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Bioactive Compound and Cytotoxic Analysis of *Premna oblongifolia* Merr. Ethanol Extract on Rat Bone Marrow-Derived MSC-like Cells

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ABSTRACT

The use of natural products in regenerative medicine holds significant promise, especially in regions where traditional medicine is prevalent. This study investigates the bioactivity of the ethanol extract of green cincau leaves (*Premna oblongifolia* Merr.) (GCE) on bone marrow-derived mesenchymal stem cell-like cells (BM-MSCs). The leaves were extracted using the maceration method, and the bioactive compounds were analysed and detected by Gas Chromatography-Mass Spectroscopy (GC-MS). The cytotoxicity tests were conducted across a concentration range of 1–1200 µg/mL using WST-1 reagent, and cell viability was measured at 24, 48, and 72 h. The results of GC-MS indicated 28 bioactive compounds with the dominant groups being terpenoids and fatty acids. The most abundant compounds in GCE were phytol (19.06%), squalene (4.32%), Urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)- (6.23%) and 8,11,14-Eicosatrienoic acid, (Z,Z,Z)- (2.88%). The GCE maintained cell viability above 80% at all concentrations, indicating low cytotoxicity. Furthermore, Alizarin Red S staining revealed calcium deposit formation at 300 µg/mL, suggesting preliminary osteogenic activity. These findings suggest GCE may have potential for supporting BM-MSCs proliferation and demonstrates early osteogenic properties. Further investigation is recommended to explore its mechanism of action and therapeutic potential in differentiation applications.

Keywords: Cytotoxicity, green cincau, MSC-like cells, *Premna oblongifolia* Merr., regenerative medicine.

INTRODUCTION

Plant-based medicines are increasingly gaining attention due to their immunomodulatory effects (Shukla *et al*., 2022), lower risk of side effects, stability, and accessibility, making them viable alternatives to conventional pharmaceuticals in natural healthcare and chronic disease prevention (Dabas *et al*., 2023). According to the World Health Organisation (WHO), approximately 80% of the global population utilises herbal medicine as part of their primary healthcare approach. Additionally, about 25% of contemporary pharmaceuticals are sourced from plant materials largely due to the wide range of bioactive primary and secondary metabolites they produce (Turvey *et al*., 2022). Plants produce a diverse array of secondary metabolites, such as alkaloids, terpenoids, flavonoids, and phenolic compounds, each of which exhibit various biological activities (Bhatti *et al*., 2022).

The use of mesenchymal stem cells (MSCs) in regenerative treatments is one of the latest biomedical advancements. MSCs are multipotent cells that can differentiate into various cell types, including osteoblasts (Zainol Abidin *et al*., 2023), chondrocytes (Li *et al*., 2020) and adipocytes (Ebrahimi *et al*., 2023), making them promising candidates for regenerative medicine and tissue engineering. Bone marrow-derived MSCs particularly are widely studied due to their accessibility and differentiation potential (Asadi *et al*., 2022; Dzobo, 2021; Melo *et al*., 2020). However, ensuring their proliferation, survival, and regulated differentiation in vitro is a significant problem. Identifying bioactive compounds that can assist these processes is critical for developing MSC-based treatments.

Premna oblongifolia Merr. or commonly known as green cincau, is a verbenaceae family plant that is commonly used as a dessert component in Indonesia (Nurdin *et al*., 2018). Green cincau (GC) is known to contain bioactive

compounds that have the potential to have positive effects on health. Aryudhani (2011) reported that GC leaves contain several components of active compounds, including alkaloid compounds, phenol hydroquinone, flavonoids and tannins. These compounds have a wide range of biological activities, including antioxidant, anti-inflammatory, and antitumor activities (Jain *et al*., 2019). Previous studies have shown that GC extract has the potential to treat several degenerative diseases such as cholesterol (Astirani & R, 2012) and high blood pressure (Nurchairina and Aziza, 2020). However, research into its impact on stem cells, particularly MSCs, is currently restricted.

Despite all of the biological activity mentioned above, the cytotoxic effects of this extract on MSCs remain unknown. The use of plants in biomedical applications, a preliminary safety assessment of the chemicals contained is required which limits its potential application in stem cell research and treatment (Jain *et al*., 2018; Morais *et al*., 2010). In vitro cytotoxicity testing is a rapid and reduced costs when contrasted with animal studies approach of assessing the hazardous potential of a chemical (Aslantürk, 2018). Although plant-based medicines are believed to be safe and minimise side effects, extracts with high bioactivity can sometimes have harmful effects on cells at certain concentrations (Anlas *et al*., 2022).

