between 8-10 weeks, and a period longer than that will decreased the quality of the callus, and the green color of callus eventually turned into creamy yellowish and then turned black. Combinations of BAP and NAA hormones gave higher growth rate of callus compared to medium supplemented with only BAP and NAA respectively.

Leaf and stem explants under certain concentration of 2.0 mg/L of IBA have exhibited adventitious root formation (Plate 3.15, Plate 3.16 and Plate 3.23). IBA is an important factor for adventitious root formation if applied exogenously. However, endogenous auxins may also play a role in the rooting process. Therefore, three mutants with altered auxin levels were investigated for the ability to form adventitious roots after IBA treatment. While IBA was able to induce lateral roots, the same response was found only at 20-fold higher concentrations of IAA (Chhun *et al*., 2003). In contrast to lateral root development, adventitious root formation has significant practical implications

because of the many plant species that are difficult to root. IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). Several possibilities exist to explain the better performance of IBA versus IAA (Epstein & Ludwig-Muller, 1993) are higher stability, differences in metabolism, differences in transport and IBA is a slow release source of IAA. There is now a great deal of evidence that IBA occurs naturally in plants (Matsuta *et al*., 1986). The higher stability of IBA, in contrast to IAA, during rooting assays was reported by Nordstrom *et al*. (1991) which affected both degradation and metabolism. It was therefore suggested that IBA may be a very simple 'conjugate' of IAA and must be converted to IAA by oxidation to have an auxin effect. The conversion of IBA to IAA occurs in many plant species, such as *Pinus sylvestris* (Dunberg *et al*., 1981), *Populus tremula* (Merckelbach *et al*., 1991). However, in microcuttings of *Malus* it was found that IBA was converted to IAA only at very low levels (1%), but IBA induced more roots than IAA.

The leaf and stem samples from *in vivo* and *in vitro* of *P.granatum* was experimented for their pigment ability. The usage of higher ratio of extracts of both leaf and stem from *in vivo* and *in vitro* samples to solvent (eg: 1:5 ratio; 3:10 ratio; 4:10 ratio of extracts to solvent) did not yield a better outcome to the absorption reading for UV-photospectrometer. The best absorption reading was taken from 1:10 ratio of sample powder to solvent (Graph 4.1 to Graph 4.4).

Effects of methanolic concentration was significant to the color value of *P.granatum* leaf and stem extracts. In this study, methanolic solvent was selected to be used as the experimental solvent. Methanolic HCl was used as the solvent in this present experiment. Figure 4.2, figure 4.4 and figure 4.5 showed the leaf and stem pigment extracts of *P.granatum* in methanolic solvent. Methanolic HCl (hydrochloric acid in

methanol) is particularly useful for preparing methyl esters of volatile (short chain) fatty acids. Fatty acids are esterified by heating them with an anhydrous alcohol (e.g., methanol) in the presence of an acidic catalyst (e.g., HCl) in a sealed vessel at a high temperature for a short time (Rousseau, 1987). In the reaction, a fatty acid molecule and an alcohol molecule are joined, with the release of a water molecule. The derivatives can be quickly and easily recovered, quantitatively, from the reaction medium (Kappus, 1991). Methanolic HCl has been used at a range of normalities, from mild to strong. The proper concentration to use is based on the conditions required to esterify the acid under study. High concentrations of methanolic HCl reduce the time necessary for complete reaction, but can create byproducts that can interfere with the analysis. Also, high concentrations of the reagent can weaken rapidly, even when refrigerated, and therefore should be prepared fresh before use (Jiang *et al*., 2009). Lower concentrations of methanolic HCl may be used, but longer time must be allowed for the reaction. If the concentration of the catalyst (HCl) is too high, concentration artifacts (polymers or unidentified derivatives) can form and interfere with the analysis. In previous study by Zhu *et al*. (2012), color value of gardenia yellow pigment increased with the ethanol concentration between 30 and 50 %, and decreased in the range of 50– 80 %. It reached the highest value at ethanol concentration of 50 %. With ethanol concentration increased, the liquid solubility of gardenia yellow pigment increased. Too high ethanol concentration leads to lower pigments solubilization, resulting in the decrement of the color value. Therefore, 40–60 % ethanol-water solution was chosen as the test solvent. Higher color value of gardenia yellow pigment was generally obtained with increasing ethanol concentration. There were rather sharp dropoffs in the color value as the ratio of liquid to material approached either 10:1 (mL:g) or 20:1 (mL:g). The homogenate extraction was performed at room temperature, which could avoid the damage of thermosensitive components and save more energy.

