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In Vitro Multiplication and Phenolic Content Quantification of *Hydrocotyle bonariensis* (Pennywort) *In Vitro* Propagated Leaves

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ABSTRACT

Hydrocotyle bonariensis is a pennywort from the family Araliaceae commonly known as “pegaga embun” among Malay community in Malaysia. *H. bonariensis* is closely related to *Centella asiatica* due to its morphological characteristics. However, both plant species are from different families. Among all the pennywort species, *C. asiatica* is the most popular with high number of research that has been undertaken compared to *H. bonariensis.* The availability of wild *H. bonariensis* in nature is unidentified due to low interest on this plant species. Hence, this study was carried out to propagate *H. bonariensis* via *in vitro* technique and to evaluate the antioxidant properties of *H. bonariensis.* The nodal segments of *H. bonariensis* were used as explants for the multiplication experiment that was conducted using Murashige and Skoog (MS) media supplemented with 6-benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (Kin) at concentrations of 0, 2.5, 5.0, 7.5 and 10.0 mg/L. After 6 weeks of inoculation, the plants were harvested and total polyphenol, phenolic acid and flavonoid contents were analysed using Folin-Ciocalteu and aluminum chloride colorimetric methods. The results of this study showed that 10 mg/L of BAP was the best cytokinin concentration for multiplication of *H. bonariensis.* For the antioxidant analysis, the highest total polyphenol content was recorded from 2.5 mg/L BAP treatment with 1.67 mg GAE/g DW. Meanwhile, the highest total phenolic acid and flavonoid contents were recorded from TDZ treatment with 3.22 mg GAE/g DW (10 mg/L) and 9.03 mg RE/g DW (7.5 mg/L), respectively. In conclusion, plant tissue culture technique would be recommended for large-scale production of plantlets and secondary metabolites for *H. bonariensis*.

Keywords: Antioxidant, *Hydrocotyle bonariensis*, plant tissue culture.

INTRODUCTION

Hydrocotyle bonariensis is a hairless and creeping perennial herb from the family Araliaceae that is commonly known as “pegaga air”, “pegaga embun”, “pegaga gajah”, and “pegaga piring” (Goh, 2007). It tends to grow mostly in tropical and subtropical regions of the world (Masoumian et al*.,* 2011). *H. bonariensis* grows well in moist and shady places. It has long stolon with roots, leaves, and inflorescences appearing at the nodes. According to Sumazian et al*.* (2010), *H. bonariensis* is one of the local herbs that contains high concentration of antioxidant compounds.

H. bonariensis is an edible herb commonly consumed fresh as salad or “ulam”, fermented vegetables, condiment, herbal tea and juice (Reihani and Azhar, 2012). *H. bonariensis* has been used for a long time to treat several diseases including tuberculosis, ophthalmic diseases, rheumatism and arthritis

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(Masoumian et al., 2011). The phytochemical screening conducted on the leaf of *H. bonariensis* detected compounds from several functional groups such as flavonoids, phenols, tannins, sterols, saponins, tannic acid and triterpenes glycosides (Marino et al., 2009; Sumazian et al., 2010; Tabopda et al., 2012; Obaseki et al., 2016; Ajani et al., 2017).

To date, there is no product-based derived from *H. bonariensis* that can be found in Malaysia, but it is normally consumed raw or as a juice. However, *Centella asiatica* or herb commonly known as "pegaga kampung" that is closely related to *H. bonariensis* was extensively studied and has a variety of skincare products such as Cicapair Dr. Jart and Cosryx Centella Blemish Ampoule. Both herbs are known as pennywort because of their similar physical appearance. It is easy to discriminate both pennyworts by the naked eyes. *H. bonariensis* and *C. asiatica* can be distinguished based on the shape of their leaves, where the leaf of *H. bonariensis* is orbicular or round while that of *C. asiatica* is kidney shaped. Furthermore, the petiole of *H. bonariensis* is attached at the center of the leaf, while the petiole of the *C. asiatica* is at the loop of its leaf blade (Goh, 2007).

