Callus Induction and Bioactive Compounds from *Piper betle* L. var nigra

Callus Induction and Bioactive Compounds from *Piper betle* L. var nigra

Junairiah[1], A Mahmuda[1], Y S W Manuhara[1], Ni'matuzahroh[1], L Sulistyorini[2]
[1]Department of Biology, Faculty of Science and Technology, University of Airlangga
[2]Faculty of Public Health, University of Airlangga

*alip.jun1@gmail.com

Abstract. Black betel is a decoration plant which also have potential as source of medicine materials and alternative of safe antiseptic. In-vitro culture method can be applied to produce secondary metabolites using culture medium and optimal supplementation of growth regulators, in this case 2,4-dichlorophenoxyacetic acid (2,4-D). This study was aimed to determine the effect of 2,4-D concentration on callus induction time, percentage of explant forming callus, callus fresh weight, dry weight, morphology, and secondary metabolites and bioactive compounds profile. The study was designed as laboratory experiment using completely randomized design with 6 concentrations treatment and replicated 12 times. Culture medium used was MS medium supplemented with various concentration of 2,4-D (0.0; 0.5; 1.0; 1.5; 2.0; and 2.5 mg/L). After callus had grown, secondary metabolites content was analyzed using phytochemical screening. 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 faster compared to other treatment, at mean induction time of 14 days. Explants supplemented with 2,4-D at 1.5 mg/L produced 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. 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 (0.5; 1.0; 1.5; 2.0; and 2.5 mg/L) found to contain terpenoids. The main component is octadecanoic acid.

1. Introduction

Infectious disease is the highest cause of morbidity and mortality, especially in developing countries, such as Indonesia. Infectious disease is a disease caused by pathogenic microbes [1-2]. Antibiotic plays important role in treatment of infectious disease [3]. Excessive application of antibiotic occurs a lot in the world, both in community and hospital environment. Application of certain antibiotic is expected to produce positive result, however irrational usage of antibiotic will result in negative effect [4].

Realization of this negative impact leads to the popularization of 'back to nature' concept, in which traditional medicine made from various plant is utilized [5]. Indonesia is country with the second largest tropical forest area in the world after Brazil, which still possesses a lot of unexplored natural potential resources for food and medicine One of the plants used as traditional medicine is betel leaves. Betel leaves contain chemicals that have antibacterial activity, such as chavicols, chavibetols, tannins, eugenols, carvacrols, caryophyllenes, and ascorbic acids [6]. One of the plants can be potentially used as medicinal plant is black betel.

Black betel (*Piper betle* L. var. Nigra) is a decoration plant which also has potential as medicinal resources. Black betel can be used as alternative of safe antiseptic. The most utilized part of this plant as medicine is the leaves, as it contains essential oils, phenyl propane, estragoles, chavicols, hydroxychavicols, chavibetols, caryophyllenes, allipyrochatecols, cineoles, cadinenes, tannins, diastases, starches, terpenes, sesquiterpenes, and sugars [7].

In-vitro culture can be applied to produce secondary metabolites as a source of aromatics and fragrances, enzymes, cosmetics, natural colorings, and bioactive components [8]. Growth regulators can affect protein synthesis and control enzymatic activity. Increasing protein synthesis as raw materials of enzymes will result in accelerating growth. This will lead in rising biosynthesis of secondary metabolites[9]. Based on previous elaboration, this study was designed with supplementation of auxin growth regulator at various concentrations to induce callus from black betel

leaf explants and to determine containing secondary metabolites in the culture.

2. Experimental Method

This study was conducted in Plant Physiology Laboratory, Department of Biology, University of Airlangga, Surabaya for 7 months, starting from January until July 2018.

Plant material used as explant was cuts of young black betel leaves. Black betel was obtained from Bratang flower market in Surabaya. Materials used for callus growth medium including Murashige and Skoog (MS) medium supplemented with 2,4-D growth regulator. Leaves sterilization used dish soap, 20% Clorox, and 70% alcohol. Other materials used included umbrella papers, sterile distilled water, spiritus, aluminum foil, pH papers, 1N KOH, 1N HCl, and methanol.