Premna oblongifolia Merr has been used both as food and medicinal products, but no research on the content of phytochemical compounds has been conducted so far. This study contributes to investigating the chemical substances that are beneficial in the field of natural product-based regenerative medicine. Considering that several studies have reported the osteogenic potential of flavonoids, which are also contained in green cincau, in promoting the differentiation of MSCs into osteogenic cells. Therefore, based on this potential, we focused on osteogenesis differentiation as preliminary research on differentiation ability. Additionally, Cytotoxic testing on the ethanol extract of *P. oblongifolia* Merr. provides information on cytotoxicity levels and adds to the potential bioactivity of the extract specifically on MSCs, this may pave the way for its use in promoting osteogenic differentiation or other therapeutic applications. By addressing this gap, this study aimed to assess the cytotoxicity of ethanol extract of green cincau leaves on bone marrow-derived MSC-like cells, providing baseline data that may inform future research on its application in stem cell-based treatments.

MATERIALS and METHODS

Preparation of Green Cincau Extract (GCE)

The leaves of *Premna oblongifolia* Merr. were collected from Lamseupeung village, Banda Aceh city (Indonesia) in March 2023. Plant identification was confirmed by Dr. Saida Rasnovi, S.Si., M.Si, taxonomist at the Herbarium of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, and verified by the official identification letter (Letter No. 758/UN11.F8.4/TA.00.03/2025). The collected leaves were cleaned and dried at room temperature. The dried leaves were ground to a fine powder for extraction. Approximately 340 g of the powdered leaves was extracted using the maceration method with 96% ethanol as the solvent with 1:10 ratio for 3×24 hr. The extract was concentrated using a rotary evaporator to remove the solvent. The extraction yield of GCE was 6.97% (w/w), with approximately 23.7 g of concentrated extract. The concentrated extract was stored at 4°C until further use. For the analyses, the extract was diluted in ethanol to prepare a stock solution of 100 mg/ml. The working concentrations used for the cytotoxic experiments ranged from 1 to 1200 µg/ml.

Gas Chromatography-Mass Spectrometry (GC-MS)

The volatile compounds present in GCE were analysed using Gas Chromatography–Mass Spectrometry (GC-MS) (GCMS-QP2010, Shimadzu, Japan), employing a capillary column (30 m×0.25 mm ID×0.25 mm). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injection volume was 1 µl, with the injector temperature set at 250°C in splitless mode. The temperature was programmed as follows: initial temperature at 50°C for 10 min, increased to 150°C at 5°C/min, then to 280°C at 10°C/min resulting in a total run time of approximately 59 min. Mass spectra of each chromatographic peak were analysed using GCMSsolution software (Shimadzu) and automated matching against reference spectrum libraries including the National Institute of

Standards and Technology (NIST) NIST17-1 version, W11main and FFNSC.

Collection, Isolation and Culture of BM-MSC like Cells

Male rats (150‑200 g) were euthanized via cervical dislocation, following ethical approval granted by the Committee of Veterinary Ethics, Faculty of Veterinary Medicine, Universitas Syiah Kuala (Reference number: 308/KEPH/VII/2024). Post-euthanasia, the femurs and tibias were excised, thoroughly rinsed with physiological saline to remove residual blood, and sterilized with 70% ethanol. Bone marrow was collected using the flushing method according to Lu *et al.* (2022). Briefly, a syringe filled with complete growth medium supplemented with 1% antibiotics and used to flush the marrow out of the bone cavity. The medium was prepared using Dulbecco's Modified Eagle's Medium (DMEM) high glucose with pyruvate and L-glutamine, supplemented with 15% fetal bovine serum (FBS) (Himedia, India), 1% antibiotic-antimycotic solution, and 1% non-essential amino acids (NEAA) (Sigma, USA). The cell suspension was centrifuged at 2000 rpm for 10 min. The pellet obtained was washed three times with phosphate-buffered saline (PBS) (Himedia, India) supplemented with 1% antibiotics (Sigma, UK). The number of cells were determined using a hemocytometer with 0.4% trypan blue staining (Sigma, USA) using this following equation (Abdul Aziz *et al*., 2022), Eq.(1):

$$Total\ cells\ (cell/mL) = \frac{Number\ of\ live\ cells\ on\ 4\ squares}{4} \times dilution\ factor\ \times 10^4 \quad Eq.(1)$$

The cells were seeded into 25 cm^2 culture flasks containing complete growth medium and incubated at 37°C with 5% CO_2. Non-adherent cells were removed on the fourth day of culture by washing with PBS, and the medium was replaced every three days. Cellular morphology was observed regularly using an inverted microscope (Olympus CKX41, Tokyo, Japan). When cell confluency reached 80%, subcultures were initiated using 0.25% Trypsin-EDTA (Gibco, USA).