Studies on the pharmacological properties conducted on the *H. bonariensis* extracts found that this plant exhibited anti-inflammatory activity, antioxidant activity, potential protective activities against cardiovascular and ophthalmic diseases, anticancer activity and anti-microbial activity (Ajani et al., 2009; Man et al., 2010; Ajani et al., 2017; Wongrakpanich et al., 2018; Kaboua et al., 2021; Barreca et al., 2021; Souza et al., 2021). In order to accumulate large amount of bioactive compounds from the plant, large amount of biomass is needed. Therefore, it is important to find an alternative method for large-scale production of plant raw materials. To date, micropropagation is one of the advance propagation techniques for large-scale production of raw materials and secondary metabolites (Haida et al., 2022).

Although *H. bonariensis* extract contains a wide range of bioactive compounds, the study related to this plant on production of phenolics through plant tissue culture method is still limited. There is no study that has been found on production of phenolics from the micropropagated leaves of *H. bonariensis.* Hence, this study was conducted to propagate *H. bonariensis* via plant tissue culture technique and to quantify the phenolic content in the tissue culture-derived sample of *H. bonariensis.*

MATERIALS AND METHODS

Location and Plant Material

The study was conducted at Plant Tissue Culture Laboratory and Postharvest Laboratory, Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia. The plant materials of *H. bonariensis* were obtained from the existing stock plant in the Plant Tissue Culture Laboratory. The stock plant was maintained in Murashige and Skoog (MS) media without an addition of plant growth regulator at 24 ± 2 °C. The nodal segments were used as the explant.

Preparation of Basal Medium

The basal medium was prepared based on the formulation of Murashige and Skoog (1962) known as MS medium. The MS medium was supplemented with 30 g/L sucrose and different concentrations of plant growth regulators and pH was adjusted to 5.75. Then, 3.0 g/L agar (Gelrite) as gelling agent was added and the medium was autoclaved at 121 °C for 20 min at the pressure 1.05 kg/cm^2.

Application of Cytokinins for Multiplication of *H. bonariensis*

The multiplication of *H. bonariensis* was conducted by supplementation of cytokinin namely 6-benzylaminopurine (BAP), N(6)-furfuryladenine (Kin) and thidiazuron (TDZ) in the MS medium at the concentrations of 2.5, 5, 7.5 and 10 mg/L (Table 1). The MS medium without supplementation of cytokinin served as control treatment. Each treatment consisted of three replications with five explants per replication (n = 15). The nodal segment was cut approximately 1 cm of stolon and 0.5 cm of the petiole. The existing leaves were removed to allow the development of new shoots and leaves. All cultures were incubated in the culture room at 24 ± 2 ºC under 16 h light and 8 h dark using white fluorescence light irradiation 2000 lux. The data on plant length, leaf width, number of leaves, number of shoots per stolon, fresh weight of leaves and number of stolons were recorded after 6 weeks of incubation.

Table 1. Treatments for shoot multiplication of *H. bonariensis*

Treatment	Type of cytokinin	Concentration (mg/L)
T1 (Control)	MS0	0.0
T2	BAP	2.5
T3		5.0
T4		7.5
T5		10.0
T6	Kin	2.5
T7		5.0
T8		7.5
T9		10
T10	TDZ	2.5
T11		5.0
T12		7.5
T13		10

Phenolic Content of *H. bonariensis*

Plant Materials

The leaves of *H. bonariensis* were harvested after 6 weeks of incubation in the culture room. The plantlets were carefully removed from the culture vessel. The culture medium attached to the plantlet was removed under running tap water. Then, the leaves were separated and cleaned thoroughly using tap water. The leaves were then dried in an oven at 50 °C for 2 days and used for the phenolic content extraction.

Sample Extraction

The extraction was carried out using the method explained by Hakiman and Maziah (2009). Briefly, 0.25 g of dried leaves of *H. bonariensis* were cut into small pieces. Then, the leaves were placed into 150 mL conical flasks for each treatment. A total volume of 12.5 mL of distilled water (room temperature, 25 °C) was added and the flask was covered with aluminum foil. The conical flasks containing the samples were then placed on an orbital shaker at room temperature for an hour in the dark. After 1 h, the extraction samples were filtered using filter paper. The mixtures were used for the phenolics content analysis.

