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Effects of light, sucrose concentration and repetitive subculture on callus growth and medically important production in *Justicia gendarussa* Burm.f.

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ABSTRACT

Objective: This research aimed at the discovering the effects of light, sucrose concentration and repetitive subculture on the growth of *Justicia gendarussa* Burm.f. calluses based on biomass, morphological characters and metabolic profiles.
Methods: The second and third leaves of young *J. gendarussa* Burm.f. were isolated and cultured in solid Murashige and Skoog (MS) medium with the addition of 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L benzyl amino purine (BAP). Different sucrose concentrations, varying from 0 to 5%, were included in the medium. The cultures were incubated under light and dark conditions for 6 weeks. Repetitive subculture was carried out every 2 weeks for a total of four times, and 3% sucrose gave the best callus growth. Dry calluses were extracted with methanol, and their metabolic profiles were analyzed using gas chromatography/mass spectrometry (GC/MS). Compound identification was performed by comparing the mass spectra to references from the WILEY version 7n.1 library.
Results: Among the 12 conditions tested for the prolonged 6-week culture, the 2–5 % sucrose treatments under light and 3–5 % sucrose treatments under dark exhibited the highest dry weight. For repetitive subculture, the highest wet and dry weights were identically detected under both light and dark conditions after the second repetitive subculture. A total of 19 metabolites was identified by GC/MS, with major compounds being taraxasterol, monoplex D, 2-methoxy-4-vinylphenol, and palmitic acid.
Conclusion: Light, sucrose concentration, and repetitive subculture all significantly impacted the growth and metabolic profiles of *J. gendarussa* Burm.f. calluses. A concentration of 3% sucrose demonstrated the best growth of callus and could be further applied for mass production.

1. Introduction

Gandarusa (*Justicia gendarussa* Burm.f.) is an Indonesian medicinal plant belonging to Acanthaceae. It is a small, erect shrub growing 0.6–1.2 m tall and is widely distributed over South and Southeast Asia, from lowlands up to 1500 m above sea level [1,2]. Gandarusa has been reported to contain saponins, flavonoids, polyphenols, essential oils, flavanol-3-glycosides, flavones, luteolins, iso-orientin, coumarin, iridoids, sterols, tannins, and calcium oxalate [3]. Traditionally, it has been used to treat muscle spasms, rheumatism, common cold, headache, pharyngitis, bronchitis, dyspepsia, eye diseases [3], antifertility [4], analgesic [5] and antihyperuricemic effects [6].

Nowadays, tissue culture approaches have been developed to produce specific plants in large quantities in order to support biotechnological researches [7,8]. Currently, gandarusa is in the public spotlight in Indonesia and is the subject of extensive research due to its medicinal potential. As a result of the increasing demand, its availability in nature has been visibly impacted. Therefore, tissue culture can be developed into a whole plant or cell suspensions to obtain only the desired secondary metabolites [9,10]. Nevertheless, the success of callus culture relies on several key factors, including the plant genotype and variety, culture medium [11], growth-controlling substances [11,12], growth environment [13], carbon source [12–14] and light [15,16]. Changes

Abbreviations: BAP, Benzyl amino purine; MS, Murashige and Skoog; 2,4-D, 2,4-Dichlorophenoxyacetic Acid; GC/MS, Gas chromatography/mass spectrometry; KOH, Kalium hydroxide /Sodium hydroxide.

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in light condition and sucrose concentration could affect the production of such molecules [17,18]. Moreover, repetitive subculture is a critical step in plant tissue culture in order for culture strategies to take into account how many times subculture is performed.

GC/MS has been successfully employed to identify metabolite patterns in a variety of medicinal plant cultures [19]. Secondary metabolite analysis has been conducted using GC/MS on both the leaf [20] and callus of gandarusa in the presence of various growth-regulating substances [21]. Some natural compound has been identified by GC/MS, such as taraxasterol which has many important pharmacological actions including anticancer activity. Palmitic acid, monoplex D and 2-methoxy-4-vinylphenol were also used as antioxidant and antimicrobial [20,21].

In this study, we measured the biomass of gandarusa callus through fresh and dry weights and used GC/MS method to characterize small metabolites produced from the callus; we then compared them among different culture conditions. Three major factors–light, sucrose concentration and number of repetitive subcultures–were varied in this study to determine which conditions were best for gandarusa callus growth. This research provided useful information about the effects of light, sucrose concentration, and repetitive subculture on the growth and metabolic profiles of gandarusa callus. In addition, it could inform the mass production of gandarusa in both agricultural and industrial settings in the future.

2. Materials and methods

2.1. Explant preparation

Gandarusa plant (*Justicia gandarussa* Burm.f.) was acquired from Taman Husada Graha Famili, Surabaya, Indonesia, and identified by botanists from the Biosystematic Laboratory, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. The second and third leaves were collected from young shoots and used as the initial explants. They were cut and washed carefully with running tap water. To sterilize the explants, the cut leaves were soaked in 2 g/L dithane fungicide for 5 min and rinsed with distilled water, three times each. They were then immersed in clorox solution (50 %, v/v) and shaken for 7 min. Lastly, the explants were rinsed three times with sterile distilled water inside a laminar airflow cabinet.