The results obtained from the reading of UV-photospectrometer in initial extraction showed that absorption of *in vivo* sample of leaf (2.914; 2.872, graph 4.2) and stem (2.523; 1.744, graph 4.4) at chlorophyll a and chlorophyll b wavelength respectively was higher and more stable than the *in vitro* sample of leaf (2.602; 2.515, graph 4.1) and stem (2.382; 2.137, graph 4.3). Therefore, in the present experiment, *in vivo* sample of leaf and stem were used. The absorption reading of chlorophyll b for *in vitro* leaf was weak compared to the absorption of *in vivo* leaf. The usage of *in vivo* sample for dye preparation will be far more convenient as the sample can be widely obtained in large quantity. *In vitro* samples are limited and expensive.

There were also effects of ratio of liquid (solvent) to material on colour value and absorption. Increased ratio of sample concentration to solvent (1:3, 1:5, 1:7) leads to unefficiency in chlorophyll absorbance. 1:10 ratio of sample and solvent yielded the optimum absorbance compared to other ratio. Decreased ratio (1:12 and 1:15) also leads to decrease in absorbancy in chlorophyll a and chlorophyll b (green pigment).

It is known that green pigment from leaf or stem of a plant consists of major active component of chlorophyll a and chlorophyll b. Robert *et al*. (2002) used a cytophotometric method for the first *in vivo* studies of the spectral characteristics of marennine. The extinction peak determined directly in isolated living cells was 663 nm, a value close to that of the peak *in vitro* at neutral pH. However, these authors did not quantify the pigment (Schubert *et al.*, 1995; Robert *et al*., 2002)

Salt test was made to replicate the rain phenomena in the real world. Acidic rain was simulated to a higher percentage of Sodium Chloride (NaCl). In salt test, the test found

that increased duration of exposure of sample on glass slide would decrease the absoption of the colourant. Similarly, when the salt concentration increased, the absorption of colorant decreased when absorption taken at chlorophyll a and b wavelength. The initial absorbance for leaf color coating from methanolic extracts at 442nm and 664nm respectively were at 1.319, 0.930 (0.5%), 1.221, 0.717 (1.0%) and 1.295, 0.901 (1.5%). After 45 minutes of exposure to salt test, the absorbance were decreased at 1.058, 0.586 (0.5%), 0.992, 0.436 (1.0%) and 0.988, 0.543 (1.5%). While leaf color coating from ethanol extracts yielded initial absorbance readings at 442 nm and 664 nm, respectively were at 1.01, 0.72 (0.5%), 0.85, 0.602 (1.0%), 0.816, 0.596 (1.5%). The absorbance after 45 minutes of exposure to salt test were 0.93, 0.665 (0.5%), 0.754, 0.581 (1.0%) and 0.739, 0.533 (1.5%). The initial absorbance for stem color coating from methanolic extracts at 442 nm and 664 nm respectively were at 0.197, 0.176 (0.5%), 0.185, 0.184 (1.0%) and 0.165, 0.165 (1.5%). The absorbance for stem color coating were observed to be decreased after 45 minutes to exposure with absorbance at 0.093, 0.126 (0.5%), 0.131, 0.112, (1.0%) and 0.106, 0.078 (1.5%). The initial absorbance for stem color coating from ethanol extracts at 442nm and 664nm respectively were at 0.212, 0.092 (0.5%), 0.311, 0.097 (1.0%) and 0.194, 0.086 (1.5%). The absorbance for stem color coating were observed to be decreased after 45 minutes to exposure with absorbance at 0.192, 0.076 (1.0%), 0.282, 0.048 (1.0%) and 0.166, 0.051(1.5%).

