Wahyuni et al, Afr., J. Infect. Dis. (2020) 14 (1): 1-7
https://doi.org/10.21010/ajid.v14i1.1

CALLUS INDUCTION OF *Sonchus arvensis* L. AND ITS ANTIPLASMODIAL ACTIVITY

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Article History

Received: 8th, May. 2019.
Revised Received: 3rd, Aug. 2019
Accepted: 8th, Aug. 2019
Published Online: 8th, Jan. 2020

Abstract

Background: Malaria is a global health problem that requires urgent need for new drugs. Tempuyung (*Sonchus arvensis* L.) possesses many potential medicinal compounds. As the plant is originally found wild, it is important to reproduce its secondary metabolites by tissue culture. The objectives of this study were to look for effective methods to induce callus from leaf explants of *Sonchus arvensis* L. and to test its *in vitro* antiplasmodial activity.
Materials and Methods: The leaves and petioles of the plant were cultured on Murashige and Skoog (MS) solid medium supplemented with indole acetic-3-acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl amino purine (BAP), in light and dark incubations. The best results obtained from callus induction were then treated by with several concentrations of sucrose (1- 5%). The best results from callus induction were then extracted with methanol for antiplasmodial test by Trager and Jensen's method. It was also tested against 3D7 strain of *Plasmodium falciparum.*
Results: The combination of 1mg/L 2,4-D and 0.5 mg/L BAP in dark incubation was the best treatment for callus induction of tempuyung. It produced the best quality of callus and the shortest period for callusing. Sucrose treatment had various effects on leaves callusing, but had no effect on petioles callusing, whereby 4% sucrose was the best treatment for leaves callusing in dark incubation. The methanol extract of the best callus had anti-plasmodial activity with IC_{50}=0.343 µg/mL.
Conclusion: Methanol extract of tempuyung callus shows potential as an antimalarial drug but more studies would be required.

Keyword: Antiplasmodial activity, Callus, Growth regulator, Methanol extract, *Plasmodium falciparum*, *Sonchus arvensis*, *tempuyung*.

List of Abbreviations: MS: Murashige and Skoog, IAA: Indole Acetic-3-Acid, IBA: Indole-3-Butyric Acid, NAA: Naphthalene Acetic Acid, BAP: Benzyl Amino Purine, 2,4-D: 2,4- Dichlorophenoxyacetic Acid, API: Annual Parent Incidence, WHO: World Health Organization, ACT: Artemisinin-Based Combination Therapy, MDG: Millennium Developmental Goal.

Introduction

Plasmodium is a single-cell protozoan parasite that causes malarial disease and transmitted to human through female *Anopheles mosquito* vector. Malaria is well known as a major disease in the world, especially in tropical areas, and endemic in Asia, Africa and Latin American countries. The spread of malaria is stable in populations with a high frequency of malaria inoculation (WHO, 2015). Malaria is a contagious disease that can cause death, anaemia and reduce work productivity (Permenkes, 2013). During the period 2005-2013, malarial incidence throughout Indonesia tended to decline. Eighty per cent (80%) of malarial cases occurred in East Indonesia region where the Annual Parent Incidence (API) value was still highest. Residents living in the Eastern region of Indonesia had a high potential of contracting (Kesehatan, 2016). Millennium Developmental Goal (MDG) aims to stop the spread of malaria and decrease its incidence through indicators that decrease the morbidity and mortality caused by the disease (WHO-World Malaria Report, 2015). Malaria eradication efforts are very important, including malaria prevention, diagnosis and treatment. The first malaria treatment approach used chloroquine. After many reports of drug resistance, malaria treatment was changed to artemisinin-based combination therapy (ACT). Malaria is a complex disease because it depends on 1) parasite aspect, *Plasmodium;* 2) vector aspect, *Anopheles*; 3) environmental aspect; 4) population behaviour; and 5) climate change that helps malarial parasites to be resistant to antimalarial drugs, and

Anopheles line to be resistant to insecticides (Ekasari, 2010). Resistance problems to first line antimalarial drugs caused various obstacles to solving malarial disease treatments. Recently, there are so many challenges and also an opportunity to overcome the spread of the disease. To reduce mortality due to malaria, it is imperative to develop new antimalarial drugs. This condition drove many researchers to find new potential drugs from both synthetic and natural sources.