2.2. Medium preparation

Murashige and Skoog (MS) medium was used for callus culture [31]. Sucrose (0%, 1%, 2%, 3%, 4% and 5%; w/v), 8 g/L agar, 1 mg/L 2,4-D and 0,5 mg/L BAP (the best growth regulator substance data for initiation that already published) were subsequently added to the medium. The pH of the medium was adjusted to 5.6–5.8 using 1 N of KOH or HCl. Prepared stock medium was divided into a small bottles (100 mL bottle, 30 mL medium), covered with aluminium foil and sterilized by autoclaving at 121 °C and 1 atm of pressure for 15 min. The medium with sucrose (3%, w/v) gave the best callus growth and was identified as the best medium used in the repetitive subculture experiments.

2.3. Callus initiation and repetitive subculture

Leaf explants, 1 cm^2 in size, were transferred to petri dishes lined with sterile filter paper. They were gently embedded on the surface of the varied sterile medium formulations inside the culture bottles. The bottles were tightly tied with a rubber band. Calluses were harvested every 2 weeks to examine their growth over a total of 6 weeks. A half gram of callus obtained from the initiation step was transferred into a subculture bottle. The first repetitive subculture was conducted on a 4-week-old callus and performed every 2 weeks afterward for total four times. Culture bottles were placed in a preservation room at 25 ± 2 °C, equipped with continuous (24 h) light of neon lamp (general electric cool white fluorescent tube) to provide 650 ± 45 lx light intensity (the lamp was off for the dark condition). The distance between the light source and culture bottles was approximately 60 cm. Every treatment was done in four times.

2.4. Callus collection and extraction

Calluses were harvested at every 2 weeks (repetitive subculture period). They were freshly weighed and measured again after complete drying in room temperature (25 ± 2 °C) with continuous light. After drying (measured until the constant weight), they were ground in preparation for extraction. Ten millilitres of absolute methanol were added to 1 g dried samples and macerated for 24 h in a closed falcon tube (15 mL, shaken every 6 h). This step was repeated three times, and the fractions were pooled together for evaporating. This methanol extract was kept at 4 °C until further analysis.

2.5. GC/MS analysis

The metabolite profiles of methanol callus extracts were determined using GC/MS. Extract of 50 mg was dissolved in 0.5 mL *n*-hexane and then filtrated by 0.45 μm of filter paper. GC/MS sample analysis was performed using an Agilent 6980 N Network GC System with autosampler and detector (Agilent 5973 inert MSD), fitted with HP-5 5% phenylmethylsiloxane capillary column (30 m ×0.32 mm, with 0.2-μm film thickness; J&W Scientific). The oven temperature was programmed from 50 °C to 280 °C at 10 °C/min and held for 10 min. Helium was used as the carrier gas at flow rate of 1.3 mL/min. The injector temperature was 280 °C and the injection volume 1 μL, with a split ratio of 1:10. The interface and MS ion source were maintained at 230 °C and 150 °C, respectively, the mass spectra were taken at 70 eV with a mass scan range of 20–700 amu. Data handling was done using GC/MS solution software. The identification of compounds was based on comparisons of their mass spectra with those of the WILEY version 7n.1 library. The relative percentage of each component was calculated by the relative percentage of the total peak area in the chromatogram.

2.6. Data analysis

Two types of data were collected, they were qualitative data which included callus morphology, including colour and texture callus, while quantitative data which included fresh and dry callus weights. The quantitative data were analyzed statistically using SPSS 20 software. They were also tested for normality using one-sample Kolmogorov-Smirnov test, followed by the Kruskal-Wallis test. The Mann-Whitney test was used to test significant differences among treatments.

3. Results

3.1. The effects of light, sucrose concentration and repetitive subculture on Justicia gendarussa Burm.f. callus morphology

One indicator of growth during *in vitro* culture is the appearance of calluses in explants. The emergence of callus begins with swelling on the side of the wound in explants. Callus formation in wound tissue was stimulated by explant growth medium, which includes growth regulators and sucrose. In this study, leaf explants were placed on MS medium with the addition of 1 mg/L 2,4-D

hormone, 0.5 mg/L BAP and sucrose at various concentrations and subsequently incubated in the dark or the light condition. In culture medium without sucrose, explants incubated in the dark did not form calluses and became brown after the first week of observation, whereas in light incubation, the calluses formed in the third week. Using culture medium with 1%–5% sucrose, callus induction started growing in the second week. The effects of different sucrose concentrations and light condition were recorded. The callus texture was friable at first, and then, when incubated in both dark and light conditions, the texture became compact in all treatments, while the callus colour varied among the treatments (Table 1 and sure 1). Overall, the dark-incubated calluses were brighter than the light-incubated calluses. The first callus appeared translucent. The calluses grew until they became enlarged and dark brown in colour. The colour of the calluses became darker with age and increase of sucrose concentration.

Callus morphology also varied during subculture. In both light and dark conditions, the first and second subcultures showed compact-textured calluses, whereas the third and fourth showed friable-textured calluses. Callus colour also changed during repetitive subculture treatments. In both light and dark conditions, the first subculture showed a white callus and the second showed a muddy white callus. In light condition, the third subculture showed a muddy white callus, but in dark condition, it showed a brown callus. In light condition, the fourth subculture remained muddy white, while in the dark they were dark brown (Fig. 2).

3.2. The effects of light, sucrose concentration and repetitive subculture on Justicia gendarussa *Burm.f. callus induction and growth*

The present study showed that calluses protruded from the leaves and were clearly visible after fourteen days of culture. Compact and light yellowish calluses were obtained after 21 days (Fig. 1C). Based on Fig. 1 and Table 1, all treatments produced callus except those without sucrose in dark condition.