Heat test was made to replicate the heat from direct sunlight in the real world. Similarly as salt test, the absorbance of colourant decreased with longer duration of exposure to heat.

The explaination of the decreasing absorption after both salt and heat test probably due to the absence of stabilizer to the colourant made. The initial absorbance for leaf color coatings for methanolic extracts at 442 nm and 664 nm respectively prior to being

exposed to heat were at 0.578 and 0.156 and the absorbance after 48 hours exposure to heat were at 0.533 and 0.118. The initial absorbance for stem color coatings for methanolic extracts at 442 nm and 664 nm respectively prior to being exposed to heat were at 0.173 and 0.146 and the absorbance after 48 hours exposure to heat decreased to 0.131 and 0.129. The initial absorbance for leaf color coatings for ethanol extracts at 442nm and 664 nm, respectively prior to being exposed to heat were at 0.581 and 0.252 and the absorbance after 48 hours exposure to heat decreased to 0.555 and 0.235. The initial absorbance for stem color coatings for ethanol extracts at 442 nm and 664 nm, respectively prior to being exposed to heat were at 0.088 and 0.044 and the absorbance after 48 hours exposure to heat decreased to 0.043 and 0.029.

Coating colors have a very simple composition. A coating color primarily consists of pigments dispersed in water plus binders, cobinders and addictives. Calculations are, as a rule, based on the dry product, even if the actual ingredients deployed are mainly liquid commercial products with differing dry contents (proportion of active substances). Pigment is the most abundant components in the coating, so pigment is naturally the most important factor affecting the properties of the coating (Chayanath *et al*., 2002). Binders serve as pigment to pigment bonders and to anchor the pigments to the base surface. Binder controls the coating color viscocity and water retention. There are driving forces, such as the pressure penetration, the capillary absorption and the heat, which causes material movement in the direction in a coating process. On the other hand, there are parameters in the coating colors to either allow or restrict the movement of particles relatively to each other. It has been shown that binder can move relative to the pigments only when the pigments are larger compared to the size, and this is not influenced by the binder level. To allow relative movement the pore size between pigments must be large. Narrow particle size distribution pigments than in the

coatings containing pigments with a broad particle size distribution. Also, the particle size distribution has been proposed to be more important than particle aspect ratio in terms of the mobility. In accordance with earlier discoveries about the pigment size and size distribution is the sort that a small particle size is reported to have a higher tendency to migrate with a large particle sizes. Water functions as a carrier for solids particles (Tamminen *et al*., 2012). A natural direction of the material movement is towards the base web during the pressure penetration (during the coating application and under the metering element), and during capillary absorption (between the application and the metering element). Many studies in the literature studies on the effect of the drying conditions on binder migration and many discrepancies between the conclusions. It has been proposed that drying, in fact, would stop the binder movement rather than cause it, and also the boundary theory was used to explain binder rich surfaces. Based on several studies, the movement of both the binder and the water would be always towards the substrate (Van der Krieken *et al*., 1993).

The DPPH radical scavenging assay is a discoloration assay that determines the activity of antioxidants to directly react with DPPH stable free radical by observing its absorbance at 517 nm. The DPPH radical is a stable organic nitrogen-centered free radical, dark purple in color that becomes colorless when it is reduced to its non-radical form by antioxidant. A purple colored 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), a stable free radical which reduces to α, α-Diphenyl-β-picrylhydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. DPPH is observed yellow in color if reacts with antioxidant. The discoloration of purple color indicates the potential of antioxidants of the samples. The activity of the extracts is attributed to their hydrogen donating ability. The stronger the discoloration occurs, the higher the antioxidant power of the sample. Methanol was used as the primary solvent

as it showed strong antioxidant capacities, apart from acetone and EtOAc (ethyl acetate). The water extract showed less antioxidant capacity at all the concentrations compared to other extracts (Singh *et al*., 2007). Variations in antioxidant capacity of methanol and water extracts may be attributed to differences in their phenolic contents. All the extracts can increase in antioxidant capacity with increase in dose, except the water extract. A similar trend has been reported in the case of olive extracts .The antioxidant activity shown by the pomegranate extracts maybe due to the presence of polyphenols, such as ellagic tannins, ellagic acid and gallic acid (Negi *et al*., 2003). Variability of phytochemical contents and their antioxidant activity in pomegranate seeds has been found due to different cultivars and growing conditions. The pomegranate seeds were compared with respect to total oils, phenolics, flavonoids, and proanthocyanidins (Pu Jing *et al*., 2012).

