Callus inducement of *Toona sinensis*: Potential agents against SARS-Corona virus replication

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Abstract. Bioprospecting of anti-SARS-corona virus phytochemical agents is an important issue today as an alternative to chemical drugs whose effectiveness has not been fully successful with no light effect. Quercetin, a component of *Toona sinensis* young leaves extract, was reported to have anti-viral activity against SARS-corona virus replication in vitro. Currently, the low efficiency of secondary metabolite production is an obstacle. Production of secondary metabolites from callus induction in vitro is considered more efficient and in a short time for commercial applications. The composition of hormones in callus media affects the secondary metabolites formed. The study was conducted to determine the effect of synthetic hormones auxin (BAP) and cytokinin (NAA) on callus percentage, fresh and dry cell weight and the average time of callus formation. The call percentage (100%) was obtained in different combinations of BAP and NAA in MS medium. The highest fresh weight of callus (165.50 gL⁻¹) and dry cell weight (28.47 gL⁻¹) were observed in MS medium (1.5 mgL⁻¹ BAP and 1 mgL⁻¹ NAA) within 7 days initial formation time of callus. The results showed that all the indices measured were positively correlated with callus induction in *T. sinensis*.

1. Introduction
The people of East and Southeast Asia have recognised *Toona sinensis* (Juss.) M.Roem [1]. Indonesian neighbourhood call is red suren, the usage of it as a woody plant with good quality (Figure 1). The Chinese have used *T. sinensis* in conventional medicine [2], the stems and leaves for digestive disorders, enteritis, pores and skin diseases [3], roots as a most cancers drug, bark for astringent and depurative and fruit for the remedy of eye infections [4]. This plant includes the primary phytochemicals terpenoids, phenylpropanoids, flavonoids and anthraquinones [2]. Modern studies additionally reviews that this species has diverse pharmacological sports towards cancer diseases, antioxidant, antidiabetic, anti-inflammatory, antibacterial and antiviral [5].

Recently, the purification of *T. sinensis* leaves for phytopharmaceutical functions has been carried out, such as methyl gallic compounds, gallic acid, kaempferol, quercetin, quercitrin, rutin, kaempferol-d-glucoside, (+)-catechin, (−)-epicatechin, beta-sitosterol, stigmasterol, beta-sitosterol-glucoside,
stigmasterol-glucoside, phytol and toosendanin [6]. Among the numerous secondary metabolites produced with the aid of using *T. sinensis*, quercetin has antiviral hobby in opposition to human immunodeficiency virus (HIV-Luc) or intense acute breathing syndrome (SARS), a breathing disorder because of the SARS-related coronavirus, with a selective index of 40 [7]. Additionally [5] pronounced that extracts from younger leaves of *T. sinensis* can inhibit the SARS-Corona virus in vitro. SARS is one of the life-threatening sicknesses global because of the SARS-corona virus (SARS-CoV) [8]. In 2003, [9] first pronounced the invention of glycyrrhizin to inhibit the replication of the SARS-Corona virus. This indicates that nature offers many capacity plant for brand spanking new medicinal substances as bioprospecting in opposition to the SARS-Corona virus, such as *T. sinensis*.

![Figure 1. Toona sinensis: seven years old trees (A), upper branches (B) and flowers (C) (Photograph: Putri, 2017 – personal documentation).](image)

Researchers accept as true with that plant tissue culture biotechnology has many advantages and feature used this technique as the principle method for generating phytochemicals. However, tissue culture nonetheless calls for extra in-intensity studies on exogenous elements as a super device as a way to produce compounds in enough portions for mass manufacturing. One of the essential elements to boom the manufacturing of phytochemical compounds in goal plant life is the kind and attention of hormones withinside the culture media. Auxins and cytokines as plant hormones play an essential position in cell growth, as a priming effect and as a bio-inhibitor in plant growth. Auxins and cytokinin as synthetic compounds in a form that are easily absorbed by plants *in vitro* will increase plant growth, protein and sugar content [11] and increase plant resistance to biological and physical stresses but have no effect on the regulation of nucleic acids, proteins, and synthetic enzymes [12].

