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CALLUS CULTURE AS THE METHOD IN PROVIDING ANTIMALARIAL COMPOUNDS OF PIPER GENUS

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Alhamdulillahi rabiil'alamin

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ABSTRACT

Malaria is still a serious cases all over the world especially in tropical country with a predict number 300-500 millions people infected per year. The resistence cases of these anti-malaria substance make a new effort to find the new strategy against malaria. This study aim to summarize the potential Piper genus as the source of potential antimalarial compound and recent research of callus induction in piper plant to obtain metabolites.The study of Piper genus represent the 11 Piper species were chemically studied and assayed against the plasmodium. The potential antimalarial compound isolates from several Piper genus were 20,60-Dihydroxy-40-methoxydihydro-chalcone,3-Farnesyl-p-hydroxybenzoic acid, Piperine, Chabamide, Benzoic acid derivatives, Guineensine, pellitorine, brachystamide B, sarmentine, and sermentosine, 5,8-Hydroxy-7-methoxyflavone, Prenylated hydroxybenzoic acid, 4-Nerolidylcatechol, Piperitone, Champor, and Viridiflorol (EO). An effort to propagate necessary antiplasmodial resources especially by callus induction has been conducted for 7 Piper species such as *Piper betle*, *P. colubrinumm*, *P. crocatum*, *P. longum*, *P. nigrum*, *P. permucronatum* and *P. solmsianum*to obtain higher content of secondary metabolites with a different plant growth hormone (PGR) supplementation while there are most of Piper genus have not been well studied. It conclude that callus culture could be the promising method to obtain antimalarial secondary metabolites as antimalarial.

Keyword:*Antimalarial, Callus, Malaria, Piper,Plant Growth Hormone (PGR), Secondary metabolites*

ABSTRAK

Malaria masih menjadi kasus yang serius di seluruh dunia terutama di negara tropis dengan prediksi jumlah 300-500 juta orang terinfeksi per tahun. Kasus resistensi zat anti-malaria membuat upaya baru untuk menemukan strategi baru melawan malaria. Penelitian ini bertujuan untuk meringkas genus Piper potensial sebagai sumber senyawa antimalaria dan penelitian terbaru tentang induksi kalus pada genusPiper untuk mendapatkan senyawa metabolit. Studi genus Piper mewakili 11 spesies Piper yang dipelajari secara kimia dan diuji terhadap Plasmodium. Isolat senyawa antimalaria potensial dari beberapa gen Piper adalah 20,60-Dihydroxy-40-methoxydihydro-chalcone, asam 3-Farnesyl-p-hydroxybenzoic, Piperine, Chabamide, turunan asam Benzoat, Guineensine, pellitorine, brachystamide B, serment, serment, serment, sermentine, 5,8-Hidroksi-7-metoksiflavon, asam hidroksibenzoat prenilasi, 4-Nerolidylcatechol, Piperitone, Champor, dan Viridiflorol (EO). Upaya untuk memperbanyak sumber daya antiplasmodial yang diperlukan terutama dengan induksi kalus telah dilakukan untuk 7 spesies Piper seperti *Piper betle, P. colubrinumm, P. crocatum, P. longum, P. nigrum, P. permucronatum* dan *P. solmsianum* untuk mendapatkan kandunganmetabolit sekunder yang lebih tinggi dengan suplementasi hormon pertumbuhan tanaman (ZPT) yang berbeda sementara sebagian besar genus Piper belum banyak diteliti. Oleh karena itu, disimpulkan bahwa kultur kalus bisa menjadi metode yang menjanjikan untuk mendapatkan metabolit sekunder sebagai antimalaria.

Keyword: *Antimalaria, Kalus, Malaria,Metabolit Sekunder,Piper, Zat Pengatur Tumbuh (ZPT)*

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I. INTRODUCTION

1.1 Background

Malaria is one of serious disease in the world which estimated cases in 2018 reached 219 million with 435 thousand deaths globally and predicted 300-500 millions of people will be infected per year (WHO, 2018). Indonesia is one of the country as the endemic of malaria in the world. Based on Riskesdas (2013) the highest prevalence rates are found in eastern Indonesia, namely in West Papua (10.6%), Papua (10.1%) and East Nusa Tenggara (4.4%).While, in West Sumatera, malaria cases showed a fluctuative number in every year, which in 2009 is estimated the highest cases reacehed 1.357 patients positive from 7.207 blood samples (Dinas Kesehatan Sumbar, 2011). This advanced number should be well managed and solve to avoid the increasing of the cases.

Malaria caused by the infecting of *Plasmodium falciparum* as the protozoan parasite distribute by the mosquitos,which transmitted by mosquito bites (TUSOM, 2020). Several chemical substance used to treat this diseasesuch as chloroquin, quinine and artemisin and its derivatives has shown the side effect and resistence cases (Cammarck, 2011). Some other compound also has been investigated as antimalaria such as cassiarine A isolated from the leaves of *Cassia siamea* and also piperidine alkaloids isolated from *Senna spectabilis* (Ekasari *et.al*, 2009; Pivatto *et.al*, 2014). According to that condition, an effort to find the effective compound against malaria need to develop (Cammarck, 2011).

Piper genus is one of potential candidate as the new source of antimalarial compound. The data shows that the biological activities of the secondary metabolites in Piper genus interestingly provide many of bioactive compound as medicinal

resources. As an example,analysis of phytochemical activity in *Piper aduncum* showed that the plant consist mainly of alkaloids and anhraquinones and an acid which is contain of anti-plasmodial and cytotoxic activity which effective against *P. falciparum* infected Red Blood Cell (RBC) (Vanegas, 2012). This plant effect against *P. falciparum* and the other protozoas also reported in Cuba by using the essential oil containing the substance which indicate that this plant could be a promising antimalarial agent (Monzote et al., 2017). Besides that, the crude methanol extract of *P. betle* leaves (50–400 mg/kg) was also investigated for its antimalarial activity against *Plasmodium berghei* (NK65). The phytochemical and antioxidant potentials of the crude extract were evaluated to elucidate the possibilities of its antimalarial effects (Adhroey *et.al*, 2011). Other recent research of crude ethanol extract of *P. guineense* investigated antiplasmodial and analgesic effects in mice. The antiplasmodial efficacy of the extract was assesed on its ability to reduce parasitemia and writhing, respectively, in mice (Kabiru, 2015).In a previous study, the fruit of *P. chaba* Hunt. was proved to exhibit promising antimalarial activity against the asexual stage of 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) strains of *P. falciparum*. This study investigated the antimalarial activity of piperine substance, the major isolated constituent of *P. chaba* Hunt. fruits against both *P. falciparum* clones (Thiengsusuk *et.al*, 2018). These studies revealed that Piper genus could be a promising resources to overcome malaria.

However, the utilization of the bioactive compound directly from the nature in a huge amount will threated the species existency. Plant tissue culture especially callus culture, can be a promising secondary metabolites production maintaining the species in the nature,also continuously with more consistent and controlled quality,

and a higher level of content compared to the wild plant (Ariati *et.al*, 2012;Sulichantini, 2015). Callus is non-diferrentiated meristematic cells which reveal only small vacuoles and lack chloroplast for photosynthesis, among other features (Efferth, 2019).Callus culture has the advantage that it can be engineered and directed to form complete organs and plants depending on the stimulus provided such as growth hormone and media composition (Rivai & Hendra, 2015). This techniques have been applied to produce various classes of chemical compounds from diverse plant species through empirical determination of ideal culture conditions and other methods (Benjamin *et.al*, 2019). Some evidence showed in callus inductionof*P. longum*using growth hormone combination recognized an increasing of alkaloid contain from wild plant extract (18,86 µg/ml) compared with callus extract of plant (34,38 µg/ml) (Siddique et al., 2019). Another research explained the phenylpropanoid content in leaves from *B. cordata* compared to that of *B. cordata* in vitro cultures found that the callus produced higherphenylpropanoid concentrations than the wild plant leaves (Zuninga et.al, 2009). TheHPLC analysis also revealed that the content ofcryptotanshinone in the callus cultured of *S. miltiorrhiza* on theMS basal medium supplemented with plant growth hormone was significantlyhigher than the marketed crude drug.Maximum yield of cryptotanshinone (4.59 mg/g) was observed in the calluscultured on MS basal medium supplementedwith 0.2 mg/L BA for sixty days (Wu et.al, 2003). The other evidence showed the substitution of KIN by 1 mg/L in culture medium combined with 0.5 mg/LNAA increased anthocyanin accumulation to 3.6 folds in *C.sinaica*. A striking increase in anthocyanin concentration reached 157.98 µg/g (11.79 folds) when culture medium contained 2mg/L BA and 1mg/L NAA (Maharik *et.al*, 2009).

