

## **Phytochemical Constituents in Leaves and Callus of *Ficus deltoidea* Jack var. *Kunstleri* (King) Corner**

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### **Abstract**

Qualitative and quantitative analysis of phytochemical constituents was done on leaves and leaf's callus of *Ficus deltoidea* Jack var. *kunstleri* (King) Corner. Tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, alkaloids, anthraquinones and polyphenols were present in the leaves and callus cultured on Murashige and Skoog (1962) media supplemented with 0.8 mg/L 2,4-D + 0.7 mg/L Kinetin (KIN). Meanwhile, cardiac glycosides only exist in the leaves but not in the callus tissue. Quantitative analysis showed that alkaloids, saponins, phenols, flavonoids and tannins exist in both samples but in lower amounts for the callus tissue. The significance of the phytochemical constituents with respect to the role of this plant in traditional medicine treatment and the future prospect for callus in the production of secondary metabolites is discussed.

**Keywords:** Callus, leaves, *Ficus deltoidea*, phytochemical constituents

### **Introduction**

*Ficus deltoidea* Jack or 'Mas Cotek' in Malay is one of many *Ficus* species that are cultivated in various parts of the world as a houseplant or an indoor or outdoor ornamental shrub [1]. There are 7 varieties of *F. deltoidea* and *F. deltoidea* Jack var. *kunstleri* (King) Corner is always said to be used in traditional medicine. In East Coast Malaysia, *F. deltoidea* is popularly used for postpartum treatment and maintaining of women's health. It is served as a health tonic or taken as herbal tea to rejuvenate and regain body strength. The use of this plant in traditional treatment can be explained scientifically by studying the presence of its phytochemical constituents. Phytochemicals are natural bioactive compounds found in plants such as vegetables, fruits, medicinal plants, flowers, leaves and roots. They work with nutrients and fibers to act as a defense system to protect the plant against disease [2].

The natural compounds that are found in plants are the basis of modern drugs such as saponins that have a wide range of biological properties and are said to make up the active major constituents of ginseng. Phenolic compounds are the most widely occurring groups of phytochemicals that have considerable physiological and morphological importance in plants. They have been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, cardioprotective and antioxidant activity [3-5]. Other low molecular weight compounds that act as antioxidants and constitute the largest group of plant phenolics accounting for over half of eight thousand naturally occurring phenolic compounds are flavonoids [6]. Other than that, tannins are also widespread in the plant kingdom and some of the most commercially available tannins are chestnut (hydrolysable tannins) and quebracho (condensed tannins) [7]. Other phytochemicals such as alkaloids also have toxicity characteristics and pharmacological activity such as for the treatment of cancer, as analgesic and anti-inflammatory agents [8,9].

Saponins, phenolics, flavonoids, tannins and alkaloids are the 5 major families of chemical constituents that are always described in phytochemical studies. These phytochemicals usually exist in low or high amounts in plants and determine the use of that plant in the medicinal activity. It is important to ensure and prove the existence of certain phytochemicals in *F. deltoidea* so the use of this plant in traditional medicine can be explained scientifically. This study is also important to identify the phytochemical constituents of this plant and callus tissue and could reveal their potential in the pharmaceutical industry. It can also be used to explain more clearly the effects of phytochemicals on callus tissue's growth and differentiation of *in vitro* propagation in this species.

## Materials and methods

### Collection of plant and callus samples

The leaves of *F. deltoidea* Jack var. *kunstleri* (King) Corner were taken from the Nursery of the Faculty of Bioresources and Food Science, Universiti Sultan Zainal Abidin (UniSZA). The phytochemical constituents were studied on stage one to stage 3 of leaves. Meanwhile the callus for this study were taken from the 60 days age of leaves callus derived from the same plant and cultured on Murashige and Skoog (1962) media [10] supplemented with 0.8 mg/L 2,4-Di-chlorophenoxy-acetic acid (2,4-D) + 0.7 mg/L Kinetin (KIN).

### Processing of plant and callus samples

The leaves were cleaned and washed with tap water and then rinsed with distilled water (dH<sub>2</sub>O). A hundred grams of leaves were dried in an oven at a temperature of 35 - 40 °C for 3 days. The dried leaves were weighed and pulverized using a clean electric blender to obtain a powdered form. The powder was stored in an airtight glass container, protected from sunlight until required for analysis. The callus sample followed the same procedure except being washed in tap water because they were derived from the tissue culture vessel, which is already clean and sterile.