Cytotoxicity Assay

The viability of cells was assessed using Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) following manufacturer's instruction and as described by Gharbaran *et al*. (2021). Briefly, after incubating the MSC-like cells (1×10^4 cells/well) on 96-well plate for 24 hr at 37°C under 5% CO_2, the medium was replaced with complete medium (100 µl) containing 1% GCE with various concentrations (1, 5, 10, 20, 40, 80, 150, 300, 600 and 1200 µg/ml). The cells were incubated in three exposure times, namely 24, 48 and 72 hr. After the cells were exposed to the extract for a predetermined time, the medium was replaced with medium containing 10 µl WST-1 and incubated for 3 hr. 70 µl of the medium was transferred into a 96-well plate with flat bottom, and the absorbance was measured at 450 nm using a microplate reader (EZ Read 400 Biochrom, UK). The percentage of cell viability can be determined using the following formula (Widiandani *et al*., 2023), Eq. (2):

$$Cell\ viability\ (\%) = \frac{Abs.treatment - Abs.blank}{Abs.control - Abs.blank} \times 100\% \quad Eq.(2)$$

Cell Differentiation

0.055×10^6 cells/well at third passage were seeded in 24-well plate and allowed to reach 80% confluence. Cells were then treated either in GCE medium which was growth medium supplemented with GCE at the concentration of 0, 150, 300, and 600 µg/ml or in osteogenic medium consist of growth medium supplemented with 0.1 µM dexamethasone (GLPBIO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, USA) and 50 µM ascorbic acid (Merck, Germany) (Bochon *et al*., 2021). Morphological examination using a microscope was carried out on days 3, 7, 11 and 14.

Alizarin Red Staining (ARS)

Staining of mineralisation was done with ARS after 14 days of treatment. Cells were washed with PBS twice and fixed in 10% formaldehyde for 30 min at room temperature. Then, the cells were stained with 2% ARS (Merck, Darmstadt, Germany) for 30 min and rinsed twice with PBS to remove any remaining staining. The cells were observed and captured by inverted microscope (Olympus CKX41, Tokyo, Japan).

Data Analysis

All experiments were done in triplicate and data were expressed as means ± Standard deviation (SD). Data were analysed using One Way

Analysis of Variance (ANOVA). P<0.05 was considered as significant difference.

RESULTS and DISCUSSION

Chemical Composition of GCE

According to the analysis of GC-MS shown in Figure 1, there were 28 peaks of different compounds in the sample (Table 1). The chromatogram was dominated by two consecutive peaks (6th and 7th peaks) as 2,4,6,8-Tetramethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocane which identified as siloxane compound. It should be noted that this compound may represent artifacts from laboratory equipment or column bleeding during GC-MS analysis rather than true plant metabolites. Siloxane has been reported as a common laboratory contaminant in GC-MS studies (English, 2022; McMaster, 2008). Therefore, in line biological significance of these compounds should be interpreted with caution. Thus, these siloxane peaks were not considered genuine metabolites of *P. oblongifolia* and were excluded from our biological interpretation.

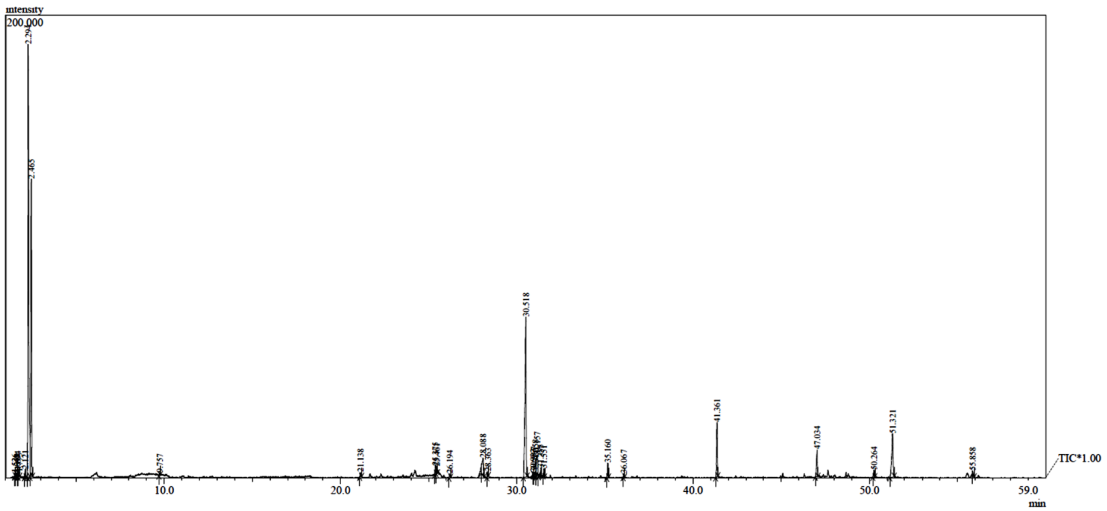

Figure 1. GC-MS chromatogram of green cincau ethanol extracts

After neglecting these artefacts, the most abundant and biologically relevant compounds were terpenoids (23%) and fatty acids (19%) (Figure 2). The chemical structures of the major identified phytochemicals are shown in Figure 3. The existence of terpenoids is particularly important, as this class of natural compounds is increasingly recognised for its potential in the osteoporosis treatment including bone health (Zhuo *et al*., 2022). In addition, certain terpenes have been identified as bioactive agents capable of directly influencing the fate and function of mesenchymal stromal cells (MSCs) (Mazzone *et al*., 2025). This may provide a convincing explanation for their role in the biological activities we have observed.