Another recent antimalarial compound production also showed the utilization of callus culture technique to obtain the substance needed. As the example, the artemisinin and its derivatives, have been produced in callus as well as suspension culture, as it is naturally very low in the natural plant improved artemisinin production has also been extensively studied. Tahir et al (2016) showed that an ideal plant growth hormone combination for callus formation in*Artemisia annua* was 0.5µM/l of both BAP and NAA, similar to results obtained by Yuliani et al (2018) although similar results were obtained by other workers with higher concentrations of BAP and different auxins. Purnamaningsih (2011) also reported the callus induction method to obtain artemisine from *Artemisia annua* with combination of NAA and BAP. Another important antimalarial is quinine. One source of quinine, *Cinchona ledgeriana,* has been grown as callus culture and showed an increasing of the quinine alkaloids production (Pratiwi *et.al*, 2018). Sumaryono & Imron (2005) also reported the in vitro technology of *Cinchonaledgeriana* Moens to obtain quinine by callus culture using BAP combination. Another *Cinchona* species such as *C. pubescens* and *C. puccirubra* also reported the utilization of callus culture to obtain the necessary anti-malarial compound (Verpoorte et.al, 1985). The secondary metabolites of *Cassia siamaea* named cassiarine A known as antimalarial compound also has been cultured by callus induction to obtain the substances by using the hormone combination and precursor (Fauziaturrahmi, 2020).

Callus cultures also potential for the sustainable and large-scale production of secondary metabolites in pharmaceuticals. As an example, the use of in vitro techniques by induction of callus in the production of secondary metabolites on an industrial scale has been proven in tread plants (*Catharanthus roseus*) as anti-cancer,

anti-diabetic and anti-diarrhea (Naeem, et al., 2017). This cases illustrated that the callus culture can be applied for sustainably production of plant secondary metabolites especially antimalarial compound. Therefore, in this present study we want to explained the recent research of Piper genus related to the potential antimalarial compund findings and recent callus induction in several Piper plants as potential technique to obtain the secondary metabolites as antimalarial.

1.2 Problem Formulation

The problem formulation of this study are:

1. How is the recent research related to the potential Piper genus as the source of potential antimalarial compound?
2. How is the recent research of callus culture in piper plant to obtain metabolite?

1.3 Objectives

1. To explain the recent research related to the potential Piper genus as the source of potential antimalarial compound.
2. To explain the recent research of callus culture in piper plant to obtain metabolite.

II. LITERATURE REVIEW

2.1 Malaria Cases

Malaria is still a public health problem can cause death especially in high risk groups, i.e. babies, children under five, and pregnant women. The higher case of malaria in the world occur in part of Africa, USA, and Asia (WHO, 2018). In 2018, an estimated 228 million cases of malaria still occurred worldwide (95% confidence interval [CI]: 206–258 million), compared with 251 million cases in 2010 (95% CI: 231–278 million) and 231 million cases in 2017 (95% CI: 211–259 million). Indonesia is one of endemic country of this disease which the east region (Papua and Nusa Tenggara) is the most higher prevalance.In addition, malaria directly cause anemia and can reduce work productivity (Riskesdas, 2013). In West Sumatera, malaria cases still showed a high number. Clinical number of Malaria in West Sumatera fluctuated in every year, which in 2009 is estimated the highest cases reacehed 1.357 patients positive from 7.207 blood samples (Dinas Kesehatan Sumbar, 2011). This advanced number should be well manage and solve to avoid the increasing of the cases.

In humans, malaria is caused by intra-erythrocytic protozoa of the genus Plasmodium. These parasites are infected by the bite of an infective female Anopheles species mosquito (Mace, 2015). The human (asexual) stage of the life cycle begins with the exoerythrocytic phase. Based on Suh (2004) whenan infected mosquito bites a human, sporozoites in the mosquito's saliva will enter the bloodstream then to the liver, where they invade hepatocytes; over a period of up to 4 weeks, the infected hepatocytes mature intoschizonts. With schizont rupture, merozoites are released into the bloodstream. Inthe erythrocytic phase, merozoites infected erythrocytes and undergo an asexual cycle of reproduction or developinto nonmultiplying sexual forms. These gametocytes are crucial for perpetuating the life cycle, as theyare

ingested by a feeding mosquito and undergo sexual reproduction within the mosquito midgut; thousands of infective sporozoites are produced, which then migrate to the salivary glands, ready to initiate another life cycle.Although, only 4 of the over 100 species of plasmodia are infectious to humans, the majority of cases and almost all deaths are caused by *Plasmodium falciparum. Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* cause less severe disease (Suh, 2004). The children infected showed some symptoms like high fever which accompanied by chills, sweats, and headaches. The other common symptoms are abdominal pain, diarrhea, vomiting, weakness, myalgia, and pallor (Metanat, 2015).

By this problem, *Plasmodium falciparum* is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7% of estimated malaria cases in 2018, as well as in the WHO South-East Asia Region (50%), the WHO Eastern Mediterranean Region (71%) and the WHO Western Pacific Region (65%). Besides *P. falciparum*, another malaria parasite, *P. vivax* also 53% occured in South-East Asia Region, with the majority being in India (47%). *P. vivax* is the predominant parasite in the WHO Region of the Americas, representing 75% of malaria cases (WHO, 2019).

The first isolate compound against malaria was quinine and followed by mepacrine, chloriquine and halofantrine which in the latest time caused sime undesirable side effects, such as the potential for levels of cardiotoxicity andanother high toxicity risks. At the latest time, artemisinine and its derivatives (artemether, artesunate and arteether) which showed a significant level to inhibit *P. falciparum* infection reporting the resistance cases in western Cambodia in 2008. Moreover, in 2018, a report was published identifying more than 30 independent cases of artemisinin resistance in southeast Asia (Tse *et.al*., 2019). The current guidelines and drug for treatment ofuncomplicated malaria are using some substances, such as *P.*

falciparum treatment by using chloroquin, atovaquone–proguanil or quinine-sulfat, *P. vivax* treated by chloroquin, atovaquone–proguanil and primaquine, *P. ovale* treated by chloroquin and primaquine, also *P.malariae* treated by chloroquin (base). Uncomplicatedfalciparum malaria may be treated with oral therapy. The choice of agent is determined by the likelihood of infectionwith a drug-resistant strain (Suh et.al, 2004). But, the resistency of antimalarial drugs is proving to be a challenging problem in malaria control in most parts of the world. Intravenous substance suc as quinine and quinidine are the most widely used drugs in the initial treatment of severe *P. falciparum* malaria, whereas artemisinin derivatives are currently recommended for quinine-resistant cases. But, the side effects mainly involve the nervous, respiratory, renal, and/or hematopoietic systems. Metabolic acidosis and hypoglycemia are common systemic complications. Furthermore, the difficulty of creating efficient vaccines and adverse side effects of the existing anti-malarial drugs highlight the urgent need for novel and well tolerated antimalarial drugs for both prophylaxis and treatment of malaria, therefore many researchers had discovered other potential antimalarial agents, which mainly from plant sources (Ghosh, 2013).