According to this method, ascorbic acid was used as a positive control in between every other sample. Between leaf and stem samples collected from *in vivo* and *in vitro* grown plants, the *in vivo* leaf sample of leaf of *Punica granatum* at 100 µg/ml concentration exhibited the lowest absorbance (0.031nm) followed by *in vitro* leaf at 100 µg/ml with a value of 0.038nm. The stem absorbance for *in vivo* and *in vitro* samples were higher compared to the leaf samples, both exhibited the lowest value at 0.063nm and *in vivo* at 0.071nm.

For percentage of the radical scavenging activity (Graph 5.1), the lower the absorbance recorded, the higher the percentage of the radical scavenging activity of the sample. The highest percentage of radical scavenging activity was found in *in vivo* leaf sample at 100 µg/ml concentration and the lowest was found in *in vivo* stem at 20 µg/ml concentration with 5.3% of radical scavenging activity. DPPH stable free radical

method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al*., 2002). The scientific community has devised a mean of evaluating antioxidant efficiencies, known as IC_{50}. This is defined as Scavenging Effect=Inhibitory Concentration or IC. The IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower the IC_{50} number, the greater the overall effectiveness of the antioxidant in question (Pourmorad *et al*., 2006). Table 5.8 shows the amount of each extract needed for 50% inhibition (IC_{50}). The highest radical scavenging activity was showed by *P.granatum* with IC_{50}=2.21 µg/ml which was the highest among all samples. The IC_{50} value of radical scavenging activity in the plant extracts decreased in the following order (Table 5.8 and Table 5.9): Peel (2.21 µg/ml) < seed (3.37 µg/ml) < *In vitro* leaf (3.47 µg/ml) < *in vivo* leaf (3.49 µg/ml) < *in vitro* stem (6.82 µg/ml) < *in vivo* stem (8.79 µg/ml). Most of the leaf, peel and seed extracts at different concentrations exhibited more than 35 % of scavenging activity. The present results proved to be comparable to literature reports by Ozgen *et al*. (2008) on pomegranate in Turkey, Zhang *et al*.(2010) on pomegranate leaves, Pu Jing *et al*. (2012) on pomegranate seeds, Tezcan *et al*. (2009) on pomegranate juices, Zhang *et al*.(2011) on flower and Kaur *et al*. (2006) on pomegranate flower extracts, that pomegranate exhibits good antioxidant capacity and is an effective scavenger of several reactive oxygen species, primarily due to its high levels of phenolic acids, flavonoids and other polyphenolic compounds.

The results showed in the present investigation indicate that the extraction with methanol gives results to high yield of extract and also high antioxidant activity to all type of extracts. This has been confirmed by previous studies by various methods used for antioxidant assay. Thus, the result in the present work shows that selective extraction of antioxidant from natural sources by appropriate solvent is very important

in obtaining fractions with high antioxidant activity. The results in this work also show the presence of compounds possessing antioxidant activity of both *in vitro* and *in vivo* leaf and stem, peel and seeds of pomegranate. Peel as an enriched antioxidants exhibiting higher activity as compared to the rest of the extracts. For comparison of the presence of antioxidant in *in vitro* and *in vivo* leaf and stem, it is observed that the *in vivo* sample of leaf and stem exhibited slightly higher antioxidant power compared to the *in vitro* extracts. The differences in the antioxidant activity of the *in vitro* and *in vivo* extracts may be due to their different phenolic compositions. Since this is the first report on the comparison of antioxidant activity between *in vitro* and *in vivo* extracts of pomegranate, further studies are needed on the report on the isolation and characterization of individual phenolic compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