Eighty per cent (80%) of the world's population still use natural products as antimalarial remedies, and 75% of malaria patients chose traditional medicines to treat the disease (Bagavan *et al*., 2011). Previously, malarial medicine was derived from *Cinchona succirubra* L., while new antimalarial drug generation, artemisinin, was obtained from *Artemisia annua* L. (Wright, 2005). Tempuyung (*Sonchus arvensis* L.) is an Indonesian traditional medicinal plant that contains various active compounds, such as flavonoids, saponins and polyphenols, which are widely used as antioxidants, hepato-protective, diuretic and potential antimalarial agents (Deylan, 2016; Sukandar and Safitri 2016). Tempuyung can be found easily, but the contents of its active compounds can vary, based on the plantation area. Furthermore, using plant material directly from nature can cause genetic loss (Thomas and Yoichiro, 2010). Tissue culture can be the best option to standardize the products, which may suggest that the amount of its secondary metabolites production is identical per mass unit. *In vitro* culture was one of the technologies used to improve the quality of plants, increase biomass and plant productivity (Sitorus et al., 2011; Ariati et al., 2012).

Plant tissue culture can produce secondary metabolites with a high economic value in a relatively short time, continuously with more consistent and controlled quality, and a higher level of content compared to direct harvest (Sitorus, 2011; Ariati et al., 2012). The factors that influence its success are basic medium (Hashemabadi and Kaviani, 2010), the combination of growth regulators (Shirin et al., 2007; Hoesen et al., 2008; Jahan et al., 2009), plant external conditions, and explant genotypes (Ibrahim et al., 2010; Reddy et al., 2011). Conservation is also the main consideration in using *in vitro* techniques, which is the way we can preserve natural genetic resources. For commercial purposes, secondary metabolites can be produced by using a bioreactor method (Radji, 2005). Based on this background, it is important to find an effective protocol for callus induction from *Sonchus arvensis* L. leaf, and examine its antimalarial activity by *in vitro* assay.

Materials and Methods

Plant material

Leaves and petioles were obtained from *Sonchus arvensis* L. located at the park of Faculty of Science and Technology, Airlangga University, and authenticated by Purwodadi Botanical Garden, Indonesian Science Institute. The 2nd and 3rd leaves of the plants were collected to be used as explants.

Explants surface sterilization

Fresh leaves and leaf stalks were washed carefully with running tap-water for 5 minutes. Then, emphasized using a fungicide (500 mg fungicide/500 ml distilled water), shaken continuously for 10 minutes and immediately rinsed twice using distilled water. Finally, the leaves were sterilized for 10 minutes in 10% Chlorox (NaOCl). Explants were washed with sterile distilled water three times.

Media preparation for growth regulator on callus induction and sucrose level on callus biomass

Medium MS (Murashige and Skoog, 1962) was used in the study. For callus induction, MS medium was enriched with 3% sucrose and 0.8 g agar. Furthermore, growth regulators of 1 mg/L Indolyl-3-acetic acid (IAA), Indolyl-3-butyric acid (IBA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy-acetic acid (2,4-D) alone and their combinations yellowish-green with 0.5 mg/L benzyl amino purine (BAP). Media acidity was adjusted to pH 5 – 6 by pH paper. Then autoclaved at 121° C and 1 atm pressure for 15 minutes.

The best growth regulator treatment was used for the next callusing and supplemented with various sucrose levels. They were 1%, 2%, 3%, 4%, and 5% of sucrose. All experimental treatments were repeated 5 times. Callus formation time data and callus-forming percentages were recorded every week until the 4th week of culture.