### Preparation of methanolic and aqueous extract of plant samples

The dried samples were weighed before being soaked for 3 days in methanol (MeOH, 99.5 %, v/v) and put in the dark. The sample was vacuum-dried on a rotary evaporator (Buchi, Switzerland) at 40 °C until the solvent was evaporated and only a dark green/yellow viscous mass left which is termed the crude extract. The method was repeated 3 times and the crude extract was measured in percentage relative to dried sample. The aqueous extract of samples was prepared by soaking 10 g of powdered samples in 200 ml of distilled water for 12 h. The extracts were filtered using Whatman filter paper.

### Qualitative analysis of phytochemical constituents

Chemical tests were carried out on the aqueous extract and on the powdered samples using standard procedures to identify the constituents as described by Edeoga *et al.* [11].

#### Test for tannins

Boiled 0.5 g of powdered sample in 20 ml dH<sub>2</sub>O in a test tube and filtered the mixture. Add 0.1 % ferric chloride (FeCl<sub>3</sub>) to the filtered samples and observed for brownish green or a blue black coloration which shows the presence of tannins.

#### Test for phlobatannins

Boiled 10 ml of the aqueous extract with 1 % hydrochloric acid (HCl) in a test tube or conical flask. A deposition of a red precipitate will occur in the presence of phlobatannins.

#### Test for saponins

Boil 2 g of powdered sample with 20 ml of dH<sub>2</sub>O in a water bath and filter the sample. Ten ml of the filtered sample was mixed with 5 ml of dH<sub>2</sub>O in a test tube and shaken vigorously to obtain a stable

persistant froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of an emulsion which indicates the presence of saponins.

#### **Test for flavonoids**

A few drops of 1 % ammonia ( $\text{NH}_3$ ) solution was added to the aqueous extract of the sample in a test tube. A yellow coloration was observed in the presence of flavonoid compounds.

#### **Test for steroids**

Add 2 ml of acetic anhydride ( $(\text{CH}_3\text{CO}_2)_2\text{O}$ ) to 0.5 g methanolic extract of each sample with 2 ml sulfuric acid ( $\text{H}_2\text{SO}_4$ ). The color changed from violet to blue or green in some samples indicating the presence of steroids.

#### **Test for terpenoids**

Mix 5 ml of aqueous extract with 2 ml of chloroform ( $\text{CHCl}_3$ ) in a test tube. Add 3 ml of concentrated  $\text{H}_2\text{SO}_4$  to the mixture to form a layer. An interface with a reddish brown coloration was formed if terpenoids were present.

#### **Test for cardiac glycosides**

Prepare 1 ml of concentrated  $\text{H}_2\text{SO}_4$  in a test tube. Mix 5 ml of the aqueous extract from the sample with 2 ml of  $\text{CH}_3\text{CO}_2\text{H}$  containing one drop of  $\text{FeCl}_3$ . The above mixture was carefully added to the one ml of concentrated  $\text{H}_2\text{SO}_4$  so that the concentrated  $\text{H}_2\text{SO}_4$  was underneath the mixture. A brown ring will appear indicating the presence of cardiac glycosides.

#### **Test for alkaloids**

Stirr 0.5 g of sample with 5 ml of 1 % aqueous HCl on a steam bath and filtered. Treat 1 ml of the filtrate with a few drops of Mayer's reagent. Precipitation was taken as evidence for the presence of alkaloids in the extracts.

#### **Test for anthraquinones**

Five grams of powdered sample was shaken with 10 ml of benzene, filtered and 5 ml of 10 %  $\text{NH}_3$  solution was added to the filtrate. The mixture was shaken and the presence of a pink or violet color in the ammonia (lower) phase indicated the presence of free hydroxyl anthraquinones.

#### **Test for polyphenols**

Heat 2 ml of aqueous extracts for 30 min in a water bath. Add one ml of 1 %  $\text{FeCl}_3$  to the mixtures followed by the addition of one ml of 1 % of potassium ferrocyanide ( $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ ). The mixture was filtered and the formation of a green-blue color indicates the presence of polyphenols.

