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# Research Article
# Effect of Sucrose and Immersion Frequency on Production of Adventitious Roots and Secondary Metabolites of *Gynura procumbens* (Lour.) Merr in Temporary Immersion Bioreactors

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## Abstract

**Background:** *Gynura procumbens* was usually used as traditional medicine in Indonesia, such as inflammation diseases, diabetes, cancer and hipertency. Production of biomass and secondary metabolite of this plant have potential to fulfill raw materials in pharmaceutical industry but harvesting of roots is destructive for the plants. This study was conducted to enhance biomass and secondary metabolite of adventitious roots in temporary immersion bioreactors. **Materials and Methods:** Adventitious roots of *G. procumbens* were cultured on liquid MS medium supplemented with various concentration of sucrose (1, 3 and 5%) and various immersion frequency (15 min each 12 h; 5 min each 3 h). Cultures were maintained for 21 days and fresh weight, dry weight and secondary metabolite profile were measured at the end of culture. Secondary metabolites were analyzed by Thin Layer Chromatography (TLC), TLC-scanner and Gas Chromatography-Mass Spectrophotometry (GC-MS). **Results:** The highest increasing of adventitious roots biomass were achieved on MS medium supplemented with 5% sucrose and immersion frequency 15 min each 12 h. Detection by TLC resulted two spot with different Rf ($Rf_1$ and $Rf_2$). Detection of maximum wave length by TLC-scanner resulted spectrum with $\lambda_{max}$ of band II is 261-270 nm and shoulders band I is 302-314 nm. These wavelength range was suspected of isoflavone group were corresponding from flavonoid compounds. The GC-MS analyzed showed that all treatment including adventitious roots *ex vitro* have volatile compound, which were known as adipic acid and bis (2-ethylhexyl) ester. **Conclusion:** Biomass production and secondary metabolite of adventitious root could increased significantly in temporary immersion bioreactor, so this technology have potential to develop in large scale.

Key words: *Gynura procumbens* (Lour.) Merr., temporary immersion bioreactors, isoflavon, flavonoid, adventitious roots, medicinal plant, secondary metabolite

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Gynura procumbens* (Lour.) Merr is a traditional medicinal plant which was used in Indonesia, Malaysia, Thailand and another region of South East Asia[1]. Ethyl acetate fraction of leaves have potential as co-chemotherapy agent[2], leaves and callus have antioxidant activity[3,4] and flavonoid of its plant have activity as vasodilatation[5,6]. This plant contain many compounds which useful as source of medicine like flavonoid, saponin, tannin, alkaloid, terpenoid and sterol glycoside[7]. Many plant secondary metabolite of interest are accumulated in roots. Harvesting of roots is destructive for the plants. Beside that, production of secondary metabolite is generally higher in differentiated tissue. So, there has been increasing interest in developing adventitious roots from medicinal plant species in *in vitro* culture. Many researcher used adventitious roots to produce secondary metabolite in liquid culture, such as in *Glycyrrhiza uralensis* Fisch, *Eurycoma longifolia*, *Hypericum perforatum*, *Prunella vulgaris* L. and *Eleutherococcus koreanum* Nakai[8-12].

Liquid culture method has many advantage but has problems such as on less oxygen (asphyxia) and hyperhidricity because of long immersion[13,14], so growth explants were limited. Temporary immersion bioreactor could solved problems by way of the immersion frequency. Immersion frequency was suggested is 5-10 min immersed and 1-12 h frequency[14,15]. Temporary Immersion System (TIS) have been used to improve biomass and secondary metabolite content, such as saponin in *Panax ginseng*[16], saponin in *Talinum paniculatum*[17], betalains in *Beta vulgaris*[18] and secoiridoid glycoside in *Centaurium maritimum*[19] but in *Gynura procumbens* had not been done yet. Concentration of sucrose, in many cases could influence production of biomass and secondary metabolite, such as adventitious roots culture of *Morinda citrifolia* L.[20] and *Iris germanica*[21]. In this study, we want to know the effect of sucrose and immersion frequency on biomass production and secondary metabolite of adventitious roots of *Gynura procumbens* (Lour.) Merr in temporary immersion bioreactors. We expected concept of temporary immersion system can be further efficiently extended to a large-scale volumes.

## MATERIALS AND METHODS

**Materials:** *Gynura procumbens* (Lours.) Merr was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java, Indonesia. Adventitious roots was obtained from leaves, which were grown in MS (Murashige and Skoog) medium supplemented Indol Butyric Acid (IBA) 5 mg $L^{-1}$, sucrose 3% and agar 8 g $L^{-1}$.

**Induction of adventitious roots:** Leaves of *G. procumbens* were sterilized by clorox 10% (v/v) for 5 min and were rinsed by steril aquadest three times, then cut 4 $cm^2$ and were planted in MS[22] supplemented with IBA 5 mg $L^{-1}$ and sucrose 3%. Cultures were maintained in dark condition at 25±3°C for 21 days.

**Cultivation of adventitious roots in temporary immersion bioreactor:** Twenty first old of adventitious roots were weighed 0.5 g and were sterilized with clorox 2% (v/v) for 2 min and were rinsed by sterile distilled water 3 times, then were used as an early inoculum. Two hundred milliliters liquid MS medium containing Indol Butyric Acid (IBA) 5 mg $L^{-1}$ were used to cultivation of adventitious roots in temporary immersion bioreactor. Medium were set at pH 5.8, conductivity 4.85 mS $cm^{-1}$. There are six bioreactor which have combination treatment of immersion frequency (15 min each 12 h; 5 min each 3 h) and various concentration of sucrose (1, 3 and 5%). Treatments were replicated 4 times. Culture were incubated at 25±3°C using 3 h light (General electric cool white fluorescent tubes) and 21 h in the dark for 21 days. Temporary immersion bioreactor were designed by modification of BIT® (Fig. 1)[23,24]. Physic condition of medium (pH, total sugar and conductivity) were measured before sterilized, early cultivation and the end of cultivation.

Fig. 1: Temporary immersion bioreactor modified from[23] BIT®

Total sugar were measured using hand refractometer (Atago, Master10T), whereas the conductivity were measured using conductometer (Ezdo, Condo5021).

**Extraction and identification of flavonoid:** Exctraction of flavonoid was done by modification of Wagner and Bladt method[25]. Adventitious roots from every treatment were dried at 50°C for 5 days and then were grinded. One hundred gram of dry roots were immersed in 10 mL ethanol (Merck) and were heated at 60°C for 5 min and then were filtered. Extracts were concentrated until 2 mL and then were analyzed by thin layer chromatography. Ethanol extract were taken 900 µL and were added 100 µL aquadest so the final volume of 90% ethanol extract was 1 mL. Twenty µL of each sample were spotted on silica gel 60 $F_{254}$ (Merck) and eluted using ethyl acetate: acetic acid glacial: formic acid: aquades (100:11:11:26). Spots were identified using UV-254 and UV-366 (Camag) and then were analyzed using TLC Scanner (Shimadzu CS-930) to determine of Rf value, absorbancy value and $\lambda_{max}$ of each sample.