Inoculation of explants material

Inoculation was conducted in the Laminar Air Flow Cabinet. Sterile leaves and petioles were initially cut into many small pieces. Explant size was 1 cm^2. The surface-sterilized explants were then planted on the media with an abaxial position exposed to the media. The culture bottle was closed using aluminium foil. Next, the culture was incubated in a dark and light place. All callus cultures were harvested at 4 weeks old.

Extractions and In Vitro Antiplasmodial Assays

The best callus was extracted with methanol at room temperature. The methanol extracts were evaporated and used in the *in vitro* anti-plasmodial assays at concentration of 100, 10, 1, 0.1, 0.01µg/L. The 3D7 strain of *Plasmodium falciparum* was used in the *in vitro* anti-plasmodial assays by Trager and Jensen's method (Trager and Jesen, 1967), that was adopted by Ekasari *et al*. (2009).

Results

Callus Induction

The combinations of plant growth regulators affected tempuyung culture. Various morphologies of the cultures were shown in different combinations. Almost all combinations induced callus and the callus grew into plantlet at the end of the culture. Combination of 2,4D 1 mg/L and BAP 0,5 mg/L was the best combination for tempuyung callusing, and it resulted in the shortest callusing time of 2 weeks in dark incubation (Table 1, Figure 1). At the first growth, callus was coloured yellow with friable texture, but at the end of growth, callus was coloured brown.

Table 1: Time and percentage degree of callusing at 4th week of culture

Growth regulator hormone treatment	Time of callusing (week)		Percentage of callusing (%)	Degree of callusing	Morphology of callus
	Dark incubation	Light incubation			
MS_0	0	0	0	-	No callus
MS_1	2	3	100	++	Compact callus, green, the end of the week callus became plantlet
MS_2	2	2	100	++	Compact callus, green
MS_3	2	3	100	++	Compact callus, green, at the end of the week callus became plantlet
MS_4	2	3	100	++	Compact callus, green, at the end of the week callus become plantlet
MS_5	2	2	100	++	Compact callus, green, at the end of the week callus became plantlet
MS_6	2	3	100	+	Compact callus, green
MS_7	2	3	100	++	Compact callus, at the end of the week callus became plantlet
MS_8	2	3	100	+	Compact callus, green
MS_9	2	3	100	++++	Friabel callus, yellow

Note: MS_1= BAP 0,5 mg/L; MS_2 = IAA 1 mg/L; MS_3=IAA 1mg/L +BAP 0,5 mg/L; MS_4 = IBA 1 mg/L; MS_5 = IBA 1 mg/L + BAP 0,5 mg/L; MS_6 = NAA 1 mg/L; MS_7 =NAA 1 mg/L + BAP 0,5 mg/L; MS_8 = 2,4D 1 mg/L; MS_9 = 2,4D 1 mg/L + BAP 0,5 mg/L; MS_0 = without growth regulator hormon

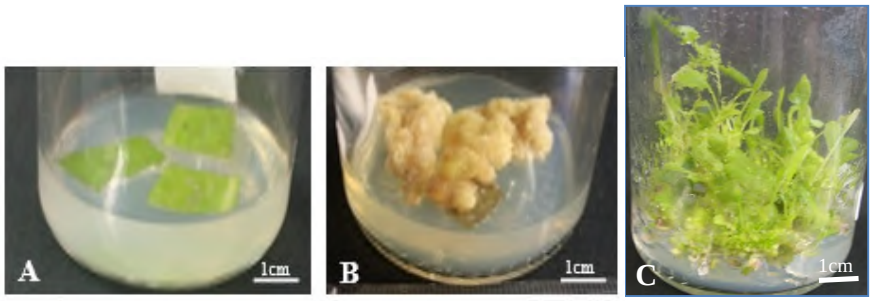

Figure 1: Callus growth of *Sonchus arvensis* L. on MS medium supplemented with2,4D 1 mg/L + BAP 0,5 mg/L treatment. (A) the first week of culture; (B) the 4th week of culture; (C) the 4th week of culture on MS medium supplemented with BAP 0,5 mg/L