### **Quantitative analysis of phytochemical constituents**

#### **Determination of total phenolic content**

The quantity of phenols was determined according to Folin and Ciocalteu's procedure. In this procedure, gallic acid was used as a standard where concentrations of 0.01, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg/ml of gallic acid were prepared in MeOH. Concentrations of 1 mg/ml of leaves and callus extract were prepared in MeOH. Mix 0.125 ml with 0.625 ml of a 10-fold diluted Folin-Ciocalteu's reagent and 0.5 ml of 7.5 % sodium carbonate ( $\text{Na}_2\text{CO}_3$ ). The mixture was allowed to stand for 30 min at room temperature before the absorbance was read at 760 nm using a Tecan infinite M200 spectrometer. All determinations were performed in triplicate. The total phenolic content was expressed as percentage in mg gallic acid equivalent (GAE) per gram of plant material on a dried basis.

#### Determination of tannins

The quantity of tannins was determined using a spectrophotometric method. In this procedure, catechin was used as a standard where concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of catechin were prepared in dH<sub>2</sub>O. Weight 5 g of dried sample into a 50 ml conical flask. Add 50 ml of distilled water and stirred for 1 h. The sample was filtered into a 50 ml volumetric flask and made up to the mark. Take out 0.625 ml of the filtered sample into test tube and mixed with 0.125 ml of 0.1 M FeCl<sub>3</sub> in 0.1 M HCl and 0.008 M K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O. The absorbance was measured with a spectrophotometer at 395 nm wavelength within 10 min. The tannins content was expressed as percentage in mg catechin equivalent per gram of plant material on a dried basis.

#### Determination of alkaloids

Prepare 3.5 g sample in a beaker and add 200 ml of 10 % glacial acetic acid (CH<sub>3</sub>CO<sub>2</sub>H) in ethanol (C<sub>2</sub>H<sub>5</sub>OH). The mixture was covered and allowed to stand for 4 h. The mixture then filtered and the extract was allowed to become concentrated in a water bath until it reached one quarter of the original volume. Concentrated ammonium hydroxide (NH<sub>4</sub>OH) was added until precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH<sub>4</sub>OH and then filtered. The residue contained alkaloids which were then dried and weighed.

#### Determination of flavonoids

Total flavanoid content was determined using Aluminium Chloride (AlCl<sub>3</sub>) as described by Miliauskas *et al.* [12]. Rutin was used as standard in concentrations of 0.01, 0.02, 0.04, 0.06, 0.08, 0.10 mg/ml rutin in MeOH. Concentrations of 1 mg/ml of leaves and callus extract were prepared in MeOH. Add 0.5 ml sample with 0.5 ml of 2 % AlCl<sub>3</sub> in MeOH. The mixture was allowed to stand for 1 h at room temperature before the absorbance was read at 420 nm. All determinations were performed in triplicate. The total flavonoids content was expressed as percentage in mg rutin equivalent per gram of plant material on a dried basis.

#### Determination of saponins

The sample was ground and 10 g of sample was put into a conical flask. Then, 100 ml of 20 % C<sub>2</sub>H<sub>5</sub>OH was added to the sample. The sample was heated over a hot water bath for 4 h with continuous stirring at about 55 °C. The mixture was then filtered and the residue re-extracted with another 200 ml of 20 % C<sub>2</sub>H<sub>5</sub>OH. The combined extracts were reduced to 40 ml over a water bath at about 90 °C. The concentrate was then transferred into a 250 ml separating funnel and 20 ml of diethyl ether (CH<sub>3</sub>CH<sub>2</sub>)<sub>2</sub>O was added to the extract and shaken vigorously. The aqueous layer was recovered while the (CH<sub>3</sub>CH<sub>2</sub>)<sub>2</sub>O layer was discarded and the purification process was repeated. Add 60 ml of n-butanol (n-C<sub>4</sub>H<sub>9</sub>OH) and washed twice with 10 ml of 5 % Sodium chloride (NaCl). The remaining solution was then heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight.