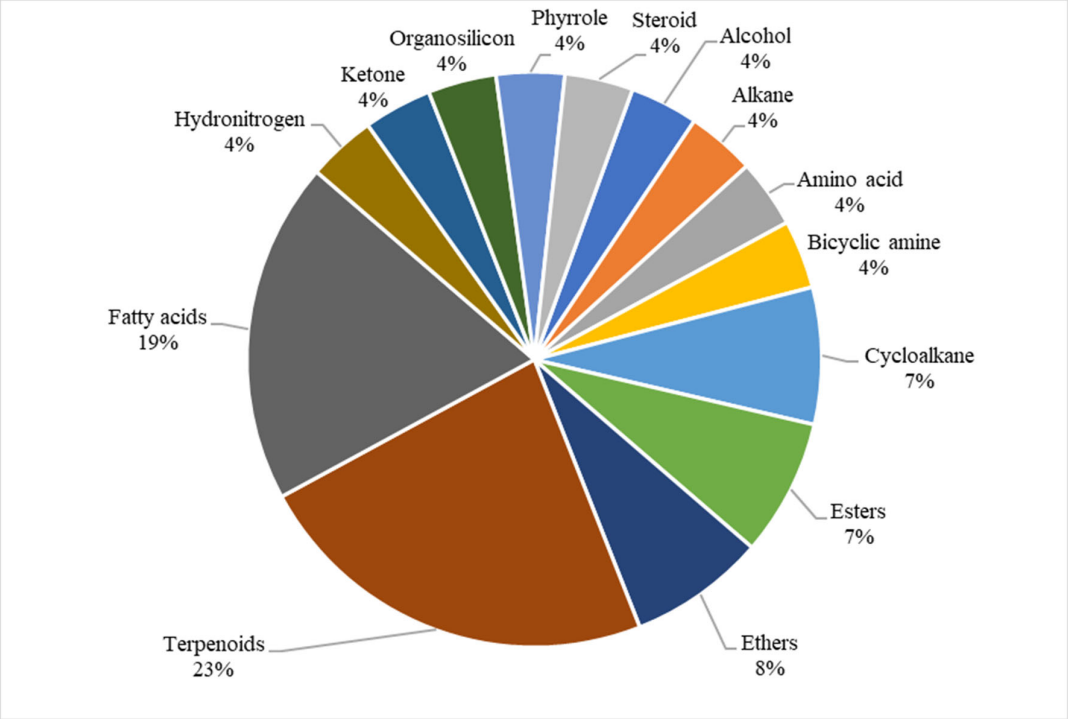

Figure 2. The group of chemical compounds found in GCE

The predominant compounds identified were phytol (19.06%), urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)- (6.23%), and squalene (4.32%). Other terpenoids identified include neophytadiene (0.7%), Olean-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)- (0.62%), and Cyclopentane, 1-ethenyl-3-ethyl-2-methyl- (0.38%). The identified fatty acids include hexadecanoic acid (2.49%), 8,11,14-(Z,Z,Z)-eicosatrienoic acid (2.88%), 7,10,13-(Z,Z,Z)-hexadecatrienoic acid (0.77%), 8-methylnonanoic acid (0.51%), and undecanoic acid, ethyl ester (0.35%).

Importantly, these specific compounds exhibit documented activity, providing a reasonable mechanistic link to the observed biocompatibility and osteogenic potential. The most abundant metabolite, Phytol (19.06%), shows direct relevance to mesenchymal stem cell (MSC) biology. In addition to its established anti-inflammatory and antioxidant properties (Naikwadi *et al*., 2022; Ren *et al*., 2023), the compound has been shown to promote osteoblast differentiation in mouse mesenchymal stem cells (C3H10T1/2) (Sanjeev *et al*., 2020). Its ability to activate the PI3K/Akt signalling pathway (Wang *et al*., 2017) is significant, as this pathway is a crucial regulator of cell survival and differentiation and indicates a key mechanistic pathway through which it can enhance MSC commitment to the osteogenic lineage.

Furthermore, the presence of urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)- (6.23%) provides a potent association with osteogenic mechanisms. While these compounds exhibit the known activities including antibacterial and anti-inflammatory (Sujatha *et al*., 2017), their significant role in bone regeneration should be highlighted. Structurally similar ursolic acid compounds have been demonstrated to upregulate the key osteogenic marker significantly including Runx2, Osterix, ALP, and Osteopontin (D. E. Lee *et al*., 2023), while protecting osteoblasts from oxidative stress through the IER3/Nrf2 pathway (S. Lee, 2019). This dual role in promoting the bone formation and ensuring cell survival positions these derivatives as important bioactive components contributing to the extract's osteogenic potential.

Squalene (4.32%) is known for its strong antioxidant properties. According to Du *et al*. (2024), the compound has been reported to protect cell membranes from free radical damage and improve lipid stability. Picón and Skouta

(2023) added that this compound also plays a role in regulating lipid peroxidation contributing to its protective effect against oxidative damage. In line with these findings, this function is very important because oxidative stress is a major factor that disrupts cellular energy metabolism, which is a determining factor in directing MSC differentiation (Yan *et al*., 2021). By reducing oxidative damage, squalene helps maintain metabolic homeostasis. Importantly, squalene has been identified as a component in olive oil fraction that specifically enhances osteoblast differentiation over adipogenesis in MSCs (Casado-Díaz *et al*., 2019), indicating an active role in cell fate determination beyond mere cell protection.