2.2 Piper genus

Based on the Plant List (2013), the Piperaceae consists of 13 genera and estimated at around 2,658 names of types that are valid. Piper species are spreaded pantropically and take the form of herbs, shrubs, and lianas common in the understory of lowland wet forests. The study by Chaveerach*et.al* (2006) showed that each species often has three plant forms which include creeping, climbing and branching stems. Plants with creeping and climbing stems have a few different leaf forms or are all the same. Leaf morphology (e.g. color and shape) for all plant forms is very different. These qualities make it quite challenging to correctly identify Piper species without an inflorescence. However, Piper with an inflorescence can be easily identified by number and shape of

stamen and stigma, bract morphology, and leaf form characteristics, such as the number and arrangement of veins, decorative design and colors. The highest diversity of this species occurs in the tropical region of America (700 spp.), followed by Southern Asia (300 spp.), where the well-known economically important species *P. nigrum* L. (black pepper) and *P. betle* L. (betel leaf) originated. Patterns of distribution of Piper species vary from being locally endemic to widespread. There are several species restricted to a specific center of diversity (e.g., Andes, Central America) and others occur throughout the Neotropics or the Paleotropics (Jaramillo, 2001).

Some species of the genus Piper also found in Mexico and they are already used ethnomedically, such as *P. auritum, P. aduncum* L. H.B. & K.; *P. nudum* C. DC., *P. hispidum* Swartz,*P. sanctum* Schiltdl. ex Miq., *P. umbellatum* L., *P. psilorhachis* C. DC., *P. diandrum* C. DC., and *P. amalago* L. Phytochemical investigation on Piper genus has found alkaloids, pterocarpans, sterols, flavonoids, triterpenoid, saponines, phenylethylamines and amines (Pelayo, 2016). In each region such as Thailand, local people used Piper plants in different ways depending on religious belief, culture, ceremony, topography, vegetation and species diversity for each community. Found that there are eight species were most popularly used. These are *P. betle* L., *P. longum* L., *P. nigrum* L., *P. pendulispicum* C.DC., *P. chaba* Hunt., *P. sarmentosum* Roxb., *P. wallichii* (Miquel) Handel Mazetti and a new species *Piper maculaphyllum* A. Chaveerach & R. Sudmoon . All of these plants utilized for vegetables, spices, decorations, medicines and for traditional ceremonies (Chaveerach *et.al*, 2006). The large leafed perennial plant Piper is used for its spicy aromatic scent and flavor. It has an important presence in the cuisine of different cultures. Another quality of these plants is their known medicinal properties. It could used as emollient, anti-rheumatic, diuretic, stimulant, abortifacient, anti-inflammatory, antibacterial,

antifungal and antidermatophytic. A survey of the literature shows that the genus Piper is mainly known for its alkaloids compound with cytotoxic, chemopreventive, anti-metastatic and antitumor properties in several type of cancer.The result of a survey of structural diversity and bioactivity indicated that groups of species specialize in the production of amides, phenylpropanoids, lignans and neolignans, benzoic acids, chromenes, alkaloids, polyketides, and a plethora of compounds with mixed biosynthetic origin (Kato & Maysa, 2007). Modern pharmacology studies also demonstrated that its crude extracts and active compounds proofing a wide pharmacological activities, especially as antioxidant, anti-depressive, hepatoprotective, antimicrobial, anti-obesity, neuropharmacological, to treat cognitive disorders, anti-hyperlipidemic, anti-feedant, cardioactive, immunoenhancing, and anti-inflamatory (Gutiérrez *et.al*, 2013).

The study of some chemical substance effectivity in Piper plant for anti-malaria showed the significant result proved by the great number of IC_{50} of some potential compound such as 20,60-Dihydroxy-40-methoxydihydro-chalcone, 3-Farnesyl-p-hydroxybenzoic acid, Piperine, Chabamide, Benzoic acid derivatives, Guineensine, pellitorine, brachystamide B, sarmentine, and sermentosine, 5,8-Hydroxy-7-methoxyflavone, Prenylated hydroxybenzoic acid, 4-Nerolidylcatechol, Piperitone, Champor, and Viridiflorol(Portet *et.al*, 2007; Vega et.al, 2008; Thiengsusuk *et.al*, 2018; Rukachaisirikul *et al*., 2002; Flores *et al*., 2008; Rukachaisirikul *et al*., 2004; Vanegas et.al., 2018; Flores et.al, 2009; Pratoko, 2013; Lin et.al, 2007; Silva et.al, 2015; Monzote et.al, 2017). Moreover, some of the potential compound has a higher IC_{50} value than the latest antimalarial compound used until today, such as 4-Nerolidylcatechol with IC_{50} 0,67µg/ml compared with chloroquin 3,9 µg/ml to inhibit 3D7 strain of *P. falciparum* (Silva *et.al*, 2015; Thiengsusuk *et.al*, 2018).

2.3 Secondary Metabolites as Pharmaceutical Resources

Secondary metabolites is an intermediete metabolic or specific plant product restricted taxonomic groups which is not essential for growth and biosynthezised from one or more primary metabolites by a wider pathways. Usually secondary metabolites play an important role for plant protection and survival. Secondary metabolites can be classified into major routes, they are terpenoid and shikimate pathways (Verpoorte, 2000).

Secondary metabolites are organic molecules that are not necessary in the normal growth and development of an organism. Absence of secondary metabolites does not result in immediate death, but rather in long-term effect of the organism's survivability, often playing an important role in plant defense. These compounds are an extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae, and animals. Most of secondary metabolites, such as terpenes, phenolic compounds and alkaloids are classified based on their biosynthetic origin. Different group of these compounds are often associated to a narrow set of species within a phylogenetic group and constitute the bioactive compound in several medicinal, aromatic, colorant, and spice plants and common foods (Costa, 2012).

Piperaceae is the plant family which contain of many types of secondary metabolites. The analysis of biochemical compound showed that groups of species specialize in the production of amides, phenylpropanoids, lignans and neolignans, benzoic acids and chromenes and alkaloids which differ between the biosyntesis origin (Kato, 2007). As the example, *P. nigrum* L. which called as the king of species worldwide by virtue of its principle piperine. It is one of the important alkaloids of Pepper fruits (Family Piperaceae) and has been found to have numerous medicinal properties such as antioxidant, antiplatelet, anti-inflammatory, antihypertensive,

hepatoprotective, antithyroid, antitumor, antiasthmaticactivity and also have important role as fertility enhancer (Chopra, 2016). Another recent study about phytochemical investigations have shown that *S. spectabilis* produces around 40 constituents from different biosynthetic pathways, including piperidine alkaloids, pentacyclic terpenoids and anthraquinones, displaying antiproliferative, antitumoral and antifungal activities. Moreover, studies have also been conducted to identify endophytic and rizhospheric microorganisms associated to *S. spectabilis* and their chemical composition, which allowed the production of cadinane sesquiterpenoids, cytochalasins, depsipeptides and dibenzopirones (Selegato, 2017).

2.4 Callus Culture as Potential Method to Obtained Metabolites

Plant tissue cultures is one of the technique represent a potential source for producing secondary metabolites (Estrada, 2009).There are two ways of tissue culture utilization based on the purposed, they are for propagation and metabolite production (Smith, 2013). The example of propagation are; the bud induction of *Agave sisalana* Perrine which produce a huge number of identical and disease free plant, root induction of *Rhododendron radians* as commercial ornamental plant, also the root formation in exotic orchid *Grammatophylum scriptum* for conservation purposed and there are many other succeed research of tissue culture (Ridhawati *et.al*, 2017; Warseno *et.al*, 2018, Isda *et.al*, 2014). Several effort for metabolite production also has been conducted and showed a bright result especially by callus induction.Callus is a wound tissue produced in response to injury. The callus is a proliferation of cells from the wounded or cut region of an explant. Callus is generally made up of friable, large,vacuolated cells that are highly differentiated,but are unorganized. Callus can be hard and compact,and can contain regions of small meristematic cell clusters. It is generally the meristematic, undifferentiated cells that are competent toregenerate via somatic embryo or organ initiation (usually shoot or root development). Not all cells

in an explant contribute to the formation of callus and,more importantly,certain callus cell types are competent to regenerate organized stuctures. Other callus cell types do not appear to be competent to express totipotency (Smith, 2013).Micropropagation-based quality control in medicinal species also performs an encouraging role to guarantee the protection and efficiency of crude drugs before final pharmaceutical production. The World Health Organization (WHO) provides broad guidelines starting from precise authentication to the post-harvest processing of materials (World Health Organization, 2003). Analysis of fingerprint for quality control was announced and acknowledged by the WHO as an approach for the investigation of plant-based product and to standardize traditional knowledge of medicines. From this we conclude that in vitro cultures of valuable medicinal species offer dependable quality control and existence without any environmental changes and disturbance (Liu, 2008).