Callus biomass accumulation was recorded for 6 weeks, at 2-week intervals, under various concentrations of sucrose, and incubated either in the dark or light. The different sucrose concentrations affected both the fresh weight and dry weights

Table 1
Formation time, morphology, and intensity of callus from *Justicia gendarussa* Burm. f., with variation in sucrose concentration for 6 weeks in dark and light conditions.

Week	Sucrose concetration (%)	Dark					Light				
		Eficiency of callus initiation (%)	Callus formation time (week)	Callus morphology		Callus intensity	Eficiency of callus initiation (%)	Callus formation time (week)	Callus morphology		Callus intensity
				Colour	Texture				Colour	Texture	
1	0	–	–	–	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–	–	–	–
	40	–	–	–	–	–	–	–	–	–	–
	50	–	–	–	–	–	–	–	–	–	–
2	0	–	–	–	–	–	–	–	–	–	–
	10	100	2	White	Friable	+	100	2	White	Friable	+
	20	100	2	White	Friable	+	100	2	White	Friable	+
	30	100	2	White	Friable	+	100	2	White	Friable	+
	40	100	2	White	Friable	+	100	2	White	Friable	+
	50	100	2	White	Friable	+	100	2	White	Friable	+
3	0	–	–	–	–	–	100	3	White	Friable	+
	10	100	2	White	Friable	+	100	2	White	Friable	+
	20	100	2	Yellowish	Compact	++	100	2	Yellowish	Compact	++
	30	100	2	Yellowish	Compact	++	100	2	Yellowish	Compact	++
	40	100	2	Yellowish	Compact	++	100	2	Yellowish	Compact	++
	50	100	2	Brown	Compact	++	100	2	Brownish	Compact	++
4	0	–	–	–	–	–	100	3	White	Friable	+
	10	100	2	White	Friable	+	100	2	White	Friable	+
	20	100	2	Yellowish	Compact	+++	100	2	Yellowish	Compact	+++
	30	100	2	Yellowish	Compact	++++	100	2	Yellowish	Compact	++++
	40	100	2	Muddy white	Compact	++++	100	2	Muddy white	Compact	++++
	50	100	2	Brown	Compact	++++	100	2	Brownish	Compact	++++
5	0	–	–	–	–	–	100	3	Yellowish	Compact	++
	10	100	2	Yellowish	Compact	++	100	2	Yellowish	Compact	++
	20	100	2	Brownish	Compact	+++	100	2	Muddy white	Compact	++++
	30	100	2	Brownish	Compact	++++	100	2	Brownish	Compact	++++
	40	100	2	Brownish	Compact	++++	100	2	Brown	Compact	++++
	50	100	2	Dark brown	Compact	++++	100	2	Brown	Compact	++++
6	0	–	–	–	–	–	100	2	Brown	Compact	++
	10	100	2	Dark brown	Compact	+++	100	2	Dark brown	Compact	++++
	20	100	2	Dark brown	Compact	++++	100	2	Dark brown	Compact	++++
	30	100	2	Dark brown	Compact	++++	100	2	Dark brown	Compact	++++
	40	100	2	Dark brown	Compact	++++	100	2	Dark brown	Compact	++++
	50	100	2	Dark brown	Compact	++++	100	2	Dark brown	Compact	++++

Note: -, no callus is formed; +, 25 % callus of explants; ++, 50 % callus of explants; +++, 75 % callus of explants; ++++, 100 % callus of explant.

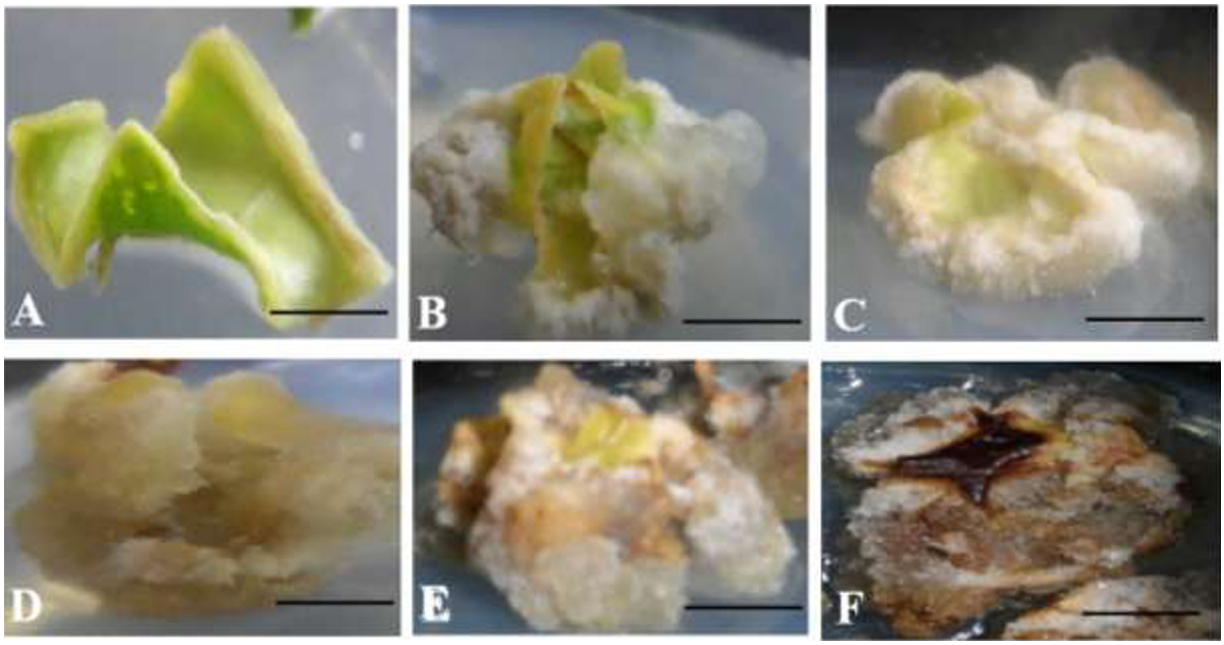

Fig. 1. Representative morphological traits of *Justicia gandarussa* Burm.f. calluses treated with 3% sucrose every week. (A) First week culture, (B) second week culture, (C) third week culture, (D) fourth week culture, (E) fifth week culture, (F) sixth week culture; (bar =1 cm).