Effect of sucrose level on callus biomass

Sucrose treatment affected callus biomass from leaves explant significantly but no significant difference was obtained on petiole explant according to *Duncan* Test ($\alpha = 0.05$). The fresh and dry weights of leaves callus varied according to the sucrose concentration added (Table 2 and Table 3). Table 2 shows that 4% sucrose produced the highest of callus dry weight (0.07±0.01g) in dark incubation, followed by 3% sucrose (0.06±0,02g), 5% sucrose (0.05±0,00g), 2% sucrose (0.04±0,01g) and 1% sucrose (0.03±0,01g). The average dry weight of leaves in light incubation was 0.15±0.09g, 0.05±0,02g, 0.03±0,00g, 0.02±0,00g, and 0.02±0,00g for 5%, 4%, 2%, 3%, and 1% sucrose, respectively.

In vitro **antiplasmodial activity of *Sonchus arvensis* L. callus against 3D7 strain of *Plasmodium falciparum***

Results of the *in vitro* anti-plasmodial assays of *Sonchus arvensis* L. callus methanol extract is presented in Table 4. Data of the *in vitro* anti-plasmodial assays were analyzed using Probit analysis to obtain the IC_{50} value, that is IC_{50}= 0.343 µg/ml.

Discussion

Callus induction is indicated by using the parameters of callus formation time, and the percentage of explant forming callus. The parameter of callus formation time was intended to find out whether the combination growth regulator is faster in callus induction and proliferation. Based on this observation, the callus formation started on 2nd week in dark condition, and 2nd -3rd weeks in the light condition. Callus formation time in the dark is faster than in the light, because callus induction is triggered by auxin that is inactive by light (Taiz and Zieger, 2013).

Table 2: Fresh and dry weights of *Sonchus arvensis* L. by 2,4D 1 mg/L + BAP 0,5 mg/L and sucrose treatment in dark incubation

Treatment	Leaf		Petiole	
	Fresh Weight (g)	**Dry weight (g)**	**Fresh weight(g)**	**Dry weight(g)**
N1	0.47^{a}±0,05	0.03^{a}±0,01	0.11^{a}±0,01	0.01^{a}±0,00
N2	0.70^{bc}±0,04	0.04^{b}±0,01	0.33^{a}±0,23	0.02^{a}±0,01
N3	0.86^{c}±0,27	0.06^{b}±0,02	0.23^{a}±0,24	0.02^{a}±0,02
N4	1.07^{d}±0,16	0.07^{c}±0,01	0.16^{a}±0,02	0.02^{a}±0,00
N5	0.61^{ab}±0,03	0.05^{b}±0,00	0.13^{a}±0,03	0.02^{a}±0,01

Note: N_1 = 1% sucrose; N_2 = 2% sucrose; N_3 = 3%sucrose; N_4 = 4%sucrose; N_5 = 5% sucrose. Number followed by the same superscript letter show no significant differences according to *Duncan* Test ($\alpha = 0.05$)

Table 3: Fresh and dry weights of *Sonchus arvensis* L. by 2,4D 1 mg/L + BAP 0,5 mg/L and sucrose treatment in light incubation

Treatment	Leaf		Petiole	
	Fresh weight (g)	**Dry weight (g)**	**Fresh weight (g)**	**Dry weight (g)**
N1	0.31^{ab}±0,07	0.02^{a}±0,00	0.72^{c}±0,21	0.03^{a}±0,01
N2	0.54^{bc}±0,13	0.03^{a}±0,00	0.49^{bc}±0,33	0.02^{a}±0,02
N3	0.21^{a}±0,00	0.02^{a}±0,00	0.56^{bc}±0,29	0.03^{a}±0,01
N4	0.70^{c}±0,26	0.05^{a}±0,02	0.35^{ab}±0,08	0.03^{a}±0,01
N5	1.30^{d}±0,25	0.15^{b}±0,09	0.18^{a}±0,05	0.02^{a}±0,00