Tools used in the study including glass tools; measuring flasks, beaker glasses, Erlenmeyer flasks, petri dishes, culture bottles, stirring rod, and pipettes. Dissecting tools used to prepare explants were scalpel, tweezer, and scissor. Sterilization instruments used were autoclave, oven, Bunsen burner, hand sprayer, and laminar airflow (LAF). Other instruments used including digital balance, pH meter, refrigerator, culture chamber, electric stove, magnetic stirrer, and camera.

Study was designed as completely randomized experiment with single factor of 2,4-D growth regulator at 6 varying concentrations replicated for 12 times for each concentration. Concentration of 2,4-D growth regulator applied to determine the difference in secondary metabolites content were presented in Table 1.

Table 1. Concentration of 2,4-D applied to each treatment group

Group	2,4-D growth regulator concentration (mg/L)
D_0	0,0
$D_{0,5}$	0,5
D_1	1,0
$D_{1,5}$	1,5
D_2	2,0
$D_{2,5}$	2,5

Medium Preparation. 1000 mL MS medium was prepared by dissolving following macronutrient chemicals; 1.650 mg/L NH_4NO_3, 1.900 mg/L KNO_3, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_2O$, 170 mg/L KH_2PO_4 into 500 mL sterile distilled water. After all macronutrients had dissolved, medium was added 5 mL iron stock solution, 1 mL micronutrient stock, 4 mL vitamin stock, and 2,4-D stock at concentration according to respective group. Next, 100 mg Myo-inositol was dissolved into solution and 30 g sucrose was added. Solution was noted of its pH using pH paper, then pH was regulated until it reached optimal MS medium pH (5.6-5.8) using 1N KOH and 1N HCl. Lastly, solution volume was adjusted by adding distilled water until it reached 1000 mL before 8 g agar powder was dissolved into it. Medium was filled into culture bottles, ± 20-40 mL per bottle. Culture bottles were closed using aluminum foil and labelled accordingly. After medium had solidified, culture bottles were sterilized using autoclave at temperature of 121°C and pressure of 1.2 atm for 15 minutes.

Explants Sterilization. Black betel leaves were washed using dish soap; both upper and lower surface rubbed clean, then leaves were submerged in soap water for 3 minutes, shaken for 2 minutes, before rinsed with running tap water to clear any remaining dirt in the leaves. Inside LAF, black betel leaves were sterilized by shaking them in 70% alcohol for 3 minutes and rinsed with sterile distilled water for 3 times. Leaves were then shaken in 20% Clorox solution for 5 minutes before lastly rinsed 3 times with sterile distilled water.

Explants Planting and Maintenance. Black betel leaves were first cut into pieces of ± 1 cm^2, leaving out the margin part. Leaf pieces were then put on culture bottles filled with callus induction medium supplemented with 2,4-D growth regulator at different concentration. Culture bottles were closed again with aluminum foil and stored in incubation chamber at temperature of 20-25°C under 20-watt neon lamp.

Callus Extraction. All callus was harvested at 8 weeks after planting. Fresh and dry weight of callus were noted, then callus was dried and crushed into powder. Callus powder was macerated using methanol solvent for 1 day.

Phytochemical Screening.

Flavonoid compounds test

1 mL of black betel callus methanol extract was put into test tube, then 0.5 mL concentrated HCl and 4 pieces of Mg bands were added. Flavonoids presence was indicated by red, orange, or green color depending on flavonoid structure contained in the sample [10].

Alkaloids compounds test

Three test tubes were filled each with 1 mL extract. Two drops of Mayer, Dragendorf, and Wagner reactor solution were added to the first, second, and third tube respectively. Positive alkaloids content was signified by white sediment in the first tube, and reddish brown in the second and third tube [11].

Terpenoid and steroid compounds test

As much as three drops of extract was put on spot test board. Three drops of anhydrous acetate (Ac_2O) and a drop of concentrated sulphuric acid (H_2SO_4) were added to it. Terpenoids content was marked by red/brown color, while steroids were indicated by blue color [11].