Total Polyphenol Content

The total polyphenol content was determined according to the method explained by Marinova et al. (2005). Briefly, 50 µL of leaf extract was added with 1.25 mL of Folin-Ciocalteu reagent which was diluted 10 times in a test tube. After 5 min of reaction, 1.25 mL of 7% sodium carbonate was added. The mixture was incubated at room temperature for 1 h before measuring the absorbance at 725 nm using a spectrophotometer (Thermo Fisher Scientific, USA). The standard curve of gallic acid absorbance against the amount of gallic acid (0, 40, 80, 120, 160 and 200 µg) was constructed to determine the total polyphenol content in the leaf extracts from different treatments of *H. bonariensis*. The total polyphenol content of leaf extracts of different treatments of *H. bonariensis* was expressed as mg gallic acid equivalents per gram plant material on a dry basis (mg GAE/g DW). Each treatment was conducted in three replications and each replication consisted of five samples.

Total Phenolic Acid Content

The total phenolic acid content was carried out following the method as described by Singleton and Rossi (1965) using Folin-Ciocalteu phenol reagent method. A total of 0.125 mL of leaf extract was added into a test tube containing 1.125 mL of distilled water. Then, 0.125 mL of Folin-Ciocalteu phenol reagent was added into each test tube, and the mixture was thoroughly mixed for 5 min using a vortex machine followed by the addition of 1.25 mL of 7% sodium carbonate. Then, the total volume of the mixture was adjusted to 3.125 mL with the addition of 0.5 mL of distilled water. After that, the mixture was incubated at room temperature for 90 mins for the reaction to complete. Finally, the absorbance was measured using a spectrophotometer at 760 nm. The standard curve of gallic acid absorbance against the amount of gallic acid (0, 40, 80, 120, 160 and 200 µg) was constructed to determine the level of total phenolic acid content in leaf extracts of different treatments of *H. bonariensis*. The total phenolic acid content of the leaf extracts was expressed as mg gallic acid equivalents per gram plant material on a dry basis (mg GAE/g DW). Each treatment was conducted in three replications and each replication consisted of five samples.

Total Flavonoid Content

The total flavonoid content was carried out according to the procedure explained by Marinova et al. (2005) using aluminum chloride colorimetric method. A total of 0.5 mL of leaf extract was added with 2 mL of distilled water in a test tube. After that, 150 µL of 5% sodium nitrite was added into each test tube. After 5 min, 150 µL of 10% aluminum chloride was added to the mixture. At the sixth minute, 1 mL of 1 M sodium hydroxide was added. Then, the mixture was made up to 5 mL by adding 1.2 mL of distilled water. The mixture was mixed thoroughly using a vortex machine and the absorbance was measured at 510 nm using a spectrophotometer. The standard curve of rutin absorbance against the amount of rutin (0, 40, 80, 120, 160 and 200 µg) was constructed to determine the level of total flavonoid content in leaf extracts of different treatments of *H. bonariensis*. The total flavonoid acid content of the leaf extracts was expressed as mg rutin equivalents per gram plant material on a dry basis (mg RE/g DW). Each treatment was conducted in three replications and each replication consisted of five samples.

Statistical Analysis

The experiments were conducted in a Completely Randomized Design (CRD) with three replications for each treatment. Analysis of variance (ANOVA) was used to analyse all data collected. Mean comparison between treatments was determined by employing the Duncan's Multiple Range Test at a significant level $p \leq 0.05$. The test was run using Statistical Analysis System (SAS) version 9.4.

RESULTS AND DISCUSSION

In vitro Multiplication of *H. bonariensis*

The nodal segment was chosen as the explant for the development of *in vitro* multiplication of *H. bonariensis*. Based on Table 2, the results showed that the highest concentration of BAP at 10.0 mg/L (T5) produced the highest number of leaves with 7.44 leaves (Figure 1). The results showed that the number of leaves of *H. bonariensis* in T5 was significantly higher in comparison to other treatments. Meanwhile the highest concentration of TDZ at 10.0 mg/L (T13) was significantly produced the lowest number of leaves with 2.33 leaves. However, statistical analysis showed that the number of leaves produced by T13 was not significantly different with a few treatments. A study by Singh et al. (2014) found that every single branch has a single leaf with an average three number of branches. The highest number of nodes was obtained from the MS medium fortified with 2.0 mg/L BAP and 0.5 mg/L Kin with an average of 16.3 nodes.