Note:N_1 = 1% sucrose; N_2 = 2% sucrose; N_3 = 3%sucrose; N_4 = 4%sucrose; N_5 = 5% sucrose. Number followed by the same superscript letter show no significant differences according to *Duncan* Test ($\alpha = 0.05$).

Table 4: Percentage growth and inhibition of *Sonchus arvensis* L. callus extract toward to 3D7 strain of *Plasmodium falciparum.*

Concentration (µg/mL)	R	% Parasetemia		Growth percentage	inhibition percentage	Average inhibition percentage
		0 hour	48 hours			
control (-)	1	1,22	9,08	7,86	-	-
	2	1,22	9,19	7,97	-	
100	1	1,22	1,45	0,23	97,47	96,59
	2	1,22	1,56	0,34	95,71	
10	1	1,22	2,34	1,12	85,86	83,33
	2	1,22	2,74	1,52	80,81	
1	1	1,22	3,99	2,77	65,03	66,48
	2	1,22	3,76	2,54	67,93	
0.1	1	1,22	6,50	5,28	33,33	33,90
	2	1,22	6,41	5,19	34,47	
0,01	1	1,22	8,12	6,90	12,88	13,13
	2	1,22	8,08	6.86	13,38	

Formation of callus is marked by the presence of cell masses, yellowish-green or light-green, on the wound. In addition, this clot will form a cell mass called "callus". Callus formation is the result of cell division in response to wounds controlled by endogenous and exogenous explants (George and Sherrington, 1992).

Combination of plant growth regulator 2,4 D and BAP was the best choice for callusing. Sen *et al.* (2014) reported that *Achyranthes aspera* L leaf explants supplemented by various concentrations of 2,4-D and BAP showed the best result in callus formation. The results of this study are also consistent with the previous work reported by Rashmi and Trivedi, 2014, which used 2,4-D and BAP at various combinations, ranging from 0.5 to 10 mg/L on *Nerium odorum* leaf explants. Arianto *et al.* (2013) reported that 2,4-D is a growth regulator substance which is commonly utilized in callus induction because its strong action stimulates differentiation of cell, suppresses organogenesis, and maintains growth of callus. This is in agreement with the statement that 2,4-D shows stronger and more optimal activities than other auxins for callus induction (Manuhara, 2014) .

Callus morphology is indicated by the colour and texture of the callus. At the beginning of the appearance, the callus is clear yellowish-green and later turns brown in the fourth week of treatment with 1 mg/L of 2,4-D and 0.5 mg/L of BAP, while in the other treatments, callus develops into buds. Changes in callus were budded in treatments other than 2,4-D, because 2,4-D is able to maintain callus growth until the end of culture. The colour change of callus indicates cell activity during cell division (Rasud, 2012). George and Sherintong (1992) also reported that the colour change of callus becomes brown because callus produces phenolic compounds which can be toxic to plants and stop the growth (Hayati et al., 2012). Further confirmation of discoloration of the callus from yellowish-white to brown shows the low cleavage activity of callus cells, so that their growth power drops (Widayanto, 2004).