Saponin compounds test

Saponin content analysis was performed using Forth method, by putting 1 mL methanol extract into test tube before 5 ml hot water and two drops of 2M HCl were added. Tube was shaken. Foam formed in the tube (± 10 minutes) indicated positive saponins content [12]. Bioactive compounds was analyzed with Gas Chromatography Mass Spectra

3. Results and Discussion

Based on result, shortest callus induction time was found from explants planted in MS medium supplemented with 2.5 mg/L 2,4-D growth regulator, at 14.83 ± 1.9462 days, while longest induction time was from 1.0 mg/L 2,4-D treatment, at 20.67 ± 0.7784 days (Table 2).

Table 2. Mean callus induction period of black betel leaf

Treatment	Mean callus induction time (days)
D_0	$0{,}00 \pm 0{,}000^a$
$D_{0,5}$	$18{,}83 \pm 0{,}8348^b$
D_1	$20{,}67 \pm 0{,}7784^c$
$D_{1,5}$	$16{,}33 \pm 2{,}0597^d$
D_2	$19{,}17 \pm 2{,}0816^c$
$D_{2,5}$	$14{,}83 \pm 1{,}9462^d$

*) Numbers followed by different letter indicating significant difference based on Mann-Whitney test at 5% significance level.

Explants supplemented with 0.5, 1.0, 1.5, 2.0, and 2.5 mg/L 2,4-D concentrations were found to be 100% forming callus, while control explants given 0.0 mg/L 2,4-D failed to grow callus (Table 3).

Table 3. Percentage of explants forming callus from various 2,4-D treatments

Treatment	Percentage of explants forming callus (%)
D_0	0%
$D_{0,5}$	100%
D_1	100%
$D_{1,5}$	100%
D_2	100%
$D_{2,5}$	100%

Based on Mann-Whitney test result, highest fresh weight was obtained from callus given 1.5 mg/L 2,4-D, which was found to be significantly different from other treatment (Table 4).

Table 4. Mean fresh weight of black betel callus

Treatment	Mean callus fresh weight(g)
D_0	$0,0000 \pm 0,0000^{a}$
$D_{0,5}$	$0,4046 \pm 0,4280^{bd}$
D_1	$0,1307 \pm 0,1021^{b}$
$D_{1,5}$	$0,8951 \pm 0,6408^{c}$
D_2	$0,2584 \pm 0,1239^{d}$
$D_{2,5}$	$0,1816 \pm 0,0840^{bd}$

*) Numbers followed by different letter indicating significant difference based on Mann-Whitney test at 5% significance level.

Similar as previous result, highest callus dry weight was found from explants given 1.5 mg/L 2,4-D at 0.0470 g and statistically different from other concentration (Table 5).

Table 5. Mean dry weight of black betel leaf callus

Treatment	Mean dry weight (g)
D_0	$0,0000 \pm 0,0000^{a}$
$D_{0,5}$	$0,0216 \pm 0,0098^{be}$
D_1	$0,0144 \pm 0,0097^{c}$
$D_{1,5}$	$0,0470 \pm 0,0187^{d}$
D_2	$0,0250 \pm 0,0088^{b}$
$D_{2,5}$	$0,0176 \pm 0,0063^{ce}$

*) Numbers followed by different letter indicating significant difference based on Mann-Whitney test at 5% significance level.

Callus morphology formed from black betel leaf explants at 8th week of culture period was presented in Table 6, while result of extract phytochemical screening was presented in Table 7, and bioactivity of methanol extract presented in Table 8.

Callus produced from in-vitro culture is formed because of injury to tissue and response towards growth regulator. Callus emerges at the wounded part of explant due to stimulus from plant tissue to close off the injured part. Cell division directed to callus formation occurs because of response to injury and endogenous or exogenous hormone supply in the explant [13].

Explants given 2.5 mg/L 2,4-D were able to induce callus growth at shortest period (two weeks). This was due to containing nutrients in MS medium had already able to induce callus growth. In addition, 2,4-D supplement at 2.5 mg/L concentration was able to stimulate cell division in the explants at optimal balance thus the cells were induced faster to continuously divide and dedifferentiate, resulting in callus formation in relatively shorter time. However, shorter induction time do not ensure higher number of callus formation at the end culture period [14].