This research become carried out to decide the impact of variations withinside the attention of exogenous hormone auxin 6-benzylaminopurine (BAP) and cytokinin 1-naphthaleneacetic acid (NAA) on callus percentage, wet and dry callus weight and callus formation in *T. sinensis* tissue culture. The composition of hormones that offer the very best callus formation is the principle awareness of the studies. The improvement and manufacturing of latest phytochemical antiviral marketers for the SARS-Corona virus is an essential issue, the manufacturing of *T. sinensis* callus cells via tissue culture can be carried out quickly, effectively and efficiently as an alternative to providing anti-coronavirus phytochemicals *in vitro*.

2. Materials and Methods

2.1. Plant material

The material source of *T. sinensis* was obtained from Blitar, East Java, Indonesia. Rejuvenated branches that were maintained in semi-sterile sand media until the rooting phase (Figure 2) were used for rejuvenation. Leaves from rejuvenated branches were used as explants for callus production. The leaves were washed with detergent in running water and then soaked in fungicide for 15 minutes, rinsed with distilled water. Sterilization was continued in laminar air flow by immersing in 70% (v/v) ethanol for 1 minute and 2.5% (w/v) sodium hypochlorite for 15 minutes, rinsed three times with sterile distilled water. Research activities are carried out in accordance with tissue culture laboratory standards.
2.2. Callus induction
The leaves cut in 1 cm² size and transferred on MS medium [13] containing 1-naphthaleneacetic acid (NAA) at 0, 0.5, 1, 1.5 and 2 mgL⁻¹ concentrations and cytokinin 6-benzylaminopurine (BAP) at 0, 1, 1.5 and 2 mgL⁻¹ concentrations. 1 mgL⁻¹ of 2,4-Dichlorophenoxyacetic acid (2,4-D) added for all treatments with 30% sucrose and solidified with 8 g/L plant agar. The cultures were maintained at growth chamber with 25 ± 2°C in the dark light and were sub cultured every 3 weeks. The percentage of callus induction, fresh and dry weight of callus [14] were recorded after 6 weeks incubation. Callus formation was calculated with following equation:

\[
\text{Callus formation} = \frac{\text{number of explants produced callus}}{\text{number of total explants}} \times 100
\]  

Fresh callus cell weight and dry callus cell weight were following method: Fresh and dry cell weights were measured at the end of the experiment by [15] method with a little modification. The fresh and dry weights were measured up to 45 days after inoculation in 6 days subcultures; this was considered to be one cycle. To determine the fresh callus weight, the cell mass was collected by Whatman No. 1 filter paper without vacuum, washed with a 3-mL distilled water, to remove the remaining agar media at callus, retained under laminar air flow for 30 second and weighed immediately. Dry callus weight was estimated by drying the collected fresh cell in the oven at 50°C for 72 h. Calluses formation were calculated with following equation:

\[
\text{Callus formation} = \frac{\text{number initial callus formation days}}{\text{number of sample}}
\]

The overall study was carried out for 12 months at tissue culture laboratory in BBPPBPTH Yogyakarta, Indonesia. The experimental units were set up in a completely randomized design (CRD). Data were subjected to analysis of variance (ANOVA), and the mean values were separated using Duncan’s multiple range test (DMRT) with level of significance at \( \alpha = 0.05 \). The statistical package SPSS (Version 24) was used for analysis.

3. Results and Discussion
The main obstacle in producing drug compounds is if it cannot produce the main compound from the target microorganism cell or chemically synthesized. Sources of renewable agents from plant cell cultures of T. sinensis can be an alternative superior system for the SARS-corona virus. In several studies that have been carried out, in vitro culture is a more efficient technique than all bioactive secondary metabolite production techniques. Plant cell callus by tissue culture offer an alternative for

**Figure 2.** Rejuvenation branches of *T. sinensis* as explant sources (A), leaves as explants (B) (Photograph: Putri, 2017 –personal documentation).
producing important metabolites [16-18]. Callus culture involves the growth of irregularly growing aggregates of cells from plant explants, by growing on a semi-solid medium containing the nutrients and hormones needed to promote cell growth. The initiation stage of T. sinensis callus cells from leaves under the influence of auxin and cytokinin hormones resulted in several forms of callus morphology (Figure 3).