Moreover, about the texture of callus, callus texture shows the quality of callus which is in accordance with the purpose of this study. Clear white callus shows cell activity that is actively dividing and is embryogenic. Furthermore, the callus will change colour to greenish and grow buds or turn brown and yellow because of its secondary metabolite contents (Manuhara, 2014). Callus texture can vary from compact texture to friable, depending on explant variant, basic medium, growth regulator, and the biotic and abiotic environments of the culture (Sitorus *et al*., 2011)

Effect of sucrose levels on callus growth varied according to the concentration added (Table 2 and Table 3). The high dry weight of callus was 0.6 g, which came from addition of 4% sucrose given to leaf explant in dark incubation. The lowest dry weight was found to be 0.01g from addition of 1% and 5% sucrose given to petiole explant. Both were incubated without light. In earlier studies, 4% sucrose was found to be the best concentration for callusing of *Tagetes spp.* (Thaneshwari and Aswath, 2018), and on 3% sucrose treatment, fresh weight of *Ficus religiosa* L. callus growth started to increase (Siwach *et al*., 2011). Conditions of lack of light increase biomass. Light affects the performance of plant hormones. Auxin hormones in the form of auxin growth regulators can work optimally to affect cell division without being blocked by light (George and Sherintong, 1992).

The resulting IC_{50} value of methanol extracts of callus of *Sonchus arvensis* L. was found at 0.343 µg/ml for 1mg/L 2,4-D and 0.5 mg/L BAP incubated without light. The extract which has IC_{50} value at the range of 1-10 (µg/ml) was categorized as a material with good anti-plasmodial activity (Weenen, 1990). In China, a plant extract with IC_{50} value at 0.008-15.38 (µg/ml) is applied as anti-plasmodial material (Aryanti *et al.*, 2006). Kayano *et al*. (2011) stated that an extract is said to be active as anti-malarial agent if it possesses IC_{50}<5 µg/ml, moderate if the IC_{50} value is 10>IC>5 µg/ml, and not active if the IC_{50} is IC>10 µg/ml.

The minimum inhibitory concentration (IC_{50}) value of methanol extract of *Sonchus arvensis* L. callus was found to be lower than the IC_{50} value of *Tamarindus indica* fruit methanol extract (IC_{50}=4.786µg/ml); aerial part of *Pavetta corymbosa* methanol extract (IC_{50}=2.024 µg/ml) (Koudouvo *et al*.,2011); *Chaetomorpha antennina* (IC_{50}=26.37µg/ml) (Ravikumar *et al*., 2011); *Phyllanthus at* leaf methanol extract (IC_{50}=4.76µg/ml) (Bagavan *et al*., 2011) against *Plasmodium falciparum*; purified compound of *Cassia fistula* against the chloroquine-sensitive strain of *Plasmodium falciparum* (phytol (IC_{50}=18.9 ± 0.60 µM), lutein (IC_{50}=12.5 ± 0.35 µM), and di-The methanol-glycerol (DLGG) (IC_{50}=5.8 ± 0.27 µM) (Gracea *et al*., 2013); and methanolic leaf extract of *Christia vespertilionid* (IC_{50}=32.0 µg/mL) against *Plasmodium falciparum* NF-54 (Upadhyaya *et al*., 2013).

The best combination of growth regulators will be used to develop methods for the production of secondary metabolites from *Sonchus arvensis* L. callus by tissue culture as an anti-malarial agent. Each type of plant will respond differently to the hormones given. The compounds produced are also different. As reported by Zhao *et al.* (2012), callus induction in *Sonchus oleraceus* L. used 2 mg/L of NAA. The utilization of callus *Sonchus arvensis* L. as an antimalarial ingredient still needs to be supported by further research. Screening of metabolites contained in the culture and strengthening the method of production of metabolites in tissue culture in order to replace the original plants that have not been cultivated also require further studies.

Conclusion

From the above discussion, it could be concluded that combination of 1mg/L 2,4-D and 0.5 mg/L BAP produced the highest callus in the shortest time, and that *in vitro* anti-plasmodial activity of methanol extract of *Sonchus arvensis* L. callus has potential to be applied as an anti-plasmodial material.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the study presented in this paper.

Acknowledgments

Financial support for this study is from Kementrian Ristek dan Dikti Republik Indonesia, for which the authors are grateful. We also thank all our colleagues for their excellent assistance.

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