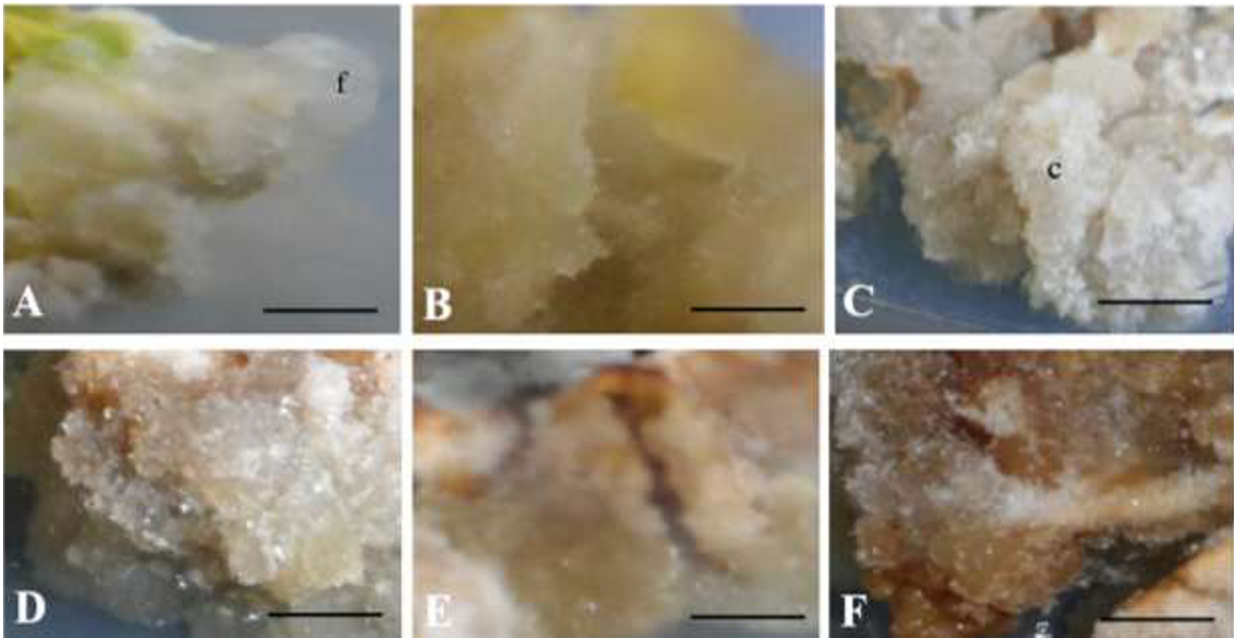

Fig. 2. Representative morphological traits of *Justicia gendarussa* Burm.f. calluses treated with different sucrose concentrations (%) with dark or light incubation. (A) Translucent white callus, (B) yellowish callus, (C) muddy white callus, (D) brownish callus, (E) brown callus, (F) dark brown callus, (f) friable callus, (c) compact callus; (bar =2 mm).

of the calluses. The highest fresh weight of callus was with 2% sucrose (light) (1.19 ± 0.18 g), 3% (light) (1.19 ± 0.18 g) and 3% (dark) (1.08 ± 0.03 g), followed by 5% (dark) (0.89 ± 0.13 g), 1% (light) (0.78 ± 0.11 g), 2% (light) (0.72 ± 0.06 g), 4 % (light) (0.72 ± 0.06 g), 4% (dark) (0.70 ± 0.12 g), 2% (dark) (0.70 ± 0.09 g), 5% (light) (0.68 ± 0.41 g), 1% (dark) (0.64 ± 0.10 g) and 0% (dark and light) (0.00 ± 0.00 g) after 6 weeks of callus culture (Table 2).

The highest dry weight of callus was with the addition of 3% sucrose (dark and light) (0.09 ± 0.00 g) and (0.09 ± 0.01 g), respectively, 4% (dark) (0.09 ± 0.03 g) and 5% (dark and light) (0.09 ± 0.01 g) and (0.09 ± 0.01 g), respectively, which did not differ significantly from 2% (light) (0.08 ± 0.01 g), followed by 4% (light) (0.06 ± 0.00 g), 1% (light) (0.05 ± 0.00 g), 2% (dark) (0.05 ± 0.00 g), 1% (dark) (0.04 ± 0.00 g) and 0% (dark and light) (0.00 ± 0.00 g) after 6 weeks of callus culture (Table 2).

The 3% sucrose media was used for subcultures, because it was the lowest sucrose level that gave the highest biomass. Callus biomass was tracked for 2 months, recorded every 2 weeks (subculture period). Calluses showed growth after the second subculture. After repeating subculture, callus growth in term of gross weight was changed (Table 3). Fresh and dry weights of the calluses increased after the first and second subculture and

Table 2
Average fresh and dry weights of *Justicia gandarussa* Burm.f. calluses after 2, 4, and 6 weeks of incubation.