In conclusion, the presence of these combined metabolites indicates varied biological activities. Through important pathways, phytol functions as a differentiation signal; derivatives of ursolic acid regulate the genetic program of bone formation; and squalene optimises the oxidative and metabolic conditions for cell function. *P. oblongifolia* extract's biological activity is supported by a strong mechanistic hypothesis due to this synergistic combination of phytochemicals, which also makes it a possible candidate for more study in the area of bone regenerative therapy.

Table 1. Phytochemical components identified in green cincau ethanol extract by GC-MS analysis

No	Name of compound	RT[a]	MF[b]	MW[c]	PA[d](%)	Classification	Function
1	Butane, 1,1'-[(1-methylethylidene)bis(oxy)]bis-	1.526	$C_{11}H_{24}O_2$	188	0.12	Ether	Unknown
2	Silane, trimethyl-	1.642	$C_3H_{10}Si$	74	0.29	Organosilicon	Unknown
3	3-Hexanol	1.694	$C_6H_{14}O$	102	0.3	Alcohol	Antifungal (Naqvi *et al*., 2022)
4	1-Aminoisopropane-1-carboxylic acid-D3	1.775	$C_4H_6D_3NO_2$	106	0.34	Amino acid	Unknown
5	Hydrazine, 1,2-dimethyl-	2.121	$C_2H_8N_2$	60	0.42	Hydronitrogen	Pharmaceutical and agricultural applications (Ramaro *et al*., 2024)
6	2,4,6,8-Tetramethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocane	2.294	$C_4H_{16}O_4Si_4$	240	35.35	Siloxane	Unknown
7	2,4,6,8-Tetramethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocane	2.465	$C_4H_{16}O_4Si_4$	240	18.13	Siloxane	Unknown
8	1-Ethyl-2-methylcyclohexane	9.757	C_9H_{18}	126	0.19	Cycloalkane	Unknown
9	1,2,5-Trimethyl-1H-pyrrole	1.138	$C_7H_{11}N$	109	0.39	Phyrrole	Unknown
10	Neophytadiene	5.375	$C_{20}H_{38}$	278	0.7	Diterpenoid	Antimicrobial and anti-inflammatory (Tawfeeq *et al*., 2024) and neuroprotective activities (Gonzalez-Rivera *et al*., 2023)
11	Phytone	5.461	$C_{18}H_{36}O$	268	0.4	Ketone	Antibacterial (Al-Shaar *et al*., 2025); analgesic, and anti-inflammatory activities (Nasrollahi *et al*., 2022)
12	3-Methylene-7,11-dimethyl-1-dodecene	6.194	$C_{15}H_{28}$	208	0.25	Alkene	Unknown
13	Hexadecanoic acid	8.088	$C_{16}H_{32}O_2$	256	2.49	Fatty acid	Anti-inflammatory agent, antimicrobial, antioxidant (Ferdosi *et al*., 2021), cell proliferation (Chen *et al*., 2010)
14	Undecanoic acid, ethyl ester	8.363	$C_{13}H_{26}O_2$	214	0.35	Fatty acid ester	Antioxidant (Uka *et al*., 2022)

15	Phytol	0.518	$C_{20}H_{40}O$	296	19.06	Diterpenoid	Induces cell differentiation (Sanjeev *et al*., 2020), antioxidant, anti-inflammatory (Naikwadi *et al*., 2022)
16	Isobutyrate <2,4-hexadienyl->	0.933	$C_{10}H_{16}O_2$	168	0.1	Esther	Unknown
17	7-Azabicyclo[4.1.0]heptane, 3-methyl-	0.967	$C_7H_{13}N$	111	0.23	Bicyclic amine	Unknown
18	1,9-Cyclohexadecadiene	1.058	$C_{16}H_{28}$	220	1.66	Cycloalkadiene	Unknown
19	8,11,14-Eicosatrienoic acid, (Z,Z,Z)-	1.157	$C_{20}H_{34}O_2$	306	2.88	Fatty acid	Antimicrobial (Naikwadi *et al*., 2022)
20	7,10,13-Hexadecatrienoic acid, (Z,Z,Z)-	1.355	$C_{16}H_{26}O_2$	250	0.77	Fatty acid	Antibacterial, antioxidant,antitumor, immunostimulant (Saha *et al*., 2024)
21	8-Methylnonanoic acid	1.551	$C_{10}H_{20}O_2$	172	0.51	Fatty acid	Unknown
22	Hexanedioic acid, bis(2-ethylhexyl) ester	5.16	$C_{22}H_{42}O_4$	370	1.03	Diester	Antimicroba (Ferdosi *et al*., 2021; Wulandari *et al*., 2024)
23	Ethanamine, 2,2'-oxybis[N,N-dimethyl-	6.067	$C_8H_{20}N_2$	160	0.2	Ether	Unknown
24	Squalene	1.361	$C_{30}H_{50}$	410	4.32	Triterpenoid	Skin emollient, antioxidant (Kumar *et al*., 2021), cytoprotective activity (Das *et al*., 2008) and metabolism-enhancing activity (Ganbold *et al*., 2020)
25	Gamma.-Sitosterol	7.034	$C_{29}H_{50}O$	414	2.28	Steroid	Antioxidant, anti-bacterial and prophylatic activies and antidiabetic (Namuga *et al*., 2024)
26	Olean-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)-	0.264	$C_{31}H_{50}O_3$	470	0.62	Triterpenoid	Unknown
27	Urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)-	1.321	$C_{31}H_{50}O_3$	470	6.23	Triterpenoid	Unknown
28	Cyclopentane, 1-ethenyl-3-ethyl-2-methyl-	5.858	$C_{10}H_{18}$	138	0.38	Triterpenoid	Unknown