The primary impediment in generating drug compounds is that if it can't produce the principle compound from the microorganism cell or chemically synthesized. Sources of renewable agents from plant cell cultures of T. sinensis is an opportunity advanced device for the SARS-corona virus. In numerous research which have been carried out, in vitro culture is a more efficient technique than all bioactive secondary metabolite manufacturing techniques. Plant cell callus by tissue culture method provide an opportunity for generating vital metabolites [16-18]. Callus culture involves the growth of irregularly developing aggregates of cells from plant explants, by growing on a semi-solid medium containing the nutrients and hormones needed to promote cell growth. The initiation stage of T. sinensis callus cells from leaves have an effect on of auxin and cytokinin hormones led to numerous varieties of callus morphology (Figure 3).

Figure 3. T. sinensis callus cells initiation after 6 weeks subculture: brown massive callus (A), whitish green massive callus (B), whitish yellow fragile callus (C) and brownish yellow fragile callus (D), and multiplication of fragile callus (D) (Photograph: Putri, 2018 –personal documentation).
Figure three indicates a few variations morphology within the colour and texture of huge and fragile callus, that is influenced through hormone treatment as proven in Table 1. Callus is a set of amorphous (undifferentiated) cells formed from cells that divide constantly in vitro. Histologically callus originates from a multiple divisions of parenchymal cells around the carrier bundle and several elements that make up the carrier bundle except xylem [19]. This callus multiplication is needed to generate somatic cell range in in-vitro culture that produces target metabolites. Callus induction of leaf explants of the *T. sinensis* turned into stricken by the interplay among BAP and NAA attention after 6 weeks incubation (Table 1).

Table 1. Effect of interaction between BAP and NAA concentration on *T. sinensis* callusing after 6 weeks incubation.

| No. | Treatments  | NAA (mgL⁻¹) | BAP (mgL⁻¹) | Callus formation (%) | Callus intensity | Color and texture of callus |
|-----|-------------|-------------|-------------|----------------------|-----------------|-----------------------------|
| 1.  | N0B0        | 0           | 0           | 100                  | +               | brown and massive           |
| 2.  | N0B1        | 0           | 1           | 100                  | +               | brown and massive           |
| 3.  | N0B2        | 0           | 1.5         | 100                  | +               | brown and massive           |
| 4.  | N0B3        | 0           | 2           | 100                  | ++              | brown and massive           |
| 5.  | N1B0        | 0.5         | 0           | 100                  | +               | brown and massive           |
| 6.  | N1B1        | 0.5         | 1           | 100                  | ++              | brown and massive           |
| 7.  | N1B2        | 0.5         | 1.5         | 100                  | ++              | brown and massive           |
| 8.  | N1B3        | 0.5         | 2           | 100                  | ++              | whitish green and massive  |
| 9.  | N2B0        | 1           | 0           | 100                  | +               | whitish green and massive  |
| 10. | N2B1        | 1           | 1           | 100                  | ++              | whitish yellow and fragile |
| 11. | N2B2        | 1           | 1.5         | 100                  | +++             | whitish yellow and fragile |
| 12. | N2B3        | 1           | 2           | 100                  | ++              | whitish yellow and fragile |
| 13. | N3B0        | 1.5         | 0           | 100                  | +               | whitish yellow and fragile |
| 14. | N3B1        | 1.5         | 1           | 100                  | ++              | whitish yellow and fragile |
| 15. | N3B2        | 1.5         | 1.5         | 100                  | ++              | whitish yellow and fragile |
| 16. | N3B3        | 1.5         | 2           | 100                  | ++              | whitish green and massive  |
| 17. | N4B0        | 2           | 0           | 100                  | +               | whitish green and massive  |
| 18. | N4B1        | 2           | 1           | 100                  | ++              | whitish green and massive  |
| 19. | N4B2        | 2           | 1.5         | 100                  | ++              | whitish green and massive  |
| 20. | N4B3        | 2           | 2           | 100                  | ++              | whitish green and massive  |

Note: + = low. ++ = intermediate, +++ = high, three explants were used for each hormone combination.