Time incubation (week)	Sucrose concentration (%)	Dark		Light	
		Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
2	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{q}	0.00 ± 0.00^{a}	0.00 ± 0.00^{q}
	1	0.09 ± 0.00^{abcd}	0.01 ± 0.00^{qrs}	0.06 ± 0.01^{abc}	0.01 ± 0.00^{qrs}
	2	0.01 ± 0.01^{abcd}	0.01 ± 0.00^{qrst}	0.12 ± 0.02^{abcde}	0.01 ± 0.0^{qrst}
	3	0.19 ± 0.06^{bcdef}	0.02 ± 0.01^{stuv}	0.11 ± 0.00^{abcde}	0.02 ± 0.00^{stuv}
	4	0.12 ± 0.01^{abcde}	0.01 ± 0.00^{qrs}	0.09 ± 0.02^{abcd}	0.01 ± 0.00^{qrstu}
	5	0.23 ± 0.06^{def}	0.03 ± 0.01^{wx}	0.18 ± 0.02^{abcdef}	0.03 ± 0.00^{uvw}
4	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{q}	0.04 ± 0.00^{abc}	0.00 ± 0.00^{q}
	1	0.07 ± 000^{abcd}	0.01 ± 0.00^{qrs}	0.16 ± 0.02^{abcdef}	0.01 ± 0.00^{rst}
	2	0.29 ± 0.06^{fg}	0.02 ± 0.00^{stuv}	0.09 ± 0.02^{abcd}	0.01 ± 0.00^{qrst}
	3	0.28 ± 0.10^{fg}	0.03 ± 0.00^{tuv}	0.13 ± 0.01^{abcde}	0.02 ± 0.00^{stu}
	4	0.05 ± 0.01^{ab}	0.01 ± 0.00^{qrs}	0.21 ± 0.03^{cdef}	0.02 ± 0.00^{tuv}
	5	0.42 ± 0.09^{g}	0.04 ± 0.00^{wx}	0.26 ± 0.03^{ef}	0.03 ± 0.00^{uvw}
6	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{q}	0.06 ± 0.01^{abc}	0.01 ± 0.00^{q}
	1	0.64 ± 0.10^{h}	0.04 ± 0.00^{wx}	0.78 ± 0.11^{hi}	0.05 ± 0.00^{xy}
	2	0.70 ± 0.09^{h}	0.05 ± 0.00^{xy}	1.19 ± 0.18^{j}	0.08 ± 0.01^{z}
	3	1.08 ± 0.03^{j}	0.09 ± 0.00^{z}	1.19 ± 0.12^{j}	0.09 ± 0.01^{z}
	4	0.70 ± 0.12^{h}	0.09 ± 0.03^{z}	0.72 ± 0.06^{h}	0.09 ± 0.00^{z}
	5	0.89 ± 0.13^{i}	0.09 ± 0.01^{z}	0.68 ± 0.41^{h}	0.09 ± 0.01^{z}

Note: Number followed by the same letter indicates no significant differences according to Mann-Whitney Test ($\alpha = 0.05$).

Table 3
Average fresh and dry weights of *Justicia gandarussa* Burm.f. calluses after 1, 2, 3 and 4 repetitive subcultures.

Treatment	First repetitive		Second repetitive		Third Repetitive		Forth repetitive	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
Light	0.90 ± 0.04^{b}	0.07 ± 0.01^{y}	1.68 ± 0.01^{d}	0.11 ± 0.00^{z}	1.30 ± 0.01^{c}	0.07 ± 0.01^{x}	0.87 ± 0.02^{a}	0.07 ± 0.00^{x}
Dark	0.88 ± 0.03^{b}	0.07 ± 0.00^{y}	1.72 ± 0.02^{d}	0.08 ± 0.00^{z}	1.27 ± 0.01^{c}	0.06 ± 0.00^{x}	0.61 ± 0.01^{a}	0.06 ± 0.01^{x}

Note: Number followed by the same letter shows no significant differences according to the Duncan test ($\alpha = 0.05$).

decreased in the third subculture. Calluses grown in both light and dark conditions demonstrated the same pattern. The highest callus weight was observed in the second subculture under dark conditions (1.72 ± 0.02 g), but this was not significantly higher than the light treatment (1.68 ± 0.01 g).

3.3. Phytochemical profile

Phytochemical profiling of *Justicia gendarussa* Burm.f. (gandarusa) calluses was carried out using GC/MS. Representative chromatograms from each condition are shown in Fig. 3. Lists of chemical identifications are reported in Table 4. A total of 19 compounds was detected from the eight different conditions. Under light conditions, the highest number of compounds was identified from the first subculture (12 compounds), followed by the third subculture (10 compounds), second subculture (8 compounds) and fourth subculture (4 compounds). Under dark conditions, the fourth subculture had the highest number of detected components (11 compounds), followed by the first and third subculture (9 compounds), and second subculture (8 compounds).

Among the 19 total compounds detected, stearic acid, palmitic acid, and linoleic acid were found in all eight tested conditions; 2-methoxy-4-vinylphenol and in seven; elaidic acid, taraxasterol, and monoplex D in five; hexadecamethyl-cyclooctasiloxane and tetrade-camethyl-cyclooctasiloxane in four; methyl oleat, methyl palmitate, and hexadecanal in three; 9,12-octadecadienoic acid (Z, Z)-, methyl ester in two of the eight tested conditions. There were 5 compounds identified from just a single condition.