Several experiment has proven the effectivity of callus initiation method to obtain essential secondary metabolites. Such as, the bright red anthocyanin pigmentation was stimulated in a protocol employed callus cultures of *Crataegus sinaica*. Anthocyanin production was affected strongly by cytokinin type hormone. Callus establishment was achieved by culturing stem and leaf explants on (Murashige and Skoog medium 1962) MS medium supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) or naphthalene acetic acid (NAA) and 6- benzyladenine (BA) (Maharik *et.al*, 2009. Another research of *Phyllanthus acidus* Skeels were established to verify whether they produce Phyllanthusol A by callus induction. The effects of various combinations of auxin and cytokinin on the growth and accumulation of Phyllanthusol A were investigated. MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg/l and 6-furfurylaminopurine (kinetin) 1 mg/l was used to support the growth of callus cultures and the maximum amount of dry biomass (613 mg) was produced after 42 days of

culture (Duangporn & Premjet, 2009). Also, cell suspension cultures were established from callus cultured on MS liquid medium with the plant growth regulators. Dicentrine substance production from *S. venosa* cell suspension cultures was obtained in the range of 15–26 mg/g dry weight. And elicitation in this cell suspension cultures by chitosan and salicylic acid significantly increased dicentrine concentration (Kitisripanyaa *et.al*, 2013).

Another investigation about callus culture, was carried out to analyze secondary metabolite (anthraquinone) production in callus cultures of *G. umbellata*. In vitro production of anthraquinone through callus cultures of *G. umbellata* was standardized using in vitro leaves, derived from the nodal explant cultures maintained in Murashige and Skoog solid medium containing 2 mg/l 6-benzylaminopurine and 3% sucrose. From this study it revealed that the callus culture is an effective method for the production of anthraquinone by in vitro technique in *G. umbellata* (Anjusha, 2016). In-vitro culture method also applied to produce secondary metabolites in *P. betle* using culture medium and optimal supplementation of growth regulators, in this case 2,4-dichlorophenoxyacetic acid (2,4-D) were utilized. Phytochemical analysis of secondary metabolites profile from 1.5 mg/L 2,4-D treatment indicated high flavonoids content, while all 2,4- D concentration treatment found to contain terpenoids in this species (Junairiah, 2019).

For industrial scale, callus culture has been widely utilized especially to provide medicinal compound and pharmaceutical uses. Some of the compound including taxol for anticancer production from *Taxus baccata* and *Taxus caspidata*using jasmonic acid to elicite the metabolite production shown a significant result in compound acquired. Also production of camptothecin (CPT) for anticancer from *Camptotheca acuminata*, *Nathophodytes foetida*, *Ophiorrhiza mungos* and

Miquelia dentate. In antibiotic production, callus culture also play an important role, such as callus culture of *Thevetia peruviana*, *Ammi visnaga*, *Venonanthura patens*, and squalene production incallus cultures of *Nilgirianthus ciliatus*(Benjamin *et.al*, 2019).

There are several factor needed for in vitro culture technique, including plant growth regulator. Plant growth regulators (PGR) have an important role in controlling plant growth and development. According to Yuwono (2006) growth regulators are needed to induce cell division and morphogenesis. According to Lestari & Hutami (2005) growth regulators are one of the important factors in callus induction and determining the direction of regeneration of callus into plants.

Evans et al. (2003) stated that the most important growth regulators inculture *in vitro* were auxin, cytokonin and exogenous gibberellins contained in the media. There are two classes of plant growth regulators which are often used in tissue culture, namely cytokinins and auxins. Which includes cytokinin groups include BA (benzil adenine), kinetin (furfuril amino purines), 2-Ip (dimethyl allyl amino purines), and zeatin. Included in the auxin group include IAA (indole acetic acid), NAA (naphtalene acetic acid), IBA (indole butiric acid), 2.4-D (2,4-dichlorophenoxy acetic acid), dicamba (3,6-dicloro-o-anisic acid) acid), and picloram (4-amino-3,5,6-tricloropicolinic acid). The use of growth regulators in tissue culture depends on the desired direction of plant tissue growth. For the formation of shoots are generally used cytokines while for the formation of roots or callus formation is used auxin (Lestari, 2011).

III. METHODOLOGY

3.1 Time

This study has been conducted in three months, April - June 2020.

3.2 Work Procedure

All of articles and scientific writing were collected and formulated based on PRISMA (Preffered Reporting Items for Systematic Reviews and Meta-Analysis) Framework (Moher *et.al*, 2009), into a brief argument in this study. Information on Piper genus was gathered via internet using five scientific databases Google Scholar, Pubmed, SciFinder, Scopus and Web of Science. The main points of this study about malaria cases, Piper genus, secondary metabolites related to anti-malarial and the callus initiation's data of Piper genus in recent research.

3.3 Data Analysis

The data analyzed descriptively and tabulated into the Tables to summarize the whole data and each important highlights were discussed.

IV. RESULT AND DISCUSSION

4.1 Anti-plasmodial Properties in Crude Extract and Sub-fraction of Piper

Table 1. Anti-plasmodial Properties in Crude Extract of Piper

Piper species	Plant Material	Extract	Inhibition Conc	Ref
P. aduncum	Leaf	Crude Extract	IC_{50} = 26,5 µg/mL	Vanegas et al., 2012
P. aduncum	Stem	Crude Extract	IC_{50} = <50 µg/mL	Vanegas et al., 2012
P. auritum	Stem	Crude Extract	IC_{50} = <50 µg/mL	Vanegas et al., 2012
P. auritum	Leaf	Crude extract	IC_{50} = <50 µg/mL	Vanegas et al., 2012
P. marginatum	Stem	Crude extract	IC_{50} = 3,75 µg/mL	Vanegas et al., 2012
P. marginatum	Leaf	Crude extract	IC_{50} =<50 µg/mL	Vanegas et al., 2012
P. obrutum	Stem	Crude extract	IC_{50} = 36,2 µg/mL	Vanegas et al., 2012
P. obrutum	Leaf	Crude extract	IC_{50} = 32,1 µg/mL	Vanegas et al., 2012
P. jericoense	Stem	Crude extract	IC_{50} = 38,7 µg/mL	Vanegas et al., 2012
P. jericoense	Leaf	Crude extract	IC_{50} = 27,9 µg/mL	Vanegas et al., 2012
P. betle	Leaf	Crude extract	IC_{50} = 12,5 µg/mL	Adhroey et al, 2011
P. chaba	Leaf	Ethanol extract	IC_{50} = 2,7 µg/mL	Rukachaisrikul et al., 2002
P. chaba	Leaf	Ethanol extract	IC_{50} = 5,3 µg/mL	Thiengsusuk et al., 2013
P. chaba	Fruits	Ethanol extract	IC_{50} = 5,9 µg/mL	Thiengsusuk et al., 2018
P. heterophylum	Leaf	Dichloromethane extracts	IC_{50} = 7,0 µg/mL	Flores et.al, 2009
P. glabratum	Leaf	Crude extract	IC_{50} = 12,7 µg/mL	Flores et.al, 2008
P. acutifolium	Leaf	Crude extract	IC_{50} = 16,3 µg/mL	Flores et.al, 2008
P. hostmanium	Leaf	Crude extract	IC_{50} = 5,64 µg/mL	Portet et. al, 2007
P. tricuspe	Leaf	Ether extract	IC_{50} = 29,78 µg/mL	Vega et.al, 2008
P. sarmentosum	Leaf	Methanol extract	IC_{50} = 4,5 µg/mL	Tuntiwachwuttikul et.al, 2006
P. piedecustanum	Leaf	Methanol extract	IC_{50} = 17,93 µg/mL	Vanegas et.al, 2018
P. nigrum	Leaf	Methanol extract	IC_{50} = 12,5 µg/mL	Kamaraj et.al, 2012
P. peltatum	Root	Crude extract	IC_{50} = 2,11 µg/mL	Silva et.al, 2011
P. cubeba ^{Tr}	Fruits	Crude extract	IC_{50} = 45,5 µg/mL	Esperandim, 2013
P. cubeba ^{L}	Fruits	Crude extract	IC_{50} = 326,5 µg/mL	Esperandim, 2013
P. demeraranum ^{L}	Leaf	Crude extract	IC_{50} = 22-86 µg/mL	Carmo et.al, 2012
P. duckeii ^{L}	Leaf	Crude extract	IC_{50} = 15-46 µg/mL	Carmo et.al, 2012