The failure of explant to grow callus is theorized due to the difference of tissue ability to absorb nutrient and growth regulator from callus induction medium. In addition, the color of explants failed to grow callus had turned from green to brown before finally dead. This is possibly occurred because of phenolic compounds discharge from the explant. According to [15] phenolic acids together with abscisic acids (ABA) were endogenous inhibitors that discourage the formation of callus.

Biomass produced by tissue culture is found to be highly dependent on division, multiplication, and augmentation rate of cells. Cell division rate can be affected by growth regulator at certain concentration based on plant species and external factors such as light intensity and temperature[16].

The best treatment to increase fresh and dry weight of callus in current study was found from 1.5 mg/L concentration of 2,4-D. This was because 2,4-D activity affecting explants. One of the working mechanisms of auxin was to elongate the cells. Auxin stimulated cell elongation in coleoptile and plant segments. Cell elongation mainly occurs to vertical direction, followed with cell augmentation and increase of fresh weight[17].

Table 6. Morphology of black betel callus at 8th week of culture period

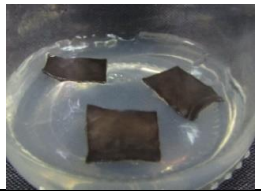
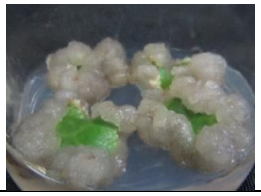
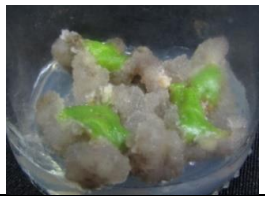
No	2,4-D concentration (mg/L)	Figure	Morphology
1	0.0		All explants planted colored black and failed to grow callus
2	0.5		Wavy explants, calli had white color and friable texture
3	1.0		Fringe of explants colored brown, explants turned wavy, calli grown colored white and brownish white with friable texture **c**
4	1.5		Wavy explants, calli grown with white or yellowish white color and friable texture
5	2.0		Explants fringe colored brown, explants wavy, calli had white and brownish white color with friable texture
6	2.5		Fringe of explants turned brown, explants curled, calli colored white and brownish white, calli had friable texture

Table 7. Phytochemical screening of black betel callus extract supplemented with various concentration of 2,4-D growth regulator

Compound	Result of phytochemical screening of callus given various 2,4-D concentration (mg/L)				
	0.5	1.0	1.5	2.0	2.5
Flavonoids	-	-	+	-	-
Terpenoids	+	+	+	+	+
Steroids	-	-	-	-	-
Alkaloids	-	-	-	-	-
Saponins	-	-	-	-	-

Table 8.Bioactive compounds of methanol extract of black betel

No	Compound	Area (%)				
		Concentration of 2,4D (mg/mL)				
		0,5	1	1,5	2	2,5
1	Ethoxymethyl Oxirene					0,09
2	Dimethylsulfoxide					8,08
3	Tetradecametyl heptasiloxane					0,05
4	5-buthyl-2-4-pentylphenyl-pyrimidine					0,05
5	Methylester hexadecanoic acid					0,14
6	Methylester 14 methyl pentadecanoic acid			0,07	0,09	
7	Tridecanoic acid					1,64
8	Hexadecanoic acid			0,16		
9	Eicosamethylcyclodecasiloxine					0,10
10	Methylester 11 Octadecanoic acid					0,33
11	Methylester 7 Octadecanoic acid			0,17	0,22	
12	Methylester 9 Octadecanoic acid		0,11			
13	Ethyloleate	0,09				
14	Methylester Octadecanoic acid					0,06
15	Octadecadienoic acid			0,07		
16	9-Octadecanoic acid					1,80
17	Octadecanoicacid					0,49
18	Semicarbazone3,3 diphenyl Acrylophenone					0,06
19	dihidrobenzothiazole					0,06

Parameters of callus morphology observed from current study including callus color and texture. Explant growth indicators in in-vitro culture, callus color, reflects the visual appearance of callus that indicate whether cells in callus still actively dividing or not. Callus texture is one of markers used to evaluate callus growth. Friable texture indicates that callus will develop to be somatic embryo[18], while compact texture which is solid and difficult to be released, indicates that callus will potentially grow into plant organs (organogenesis), such as roots or sprouts [19].