A wide range of chemical components was identified under light conditions. Taraxasterol was abundant in the first and fourth subculture, while palmitic acid and 2-methoxy-4-vinylphenol were highly observed in the second subcultures. Moreover, monoplex D was enriched in the third subculture. Under dark conditions, taraxasterol was a major constituent in the first, second and third subcultures, as was 2-methoxy-4-vinylphenol. Furthermore, in the fourth subculture, palmitic acid was redundant.

The GC/MS analyses revealed that the callus extracts were predominantly composed of fatty acids, in addition to small quantities of a phenolic (2-methoxy-4-vinylphenol), an aliphatic alcohol (homovanillyl alcohol), a sterol (taraxasterol), and ester (hexanedioic acid, bis(2-ethylhexyl) ester). Retention times of fatty acids were recorded in a range of 44–48 min. Taraxasterol was eluted last, at approximately 66 min, indicative of its nonpolar property.

4. Discussion

Although *Justicia gendarussa* Burm.f. (gandarusa) has pharmaceutical benefits, it is not a cultivated species, and the continued use of natural sources is not sustainable. *In vitro* cultivation of gandarusa is expected to replace conventional cultivation for the production of bioactive compounds. If achieved, there will be no necessity to take plants directly from nature. Furthermore, *in vitro* culture facilitates the distribution of gandarusa to other institutions around the world, as this form of plant does not require quarantine [22,23]. In this study, we examined the effects of light, sucrose concentration and repetitive subculture on growth and secondary metabolite profile in gandarusa.

The combination of light, sucrose content and repetitive subculture influenced callus morphology, which was indicated by the colour and texture of the callus. Callus colour was brighter at the first growth, and then, it became darker with age and increase of sucrose concentration. Callus colour in dark conditions was brighter than in light conditions. The colour change of calluses indicated cell activity during cell division [16]. In addition, George and Sherintong [24] also stated that the colour of calluses becomes brown because they produced phenolic compounds that could be