All combinations of auxin and cytokine treatment resulted in callus formation (100%). The irregular mass of plant cells will collectively form a callus. This process is related to tissue injury, namely the response to explant tissue stimulation to close the wound. Callus formation on *T. sinensis* leaves was only found on the abaxial surface, namely the lower cuticle layer. The response of explants to the treatment medium was initiated by swelling or elongation of the explants. The size of the explants became larger from the start, and callus began to form on the injured explants. Table 1 suggests that the low awareness of NAA has an impact at the brownish and massive texture. It is vital to do in addition studies at the impact the colour of callus and texture on the accumulation of *T. sinensis* phytochemicals produced. Callus commenced to seem on the rims of the explants and at the injured elements and persevered to develop till the stop of the observation on the 6 weeks after planting. The preliminary formation of callus is the begin of cells multiplication to provide new tissue, that is an vital reaction indicating the excessive callus cells regeneration potential of a plant [21]. The concentration of auxin and cytokine hormone added to MS basal media affected the alternate in colour and texture of *T. sinensis* callus. Effect of NAA and BAP on wet and dry weight of *T. sinensis* callus after 6 weeks incubation is proven in Table 2.
Table 2. Effect of NAA and BAP on weight of *T. sinensis* callus after 6 weeks incubation.

| No. | Treatments   | Fresh callus weight (mg) | Dry callus weight (mg) | Weight difference (mg) | Average time of initial callus formation (days) |
|-----|--------------|--------------------------|------------------------|------------------------|-----------------------------------------------|
| 1.  | N0B0         | 38.0 ± 2.3<sup>abc</sup> | 15.7 ± 2.9<sup>a</sup> | 22.3                   | 7.6                                           |
| 2.  | N0B1         | 29.0 ± 0.6<sup>ab</sup>  | 15.0 ± 2.8<sup>a</sup> | 14.0                   | 7.6                                           |
| 3.  | N0B2         | 40.0 ± 4.3<sup>abc</sup> | 18.7 ± 2.3<sup>a</sup> | 21.3                   | 8.0                                           |
| 4.  | N0B3         | 51.3 ± 3.8<sup>abc</sup> | 19.3 ± 4.0<sup>a</sup> | 32.0                   | 8.0                                           |
| 5.  | N1B0         | 29 ± 0.7<sup>ab</sup>    | 15.7 ± 2.3<sup>a</sup> | 13.3                   | 7.0                                           |
| 6.  | N1B1         | 61.0 ± 1.0<sup>ef</sup>  | 16.7 ± 4.4<sup>a</sup> | 44.3                   | 7.0                                           |
| 7.  | N1B2         | 105.0 ± 1.0<sup>gi</sup>| 22.7 ± 2.6<sup>ab</sup> | 82.3                   | 7.0                                           |
| 8.  | N1B3         | 94.3 ± 4.1<sup>bc</sup>  | 22.0 ± 2.9<sup>ab</sup> | 72.3                   | 7.0                                           |
| 9.  | N2B0         | 106.0 ± 3.2<sup>jak</sup>| 23.3 ± 1.2<sup>ab</sup> | 82.7                   | 8.0                                           |
| 10. | N2B1         | 121.3 ± 12.7<sup>k</sup>| 23.0 ± 1.0<sup>ab</sup> | 98.3                   | 8.0                                           |
| 11. | N2B2         | 165.5 ± 1.0<sup>k</sup>  | 22.8 ± 3.0<sup>ab</sup> | 142.7                  | 7.3                                           |
| 12. | N2B3         | 112.0 ± 14.1<sup>jk</sup>| 22.0 ± 1.1<sup>ab</sup> | 90.0                   | 8.0                                           |
| 13. | N3B0         | 44.0 ± 2.6<sup>bcd</sup>| 12.7 ± 1.2<sup>b</sup>  | 31.3                   | 8.0                                           |
| 14. | N3B1         | 71.7 ± 3.48<sup>ef</sup>| 12.7 ± 2.3<sup>b</sup>  | 59.0                   | 7.3                                           |
| 15. | N3B2         | 84.3 ± 2.7<sup>ab</sup>  | 12.0 ± 2.6<sup>b</sup>  | 72.3                   | 7.0                                           |
| 16. | N3B3         | 100.0 ± 5.8<sup>hij</sup>| 17.0 ± 3.6<sup>b</sup>  | 83.0                   | 7.0                                           |
| 17. | N4B0         | 25.0 ± 1.7<sup>a</sup>   | 11.3 ± 1.4<sup>b</sup>  | 13.7                   | 8.0                                           |
| 18. | N4B1         | 46.7 ± 6.3<sup>de</sup>  | 14.3 ± 3.3<sup>b</sup>  | 32.4                   | 7.6                                           |
| 19. | N4B2         | 60.0 ± 1.5<sup>ef</sup>  | 18.0 ± 2.0<sup>b</sup>  | 42.0                   | 7.3                                           |
| 20. | N4B3         | 37.0 ± 0.01<sup>abc</sup>| 14.3 ± 2.6<sup>b</sup>  | 22.7                   | 8.0                                           |