**Analysis of flavonoid using GC-MS:** Ethanol extract of adventitious roots of all samples were analyzed using GC-MS. These analysis were performed on an Agilent 6980 N GC equipped with Agilent 5973 inert mass selective detector and HP-5 (5% phenyl methyl siloxane) capillary column (30 m× 0.32 mm; 0.25 µm film thickness). The inlet temperature was 29°C. The oven temperature was programmed from 50 (held at this temperature for 2 min) to 100°C at the rate of 2°C $min^{-1}$ and then programmed from 100-256°C at a rate of 5°C $min^{-1}$. The carrier gas was Helium at a flow rate of 1.4 mL $min^{-1}$. Ion source and quadrupole temperatures were maintained at 230 and 150°C, respectively. Each samples (5 µL) was injected with solvent delay for 7 min. Further identification of compounds was done by comparing their mass spectra with those of the Wiley 8.0 version spectra data. Mass spectral data were acquired in the scan mode in the m/z range 40-450 amu.

**Statistical analysis:** Biomass of adventitious roots data were analyzed using statistical software program (SPSS 19). Each mean value represented the replicate of four determinations were analyzed using Kruskhal-Wallis test ($p<0.05$) and to determine the significant difference between treatments were then analyzed using Mann-Whitney test.

## RESULTS AND DISCUSSION

**Biomass production:** Statistical analysis of biomass data showed that there was a significant differences on fresh weight and dry weight of adventitious roots in each treatment. Differences of initial inoculum, fresh weight and dry weight of adventitious roots of *G. procumbens* data were showed in Fig. 2. The fresh weight of adventitious roots in the treatment of immersion frequency 15 min each 12 h and 5% sucrose (T1S50) not significant differences with treatment of immersion frequency 15 min each 12 h and 3% sucrose (T1S30), whereas dry weight in the treatment of T1S50 was not significant differences with treatment of T1S30 and T2S50 (immersion frequency 5 min each 3 h and 5% sucrose). The highest biomass of adventitious roots were showed in treatment of T1S50, even in fresh weight and dry weight.

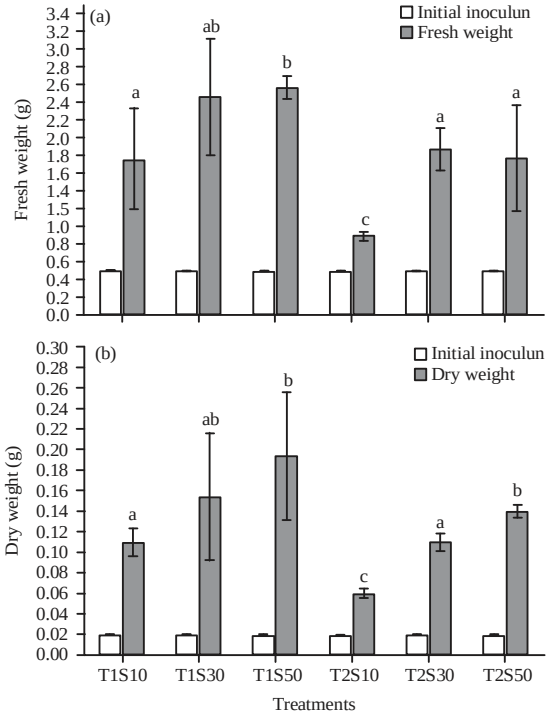



Fig. 2(a-b): Comparison of adventitious roots biomass of *Gynura procumbens* in various treatments after cultivation for 21 days, (a) Fresh weight and (b) Dry weight, T1S10: Immersion frequency 15 min each 12 h, supplemented with 1% sucrose, T1S30 : Immersion frequency 15 min each 12 h, supplemented with 3% sucrose, T1S50: Immersion frequency 15 min each 12 h, supplemented with 5% sucrose, T2S10: Immersion frequency 5 min each 3 h and supplemented with 1% sucrose, T2S30: Immersion frequency 5 min each 3 h and supplemented with 3% sucrose, T2S50: Immersion frequency 5 min each 3 h and supplemented with 5% sucrose

Morphology of adventitious roots in bioreactor changed forming aggregate in all treatments compare with initial inoculum. It was caused by formation of roots branch and callus in around the roots branch. Growth of roots hair were occurred in adventitious root tip, white color and its condition were found in two treatment, there are medium with supplemented 5% sucrose and immersion frequency 15 min each 12 h and immersion frequency 5 min each 3 h (Fig. 3/T1S50 and T2S50). In this experiment was also found phenomenon of color changed of adventitious roots in many treatment, especially in medium with supplemented 1% sucrose and immersion frequency 15 min each 12 h (Fig. 3/T1S10). In that treatment much more adventitious roots have red color than other treatments. Appeared of its color also happened in adventitious roots wild type. Wild type of adventitious roots which growth at the upper soil have violet color, whereas adventitious roots which growth in under soil have white color (Fig. 3b).

Physic condition of medium in bioreactor was investigated before sterilization, initial cultivation and final cultivation. Results showed that during sterilization, pH, total sugar and conductivity value medium changed. This phenomenon was assumed that it caused by medium which was supplemented with sucrose and without agar. In this study, the pH value constant at 5.3 in the early cultivation until the end of cultivation, except in treatment of T1S50 and T2S10 (Fig. 4a, b). The constant pH could maintain ion balance in the plant cell, so this condition can increase