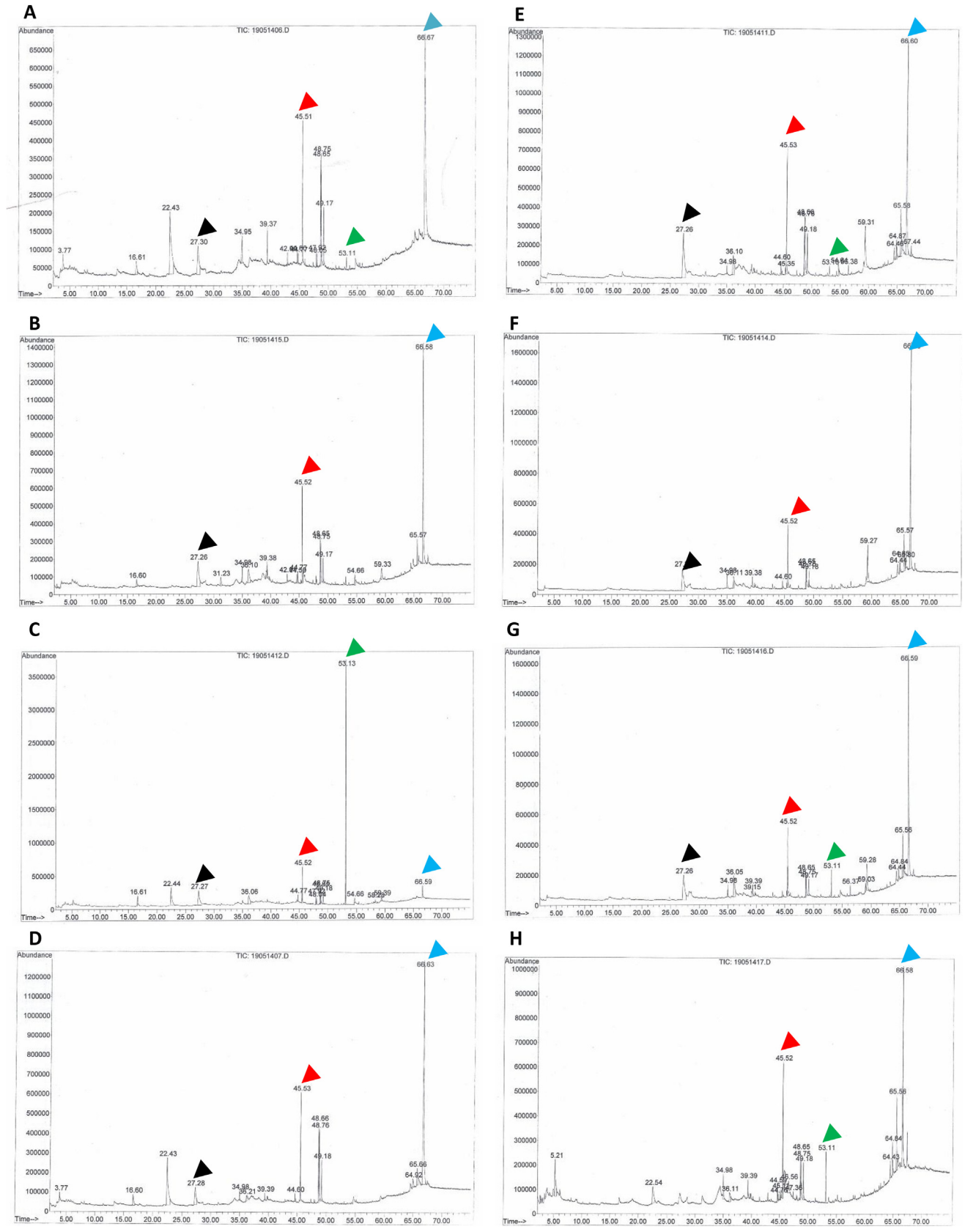

Fig. 3. Representative chromatogram of methanol extracts of *Justicia gendarussa* Burm.f. calluses among the different conditions. (A) Light, first subculture; (B) light, second subculture; (C) light, third subculture; (D) light, fourth subculture; (E) dark, first subculture; (F) dark, second subculture; (G) dark, third subculture; (H) dark, fourth subculture. black arrow 2-methoxy-4-vinylphenol; red arrow: palmitic acid; green arrow: monoplex D; blue arrow: traxasterol.

Table 4
Phytochemical components from GC/MS analysis of *Justicia gandarusa* Burm.f. calluses in every repetitive subculture.

No.	Compound	RT (min)	Relative area percentage (peak area relative to the total peak area (%)								Bioactivity
			Subculture of Light incubation				Subculture of Dark incubation				
			1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Fatty acid											
1	Stearic acid	49.13	2.80	2.21	2.071	6.26	2.97	1.726	1.75	3.26	Antimicrobial, 5α reductase inhibitor, and hypocholesterolemic agent [44]
2	Palmitic acid	45.38	7.09	10.94	6.47	0.50	0.46	7.580	8.18	12.77	Anti-inflammatory, antidiabetic, antibacterial and antioxidant [44]
3	Linoleic acid	48.27	5.41	4.42	2.67	7.98	3.89	2.576	2.52	3.81	Anti-inflammatory, acne reductive and moisture-retaining properties [44]
4	Elaidic acid	48.74	5.01	–	2.52	5.89	5.89	–	–	2.21	Decreases HDL cholesterol[44]
5	Eudesmane	44.60	–	–	–	–	1.12	–	–	–	Antioxidant [44]
6	Hexadecanal	44.59	–	–	–	0.50	–	0.891	–	1.35	Antimicrobial and antioxidant [44]
7	Heptadecanoic acid (CAS)	47.36	–	–	–	–	–	–	–	0.83	Antioxidant and antimicrobial [44]
Phenols											
8	2-Methoxy-4-vinylphenol	27.27	8.14	14.04	14.30	5.49	16.67	13.793	14.92	–	Antimicrobial and antioxidant [44]
9	Homovanillyl alcohol	36.09	–	6.32	7.15	–	5.55	3.874	8.05	2.90	Neurotransmitter dopamine, antioxidant [44]
Sterol											
10	Taraxasterol	66.61	39.52	–	–	45.89	28.66	42.437	35.34	`	Antibacterial, antiviral, anti-inflamatory, and anti-apoptosis [44]
Silosane											
11	Hexadecamethyl-cyclooctasiloxane	39.32	1.65	–	–	–	–	1.072	1.36	1.80	Antioxidant, used for treatment and prevention of hypoxia, anemia, and sickle cell disease [44]
12	Tetradecamethyl-cycloheptasiloxane	34.97	3.43	1.73	–	–	–	–	–	3.42	Antimicrobial agent, antifouling immunomodulatory, and has antitumor activities [44]
Ester											
13	Methyl palmitate	44.76	–	1.24	1.03	–	–	–	–	0.59	Anti-inflammatory, antidiabetic, antibacterial, and antioxidant [44]
14	Methyl oleate	48.28	0.48	–	0.89	–	–	–	2.78	–	Anticancer anti-inflammatory and antioxidant [44]
19	Monoplex D	53.11	0.54	–	39.22	–	0.63	–	2.32	–	Antioxidant and antimicrobial [44]
15	912-Octadecadienoic acid (Z,Z)-, methyl ester	47.92	0.66	–	1.24	–	–	–	–	–	Anti-inflammatory, anticancer [44]
16	Pentadecanoic acid, 14-methyl-, methyl ester	44.77	0.52	–	–	–	–	–	–	–	Antioxidant [44]
Others											
17	6-Aza-571,214-tetrathiapentacene	39.37	–	2.39	–	–	–	–	–	–	Antibacterial [44]
18	(+-)-15-Hexadecanolie	45.11	–	–	–	–	–	–	–	0.98	Antimicrobial [44]

toxic to the plants and stop the growth. It has also been reported that increasing the sucrose concentration of the culture medium resulted in the increased production of phenolics, leading to cell death [25].

Further discoloration of the callus, from white to dark brown, was an indicator of the low cleavage activity of callus cells [26]. Taranto et al. [23] found that callus browning was caused by polyphenolic compounds, which were present when the explants were wounded, and the enzymatic browning reaction of phenolic compounds, oxidized by polyphenol oxidase, peroxidized or exposure to air. The oxidation of phenolic compounds was enhanced by light. Light affected the performance of plant hormones. Auxin hormone in the form of 2,4-D worked optimally to improve cell division in dark conditions [27]. Furthermore, calluses would change to a yellowish colour and then turn brown because of the secondary metabolite content [28].

Callus texture showed the quality of callus that was in accordance with the purpose of the study. Friable white calluses indicated high cell activity, in-line with active division and embryogenic behaviour. Callus texture could vary from friable to compact depending on explant variant, basic medium, growth regulators and the biotic and abiotic supplements in the culture [29].