Notes: Tr = Trypanosoma, L= Leismania

The genus Piper includes more than 1000 species making it one of the largest genera of basal angiosperms (Jaramillo, 2001). In West Sumatera, showed that 25 species of Piperaceae was existing in the regions that have been identified, include 21 species consisting, 19 species of the genus Piper and two species of the Peperomia. This investigation revealed about 10 species of Piperacaee are used as medicinal plants and 4 species listed have potential for ornamental plants, while the other eleven species are unknown (Munawaroh, 2011). The large sized leaf of parennial Piper is used for its spicy aromatic scent and flavor. Another quality of these plants is their known as medicinal properties. The modern pharmacology studies have showed that its crude extracts and active compounds possess wide pharmacological activities (Gutiérrez, 2013). In this context, the crude extracts and partitioned fractions of 27 Piper species were chemically studied and assayed against the plasmodium or protozoa (Table 1).

Based on the Table 1, showed that the study of antiplasmodial and cytotoxic activity of several species including *Piper aduncum* L, *Piper auritum* Kunth, *Piper jericoense* Trel. & Yunck, *Piper obrutum* Trel. & Yunck, *Piper marginatum* Jacq which collected in Antioquia, Colombia. The results of antiplasmodial activity (IC_{50}) ranged between 26.5 and 50 µg/mL and cytotoxicity (CC_{50}) between 8.67 and 100 µg /mL. From this case, the moderate anti-plasmodial activity and low cytotoxic activity for extracts of several genus of Piper species evaluated (Vanegas *et.al*., 2012).

While, the crude methanol extract of *Piper betle* leaves was investigated for its antimalarial activity against *Plasmodium berghei* during early and established infections. The phytochemical and antioxidant potentials of the crude extract were evaluated to elucidate the possibilities of its antimalarial effects which inhibitory concentration (IC_{50}) obtained was 12,5 µg/mL. The leaf extract also demonstrated significant ($P < 0.05$) schizonticidal activity in all three antimalarial evaluation models. Phytochemical screening showed that the leaf extract contains some

important antiplasmodial chemical constituents (Adhroey *et.al*, 2011). Another research of *Piper chaba* found that the ethanol extract of *P. chaba*, among others, exhibit significant and promising activity against *P. falciparum* (strains: K 1 and 3D7) with IC_{50} values of 5.3 and 4.1 µg/mL, respectively (Thiengsusuk et al., 2013). Previously, chabamide displayed activity against *P. falciparum* (strain: KI) with an IC_{50} value 2.7 µg/mL (Rukachaisrikul et al., 2002). Recently, findings by Thiengsusuk et al. (2018) demonstrated that piperine, the major isolated constituent of *P. chaba* fruits showed activity against 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) *P. falciparum* clones with IC_{50} values of 11,15 and 5,9 µg/mL, respectively.

Flores *et.al*. (2008) analyzed *P. glabratum* and *P. acutifolium* for their content of main secondary metabolites, affording nine new benzoic acid derivatives, in addition to four known compounds, were evaluated in vitro against some protozoa *Leishmania* spp., *Trypanosoma cruzi* and *P. falciparum*. Among the evaluated compounds, there were relative great antiplasmodial activity against F-32 Tanzania (chloroquine sensitive) strains of *P. falciparum*, with IC_{50} values of 12.7 and 16.3 µM, respectively. And also fractionation of dichloromethane extracts from the leaves of *P. heterophyllum* and *P. aduncum* showed three new prenylated hydroxybenzoic acids, along with six known compounds. Evaluation of the antiparasitic activity for all isolates showed that all the compounds were considered to be moderately active (IC_{50} high than 10 µg/mL), which exhibited a positive activity against *P. falciparum* with an IC_{50} of 7.0 µM (Flores *et.al*, 2009).

Another findings also showed anti-protozoal activity of some Piper genus which indicate the potency of Piper compund to inhibit the protozoal infection. The in vitro activity of the essential oil of *Piper cubeba* against trypomastigotes of *T. cruzi* increased upon rising concentrations, giving IC_{50}values of 45.5 and 87.9 µg/ml

against trypomastigotes and amastigotes, respectively. But, the essential oil was not active against *L. amazonensis*, since it displayed lyses of only 24% at 400 µg/ml, and an IC_{50} of 326.5 µg/ml (Esperandim, 2013). Also, found that *P. demeraranum* and *P. duckei* oils exhibited biological activity, with IC_{50} values between 15 to 76 µg/ml against two Leishmania species, where *P. duckei* oil being the most active compared than *P. demeraranum* (Carmo *et.al*, 2012).

4.2 Potential Anti-malarial Compound Isolated from Piper Plants

Table 2. Potential Anti-malarial of Isolated Metabolites from Piper Plants

Active Metabolite	Piper species	IC_{50}	Ref
20,60-Dihydroxy-40-methoxydihydro-chalcone	*P. hostmannianum*	12,7 µg/ml	Portet *et.al*, 2007
3-Farnesyl-p-hydroxybenzoic acid	*P. tricuspe*	29,78 µg/ml	Vega et.al, 2008
Piperine	*P. chaba*	59 µg/ml	Thiengsusuk *et.al*, 2018
Chabamide	*P. chaba*	2,7 µg/ml	Rukachaisirikul *et al*., 2002
Benzoic acid derivatives	*P. acutifolium*	13,8 µg/ml	Flores *et al*., 2008
Benzoic acid derivatives	*P. glabratum*	15,6 µg/ml	Flores *et al*., 2008
Guineensine, pellitorine, brachystamide B, sarmentine, and sermentosine	*P. sarmentosum*	6,5 – 18,9 µg/ml	Rukachaisirikul *et al*., 2004
5,8-Hydroxy-7-methoxyflavone	*P. piedecuestanum*	7,3 µg/ml	Vanegas et.al., 2018
Prenylated hydroxybenzoic acid	*P. heterophyllum*	7,0 µM	Flores et.al, 2009
Piperine	*P. longum*	34 µM	Pratoko, 2013
Piperine	*P. nigrum*	12,5 µg/ml	Lin et.al, 2007
4-Nerolidylcatechol	*P. peltatum*	0,67 µg/ml	Silva et.al, 2015
Piperitone, Champor, Viridiflorol (EO)	*P. aduncum*	1,3 µg/ml	Monzote et.al, 2017

Even some species of Piper showed the effective compound for antimalarial agents from the crude extract, the effort to find the specific bioactive compound needed to

proceed the medicinal product. Several research has been conducted to gain the promising substance against Plasmodium spp. There the 11 compund from 12 species of Piper found as potential agents for antimalaria.