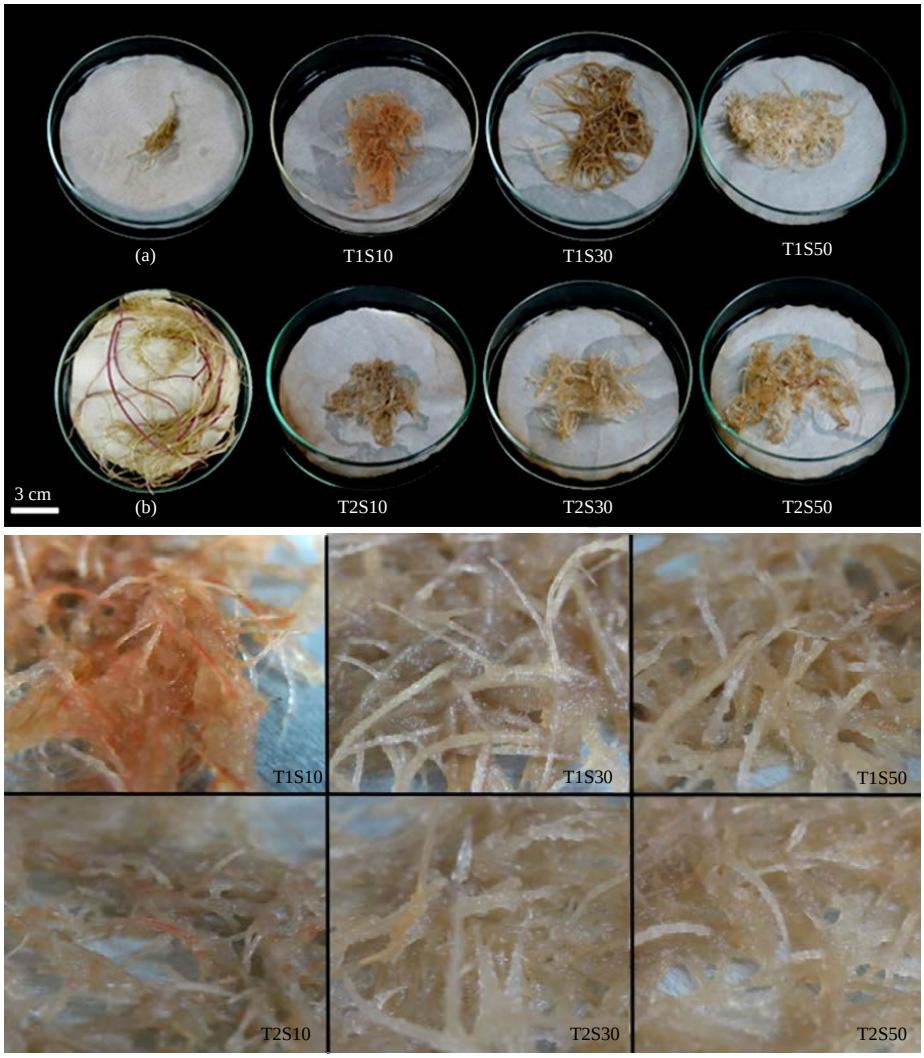



Fig. 3(a-b): Yield and morphology of adventitious roots after cultivation in temporary immersion bioreactor for 21 days, (a) Initial inoculum and (b) Adventitious roots *ex vitro*

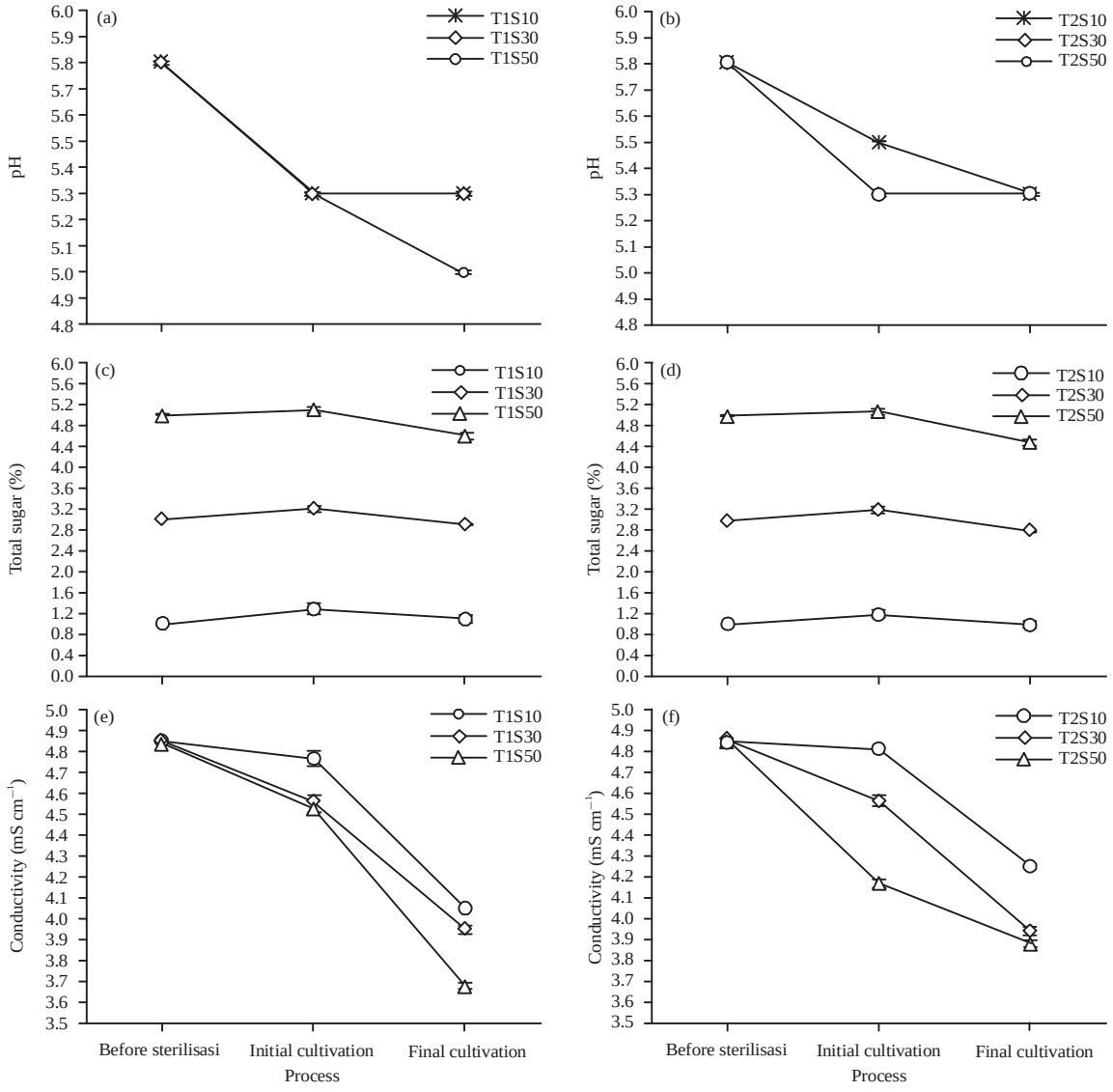



Fig. 4(a-f): (a and b) Change of pH, (c and d) Total sugar and (e and f) Conductivity medium during cultivation in various process level, (a, c, e) immersion frequency 15 min each 12 h and (b, d, f) immersion frequency 5 min each 3 h

growth of the adventitious roots. Total sugar in medium increase after sterilization (Fig. 4c, d). During sterilization, sucrose were hydrolyzed to glucose and fructose, so the monosaccharide compound can increase of total sugar in medium. Sucrose in medium will be hydrolyzed 10-15% to monosaccharide compound in pH 5.5-5.8. Decreasing of total sugar was found in the end of cultivation. Especially in treatment of T1S50 and T2S50, decreasing of total sugar occurred drastically.

Changed of medium conductivity also occurred after sterilization. During cultivation, in all treatments medium conductivity decrease drastically, especially in medium which were supplemented with 5% sucrose (T1S50 and T2S50) (Fig. 4e, f). Decreasing of the conductivity was assumed because of the high concentration of sucrose. Absorption of inorganic ion from the medium will be higher when the concentration of sucrose increase (Thorpe in 2008). The pH medium (5.3) also optimize the absorption of inorganic ion, so the growth of adventitious roots of *G. procumbens* increased significantly.