Note: a: RT=Retention time;b: MF=Molecular formula; c: MW=Molecular weight; d: PA=Peak area;

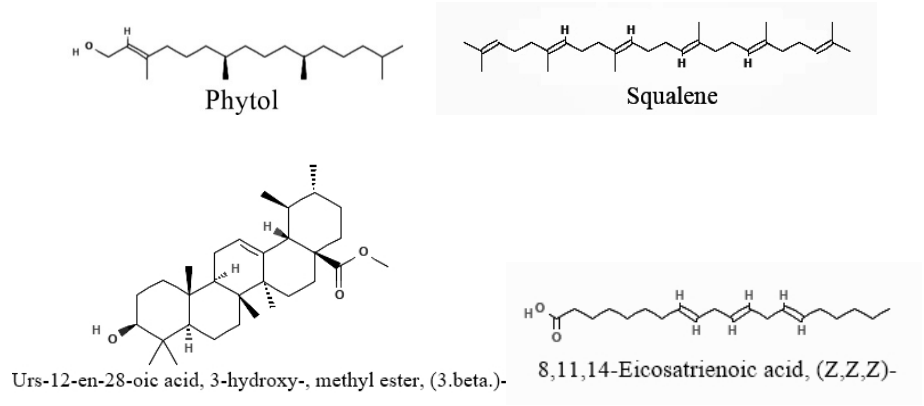

Figure 3. Chemical structure of the main phytochemical components discovered in the ethanol extract of green cincau

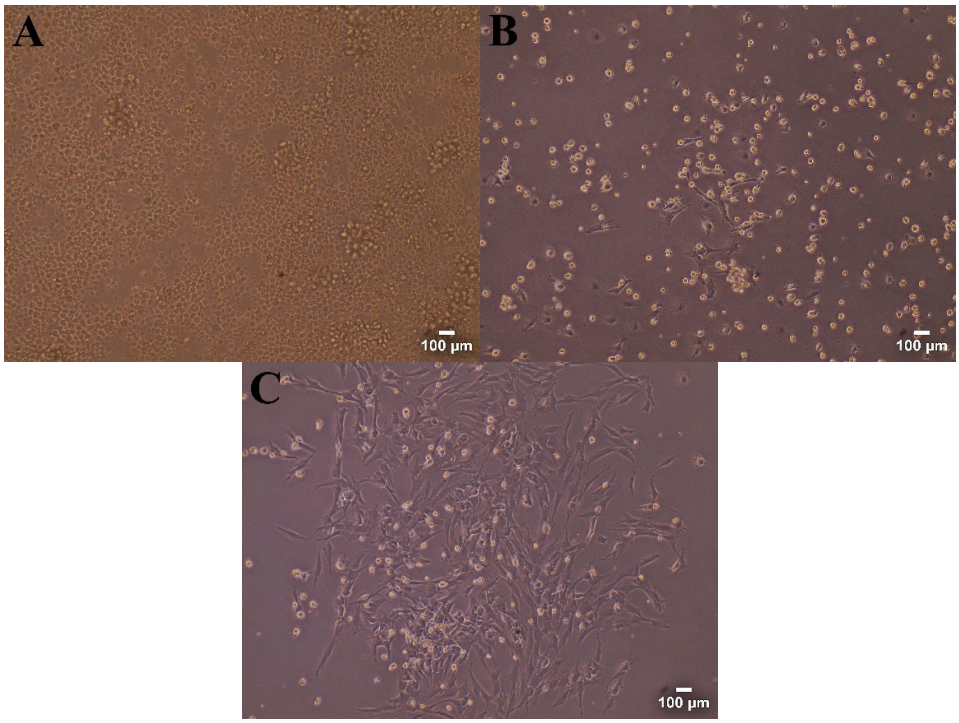

Figure 4. Cell morphology on primary isolation. (A) Cells remained spherical on the second day. (B) Spindle-shaped cells began to develop on the fifth day. (C) MSC-like cells formed colonies on the eighth day. Magnification: 100X; scale bar: 100µm.