#### Results and discussion

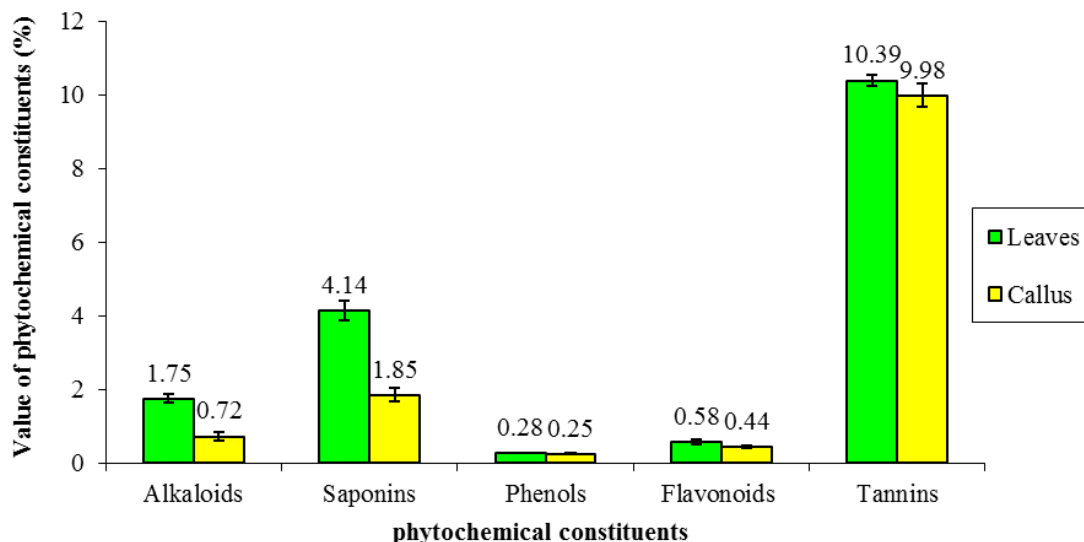
Qualitative analysis carried out on leaves and callus of *F. deltoidea* var. *kunstleri* showed the presence of phytochemical constituents with the results summarized in **Table 1**. It showed that all phytochemicals tested were present in both leaves and callus except for cardiac glycosides that are not present in the callus crude extract.

**Table 1** Qualitative analysis of phytochemicals constituent from methanolic extracts of *F. deltoidea* Jack var. *kunstleri* (King) corner leaves and callus.

Phytochemicals	Leaves	Callus
Tannins	+	+
Phlobatannins	+	+
Saponins	+	+
Flavonoids	+	+
Steroids	+	+
Terpenoids	+	+
Cardiac glycosides	+	–
Alkaloids	+	+
Anthraquinones	+	+
Polyphenols	+	+

(+) symbol indicate present of phytochemical, (–) symbol indicate absent of phytochemical

The quantitative analysis results of 5 major groups of phytochemical constituents in leaves and callus of *F. deltoidea* var. *kunstleri* is summarized and shown in **Figure 1**. Quantitative analysis was calculated as the dried weight percentage of leaves and callus extract. It was found that all the phytochemicals tested exist in both leaves and callus tissue. The value showed that the phytochemical constituent of callus tissue was slightly lower compared to leaves. The saponins content was very high in the leaves ( $4.14 \pm 0.26$  %) but very low in callus ( $1.85 \pm 0.18$  %). The tannins value was also high in both leaves and callus. Phenols showed the lowest value for both leaf and callus compared to the other phytochemical constituents.



**Figure 1** Quantitative analysis of phytochemical constituents from methanolic extracts of *F. deltoidea* Jack var. *kunstleri* (King) Corner leaves and callus dried weight (%). Bars indicate standard deviation of triplicate data.

Phytochemical screening and quantitative estimation for the chemical constituents of *F. deltoidea* var. *kunstleri* leaves and callus crude extract showed positive results with high values of tannins, alkaloids and saponins. Flavonoids and phenols are also present but in lower values compared to other chemicals. These chemicals are known to show medicinal value as well as exhibiting physiological activity. According to the quantitative analysis of phytochemical constituents, this plant and callus tissue are found to have high value of tannins, saponins and alkaloids. The presence of these chemicals is appropriate to support the public's opinion on the use of this plant in traditional medicine, to prevent and cure several other diseases like watery lungs, diabetes, kidney problems, high blood pressure, diarrhea and cancer [14].