Friable calluses were first observed with all treatments. The friable calluses were formed through the growth of cells at a small site and lost cell interactions that were affected by the presence of auxin. Previous studies have reported that 2.4-D stimulated cell elongation by increasing the plasticity of the cell wall to become loosen, allowing water to easily flow to the inner cell by osmosis, causing the cell to become elongated [3○]. Thus, friable calluses contained a lot of water because the cell wall had not yet reached lignification, and the cells could be easily separated from each other. A friable callus from an explant had loosen cell interactions and could be easily detached using tweezers [24].

Compact calluses were produced in this study after 3 weeks of culture and at the third and fourth subcultures. Compact calluses are composed of tight cells that are difficult to separate, a relatively whitish, light yellowish to brownish colour with a smooth surface. Our finding was in agreement with other reports of callus induction for secondary metabolite production [31]. Based on colour and structural morphology, the calluses obtained from the present experiment were mostly non-embryogenic (compact calluses). Thus, the calluses presented here were suitable for metabolite secondary production.

Following the study of light, sucrose concentration and subculture on callus morphology, we also evaluated their effects

on callus growth. The effects varied according to the concentrations added (Tables 2 and 3). In the second and fourth week, the addition of 5% sucrose in dark conditions and 3%–5% sucrose in light conditions gave the highest dry weight. The 2%–5% sucrose treatment under light conditions and 3%–5% sucrose treatment under dark conditions exhibited the highest dry weight at the sixth week.

Callus growth increased from the second to sixth week under dark conditions using 5% sucrose in both dark and light conditions since exogenous carbon source was the main energy sources for explants. This was in accordance with previous studies [30,32]. For the first time, the explant was unable to manufacture its own food, which was called as heterotrophism in nature [33]. The addition of sucrose increased the growth of the callus because sucrose would increase cell respiration, causing in an increase of callus biomass. Low-sucrose concentrations decreased the respiration rate and nitrogen absorption. Thus, the energy supply decreased, and protein synthesis was inhibited because the source of nitrogen decrease [34].

Furthermore, comparing callus growth in light and dark conditions, calluses in light conditions grew faster than in dark conditions at the same sucrose concentration. Our results showed that light influenced explant metabolism related to photosynthesis. Light induced callus cells with photosynthetic pigments that made the callus autotrophic. Hence, calluses might synthesize the primary or secondary metabolites. This result agreed with other reports claiming that the light condition might affect photosynthetic pigments that influenced growth and development of calluses [35–39].

Further, subculture of calluses was essential to ensure optimal growth and development. The known effects of light and subculture on callus growth are varied. Narayanaswamy [40] recommended that calluses be subcultured every 4–6 weeks. In this study, subculture was carried out every 2 weeks up to four total subcultures, and this significantly enhanced callus growth with no morphological variations, and the calluses started to brown during the third week. Among the four subcultures, the second subculture had the highest dry weight. Suman et al. [41] reported that, after 6–8 weeks of inoculation and subculture, the calluses became yellowish, compact, hard and nodular when pedicel and inflorescence parts of safed musli were used as explants. Hence, subcultures had to be carried out before the cultures entered the stationary phase in order to maintain the cell lines for prolonged durations and in a healthy condition. For the duration of the exponential phase, acceleration of cell growth and proliferation was evident since the highest number of cells was in metaphase [42]. Exhaustion of nutrients in the culture medium, drying of solid media or concentration by evaporation of liquid media, accumulation of toxic by product, tissue metabolites or dead cells, and oxygen depletion were the growth-limiting factors for *in vitro* cultures during the stationary phase [42,43].

The variation of the chemical constituents in the studied subcultures was remarkable. The nature and extent of the chemical constituents varied from subculture to subculture. Factors such as media, temperature, light, growth-regulating substance, and carbon source also affected the secondary metabolite profiles of calluses [40]. Some of the chemicals detected from gandarusa calluses in this study had pharmacological activities, bolstering the evidence for its use as herbal medicine. Taraxasterol, a major compound in the first and fourth subcultures light condition and first-third of dark condition, had been reported to possess strong antibacterial, antiviral, anti-inflammatory and anti-apoptotic functions [44,45]. Palmitic acid had anti-inflammatory, antidiabetic, antibacterial and antioxidant activity [44]) and 2-methoxy-4-vinylphenol which was highly observed in the second subculture was used as antimicrobial and antioxidant [44]). Moreover, monoplex D was enriched in the third subculture was also used as antioxidant and antimicrobial [44]).

Palmitic acid was also the main compound in the leaves and callus [20,46]. Taraxasterol was a major compound in arnica, burdock, chicory and dandelion. The distribution of taraxasterol in plants was not extensive, but the biological activity of this compound was very interesting [45]. This finding was the first to report that taraxasterol was present in calluses gandarusa. Overall, our results indicated that gandarusa calluses could be used as an alternative biotechnological resource for obtaining bioactive compounds.

5. Conclusions

In this paper, we have described the effects of light, sucrose concentration and repetitive subculture on callus growth of gandarusa (*Justicia gendarussa* Burm.f. Our results strongly suggested that 2% sucrose under light conditions could be used for callus initiation, while 3% sucrose could be used for callus subculture. This finding was a starting point for further investigation and for enhancing the production of secondary metabolites in *in vitro* callus cultures for future use in pharmaceutical applications. Additionally, the establishment of callus cultures was recommended because they could be a source of higher amounts of bioactive metabolites.

Data availability

Complete GC/MS (spectra) data is as supplementary data.

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Declaration of competing interest

No conflict of interest exists.

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