Similarly in previous studies by Gil *et al*., (2000), the MeOH (methanol) extract showed strong antioxidant capacities at 25 and 100 mg/ml concentration than acetone and EtOAc extracts. Variations in antioxidant capacity of methanol and water extracts may be attributed to differences in their phenolic contents. The antioxidant activity shown by the pomegranate extracts maybe due to the presence of polyphenols, such as ellagic tannins, ellagic acid and gallic acid. Previous report by Pu Jing *et al*. (2012) reported that 50% acetone extracts had an IC_{50} range from 6.4 to 13.1 mg flour equivalents/ml and significantly differed among cultivars of China-grown pomegranate. The 80% methanol extracts had an IC_{50} range from 11.2 to 19.8 mg flour/ml, which was higher than the corresponding 50% acetone extract, indicating a lower ability to quench DPPH radicals. The DPPH scavenging capacity of pomegranate seed flours was comparable to that of Maryland-grown soybeans (7–10 mg seed flour equivalent/ml and lower than

that of red grape seeds (2.7–4.6 lg/ml). The high concentration of total phenolics and flavonoids in young leaves indicated that the substrates of those metabolites came from the stem and/or root, and the synthesis of these metabolites accompanies bud germination. The lower concentration of alkaloids in the shoots suggests that the course of alkaloid synthesis follows leaf growth and development and that the substrates come from intermediates of primary or photosynthetic metabolism (Zhang *et al*., 2010).

It is well-known that free radicals cause antioxidation of unsaturated lipids in food. Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS (Viuda-Martos *et al*., 2010). On the other hand, antioxidants can intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, forming a stable end-product, that does not initiate or propagate further oxidation of the lipid (Tzulker *et al.*, 2007). The data revealed that the extracts were free radical inhibitors and primary antioxidant that react with free radicals. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Hochestein & Atallah, 1988). This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. Additionally, this species is believed to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid-iron EDTA.

Phytochemicals constituent of *P.granatum* was studied using several tests to detect the presence of saponin, terpenoid, tannin, glycoside, flavonoid and reducing sugar. This test was conducted between leaf and stem of *in vitro* and *in vivo* sample, peel, pulp and

seed from pomegranate fruit. From the results, presence of cathetolic tannins for both leaf and stem from *in vitro* and *in vivo* sample of *P.granatum*. Presence of terpenoid only exists in *in vivo* leaf and stem (Positive match; steroid). No glycoside detected in all *in vitro* and *in vivo* samples. Flavonoid was detected in all sample of leaf and stem of *in vitro* and *in vivo*. Reducing sugar was detected in leaf sample of *in vitro* and *in vivo*, but absent in stem sample *in vitro* and *in vivo* (Table 6.1).

For peel and seed sample, only peel sample gave positive results in all three phytochemicals test conducted (terpenoid, tannin and reducing sugar). Tannin and reducing sugar tests conducted exhibited negative results for seed sample. Absence of reducing sugar (mainly fructose and glucose) in seed might because of juice has been removed and long hours of exposure to alcohol solvent that cleanse the trace of juice from the aril of the seed (Ayaz *et al*., 2000). Overall results showed that the phytochemical tests conducted *on in vitro* sample have the same and stable results to *in vivo* sample of *P.granatum* L. This proves that the *in vitro* sample from the regeneration experiment in chapter 2 has the same phytochemical constituents with *in vivo* sample of normal *P.granatum* L plant.

The present results are similar to tests conducted by Tezcan *et al*. (2009) where fructose (F) and glucose (G), a component of reducing sugar were found as the major sugars in commercial pomegranate juices. The combination and the ratio of sugars and organic acids have been related to flavour quality of fruits. In a study by Ozgen *et al*. (2008), revealed that fructose and glucose were found to be dominant sugars in all cultivars of pomegranate. The amount of sucrose found in pomegranate samples was almost negligible. It is well-known that the sugar/acid ratio in many fruits is a primary driver of

flavour quality. In general, there seems to be a modest variation in sugar contents among the cultivars tested, but there was substantial variability in acidity.