Table 2. The effect of different concentrations of cytokinins on *H. bonariensis* multiplication

Treatment	Number of leaves	Number of stolons	Number of shoots per stolon	Length of petioles (cm)	Width of leaves (cm)	Fresh weight (g)
T1	3.22^{de}	1.78^{cd}	1.97^{a}	0.82^{ab}	0.82^{a}	0.04^{e}
T2	4.11^{bcd}	3.22^{abc}	2.39^{a}	0.57^{cd}	0.31^{d}	0.06^{cde}
T3	5.67^{b}	4.00^{a}	1.82^{a}	0.43^{d}	0.27^{d}	0.07^{bcde}
T4	5.11^{bc}	3.45^{ab}	1.51^{a}	0.41^{d}	0.32^{d}	0.11^{b}
T5	7.44^{a}	4.44^{a}	1.73^{a}	0.43^{d}	0.36^{d}	0.15^{a}
T6	4.33^{bcd}	3.45^{ab}	1.78^{a}	0.53^{d}	0.70^{bc}	0.05^{de}
T7	3.56^{cde}	2.22^{bcd}	1.65^{a}	0.54^{d}	0.65^{bc}	0.04^{de}
T8	4.22^{bcd}	3.22^{abc}	1.24^{a}	0.47^{d}	0.62^{c}	0.05^{de}
T9	5.00^{bc}	4.56^{a}	1.11^{a}	0.46^{d}	0.75^{ab}	0.07^{bcde}
T10	4.00^{cd}	2.33^{bcd}	1.75^{a}	0.83^{ab}	0.35^{d}	0.07^{bcde}
T11	3.56^{cde}	1.78^{cd}	2.24^{a}	0.71^{bc}	0.32^{d}	0.09^{bc}
T12	3.67^{cde}	1.67^{d}	2.41^{a}	0.85^{ab}	0.35^{d}	0.07^{bcd}
T13	2.33^{e}	1.44^{d}	2.22^{a}	0.93^{a}	0.30^{d}	0.08^{bcd}

Means with different alphabet in each column are significantly different at $p < 0.05$ using Duncan's Multiple Range Test (n=15).

Figure 1. A well-developed *H. bonariensis* grown on the MS medium supplemented with 10 mg/L BAP. The scale bar represents 1.0 cm of actual size.

Based on Table 2, the highest concentration of Kin at 10.0 mg/L (T9) produced the highest number of stolons, followed by the highest concentration of BAP at 10.0 mg/L (T5) with a slight difference of stolon number with 4.56 and 4.44 (Figure 2), respectively. The statistical analysis showed that the mean number of stolons for multiplication of *H. bonariensis* was not significantly different between a few treatments. Meanwhile, the highest concentration of TDZ at 10.0 mg/L (T13) showed the lowest number of stolons produced with 1.44. To date, no research has been conducted in collecting the number of stolons as one of the parameters from *in vitro* multiplication of *H. bonariensis,* but most studies revealed the result of the shoots per node. However, number of stolons is a relevant parameter for future study because the nodal segment grown from the stolons will affect the number of explants that can be cultured to generate more shoots. The stolons of the pennywort plant are the vegetative part which can be used as an explant for multiplication purposes. Hence, high number of stolons could provide high number of explants for further multiplication of the plant.

Figure 2. The stolons produced from the *H. bonariensis* grown on the MS medium supplemented with 10 mg/L BAP. The scale bar represents 1.0 cm of actual size.

From Table 2, the treatment of 7.5 mg/L TDZ (T12) produced the highest number of shoots per stolon with 2.41 shoots, followed by 2.5 mg/L BAP (T2) with 2.39 shoots. It showed that a low concentration of BAP could also produce a high number of shoots per stolon compared to the high concentration of TDZ. Meanwhile, the highest concentration of Kin at 10.0 mg/L (T9) showed the lowest number of shoots per stolon with a mean of 1.11. However, there was no significance difference between the treatments on the number of shoots per stolon. The number of shoots per stolon produced ranged between 1.11 to 2.41 shoots. Several studies found that BAP at optimum concentration would induce the highest number of shoots. Alagumanian et al. (2015) revealed that MS media with 17.76 µM BAP and 1.44 µM GA_3 produced the mean number of shoots with 21 shoots per nodes after 45 days of inoculation in *C. asiatica*. Another study conducted by Karuppusamy et al. (2007) for micropropagation of *Hydrocotyle conferta* claimed that MS media with 6.66 µM BAP and 5.37 µM NAA induced a high frequency of bud break (88%) and multiple shoot formations.