**Secondary metabolite profile:** Identification of spots using UV light at $\lambda_{366}$ and $\lambda_{254}$ showed two spots with different Rf value, there are $Rf_1 = 0.85$ and $Rf_2 = 0.64$. Spot analysis of each Rf using TLC scanner obtained $\lambda_{max}$ for each spot were 261-270 nm (band II) and 302-314 nm (band I). Based on its

Table 1: Identification of compounds in ethanol extract of adventitious roots using GC-MS compared with Wiley spec data (Wiley version 8.0)

| | | | | T2S10 | | T2S30 | | T2S50q | | T1S10 | | T1S30 | | T1S50 | | *Ex vitro* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peak No. | RT (min) | Mol. Ion (m/z) | Compounds | % | Qly | % | Qly | % | Qly | % | Qly | % | Qly | % | Qly | % | Qly |
| 1 | 8.7 | 121 | S-collidine | - | - | 1.7 | 94.0 | - | - | - | - | - | - | 3.6 | 91 | - | - |
| 2 | 18.7 | 128 | Naphthalene | 21.7 | 93 | 13.5 | 94 | 19.9 | 91 | 8.9 | 91 | 25.3 | 94 | 26.1 | 91 | 18.6 | 90 |
| 3 | 31.8 | 204 | Trans-caryophyllene | - | - | - | - | - | - | - | - | - | - | - | - | 1.6 | 95 |
| 4 | 33.0 | 204 | α-Humulene | - | - | - | - | - | - | - | - | - | - | - | - | 1.9 | 97 |
| 5 | 35.4 | 204 | δ-cadinene | - | - | - | - | - | - | - | - | - | - | - | - | 1.8 | 98 |
| 6 | 37.0 | 200 | Lauric acid | - | - | - | - | - | - | - | - | 1.7 | 91 | - | - | - | - |
| 7 | 44.8 | 270 | Metil palmitate | 3.4 | 98 | 2.4 | 99 | 3.3 | 98 | 1.7 | 98 | 4.2 | 98 | 5.4 | 99 | 5.3 | 99 |
| 8 | 45.7 | 256 | Palmitic acid | 32.1 | 99 | 24.8 | 99 | 32.9 | 99 | 17.0 | 99 | 32.0 | 99 | 31.3 | 99 | 27.6 | 99 |
| 9 | 48.8 | 280 | Linoleic acid | 1.1 | 96 | 1.6 | 99 | 2.5 | 99 | 1.1 | 99 | 2.1 | 98 | 3.4 | 98 | - | - |
| 10 | 48.9 | 282 | Oleic acid | 1.4 | 97 | 2.8 | 99 | 2.2 | 99 | 2.4 | 93 | 3.1 | 99 | 2.8 | 99 | - | - |
| 11 | 49.3 | 284 | Stearic acid | 3.3 | 98 | 4.1 | 98 | 4.7 | 99 | 3.7 | 98 | 6.5 | 98 | 5.9 | 98 | - | - |
| 12 | 53.2 | 370 | Adipic acid, bis (2-ethylhexyl) ester | 23.4 | 91 | 42.4 | 91 | 22.4 | 91 | 62.6 | 91 | 6.3 | 91 | 10.5 | 91 | 26.8 | 91 |
| 13 | 55.2 | 212 | Tetradecanal | - | - | 2.4 | 90 | - | - | - | - | - | - | - | - | - | - |
| 14 | 55.2 | 278 | 1.19-Eicosadiene | 3.1 | 90 | - | - | 3.6 | 95 | - | - | - | - | 3.4 | 94 | - | - |

spectrum it was assumed that secondary metabolite type in ethanol extract of adventitious roots was isoflavon glicoside, which appropriate with isoflavon spectrum in reference ($\lambda_{max}$ band II = 245-275 nm and $\lambda_{max}$ shoulder band I = 310-320 nm). Analysis using TLC scanner showed the difference of absorbance in each sample (Fig. 5). The highest absorbance was found in treatment of immersion frequency 15 min each 12 h and sucrose 1%, although biomass in this treatment was low.

Analysis of ethanol extract of adventitious roots using GC-MS (Fig. 6, Table 1) showed 14 compounds which appropriate with library (Wiley version 8.0). Based on GC-MS analysis, ethanol extract of adventitious roots contain of naphthalene and sesquiterpene hydrocarbons, such as trans-caryophyllene, α-humulene and δ-cadinene. Fatty acid compounds were also identified such as lauric acid, methyl palmitate, palmitic acid, linoleic acid, oleic acid and stearic acid, whereas another volatile compounds were identified as adipic acid, bis (2-ethylhexyl) ester, tetradecanal and 1,19-eicosadiene.

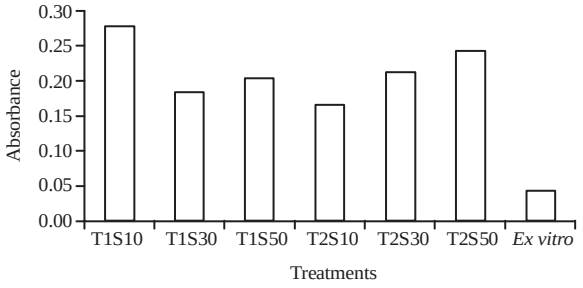



Fig. 5: Absorbance profile of spots on chromatograme analyzed by TLC-scanner. Absorbance was obtained from band II of spot 2 of each treatment

**Effect of sucrose and immersion frequency on production of adventitious roots:** Adventitious roots culture in temporary immersion bioreactor enable explants to direct contact with high concentration of sucrose, so explants did not stress caused by high osmoticum of sucrose. Supplementation of sucrose increased growth of adventitious roots, because increasing of sucrose will be induce cell respiration, so high sucrose could increase respiration of cells and finally could increased growth of adventitious roots. In contrary, adventitious roots in medium supplemented with low sucrose showed the lowest biomass. It could be occurred caused by concentration of sucrose decreased absorption of nitrogen, so protein synthesis in plant cells was low and finally growth of the adventitious roots was achieved in early phase of stationer.

Immersion frequency was also influenced growth of adventitious roots of *G. procumbens* during cultivation, because immersion frequency influenced absorption of nutrient and water for growth process. This study result could increase biomass 5 fold from initial explants better than adventitious roots of *Talinum paniculatum* which obtained the highest biomass 1.5 fold from initial explants[17]. In preliminary study we obtained adventitious root biomass in flask shake culture only 3.9 fold from initial explants. Immersion frequency for 15 min is the best condition to increase biomass production, which was also obtained in hairy roots of *Beta vulgaris* (L.)[18] and in the hairy roots of *Centaurium maritimum* (L.)[19]. Immersion interval 12 h provided oxygen supply much more than immersion interval 3 h. Oxygen supply will increase of aerobic respiration to provide ATP in catabolic reaction such as glucose reduction in glycolysis. Explants in treatment 15 min immersion each