From the Table 2 several species of Piper has been identified and bioactive compound obtained as antimalarial agent. The quantity of the compound effectivity measured by IC_{50} value which mean the lower the IC_{50} means the more potent the molecule (<50). The bioassay assesment for purification of an n-hexane extract from the leaves of *P. hostmannianum* var. berbicense resulting to the isolation of four monoterpene or prenyl-substituted dihydrochalcones as well as the identified compounds 2′,6′-dihydroxy-4′-methoxydihydrochalcone, linderatone, strobopinin, adunctin E and (−)-methyllinderatin. Their structures were established on the basis of NMR and X-ray analysis. (−)-Methyllinderatin, linderatone and 2′,6′-dihydroxy-4′-methoxydihydrochalcone exhibited the most potent antiplasmodial activity with IC_{50} values of 5.64, 10.33 and 12.69 µM, respectively against both chloroquine-sensitive and resistant strains of *Plasmodium falciparum* (F32, FcB1). The activity of (−)-methyllinderatin also confirmed in vivo against *Plasmodium vinckei* petteri in mice (80% of reduction of parasitemia) at a dose of 20 mg/kg/day (Portet et.al, 2007).

Dictyochromenol whis is an unusual compound, rarely found in plants, and two known compounds obtained from petroleum ether extract of the whole plant *P. tricuspe* showed antimalarial and antioxidant activity as well as cytotoxicity. The results show that the compounds are active against several strains of *P. falciparum* with IC_{50}´s ranging from 1,37 to 29,78 µM and cytotoxic effects with IC50´s ranging from 1,07 to 40,95 µM; the selectivity index based on IC_{50}´s suggests a high toxicity of the compounds (Vega et.al, 2008).

In a previous study, the fruit of *P. chaba* Hunt. was proved to exhibit promising antimalarial activity against the asexual stage of 3D7 (chloroquine-

sensitive) and K1 (chloroquine-resistant) strains of *P. falciparum*. This study aim to investigate the antimalarial activity of piperine, the major isolated constituent of *P. chaba* Hunt. fruits against both *P. falciparum* clones. The antimalarial activity was analyzed using SYBR green-I-based assay and some morphological change was observed under the light microscope with Giemsa staining. The moderate IC_{50} (concentrationthat inhibits parasite growth by 50%) values of piperine against 3D7 and K1 *P. falciparum* were 111.5 and 59 μM, respectively (Thiengsusuk *et.al*, 2018). A novel piperine dimer, named chabamide, was isolated from stems of *P. chaba* Hunter and its structure was explained on the basis of spectroscopic evidence. Chabamide showed antimalarial activity with an IC_{50} value of 2.7 μg/ml and antituberculosis activity with the minimum inhibitory concentration (MIC) of 12.5 g/ml (Rukachaisirikul et al., 2002).

P. glabratum and *P. acutifolium* were analyzed for their content of main secondary constituents, affording nine new benzoic acid derivatives, in addition to four known compounds. Among the evaluated compounds, methyl 3,4-dihydroxy-5-(3'-methyl-2'-butenyl) benzoate exhibited leishmanicidal effect (IC_{50} 13.8-18.5 μg /ml) against the three Leishmania strains used, and methyl 3,4-dihydroxy-5-(2-hydroxy-3-methylbutenyl)benzoate , methyl 4-hydroxy-3-(2-hydroxy-3-methyl-3-butenyl)benzoate , and methyl 3,4-dihydroxy-5-(3-methyl-2-butenyl) benzoate showed significant trypanocidal activity, with IC_{50} values of 16.4, 15.6, and 18.5 μg /ml, respectively (Flores *et.al*., 2008).

Eight amides, pellitorine, guineensine, brachystamide B, sarmentine , brachyamide B , 1-piperettyl pyrrolidine , 3',4',5'-trimethoxycinnamoyl pyrrolidine and sarmentosine, two lignans, (+)-asarinin and sesamin, and four other compounds, 1-(3,4-methylenedioxyphenyl)-1E-tetradecene , methyl piperate and a mixture of beta-sitosterol and stigmasterol, were isolated from the fruits of *P. sarmentosum*.

These compounds were evaluated in antituberculosis and antiplasmodial tests with IC_{50} between 6.5 - 18.9 µg/ml (Rukachaisirikul et al., 2004).

In addition, from *P. piedecuestanum* species were isolation and characterization five metabolites 5,8-Hydroxy-7-methoxyflavone, 6,7-dimethoxy-5,8-dihydroxyflavone, 6,7-dimethoxy-5-hydroxyflavone (mosloflavone) , 5,6-dihydroxy-7-methoxyflavone (negletein) , 5-hydroxy-7- methoxyflavone and a brominated derivative from named 6,8 bromo-5-hydroxy-7-methoxyflavone. While, 5,8-Hydroxy-7-methoxyflavone presented promising antiplasmodial activity with an IC_{50} = 7.325 µg mL (Vanegas et.al., 2018).

Fractionation of dichloromethane extracts from the leaves of *P. heterophyllum* found a new prenylated hydroxybenzoic acid. Evaluation of the antiparasitic activity for all isolates showed that the compounds were considered to be moderately active which exhibited a good activity against *P. falciparum* with an IC_{50} of 7.0 µM.Another investigation of piperine, a compound contained in *P. longum* (L.) has activity antimalarial with the mechanism of deregulation of the Ubiquitin Proteasome System. 2BJU, as the enzyme Plasmepsin II with IH4 ligand has antimalarial activity with IC_{50} 34 µM. IH4 is an inhibitor of Plasmepsin II which has a piperidine ring on the structure which also belongs to the piperin compound and several alkaloid compounds in *P. longum* (L.) (Pratoko, 2013).

P. nigrum which has been used by South Indian to treat fevers in general, malaria asthma, cold, intermittent fever, cholera, colic pain and diarrhoea proved the ethyl acetate seed extract showed promising in vitro antiplasmodial activity against *P. falciparum* 3D7 and INDO strains with IC_{50} values of 12.5 and 12.0 µg /ml, respectively with a low cytotoxicity (TC_{50} = 87.0 µg /ml) and a significant therapeutic index of 7.0. alkaloids piperine, guineensine, pipericide, N-feruloyltyramine and N-

isobutyl-2E, and 4E-dodecadienamide have been isolated from *P. nigrum* and piperine has been reported as a potential compound against Plasmodium (Lin *et al*., 2007). Also, 4-Nerolidylcatechol known as abundant antiplasmodial metabolite that is isolated from *P. peltatum* roots. O-Acylation or Oalkylation of compound 1 provides derivatives showed stability and significant in vitro antiplasmodial activity (Silva *et.al*., 2015). While, a total of 90 compounds were identified in the essential oil (EO) of *P. aduncum*, of which camphor (17.1%), viridiflorol (14.5%), and piperitone (23.7%) were the main components. The cluster analysis revealed at least nine different chemotypes. The EO did not show notable activity against bacteria or fungi, but was active against parasitic protozoa (*P. falciparum* rise IC_{50}= 1,3 µg/ml) (Monzote et.al, 2017).

These all recent studies about the potential chemical substances showed the great opportunity to develop the technique for secondary metabolites acquistition from Piper genus to be applied for pharmaceutical and medicinal as the future prospective. The effective technique to obtain the compound of interest in large-scale production should be well studied and develop.

4.3 Callus Culture of Piper to Obtained Metabolites

Callus culture is one of important method of plant tissue culture engineering to obtained high quantity of secondary metabolites. Callus culture also the bridge of many technique to obtain the specific chemical compound through cell culture or organ initiation in vitro (Bourgaud, 2001). Callus is the middle stage of many purpose in secondary metabolites acquisition in many plants including Piper. This method also affected by several factor involved such as plant growth regulator (PGR) and suitable medium. Table 3. showed the data of recent research of callus induction in Piper genus including (*P. betle, P. colubrinum, P. crocatum, P. longum, P. nigrum, P. permucronatum* and *P. solmsianum*).