Primary Culture of Bone Marrow MSC-like Cells

Isolation of MSC-like cells from rat bone marrow was carried out using the flushing method, in which bone marrow was extracted from the femur and tibia of adult rats. The total number of cells isolated from each mouse was approximately 3.152×10^8 cells. The primary cell in this study initially showed heterogeneous morphology as shown in Figure 4A. According to Chong *et al*. (2018), primary isolation from bone marrow (BM-MSC) can be contaminated with hematopoietic cells during the bone marrow collection process and can be mixed with peripheral blood or lipids, causing a mixture of cell types and potential contamination. However, as the passages proceed, the non-adherent hematopoietic cells will disappear (Baustian *et al*., 2015; Rennerfeldt *et al*., 2019).

On the early day of culture (Figure 4A) most cells still were mononuclear. Some cells began to adhere to the culture surface, while others have not yet adhered and formed separate deposits on the surface of the culture vessel. Joujeh *et al*. (2021) stated that spindle-shape morphology increased since day three of primary culture, which in this study appeared on the fifth day (Figure 4B). The cells exhibited fibroblast-like appearance and started to form colonies on the eighth day (Figure 4C). Primary cells reached confluence on day 27. This duration was shorter than the study conducted by Pissarra *et al*. (2022) where it took 35-56 days for the cells to reach confluence.

Effect of GCE on Cellular Viability

Cytotoxic examination was performed using the WST-1 method. The principle of WST-1 is based on the conversion of WST-1 tetrazolium salt into a coloured dye by the mitochondrial dehydrogenase enzyme (Gharbaran *et al*., 2021). Cell viability was evaluated after treatment with 10 different concentrations of GCE (1-1200 µg/ml) for 24, 48, and 72 hr. The findings revealed that as the concentration of the extract increased the percentage of cell viability remained relatively constant across most concentrations, however, as the exposure duration of the extract increased, the percentage of viability showed a decreasing trend (Figure 5). Statistical analysis indicated that GCE concentrations demonstrated no significant cytotoxic activity ($p>0.05$) at any specific time point, although the variation in viability with exposure duration was considerable ($p<0.05$).

Detailed observation of the data revealed a time-dependent and biologically significant decline in viability. Cell viability decreased by 21%, 29%, and 27% after 48 hr of exposure to GCE concentrations of 80, 600, and 1200 µg/ml, respectively. After 72 hr of exposure to the extract at 300 µg/ml concentration, viability decreased by more than 20%. It is important to note that while cell viability reduced as exposure duration increased, it remained above 70-80% across all tested conditions. Moreover, a standard half-maximal inhibitory concentration (IC_{50}) could not be calculated for any exposure time because the cell viability did not fall below 50%, even at the highest concentration of 1200 µg/ml. This result indicates that the IC_{50} value for GCE is definitively greater than 1200 µg/ml. Based on established toxicity categories, such as that of Saputri *et al*. (2019), this places GCE in the "no significant cytotoxicity" classification ($IC_{50}>1000$ µg/ml). The decrease in viability observed with longer exposure may be due to the cumulative action of bioactive chemicals, which can result in the accumulation of toxic metabolites and initiate cellular stress responses. The duration of exposure in cytotoxic is crucial since it influences the amount of cell damage and the efficiency of the cytotoxic substance (Byrne and Maher, 2019). Prolonged exposure of extracts may result in higher toxicity, but shorter intervals may not result in meaningful cellular reactions. Other factors that are suspected to influence the results are the proportion of bioactive chemicals extracted in plant extracts may vary depending on the extraction solvent and technique used (Anlas *et al*., 2022).

Based on the results, it can be concluded that in the concentration range of 1-1200 µg/ml and incubation times of 24, 48, and 72 hr of GCE did not exhibit severe toxicity. The inability to calculate an IC_{50} within this range confirms its low cytotoxic potential. Nevertheless, the time-dependent declines in viability of 20-30% indicate that the claim of GCE being completely non-toxic requires caution. Hence, while GCE has a favourable safety profile for possible use as an active ingredient, particularly in situations involving shorter periods, its effects on prolonged exposure require further investigation.