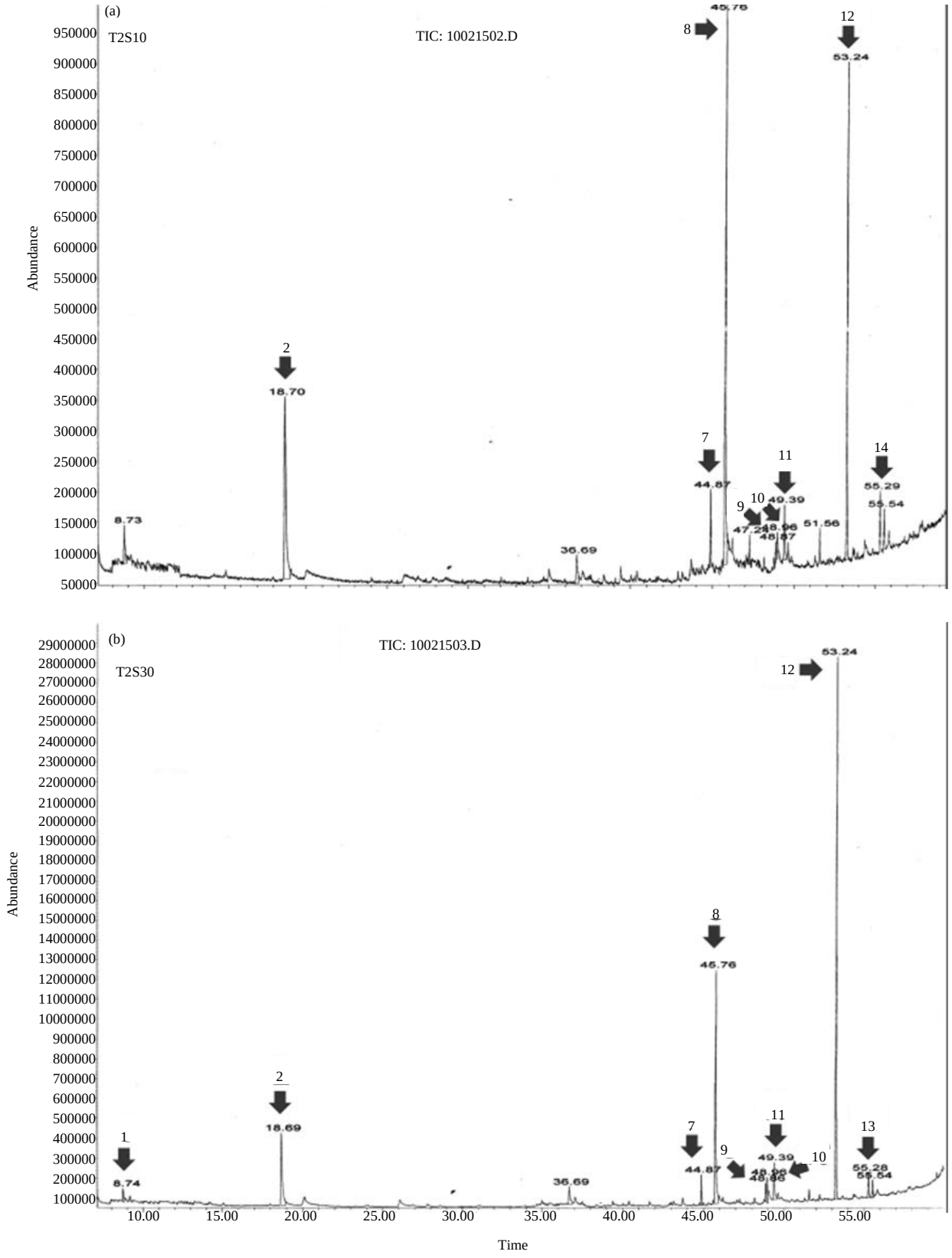
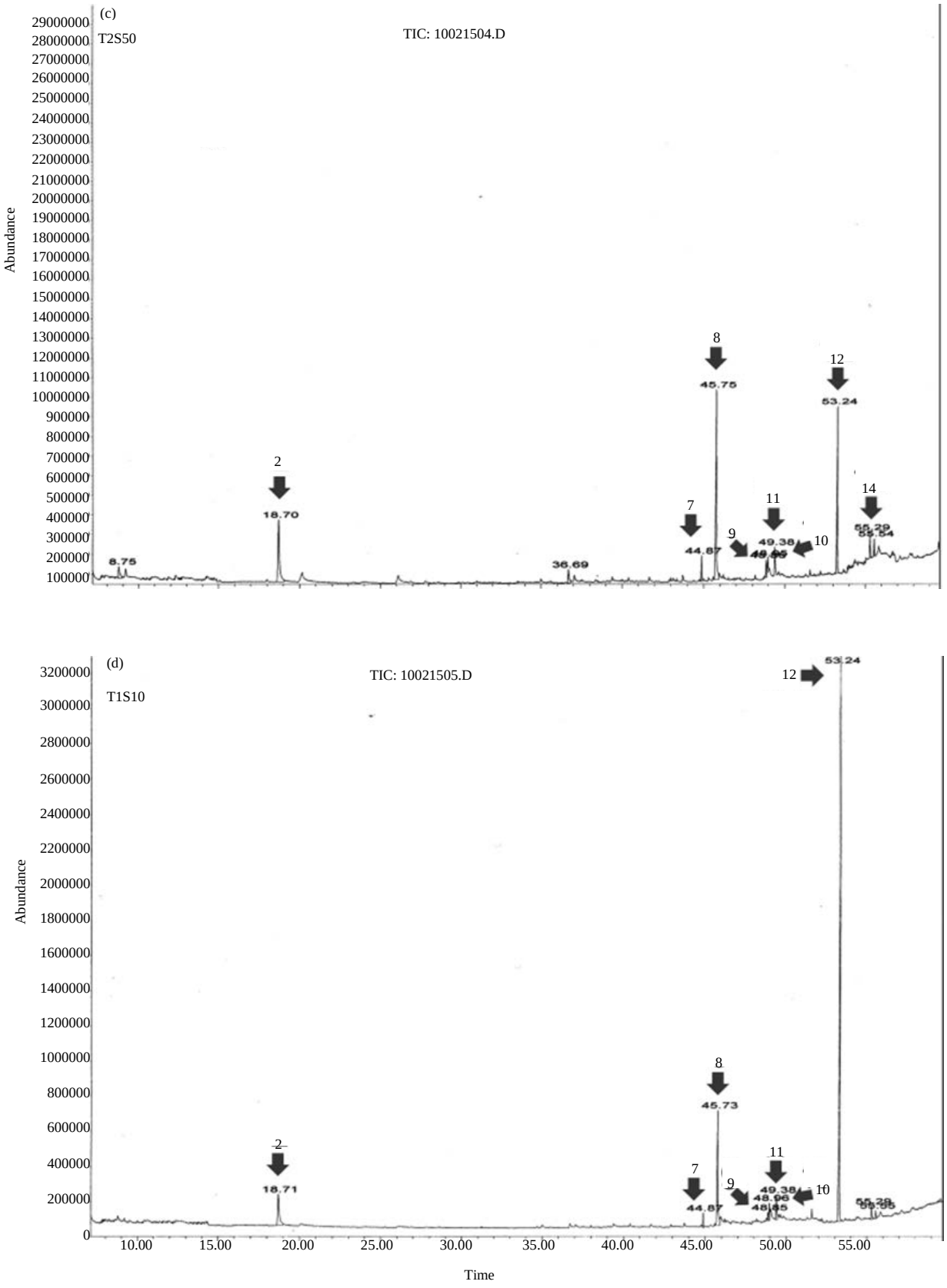