Table 3. Callus Induction in Piper to obtained Metabolites

Piper species	PGR Conc.and Media	Explant	Metabolites	Callus morph.	Ref.
P. betle	1,5 mg/L 2,4-D (MS)	Leaf	Flavonoid, Terpenoid, octadecanoic acid	Friable texture and yellowish white color	Junairiah et.al, 2019
P. betle	1,0 and 1,5 mg/L kinetin (MS)	Leaf	(Not reported)	White, Greenish color	Junairiah et.al, 2017
P. colubrinum	2.2 µM BA and 0.46 µM kinetin (MS)	Leaf	(Not reported)	White	Yusuf et.al, 2001
Piper crocatum	0,5 mg/L 2,4-D (MS)	Leaf	Euganol	Compact, crumbs, white translucent yellowishgreen	Kartika, 2007
P. longum	1 mg/l BAP and 0.5 mg/l Kinetin (MS)	Nodus	(Not reported)	Greenish white	Padhan, 2015
P. longum	0.5 mg/l BAP, 1.0 mg/l kinetin, 0.5 - 2.0 mg/l 2,4-D(MS)	Leaf	Piperine	(Not reported)	Siddique et.al, 2019
P. longum	3.0-4.0 mg/ l 2,4-D (MS)	Leaf	(Not reported)	Hard and compact yellow colored	Wasti & Krishna, 2019
P. nigrum	0.5 or 1.5 mg/l BA and 1.0 mg/l NAA (MS)	Leaf	1, 1-diphenyl-2-picryl-hydrazy	Greenish, Yellowish	Ahmad *et.al*, 2010
P. nigrum	4.0 mg/l TDZ and BA 1.5 mg/l (MS)	Leaf	Piperine	(Not reported)	Ahmad *et.al*, 2014
P. nigrum	1.0 mg/l BA and 0.5 mg/l GA	Leaf	(Not reported)	Greenish, Yellowish	Ahmad *et.al*, 2015
P. nigrum	1 mg/l of NAA and 1.5 mg/l of Kinetin (MS/ ½ MS)	Leaf	(Not reported)	Whitish brown	Dissanayake et.al, 2015
P. permucronatum	4.52 µM 2,4-D and 4.44 µM BA (MS)	Leaf	(Not reported)	Friable and whitish	Santos et.al, 2015
P. solmsianum	0.2 mg.l-1 2,4-D and 2 mg.l-1 BA (MS)	Leaf	(Not reported)	Friable and whitish	Balbuena et.al , 2009

P. betle (Black betel) known as decoration plant which also have potential as source of medicine materials and alternative of safe antiseptic. In vitro culture method applied to produce secondary metabolites using culture medium and optimal supplementation of growth regulators, in this case 2,4-dichlorophenoxyacetic acid (2,4-D). Result showed that 2,4-D growth regulator affected callus grown from black betel leaf explants. Growth regulator 2,4-D given at 2.5 mg/l was able to induce callus early compared to other treatment, which induction time mean of 14 days. Explants supplemented with 2,4-D at concentration 1.5 mg/L produced the highest fresh and dry weight, at 0.8951 g and 0.0470 g respectively. Callus morphology with friable texture and yellowish white color was resulted from 1.5 mg/L 2,4-D treatment. In the same research, phytochemical analysis of secondary metabolites profile from 1.5 mg/L 2,4-D treatment indicated flavonoids content, while all 2,4-D concentration treatment found to contain terpenoids. The main component obtained was octadecanoic acid (Junairiah et.al, 2019).

Another experiment of *P. betle* showed that the combination of growth regulators IAA (auxin) with BAP (cytokins) and kinetin had effects on leaf growth of *P. betle* L. Var Nigra. During the observation, it indicated that the combination of concentration IAA 0.5 mg/L and BAP 2.0 mg/L showed the earliest callus formation at 8.5 days. Based on this current study, 1.0 mg/L IAA and 1.5 mg/L kinetin was the best combination of plant growth regulators for induction of callus from leaf of *P. betle* L. Var Nigra, as it resulted in callus with highest fresh weight of 0.2972 g and dry weight of 0.1660 g. Callus of *P. betle* L. Var Nigra had two textures, that were compact and friable, and also showed various kind of colors, like white, greenish white, yellowish white, tanned white, brown and black.

Callus induction forsomatic embryogenesis of multiple disease-resistant pepper, *P. colubrinum* L also investigated. Somatic embryos were initiated on

Murashige and Skoog's (1962) basal medium containing 2.2 µM benzyladenine and 0.46 µM kinetin and multiplied profusely through secondary embryogenesis on the same medium. This cultured through the formation of callus which is formed the white callus before becoming embryos (Yusuf, 2001). The reasearch of Kartika (2007) about *P.crocatum* showed the highest fresh weight of callus was found in the addition of 2,4-D 0,5 mg/L. Morphology of callus formed on all treatments are compact, crumbs, then turned into a white translucent yellowish green to clear. Also this study found that the content of eugenol on Red Betel callus tend to be higher than the content of eugenol on Red Betel leaf where the highest content of eugenol obtained from the addition of BA 2 mg/L.

Plantlet regeneration in *P. longum* L. also has been achieved from nodal segments excised from in vivo grown plantlets cultured on MS medium supplemented with growth regulators. This present investigation was carried out to regenerate plantlet of *P. longum* L through in vitro culture. The nodal explants were cultured on MS medium supplemented with different concentration and combination of cytokinines and auxines for primary shoot proliferation. The best shoot proliferation was observed in MS medium containing 1.0 mg/l Kinetin and 1.5 mg/l BAP where 98 % of explants showed proliferation with highest rate of shoot multiplication. Callus induction occurred in (1 mg/l) BAP and (0.5 mg/l) Kinetin and 10-15 days of callus subculture initiation of greenish white shoot buds was observed (Padhan, 2015).

In Nepal, the young leaves of *P. longum* were cultured in vitro on different concentraton of 2,4-D (0.5-5.0 mg l-1) for callus inducton. Among them, 3.0-4.0 mg/l 2,4-D gave hard and compact yellow colored callus. These 2,4-D mediated calli were transferred to thirty different media (BAP, NAA, Kinetn, coconut water) for organogenesis (Wasti & Krishna, 2019).

Piperine as chief bio-molecular active compound of *P. longum*, exhibited various pharmacological activities. In addition it improves the bioavailability of other nutritive substances. The study to obtained Piperine from *P. longum* by callus induction has been conducted, and compared the quantification of piperine in callus and wild grown *P. longum*. The callus were produced by transferring the sterile leaves on MS medium containing different concentration of cytokinins like 0.5 mg/l BAP, 1.0 mg/l KN (kinetin) with auxins like 0.5 - 2.0 mg/l 2,4-D (2 4-dichlorophenoxyacetic acid). The petroleum ether, ethanol and aqueous extracts of *P. longum* plant and callus were prepared. The content of piperine in plant extract and callus extract were performed by HPLC method. The phytochemical study uncovered the nearness of different secondary metabolites in various extract of plant. The HPLC chromatogram displayed that content of piperine present in callus extract was higher compared to field grown plants (Siddique *et.al*., 2019).

Another study of*P. nigrum* L. (black pepper) to obtain the organogenic potential and antioxidant potential (1, 1-diphenyl-2-picrylhydrazyl-scavenging activity) of the medicinal plant were investigated. Callus induction and shoot regeneration were induced from leaf explants of potted plants cultured on MS medium supplemented with different plant growth regulators. The best callogenic response was observed on explants cultured for 30 days on MS medium supplemented with either 0.5 or 1.5 mg l-1 6-benzyladenine (BA) 1.0 mg l-1 a-naphthaleneacetic acid (Ahmad *et.al*, 2010). By using the same plant in 2014, a novel approach for in vitro regeneration of *P. nigrum* L. has been applied in order to increase healthy biomass, phytochemicals and piperine production via reverse photoperiod (16hD/8hL). Leaf portions of the seed-derived plants were placed on an MS-medium fortified with different PGRs. Under 16hD/8hL, thidiazuron (TDZ; 4.0 mg/l) and BA (1.5 mg/l) was found to be the most effective (< 90%) in callus induction (Ahmad *et.al*, 2014). Also,

in 2015 callus and shoot regeneration of the same species (*P. nigrum*) was encouraged from leaf portions on Murashige and Skoog (MS) medium augmented with varied concentrations of plant growth regulators. A higher callus production (90 %) was observed in explants incubated on MS medium incorporated with 1.0 mg/l 6-benzyladenine (BA) along with 0.5 mg/l gibberellic acid (GA) after 4 weeks of culture (Ahmad *et.al*, 2015).