Based on result, initial formation of calli from black betel leaf explants occurred at various time, however callus from respective treatment possessed the same characteristic initial color of white. Along culture period up to observation at 8th week, forming calli had white, yellowish white, and brownish white color. The difference of color is thought to be caused by varying chemicals content according to secondary metabolites each callus contained.

Extraction of black betel callus in current study was performed using methanol solvent, as conducted in many other studies isolating organic compounds from natural sources, because of its ability to dissolve secondary metabolite compounds[20]. Based on result [21], phytochemical screening of black betel leaf methanol extract contained flavonoids, terpenoids/steroids, and alkaloids.

Flavonoid test performed in current study produced change of color, which indicated positive flavonoid content. Extract of callus given 1.5 mg/L 2,4-D had color alteration from brownish yellow into green, while other 2,4-D concentration (0.5, 1.0, 2.0, and 2.5 mg/L) produced negative result. Based on previous finding [21], methanol extract of black betel leaves positively contained flavonoids.

Terpenoids and steroids screening from callus extract indicated positive result. Callus given 0.5 and 2.5 mg/L 2,4-D produced color change brownish yellow into red, while callus given 1.0, 1.5, and 2.0 mg/L concentration had color alteration from brownish yellow into brown. Both color changes indicated positive terpenoids/steroids content. Based on previous study [21] methanol extract of black betel leaves positively contained terpenoid/steroid.

Result of alkaloids screening did not produce sedimentation, either for Meyer, Dragendorf, or Wagner tests, indicating negative alkaloids content. Based on previous study methanol extract of black betel leaves contained alkaloids [21]. As callus resulting from current study was found to have no or low level of alkaloids, nitrogen in the medium was not used to form coordinate covalent bonds with K+, resulting in orange sediment [21]. Saponin screening to callus extract did not form foam. Based on

previous study [20], methanol extract of black betel leaves did not contain saponins. First, it could be caused by not all bioactive compounds could be dissolved by methanol. In addition, foam did not form might also because callus extract could not dissolve in water, thus saponins contained in the extract was not hydrolyzed and foamed when shaken [23]. Stearic acid, also called octadecanoic acid, is one of the useful types of saturated fatty acids that comes from many animal and vegetable fats and oils, this compound has antimicrobial activity [24].

The difference of chemical content in the methanol extract of black betel callus and leaves was that callus only contained flavonoids and terpenoids, while leaves contained flavonoids, terpenoids/steroids, and alkaloids. Difference of chemical content between callus and leaves can occurs as secondary metabolites synthesis was affected by several factors, such as extraction method, sample particle size, condition and time during extraction process, and size ratio of sample with solvent (Harborne, 2006). The role of growth regulator is also a critical factor in plant metabolism.

4. Conclusion

Variation of 2,4-D growth regulator concentration affected callus induction time, percentage of explant forming callus, callus fresh weight, dry weight, and morphology. Phytochemical screening result of black betel callus secondary metabolites profile resulting in positive flavonoids content of callus supplemented with 1.5 mg/L 2,4-D and terpenoids from callus given 0.5, 1.0, 1.5, 2.0, and 2.5 mg/L 2,4-D concentrations. The main component is octadecanoic acid.