Fig. 6(a-i): Continue

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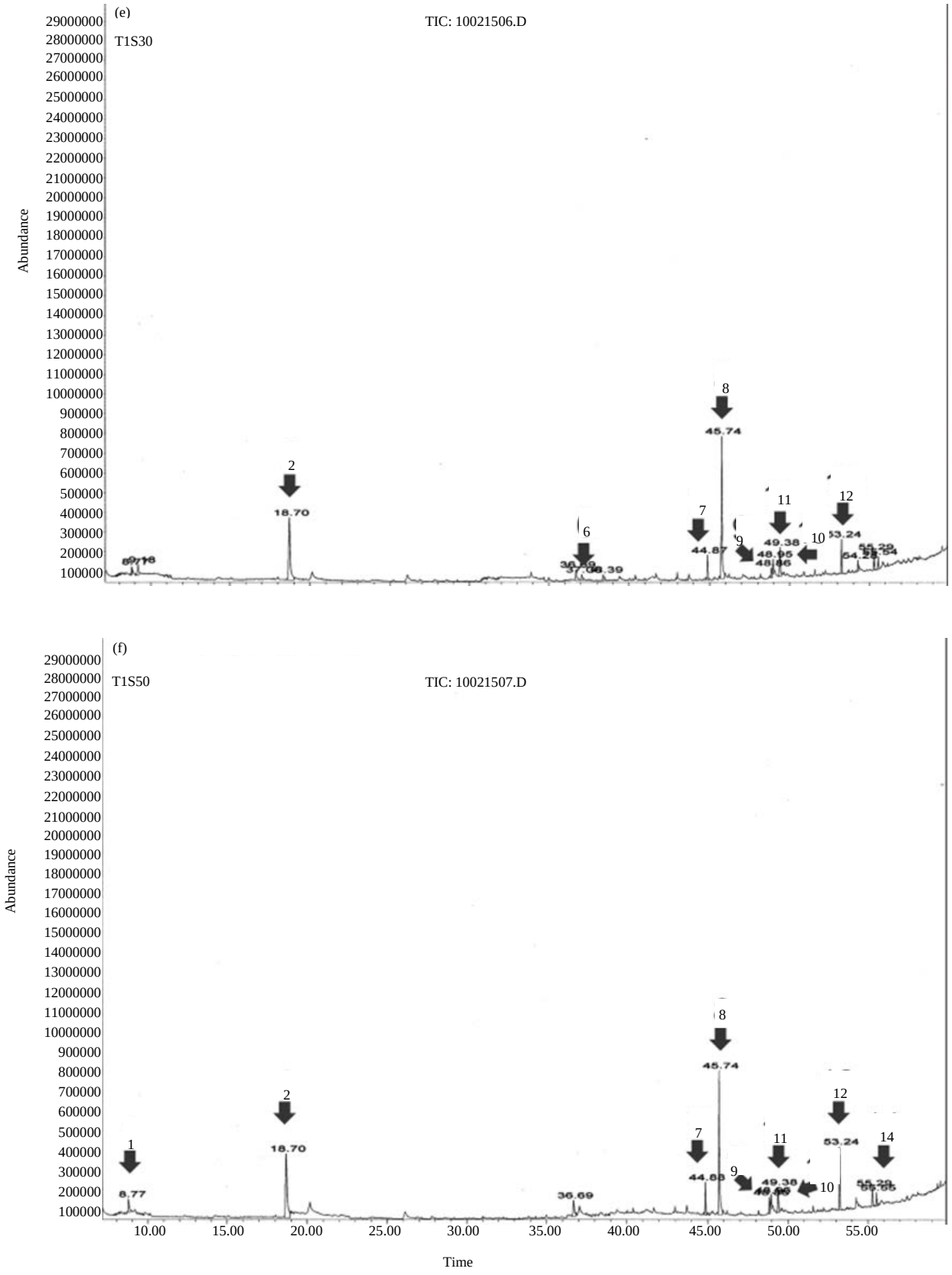


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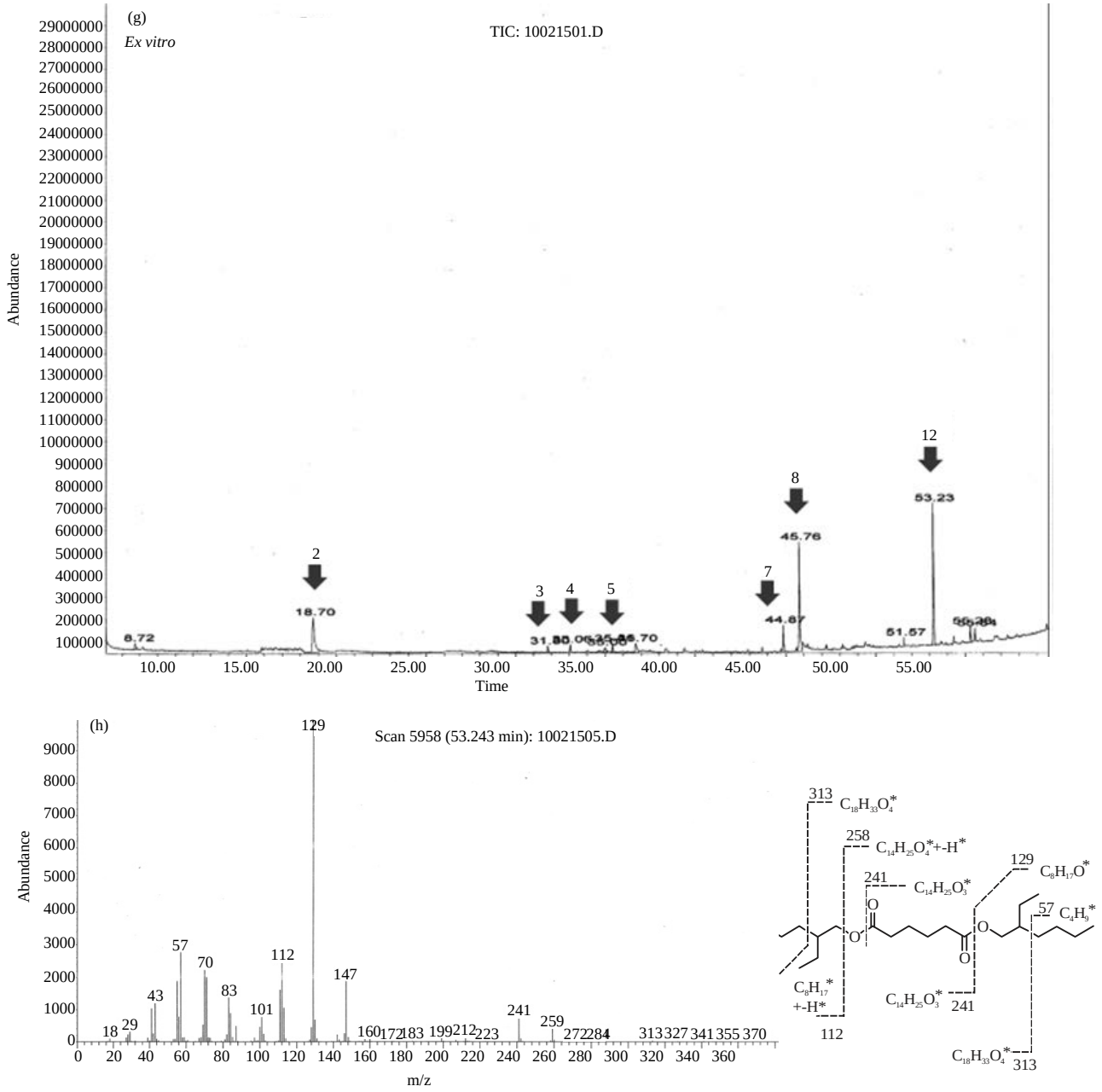