According to Goh (2007), the petiole height was measured directly from the surface of the media to the bottom of the leaf blade. Grąbkowska et al. (2014) mentioned that length of petioles is important for their capacity to form roots. In addition, the quality of regenerated shoots in terms of their length and morphology was affected by the type of cytokinin used. A study by Singh et al. (2014) found that 2.0 mg/L BAP + 0.5 mg/L Kin showed the highest mean length of petioles of *C. asiatica* with 4.6 cm after 4 weeks of inoculation. However, the result obtained was in contrast with the findings of this current study. Table 2 showed that 10.0 mg/L of TDZ (T13) demonstrated significantly the highest mean of total length of petioles compared to other treatments with 0.93 cm, while 7.5 mg/L of BAP (T4) demonstrated the lowest mean total length of petioles compared to other treatments with 0.41 cm. The difference in results may be due to the different requirements for growth by different species. The statistical analysis showed that the means length of petioles for multiplication of *H. bonariensis* were not significantly different between a few treatments.

Based on Table 2, MS0 (T1) showed significantly the highest mean total width of leaves with 0.82 cm, while 5.0 mg/L of BAP (T3) showed the lowest mean of total width of leaves compared to other hormone treatments with 0.27 cm. From a study by Shukla et al. (2007), *Curcuma angustifolia* that was maintained in MS medium supplemented with 3.0 mg/L BAP produced maximum width of leaves with 18 mm per leaf. However, they also mentioned that further increase in BAP concentration in the medium could give adverse effects and would show poor growth. As the result obtained, we found that the total width of leaves was higher than T1 (control) and 10.0 mg/L of Kin (T9) compared to all the BAP treatments. It may be concluded that different species of plants may need different requirements of plant growth regulators. The statistical analysis using ANOVA showed that the mean total width of leaves for multiplication of *H. bonariensis* was significantly different between T1 and T2, T3, T4, T5, T6, T7, T8, T10, T11, T12 and T13.

From Table 2, the highest concentration of BAP at 10.0 mg/L (T5) showed significantly the highest mean of fresh weight with 0.15 g, while T1 (control) showed significantly the lowest mean of fresh weight with 0.04 g mean of fresh weight. The fresh weight is influenced by the size and thickness of the whole explant of *H. bonariensis.* Based on the study on the effects of varying concentrations of cytokinins using BAP, Kin and TDZ on shoot development and biomass of *Thymus leucotrichus*, Bekircan et al. (2018) found that TDZ demonstrated the highest fresh (697.0 ± 59.7 mg) and dry (61.3 ± 6.8 mg) weight of biomass production. They also mentioned that the appearance of cultured shoots was poor when using high concentrations of TDZ due to hyperhydricity. This is in line with the observation of the appearance of *H. bonariensis* when TDZ was supplemented in the medium. However, in the context of fresh weight, the results obtained were in contrast with their study. The result showed that BAP was the optimum cytokinin for fresh weight and the mean of fresh weight increased as the concentration of BAP increased. According to statistical analysis, there was a significant difference between T5 and other treatments.

From this study, BAP at 10 mg/L (T5) was observed to produce optimal response for the highest number of leaves, number of stolons and fresh weight with 7.44, 4.44 and 0.15 g, respectively. Lower concentration of BAP at 2.5 mg/L (T2) was observed to produce optimal response for the highest number of shoots per stolon with 2.39. Moreover, TDZ at 10.0 mg/L produced the best response for total length of petioles at 0.93 cm. Last but not least, the control treatment was observed to produce the highest total width of leaves at 0.82 cm.

Antioxidant Content of *H. bonariensis*

The aqueous extraction of *H. bonariensis* was used to determine the total polyphenol, phenolic acid and flavonoid content. Based on Table 3, the treatment of 2.5 mg/L BAP (T2) significantly exhibited the highest total polyphenol content with 1.67 mg GAE/g DW. Meanwhile, 2.5 mg/L TDZ (T10) significantly exhibited the lowest total polyphenol content with 0.34 mg GAE/g DW. In a study by Grzegorczyk-Karolak et al. (2017), the shoots of *Scutellaria altissima* cultured with low concentration of BAP produced a lower amount of polyphenol at 7.13 mg GAE/g DW, while the control shoots cultivated on MS medium only with auxin (0.57 mM IAA) produced 8.58 mg GAE/g DW. They also mentioned that 1 µM of TDZ was optimal cytokinin for polyphenol accumulation. The result contradicts with the present study which indicated that T2 was the best cytokinin for the highest total polyphenol content of *H. bonariensis.* It can be concluded that the requirement of cytokinin was different between species. The statistical analysis also showed that there was significant difference between T2 and other treatments.