References

[1] Nascimento G.G.F., Locatelli J., Freitas P.C., Silva G.L. 2000 Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria *Brazilian Journal of Microbiology* 31 247-256.
[2] Alavijeh P.K., Alavijeh P.K. Sharma P.2012 A study of antimicrobial activity of few medicinal herbs *Asian Journal of Plant Science and Research* 2 4 496-502.
[3] Amenu. 2014 Antimicrobial activity of medicinal plant extracts and their synergistic effect on some selected pathogens *American Journal of Ethnomedicine.* 1 1 018-029
[4] Tunger, O., Karakaya, Y., Cetin, C.B. 2009 Rational antibiotic use *J Infect Developing Countries,* 3 2 88-93.
[5] Abdallah E.M. 2011 Plants: An alternative source for antimicrobials *Journal of Applied Pharmaceutical Science* 01 06 16-20.
[6] Jenie, B.S.L. 2015 Antimicrobial activity of *Piper betle* L. extract towards foodborne pathogens and food spoilage microoragnisms *FT Annual Meeting*, New Orleans, Louisiana.
[7] Yanti. 2012 *Piper betle* var. Nigra. www.thebest-healthy-foods.com. Diakses pada tanggal 02 Oktober 2017 pukul 09.55 WIB.
[8] Karuppusamy, S. 2009 A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell Culture *Journal of Medicinal Plants Research*, 3 13 1222-1239.
[9] Taiz, L., Zeiger E. 2006 *Plant physiology* Sinaur Associates, Inc, Publishers Sunderland, Massachusetts.
[10] Kristanti, A.N., Aminah, N.S., Mulyadi, T., Kurniadi, B. 2008 *Buku ajar fitokimia*n Airlangga University Press, Surabaya.
[11] Harborne, J.B. 2006 *Metode fitokimia penuntun cara Modern menganalisis tumbuhan* ITB, Bandung.
[12] Arief, D.A., Sangi, M.S., Kamu, V.S. 2017 Skrining fitokimia dan uji toksisitas ekstrak biji aren (*Arenga pinnata* MERR.) *Jurnal MIPA UNSRAT,* 6 2 12-15.
[13] Takatsuka H. and Umeda M. 201. Hormonal control of cell division and elongation along differentation trajectories in roots *Journal of Experimental Botany*. 65 10 2633-2643
[14] Abu Bakar D., Ahmed B.A., Mat taha R. 2014 In vitro callus induction and plant regeneration of *Celosia argenta*-an important medicinal plant *Brazilian Archieves of Biology and Biotechnology* 57 6 860-866.
[15] Castro A.H.F., Braga K.Q., Sousa F.M., Colimbra M.C., Chagas R.C.R. 2016 Callus induction and bioactive phenolic compounds production from *Byrsonima verbascifolia* (L)DC. (Malphigiaceae) *Revista Ciencia Agronomica.* 47 1 143-151.
[16] Chen. 2004 Humidity in plant tissue culture vessels Biosystems Engineering 88 92 231-241.
[17] [17]Velasquez, S.M. Kleinvhen, J., Barbez, E. Estevez, J.M. 2016 Auxin and Cellular Elongation

Plant Physiology 170 3.
[18] Tiwari, S and Tripathi, M.K. 2005 Comparison of morphogenic ability of callus types induced from different explants of soybean (Glycine max L.Merrill) *Legume Res* 28 2 115-118.
[19] Wahyuni, D.K., Prasetyo D., dan Hariyanto S. 2014 Perkembangan kultur daun *Aglaonema sp.* dengan perlakuan kombinasi zat pengatur tumbuh NAA dan 2,4-D dengan BAP *Jurnal Bioslogos* 4 1 9-16.
[20] Prashant. 2011 Phytochemical screening and extraction *Internationale Pharmaceutica Sciencia*, 1 1 1-9.
[21] Junairiah., Ni'matuzahroh, Zuraidassanaaz, N.I., Sulistyorini L. 2017 Isolation and identification of secondary metabolites of black betel (*Piper betle* L. var. Nigra) *The 2nd Molecular and Cellular Life Sciences*., Institute of Tropical Disease and Department of Chemistry, FST, Universitas Airlangga.
[22] Sangi, M.S., Momuat, LI., dan Kumaunang, M. 2013 Uji toksisitas dan skrining fitokimia tepung gabah pelepah aren (*Arange pinnata*) Manado: Universitas Sam Ratulangi.
[23] Rustina., dan Tasminatun, S. 2016 Uji aktivitas antioksidan dan antibakteri ekstrak etil asetat biji labu kuning (*Cucurbita moschata* Duch. Poir) Farmasi FKIK, UMY.
[24] Pu, Z., Zhang, Y., Yin, Z., Xu J., Jia, R. Lu, Y., Yang, F.2010 Antibacterial activity of 9-octadecanoic acid-hexadecanoic acid-tetrahydrofuran-3,4-diyl ester from neem oil Agricultural sciences in china 9 8 1236-1240.

Acknowledgement
We acknowledged RISTEK DIKTI (Grant: Penelitian Terapan Unggulan Perguruan Tinggi (PTUPT) 2018.