Each value is a mean of three replicates with standard error (Mean ± S.E). Mean with different letters (a,b,c,…) are significantly different from each other at 0.5 probability level by Duncan multiple range test.

Based at the fresh callus weight, dry callus weight and the distinction in weight among the two (Table 2), the satisfactory impact of auxin and cytokinin became on *T. sinensis* callus induction with 1.5 mgL<sup>-1</sup> BAP and 1 mgL<sup>-1</sup> NAA (N2B2). The ANOVA results confirmed a significant difference (P 0.05). The decrease or better the combination of the 2 hormones, the tendency to lower callus weight. The combination of auxin and cytokinin hormones in MS media can induced the callus in single or mixed hormone treatments. The efficiency of callus cells in forming secondary metabolites will increase with the proper hormone concentration. This is influenced by the increased multiplication and differentiation of cells as in a study conducted by [14]. Meanwhile, BAP and NAA did not longer have an effect on the suggest baseline (seven to eight days). Fresh and dry weights of callus cells, in addition to hormones impact on callus and the callus formation are proven in Figure 4.

Callus induction of *T. sinensis* is a potential agent that has high efficiency to deal with SARS-Corona virus replication because it has the advantages of a short growth cycle, relatively easy separation of secondary metabolites with more controlled environmental conditions. Specific drug products from secondary metabolites produced by nature are more efficiently carried out by callus culture techniques through [22].
Figure 4. The effect of hormone treatment on callus weight on fresh and dry weights of *T. sinensis* callus cells.

Figure 4 suggests a linear prediction of callus weight measurements for six weeks of subculture which shows the opportunity that callus weight will increase with the addition of auxin and cytokinin concentrations. The impact of this mixture of BAP and NAA hormones can offer extra entire information with a much wider attention range. Controlled condition in *vitro* by tissue culture technique will optimally produce callus growth, cell differentiation and accumulation of phytochemicals, as a consequence callus propagation may be performed efficiently and may be directed to induce, synthesize and accumulate secondary metabolites towards COVID 19 [19]. Callus tissue culture is the promising biosynthetic approach for generating plant secondary metabolites that has been implemented globally, however, research on callus culture and detection of *T. sinensis* secondary metabolites have been broadly reported. Callus induction of *T. sinensis* is a capability agent that has excessive performance to address SARS-Corona virus replication as it has the benefits of a brief growth cycle, smooth separation of secondary metabolites with extra managed environmental situations. Specific drug from secondary metabolites produced by nature are more efficiently carried out by callus culture techniques through [22].

4. Conclusion

Callus induction of *Toona sinensis* leaves were successfully carried out in this study, the best callus multiplication using 1 mgL⁻¹ synthetic auxins 1-naphthaleneacetic acid (NAA) and 1.5 mgL⁻¹ synthetic cytokinin 6-benzylaminopurine (BAP) has the highest callus weight.

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