Fig. 6(a-i): GC-MS chromatograme profile of ethanol extract of (a-f) Adventitious roots *in vitro*, (g) Adventitious roots *ex vitro*, (h) Fragmentation profile of peak at retention time of 53.2 m in T1S10 treatment and (i) Adipic acid, bis (2-ethylhexyl) ester. Number and arrow showed the peak number in Table 1

12 h was assumed could absorb nutrient effectively but in immersion time 5 min each 3 h, explants were immersed in liquid medium more often, so the oxygen supply was low.

In the end of cultivation, some of the adventitious roots have red color. Pigmentation of adventitious roots could happened cause by drought condition dan light. Red color of adventitious roots founded in medium with supplemented 1% sucrose, immersion time 15 min each 12 h and. Longer interval of immersion could cause adventitious roots experiencing drought and obtained much more light. In natural condition adventitious roots of *G. precumbens* in the upper soil have violet color but adventitious roots in soil have white color. This phenomenon also happened in adventitious roots of *Raphanus sativus* L. cv. Peking Koushin which have red color[24] and green color in *G. procumbens*[25].

**Effect of sucrose and immersion frequency on production of secondary metabolite:** Sucrose was assumed influence of biosynthesis pathway of isoflavonoid in the metabolism of phenylpropanoid pathway. Sucrose take place as an essential carbon back bone of phenylpropanoid metabolism and signal molecul to increase phenylpropanoid biosynthesis. Another study showed that supplementation of sucrose, fructose and glucose increase genistein and isoflavon in embrio axis of *Lupinus luteus* L., cv Juno[26].

Absorbance value was directly proportional with concentration of compound, so absorbance value was used to determined how much of bioactive compound concentration. In Fig. 5 showed that highest absorbance was found at treatment of sucrose 1%, although biomass in this treatment was low. Ability of sucrose to increase secondary metabolite production was different in each plant species. In adventitious roots culture of *Morinda citrifolia* (L.) anthraquinon content, such as fenolic and flavonoid increased at sucrose 1% treatment[20]. Accumulation of fenolic, flavonoid, chlorogenic acid and hypericine total at *Hypericum perforatum* increased caused by osmotic stress in the sucrose 5, 7 and 9% treatments[11]. Osmotic stressed caused by concentration of sucrose in the medium also increased of saponin at *Panax notoginseng* and ginsenoside at *Panax ginseng*[27,28]. Increasing isoflavon in this study was assumed play a role in key enzyme signaling of isoflavon synthesis. Its assumed was also occurred in expression of Phenylalanine Ammonia Lyase (PAL) at *Morinda citrifolia* (L.) and this enzyme was also responsible to started fenilpropanoid metabolism[29,30].

Treatment using long immersion interval (12 h) and immersion duration 15 min in low sucrose concentration provided the highest isoflavon content. Adventitious roots could absorb oxygen optimally in glycolysis to produce phosphoenolpyruvate (PEP). The PEP with erythrose-4-phosphat will start shikimic pathway to produce phenilalanine[31] but next research will be important to know and prove role of transduction signal enzyme in its hypothesis.

Information about volatile compound at *G. procumbens* was limited. Research at *Gynura bicolor* founded the volatile compound was group of monoterpen and sesquiterpen hidrocarbon, such as (E)-caryophyllene, α-humulene and bycyclogermcrene, whereas (Z,E)-α-farnesene, (E)-caryophyllene, δ-cadinene and α-copaene was founded at *in vitro* plant[32]. Sesquiterpen hidrocarbon was found more diverse at adventitious root *ex vitro* (wild type) compared with adventitious root *in vitro*, except naphthalene.

Adipic acid and bis (2-etylhexyl) ester have higher concentration in *in vitro* adventitious roots compared with *ex vitro* adventitious roots. Adipic acid was produced in large scale and was used as raw material to make nylon. Tetradecanal and 1,19-eicosadiene only were found at *in vitro* adventitious roots. Tetradecanal was known as myristyl aldehyde and useful as flavoring materials, whereas 1,19-eicosadiene was known as volatile oil which useful as aroma boosters and antibacteria[33]. Increasing of volatile compound in adventitious roots of *G. procumbens* in temporary immersion bioreactors, especially in T1S10 treatment was assumed caused by water stress in the culture environment. Less of water and nutrition can increase production of volatile compounds. Based on previous study, it was known that adipic acid was synthesized from intermediate compound at the aromatic amino acid which originated from glucose which was discharged to PEP and erythose-4-phosphat. Generally, volatyl C6 was synthesized through many biosynthesis pathway, such as biosynthesis pathway of lipoid acid that was preceded by formation of linoleic, shikimic acid pathway, isoprenoid pathway and derivate of amino acid. During metabolism in the shikimic acid pathway was produced adipic acid as an intermediated compound but did not known yet how is the relation between flavonoid biosynthesis and adipic acid in *G. procumbens*.

## CONCLUSION

Biomass production of adventitious roots of *G. procumbens* in temporary immersion bioreactor increased 5 folds from initial explant. Secondary metabolite, especially isoflavon was found in adventitious roots at low sucrose treatment, whereas volatile compound and adipic acid were identified in all treatments. It could be interesting to develop in large scale.