Table 3. Analysis of antioxidant contents of *H. bonariensis*

Treatment	Total polyphenol content (mg GAE/g DW)	Total phenolic acid content (mg GAE/g DW)	Total flavonoid content (mg RE/g DW)
T1	1.00^{b}	1.97^{f}	5.20^{c}
T2	1.67^{a}	2.23^{d}	3.95^{f}
T3	0.72^{g}	1.30^{l}	4.37^{ef}
T4	0.57^{i}	1.36^{k}	5.51^{bc}
T5	0.86^{d}	1.86^{h}	5.84^{b}
T6	0.40^{l}	1.43^{j}	3.11^{g}
T7	0.55^{j}	1.94^{g}	4.53^{de}
T8	0.60^{h}	1.79^{i}	4.63^{de}
T9	0.53^{k}	2.03^{e}	2.70^{gh}
T10	0.34^{m}	1.28^{l}	2.29^{h}
T11	0.76^{f}	2.40^{c}	5.04^{cd}
T12	0.82^{e}	2.89^{b}	9.03^{a}
T13	0.97^{c}	3.22^{a}	5.95^{b}

Means with different alphabet in each column are significantly different at $p < 0.05$ using Duncan's Multiple Range Test (n=15).

From Table 3, T13 with the highest concentration of TDZ at 10 mg/L showed significantly the highest total phenolic acid content at 3.22 mg GAE/g DW, while T10 with the lowest concentration of TDZ at 2.5 mg/L showed significantly the lowest total phenolic acid content with 1.28 mg GAE/g DW and BAP at 5.0 mg/L with 1.30 mg GAE/g DW. As mentioned by Baskaran et al. (2014), different type of cytokinin has a significant effect on total phenolic acid content. In their study on the phenolic production of *Coleonema pulchellum*, they found that a combination of benzyladenine and glutamine significantly increased the *in vitro* accumulation of phenolic acid content, compared to when the compounds were used singly. Glutamine is an organic elicitor that can accelerate shoot proliferation and enhance the phenolic acid content of plants (Sena et al., 2023). As conclusion, the results of the present study are in contrast to that of Baskaran et al. (2014). Based on the statistical analysis, application of cytokinins significantly affected the total phenolic acids content of *H. bonariensis.* This indicated that different concentrations and types of cytokinin directly influenced the production of phenolic acids of *H. bonariensis.*

Based on Table 3, treatment of 7.5 mg/L TDZ (T12) revealed significantly the highest total flavonoid content with 9.03 mg RE/g DW. In comparison with other treatments, the second highest flavonoid content was using TDZ with 10 mg/L at 5.947 (T13) followed by 10 mg/L of BAP (T5) with 5.84 mg RE/g DW. Meanwhile, the lowest total flavonoid content was recorded in 2.5 mg/L of TDZ (T10) with 2.29 mg RE/g DW. In a study by Masoumian et al. (2011) on flavonoids production in *H. bonariensis* callus tissues, they reported that Kin and TDZ at 1 mg/L supplemented to MS medium showed a very high production of flavonoids and enhanced the flavonoid accumulation compared to BAP. Grzegorczyk-Karolak et al. (2017) found that MS medium supplemented with 1 μM TDZ was the optimal supplementation for flavonoid production. Therefore, it was proven that TDZ can produce high flavonoid content. The statistical analysis showed that T12 was highly significant compared to the other treatments.

CONCLUSION

In this study, the effect of cytokinins directly influenced the growth and multiplication of *H. bonariensis.* Among all the treatments, 10 mg/L BAP was the most effective cytokinin for the multiplication of *H. bonariensis*. However, the study on antioxidant content found that TDZ produced the highest total phenolic acid and flavonoid contents in the leaf extract of *H. bonariensis*. Therefore, BAP treatment may be recommended for better yield while TDZ treatment may be recommended for secondary metabolite production of medicinal plants.

ETHICAL APPROVAL

No ethical approval was required.

AUTHORS CONTRIBUTION

MH, NN, NSZ conceived and designed the analysis. NN and NSZ collected the data. NN, NSZ and ZH performed the analysis. ZH and MH wrote the paper. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

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Not applicable.

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