Presence of steroid from terpenoid test only detected in *in vivo* sample, and absence in *in vitro* sample. Previous study was previously conducted by Choi *et al*. (2006) suggested that there were no steroid estrogens including estrone, estradiol and testosterone in pomegranate seed, fruit juice and preparations. Consequently, the previously reported analysis of pomegranate were misunderstood due to their analytical results according to either the estrogen-like effects or similarity of peak retention time and Rf values in experiments. Therefore, the result for steroid obtained in the present terpenoid test for *in vivo* sample might be suggesting to the false positive results.

Another previous report was undertaken to investigate changes in the major chemical composition in arils and peels during fruit maturation in two commercial accessions, showed that hydrolysable tannins were reduced in the peels during maturation, while the anthocyanin level increased. The results show that the sugar content in the aril juice increased in both accessions while the levels of acidity and of citric acid decreased. The high correlation between antioxidant activity and the level of hydrolysable tannins, such as punicalagin, punicalin and gallagic acid, which are found mainly in peels, suggest that these latter compounds contribute significantly to the antioxidant activity of peels (Tzulker *et al*., 2007).

During fruit development and maturation, significant changes were found in the physical parameters (fruit weight and volume) and chemical profile of pomegranate arils and peels. For example, the levels of antioxidant activity, total phenolics compounds and citric acid were reduced during maturation in the aril juice, while the levels of glucose and fructose, as well as of malic and ascorbic acids, increased (Gil *et*

al., 2000). Some trends can be found in one accession but not in the other. Changes between the two accessions were also found in the relationship between colour development of the fruit's skin and aril juice quality (Shwartz *et al*., 2009)

Selection of ethanol and methanol as solvents used for this present phytochemical test was due to its polar protic properties, where it can act as solvent that share ion dissolving power but have an acidic hydrogen (Laura *et al*., 2010). In general, ethanol and methanol have high dielectic constants and high polarity (Rousseau, 1987). An earlier study revealed that the Total Flavonoid Compound value of pomegranate leaves higher than that of the pomegranate seeds in this study using 80% methanol extracts. In the study by Pu Jing *et al*. (2012), the 80% methanol significantly increased the flavonoid yields by 12–36% in tested seed of pomegranate samples, compared to the 50% acetone extraction. This suggests that a change in solvent polarity might alter the ability to extract compounds of interest from a selected botanical sample. The 50% acetone and 80% methanol were suitable solvents for flavonoid and total phenolic extraction from pomegranate seeds, respectively, possibly due to the high polarity of flavonoids compared to the overall phenolic compounds. (Pu Jing *et al*., 2012). Yield and efficiency of the extraction depends on the type of the solvent as well on the flavonoids, which is being isolated. For total phenolics and flavonoids extraction from ginger parts methanol was more efficient compare to acetone and chloroform. In the conventional solvent extractions, methanol showed the greatest capability in extracting antioxidants and inhibiting the free radicals among the three solvents, methanol, acetone and chloroform (Ghazemsadeh *et al*., 2011).

CHAPTER 8

8.0 CONCLUSION

In tissue culture studies of *Punica granatum* L, the best explant source for the production of multiple shoots was nodal stem. Optimal shoot formation resulted from nodal stem of *P.granatum* cultured in MS medium enriched with 1.5 mg/L BAP (3.8±0.2), meanwhile the optimal shoot length (1.9±0.1cm) was obtained from MS medium supplemented with 2.0 mg/L BAP from shoot tip explants. Nodal stem explant exhibited a higher shoot number while shoot tip explant yielded a better shoot length when cultured *in vitro*. The optimum sucrose concentration for highest shoot number was 40 g/L that yielded 4.1 ±0.2 shoots. In scanning electron microscopy studies, *in vitro* leaf sample was observed to have a higher density of stomata compared to *in vivo* leaf sample and no somaclonal variation indication of *in vitro* samples based on SEM observations.