## SIGNIFICANT STATEMENTS

- This study was necessary to develop *in vitro* organ culture in order to fulfill pharmaceutical industry demand
- Gynura procumbens have potential to develop as a source of bioactive compound which was needed for medicine
- Secondary metabolite could be increased through *in vitro* organ culture

## ACKNOWLEDGMENTS

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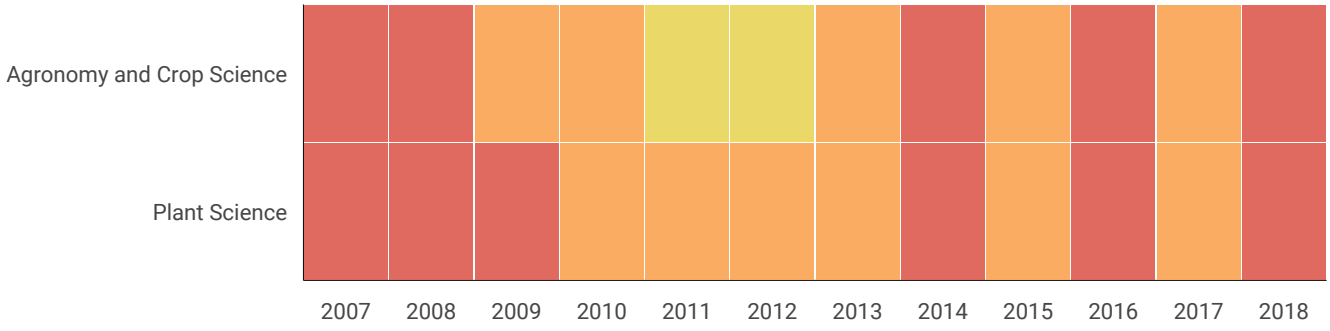


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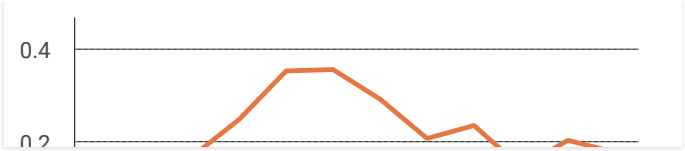


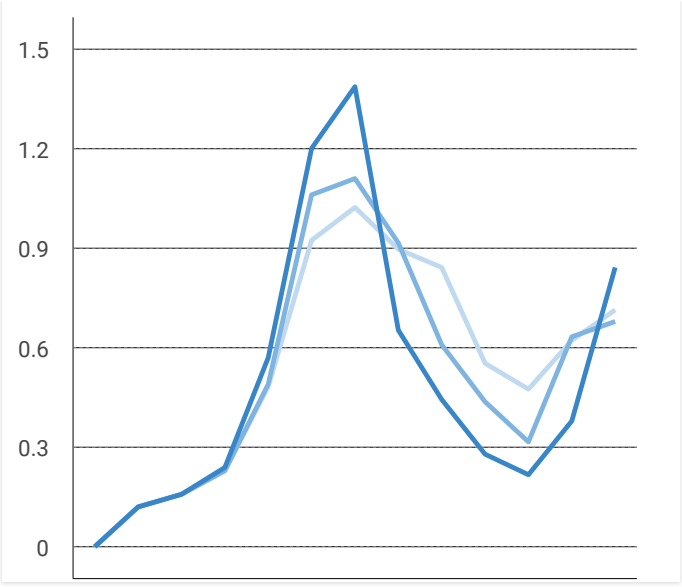


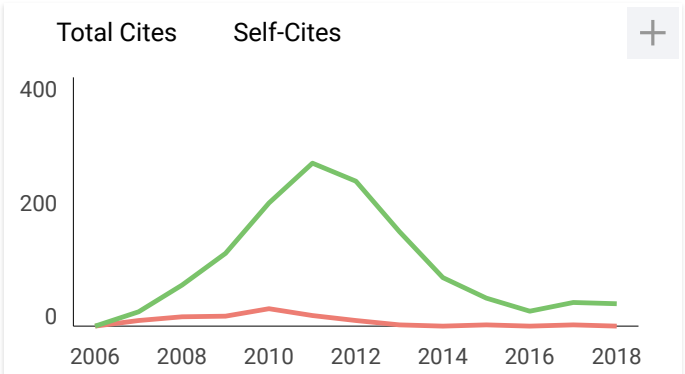


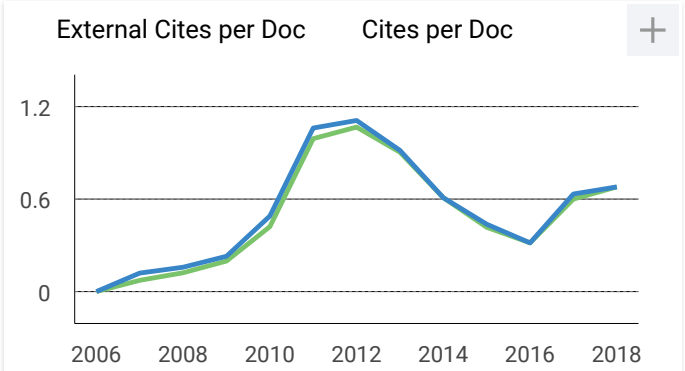


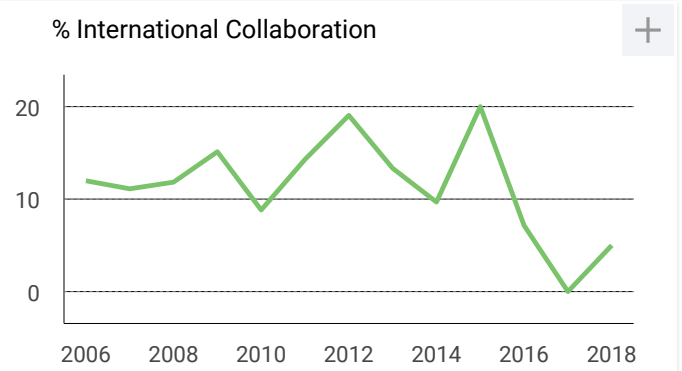


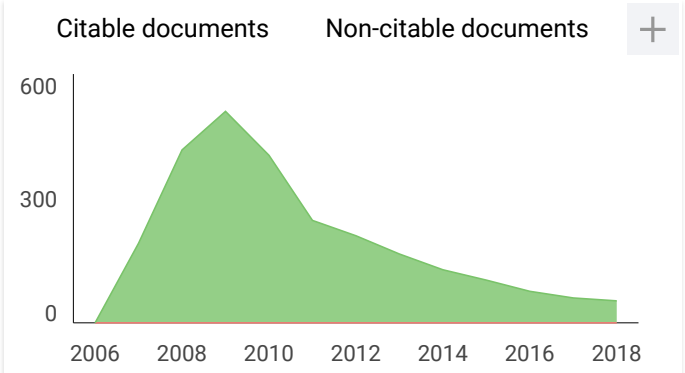


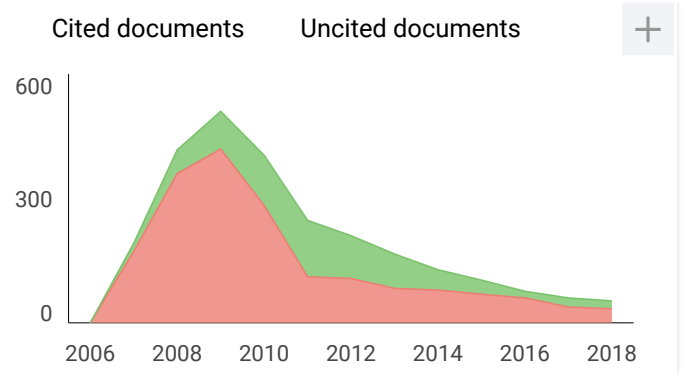



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