All of the tested phytochemicals have high value in the medical field. There are a diversity of uses in traditional medicine related to the presence of various types of phytochemicals in this plant. These phytochemicals have been proven to act as antioxidants by inhibition of peroxidation by free radical scavenging activities that neutralize highly unstable and extremely reactive molecules called free radicals which attack human cells everyday and contribute to a variety of health problems including cancer, heart disease and aging [15]. Moreover, some of the phytochemicals also showed cytotoxic properties, cardioprotective, anti-inflammatory, antitumor, antiviral and antimicrobial characteristics [3-5].

It was shown that callus tissue has the same phytochemicals as the intact plant. Therefore, other than the leaves, callus tissue could be an alternative source of phytochemicals. Even though the quantity of phytochemicals in callus tissue was lower compared to the leaves, its production is very economic in terms of time and space. Callus tissue can be produced in large quantities starting only from leaf cuttings that place on the nutritional media (**Figure 2a**). The cells will begin to form after 2 weeks incubation and rapid growth can be obtained by doing subculture in 4 - 5 weeks and it can be done repeatedly (**Figure 2b**). Its production does not require a large space as they can be grown in petri dishes or test tubes and stacked on shelves in incubation room.

All phytochemicals that exist in leaves and callus of *F. deltoidea* var. *kunstleri* have their own pharmacological properties that are related to the use of this herb in traditional treatments. Phenolics, tannins, saponins, alkaloids and flavonoids have been linked or suggested to be involved in scavenging the free radical activity and for the treatment of many ailments. From clinical studies, terpenoids have been found to strengthen the skin, increase the concentration of antioxidants in wounds and restore inflamed tissue by increasing blood supply, thus playing an important role in wound and scar healing [16]. Other than that, cardiac glycosides were also used for the therapy of chronic congestive heart failure.

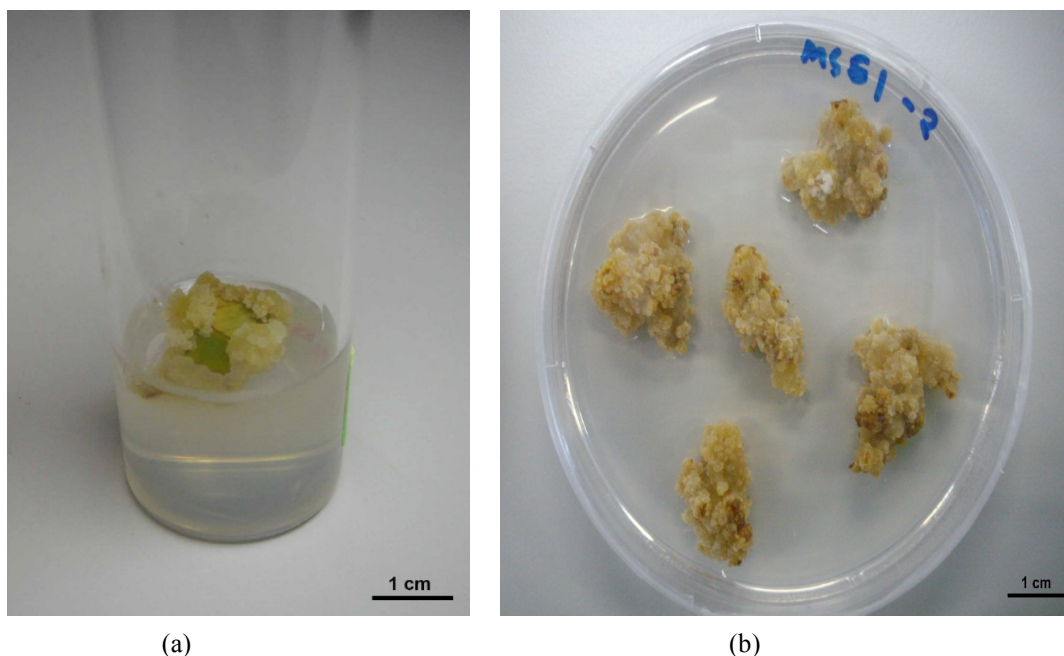
It was found that *F. deltoidea* var. *kunstleri* has similar amounts of phytochemical constituents compared to other medicinal plants like *Azadirachta indica*, *Moringa oleifera*, *Hibiscus rosa-sinensis*, *Eurycoma longifolia* and *Labiata pumila*. Some phytochemicals like phenolic compounds and flavonoids are higher in *F. deltoidea* compared to *E. longifolia* and *L. pumila* [17,18], and lower compared to *A. indica*, *C. asiatica* and *H. rosa-sinensis* [2]. However, the amount of alkaloids and saponins were quite high in *F. deltoidea* var. *kunstleri*. Many reports have said that large doses of saponins could cause serious effects such as dizziness, headache, nausea, itching, diarrhea and vomiting. Perhaps this is the reason why people claim that over consumption of leaves of this variety may cause headaches.

The radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) showed that *F. deltoidea* has high total percentage of antioxidant and total polyphenol and flavonoid content are well correlated with total antioxidant activities [19]. The total polyphenol content for female flower of 10 accession lines of *F. deltoidea* were between 0.81 to 1.30 mg/g dry weight and total flavonoid content between 2.44 to 27.36 mg/g fresh weight [19]. Our finding for the total polyphenols and flavonoid content were slightly low but still in the range of the previous study by [19]. These results indicate that low amounts of polyphenols and flavonoids content caused low antioxidant activities in our line. Moreover, the amount of polyphenols and flavonoids content in the callus were lower than in the leaves. It could cause the antioxidant activities to be low and could be related to the callus recalcitrant of this species that cannot be differentiated after many manipulations.

Plant cell cultures have great potential for the production of secondary metabolites. Plant cells grown in culture have potential to produce and accumulate chemicals similar to the parent plant from which they were derived. However, in most cases production has remained far lower than the intact plant

and this is also happened in our study. Our study has shown that callus tissue does have similar chemicals to the parent plant but in lower amounts. Even though cell culture produces low amounts of secondary metabolites, there are many approaches to enhance the production of the active principles. Growth and production of secondary metabolites are optimized by manipulating the physicochemical factors (media and culture environment) followed by selection of highly productive cells [20]. Afterwards, the use of the production medium and specific techniques such as elicitation, hairy roots and immobilization are applied.

*In vitro* cultured material can be elicited by bacterial or fungal lysates or stress response mediators such as salicylate or jasmonic acid/methyl jasmonate and hydrogen peroxide as well as by environmental factors like metals or irradiation [21]. Elicitors are mediator compounds of microbial stress or other stress agents like UV light, alkalinity, osmotic pressure or heavy metal ions. Addition of jasmonic acid successfully showed the elicitation response in the enhancement of the  $\alpha$ -tocopherol in *Arabidopsis* and sunflower cell cultures [22]. Other than that, thousands of species have been transformed with *Agrobacterium rhizogenes* with the aim to get transformed roots induction thus increasing the production of secondary metabolites level in the transformed hairy roots culture [21]. As an example, production of rosmarinic acid in the transformed roots of *Salvia officinalis* was much higher than untransformed organs [23].



**Figure 2** Callus tissue of *F. deltoidea* Jack var. *kunstleri* (King) Corner on Murashige and Skoog (1962) medium supplemented with 0.8 mg/L 2,4-D + 0.7 mg/L KIN at  $25 \pm 2$  °C under dark condition. (a) Callus formation around leaf explant after 4 weeks incubation and (b) Callus tissue after 8 weeks incubation.

## Conclusions

The qualitative analysis showed that all phytochemical tested (tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, alkaloids, anthraquinones and polyphenols) except cardiac glycosides were present in both leaves and callus extract. The quantitative analysis of 5 major groups of phytochemical constituents in leaves and callus also showed positive results. All phytochemicals tested (phenols, flavonoids, tannins, alkaloids, saponins) in the quantitative analysis showed that the callus tissue have the same phytochemicals as the intact plant but in lower amounts. Therefore, other than the leaves itself, callus tissues could be an alternative source of secondary metabolites. However, further study is needed to increase production and optimize the process. Since the study on secondary metabolites of *F. deltoidea* varieties and callus tissues are still limited, it is hoped that this study will be the starting point for further study and research on the production of secondary metabolites by *in vitro* culture. This is because production of *in vitro* secondary metabolites which is rapid and economical is very encouraging and beneficial for future pharmaceutical purposes.

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