Healthy and optimum callus growth produced from leaf explant (95.44%), followed by stem explant (94.67%) and root explant (83.44%). Highest callus weight obtained from MS medium supplemented with BAP and NAA and their combinations; 0.5 mg/L BAP and 0.5 mg/L NAA for leaf explants (0.22± 0.01g), 2.5 mg/L BAP and 1.0 mg/L NAA for stem explants (0.37 ± 0.03g) and 1.5 mg/L BAP and 1.5 mg/L NAA for root explant (0.47 ± 0.07 g), while the optimum callus weight for MS medium supplemented with other hormones (IAA, IBA 2,4-D and Kinetin); 1.5 mg/L 2,4-D (0.18 ±0.01g) for leaf explants, 2.0 mg/L IBA (0.28 ± 0.01g) for stem explants and 1.0 mg/L 2,4-D (0.062 ± 0.06g) for root explants.

In vivo leaf and stem color extraction exhibited a higher and more stable absorbance of chlorophyll wavelength, with 1:10 ratio of material and solvent is the best ratio for wavelength determination. Absorbance values for leaf and stem methanolic extracts decreased when subjected to salt test and heat test for 45 minutes and 48 hours, respectively. Absence of stabilizer in color coating played a major role in the degradation of color coatings in salt test and heat test. Pigments from *P.granatum* leaf and stem have the potential in producing natural color coatings in presence of good stabilizer and binder.

At the highest concentration of *in vivo* leaf extracts and *in vitro* stem extracts of *P.granatum* (100µg/ml), the percentages of radical scavenging activity were 67.4 % and 33.7% (100µg/ml) respectively. The highest percentage of radical scavenging activity between peel, pulp and seed sample was obtained from peel with 77.6% at 100µg/ml extract concentration. The best IC_{50} values were obtained from *in vitro* leaf sample and peel sample with the values of 3.47 µg/ml and 2.21 µg/ml, respectively.

In phytochemical studies, Saponin was detected in both *in vitro* and *in vivo* samples of leaf and stem of *P.granatum*. Terpenoids were detected in *in vivo* sample of leaf and stem, peel, pulp and seed sample. Tannins were detected in all *in vitro* and *in vivo* samples and in peel sample. Tannins were not detected in pulp and seed samples. Glycoside was not detected in any of the samples while flavonoids were detected in all *in vitro* and *in vivo* samples. Reducing sugar was detected in leaf sample of both *in vitro* and *in vivo* and peel sample. Reducing sugar was not detected in both *in vitro* and *in vivo* stem, pulp and seed samples.

Further studies in tissue culture and biological activities of this species are vital and should be carried out in the future, focusing more on the callus, rooting, acclimatization and uses of pigment extraction of the species. Studies on the phytochemicals and antioxidant potential of *P.granatum* L should be carried out to a wider range of *in vivo* and *in vitro* samples not particularly on *P.granatum* L parts only, but also to other species.

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APPENDIX A

Table 2.1 (b): Anova analysis of IAA hormone to leaf, stem and root explant in callus weight

		Leaf callus (g)	Stem callus (g)	Root callus (g)
	df	MS	MS	MS
IAA	3	0.0063**	0.0015**	0.0013**
rep	29	0.001*	0.0003	0.00004
error	87	0.0006	0.0002	0.00005

Table 2.2 (b): Anova analysis of IBA hormone to leaf, stem and root explant in callus weight.

		Leaf callus (g)	Stem callus (g)	Root callus (g)
	df	MS	MS	MS
iba	3	0.11**	0.27**	0.006*
rep	29	0.002	0.007	0.002
error	87	0.002	0.005	0.002

Table 2.3 (b): Anova analysis of IBA hormone to leaf, stem and root explant in callus weight.

		Leaf callus (g)	Stem callus (g)	Root callus (g)
	df	MS	MS	MS
kin	3	0.007*	0.04**	0.002**
rep	29	0.001	0.0005	0.0002
error	87	0.002	0.0006	0.0003

Table 2.4 (b): Anova analysis of BAP and NAA hormone to stem explant in callus weight and shoot number

		Callus weight (g)	Shoot number
	df	MS	MS
bap	5	0.88**	28.4**
naa	4	0.35**	14.6**
bap*naa	20	0.21**	7.2**

rep	29	0.02	0.1
error	841	0.02	0.07

Table 2.5 (b): Anova analysis of BAP and NAA hormone to root explant in callus weight.