Dissanayake (2015) in development of a protocol for in vitro propagation of black pepper (*P. nigrum* L.) local selections, following methods for surface sterilization, culture establishment, shoot multiplication and callus induction are established. Either full or half strength MS medium supplemented with 1mg/L of NAA and 1.5 mg/L of Kinetin is better to use for callus induction from leaves of black pepper local selections. Other experiment of combinations of 2,4-D and BA also resulted in callus induction and proliferation of *P. permucronatum*. The highest percentage of callus induction was observed with the combination of 4.52 µM 2,4-D and 4.44 µM BA. The calluses thereby produced were friable and whitish. The callus growth pattern followed a sigmoid shape.

In Southeast Brazil, *P. solmsianum*, a shrub in which many biologically active compounds were identified. This work established a cell suspension culture system for this species. With this in mind, petiole and leaf explants obtained from in vitro plantlets were cultured in the presence of different plant growth regulator combinations (IAA, NAA, 2,4-D and BA). Root and indirect shoot adventitious formation, detected by histological analysis, was observed. Besides the different combinations of plant growth regulators, light regime and the supplement of activated charcoal (1.5 mg/l) were tested for callus induction and growth. Cultures maintained in light, on a 0.2 mg/l 2,4-D and 2 mg/l BA supplemented medium, and in the absence of activated charcoal, showed the highest calli fresh matter increment. From a callus

culture, cell suspension cultures were established and their growth and metabolite accumulation also studied indicated the existed active compound (Balbuena *et.al* , 2009).

There are various suitable condition including plant growth regulator for each plant species of Piper. The different of this concentration and treatment did not happen only in the differ spesies but also the same species, illustrated in *P. betle* (2 suitable concentration), *P. longum* (3 suitable concentration) and *P, nigrum* (4 suitable concentration). This indicated the different individual has a different needs of growth regulator and condition. This case may related to the role of endogenous hormone in plants. The endogenous hormonal system plays a leading role in the regulation of growth and development of plants. This regulatory system responds sensitively to even slight changes in the plant environment, which is manifested in reorganization of the hormonal status. It is necessary to know that simultaneous analysis of different groups of phytohomones allowed us to reveal a complex pattern of changes in plant hormonal system in response to treatments with exogenous growth regulators and to evaluate their contribution to the control of resistance to stress factors (Shakirova *et.al*, 2010).

Plant hormones rarely act alone, and for most processes, at least those that are observed at the organ level, many of these regulators have interacted in order to produce the final effect. Classical plant hormones like auxins, cytokinins, gibberellins, abscisic acid, ethylene and growth regulatory substances with similar biological effects. A better knowledge of the uptake, transport, metabolism, and mode of action of phytohormones and the appearance of chemicals that inhibit synthesis, transport, and action of the native plant hormones has increased our knowledge of the role of these hormones in growth and development (Gaspar *et.al*, 1996).

Based on the literature above, some factors may affected the succeed of Piper plant callus culture method mainly the suitable plant growth hormone (PGR) concentration occur in cells and external supplementation. The callus formed depends on the balanced ratio of plant growth hormone, in general auxin and cytokinin. We could not expect the endogenous hormone occur in plant cells, so variation of PGR concentration needed in specific interval. As an example various concentration of 2,4-D (0.0; 0.5;1.0; 1.5; 2.0; and 2.5 mg/L) to induced *P. betle* callus (Junairiah et.al., 2019). This PGR supplementation also impacted to the secondary metabolites accumulation in cells.The optimum condition of PGR seems to stimulate the secondary metabolites production (Raj et.al, 2015). But the latest technique also utilized another substance to induce plant stress to increase the metabolite production called as elicitors and also supplemented the substance based on biosyntesis pathways as precursors. As the example, the *P. betle* callus supplemented with elicitor (1,0 mg/l $CoCl_2$) in two weeks has a higher percentage of terpenoid compared to the control (Junairiah et.al, 2020).

There is considerable interest in callus culture of Piper as a means of producing therapeutic compounds especially as antimalaria. Some species has been investigated as potential source to produce specific anti-malarial compound such as *P. hostmannianum, P. tricuspe, P. chaba, P. acutifolium, P. glabratum, P. sarmentosum, P. piedecuestanum, P. heterophyllum, P. longum, P. nigrum, P. peltatum* and *P. aduncum* (Table 2). Several Piper plant has been utilized callus culture technique to obtain metabolites for antimalaria, such as *P. longum* and *P.nigrum* callus cultured to obtain piperine (Ahmad et.al 2014; Siddique et.al, 2019). However, the effort to culture the other potential Piper plant for antimalarial compound production still very lacking such as *P. hostmannianum* (20,60-Dihydroxy-40-methoxydihydro-chalcone), *P. tricuspe* (3-Farnesyl-p-hydroxybenzoic acid), *P.*

chaba (Chabamide), *P. sarmentosum* (sermentosine), *P. piedecuestanum* (5,8-Hydroxy-7-methoxyflavone), *P. heterophyllum* (Prenylated hydroxybenzoic acid), *P. peltatum* (4-Nerolidylcatechol) and*P. aduncum* (Piperitone).

Callus culture known as the middle stage of biotechnological applications including for generation of genetically modified plants in agriculture and horticulture (Efferth, 2019). Callus culture systems represent a potential renewable source of valuable antimalarial compounds which cannot be produced by microbial cells or chemical synthesis. Biotechnological applications of callus cultures presents the most updated reviews on current techniques in plant culture in the field. The principle advantage of this technology is that it may provide continuous, reliable source of antimalarial compound and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. HPLC known as the effective method to analyzed the amount of specific compound needed after extraction and each HPLC results the increasing of natural products after application of callus culture. This increasing profitable for medicinal purposes,even more the low product yields and supply concerns of plant product harvesation has renewed interest in large-scale plant cell culture technology in the future.

V. CONCLUSION

5.1 Conclusion

Based on this study, we can conclude that:

1. The recent study of crude extract of several Piper genus represent the 11 Piper species were chemically studied and assayed against the plasmodium or protozoa including*Piper aduncum, P. auritum, P. marginatum, P. obrutum, P. jericoense, P. betle, P. chaba, P. heterophylum, P. glabratum, P. acutifolium, P. cubeba, P. demeraranum and P. duckeii*with potential secondary metabolites isolates from several Piper genus were 20,60-Dihydroxy-40-methoxydihydro-chalcone, 3-Farnesyl-p-hydroxybenzoic acid, Piperine, Chabamide, Benzoic acid derivatives, Guineensine, Pellitorine, Brachystamide B, Sarmentine, Sermentosine, 5,8-Hydroxy-7-methoxyflavone, Prenylated hydroxybenzoic acid, 4-Nerolidylcatechol, Piperitone, Champor, and Viridiflorol (EO).
2. Callus culturecould be the promising method for antimalarial secondary metabolites acquisition and proved to increase the content of metabolites, which has been proved for 7 Piper species such as *Piper betle, P. colubrinumm, P. crocatum, P. longum, P. nigrum, P. permucronatum* and *P. solmsianum* indicated the different suitable concentration of plant growth regulator (PGR) to induce the callus formation even the same species which related to the various amount of endogenous hormone.

5.2 Suggestion

Furthure research and investigation about the Piper as an antimalarial agent should lead to the discovery of Piper species with high content of molecules of interest in case the lack of Piper studies, and callus culture for Piper species can be further improved by the research of optimization processes for easy and cost-effective of callus culture products should receive commensurate attention.

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