		Callus weight (g)
	df	MS
bap	5	0.63**
naa	4	0.52**
bap*naa	20	0.16**
rep	29	0.02
error	841	0.01

Table 2.6 (b): Anova analysis of BAP and NAA hormone to nodal stem explant in shoot number and shoot height.

		Shoot number	Shoot height (cm)
	df	MS	MS
bap	5	41**	0.49**
naa	4	36.6**	1.66**
bap*naa	20	9.5**	0.14
rep	29	1.1	0.04
error	841	0.8	0.09

Table 2.7 (b): Statistical analysis of the influence BAP and NAA root explants

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Callus weight	0.10685	0.00486	0.14578	136.44	0	1.2586

Table 2.8 (b): Statistical analysis of the influence BAP and NAA leaf explants

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Callus weight (g)	0.09753	0.00189	0.05657	58	0	0.346
Shoot number	0.2511	0.0158	0.4731	188.42	0	2

Table 2.8 (b): Statistical analysis of the influence BAP and NAA nodal stem explants

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Shoot number	2.3656	0.0392	1.1748	49.66	1	7
Shoot height (cm)	0.6418	0.0103	0.3085	48.07	0.1	1.8

Table 2.9 (b): Statistical analysis of the influence IAA on leaf, stem and root explants to callus weight

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Leaf callus	0.06939	0.00261	0.02861	41.23	0.0213	0.1452
Stem callus	0.04882	0.00146	0.01599	32.75	0.0183	0.0795
Root callus	0.020609	0.000811	0.008888	43.12	0.0078	0.0492

Table 2.10 (b): Statistical analysis of the influence IBA on leaf, stem and root explants to callus weight

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Leaf callus	0.09076	0.00622	0.06809	75.02	0.018	0.3262
Stem callus	0.1214	0.0102	0.1115	91.81	0.0136	0.789
Root calusl	0.04482	0.00388	0.04247	94.75	0.0112	0.1696

Table 2.11 (b): Statistical analysis of the influence IBA on leaf, stem and root explants to callus weight

Variable	Mean	SE Mean	StDev	CoefVar	Minimum	Maximum
Leaf callus	0.09235	0.00427	0.04675	50.62	0.0136	0.1657
Stem callus	0.09651	0.00351	0.03843	39.81	0.0238	0.1746
Root calusl	0.04104	0.0016	0.01748	42.6	0.0172	0.0951

APPENDIX B

Stock Solution for MS Media:

A. Macro Stock (10X) Soln.:

Ingredient	Amount Per litre in MS (g/L)
NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2•2H_2O$	0.44
$MgSO_4•7H_2O$	0.37
KH_2PO_4	0.17

B. Micro Stock (100X) Soln.:

Ingredient	Amount Per litre in MS (g/L)
H_3BO_3	0.0062
$MnSO_4•4H_2O$	0.0223
$ZnSO_4•H_2O$	0.0086
KI	0.000083
$NaMO_4•2$ H2O	0.00025
$CuSO_4•5H_2O$	0.000025
$CoCl_2•6$H2O	0.000025

C. Iron Stock (10X) Soln.:

Ingredient	Amount Per litre in MS (g/L)
$FeSO_4•7H_2O$	0.0278
$Na_2EDTA•2H_2O$	0.0373

D. Vitamin Stock (100X) Soln.:

Ingredient	Amount Per litre in MS (g/L)
Nicotinic acid	0.0005
Pyridoxine•HCl	0.0005
Thiamine•HCl	0.0001
Myo-inositol	0.01

E. Others

Ingredient	Amount Per litre in MS (g/L)
Glycine (Amino Acid)	0.002
Sucrose (Carbon Source)	30
Agar (Solidifying Agent)	9