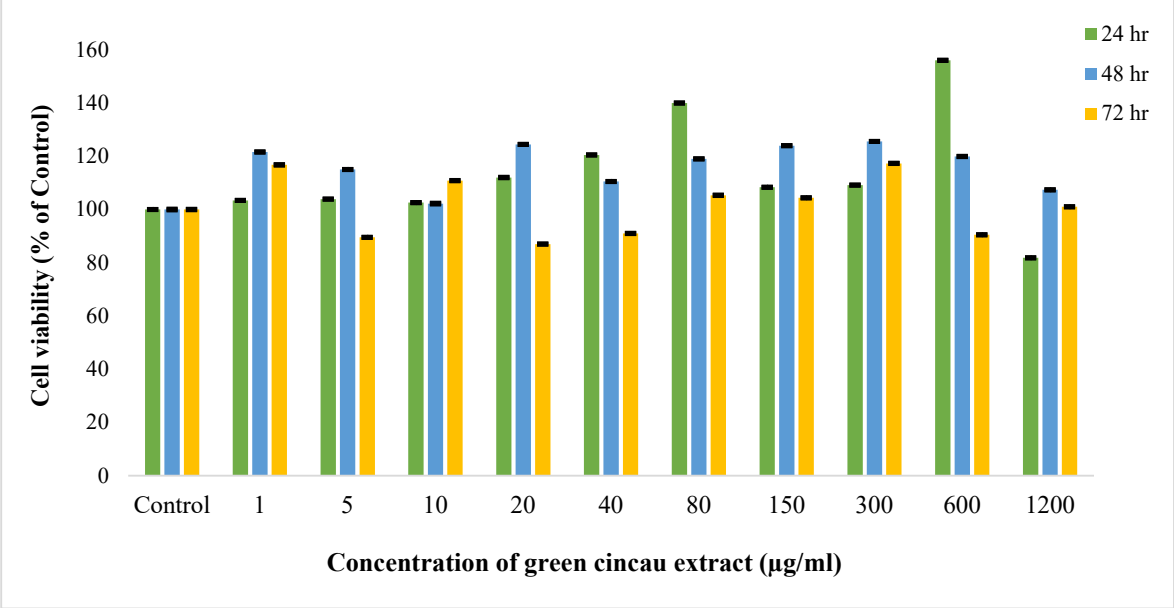

Figure 5. Percent cell viability of BMMSC-like cells were measured at 24, 48, and 72 hr after treatment with varying concentration of GCE by WST-1 assay. Data were presented as mean ± standar deviation (n=3).

The Effect of GCE on Cell Differentiation

The observation of cell morphology development during the differentiation process with treatment using GCE (Figure 6) at different concentrations (150, 300, and 600 µg/ml) as well as positive control (K+) and negative control (K-) on the third day showed cell morphology resembling fibroblasts with relatively uniform distribution. There was no morphological difference in the positive control treatment with other treatment groups until day 14. Calcium deposit examination was conducted on day 14 based on Hanna *et al*. (2018), Ca^{2+} deposits begin to accumulate at the end of the second week and become increasingly dense. Alizarin Red S (ARS) is a staining standard commonly used for the detection and quantification of mineralisation, which is a marker of osteoblast differentiation (Liu *et al*., 2021).

The osteogenic medium-treated group showed orange to red stains (Figure 7B), which are most likely ARS that binds to calcium ions, allowing visualisation of mineralised structures in the tissue. The mixture of ingredients used to induce osteocyte differentiation was dexamethasone, ascorbic acid and β-glycerophosphate. The combination of these ingredients is commonly used in osteogenic differentiation studies (Azizsoltani *et al*., 2018; Colter *et al*., 2001; Harsoyo *et al*., 2020). Dexamethasone triggers Runx2 expression and activity through activation of FHL2/β-catenin, TAZ, and MKP-1 pathways to support the osteogenic pathway (Bella *et al*., 2021; Langenbach and Handschel, 2013). Ascorbic acid stimulates the formation of type I collagen (Col1). β-GP contains the inorganic phosphate required for the production of hydroxyapatite, the main mineral component of bone (Freeman *et al*., 2016; Langenbach and Handschel, 2013). The positive control indicated that the osteogenic pathway was successfully activated, although not optimally.

The GCE treatment group with a concentration of 300 µg/ml showed indications of possible mineral deposition through red staining (Figure 7D). In addition, the fibroblastic-like characteristics on the cell morphology still showed. The phytol content in GCE could potentially contribute to this process, based on literature report that has been conducted by Sanjeev *et al*. (2020), treatment with phytol enhanced osteoblast differentiation in C3H10T1/2 cells by promoting Runx2 expression, a process linked to the downregulation of Smad7 through miR-21a activity.

Treatments with other GCE concentrations (150 and 600 µg/ml) displayed relatively stable shape, this is thought to be due to the presence of several bioactive ingredients of GCE that function as antioxidants which may support the differentiation of damaged stem cells. The substances that may function as antioxidants and anti-inflammatories include neophytadiene (Tawfeeq *et al*., 2024), phytone (Nasrollahi *et al*., 2022), hexadecanoic acid (Ferdosi *et al*., 2021), undecanoic acid, ethyl ether (Uka *et al*., 2022), phytol (Naikwadi *et al*., 2022), 7,10,13-hexadecatrienoic acid, (Z,Z,Z)- (Saha *et al*., 2024), squalene (Kumar *et al*., 2021) and Gamma.-Sitosterol (Namuga *et al*., 2024). However, it is crucial to acknowledge that the evidence for the differentiation in this study is still preliminary. Qualitative morphological findings and ARS staining indicate mineral deposition at 300 µg/ml, but cannot be considered final confirmation. These findings require to be validated using quantitative approaches such as measuring alkaline phosphatase (ALP) activity, analysing mineralisation quantitatively, and using qPCR to assess the expression of osteogenic marker genes Runx2 and Osteocalcin. In a result, while GCE has the potential to influence osteogenic differentiation, further research is essential to provide solid evidence.

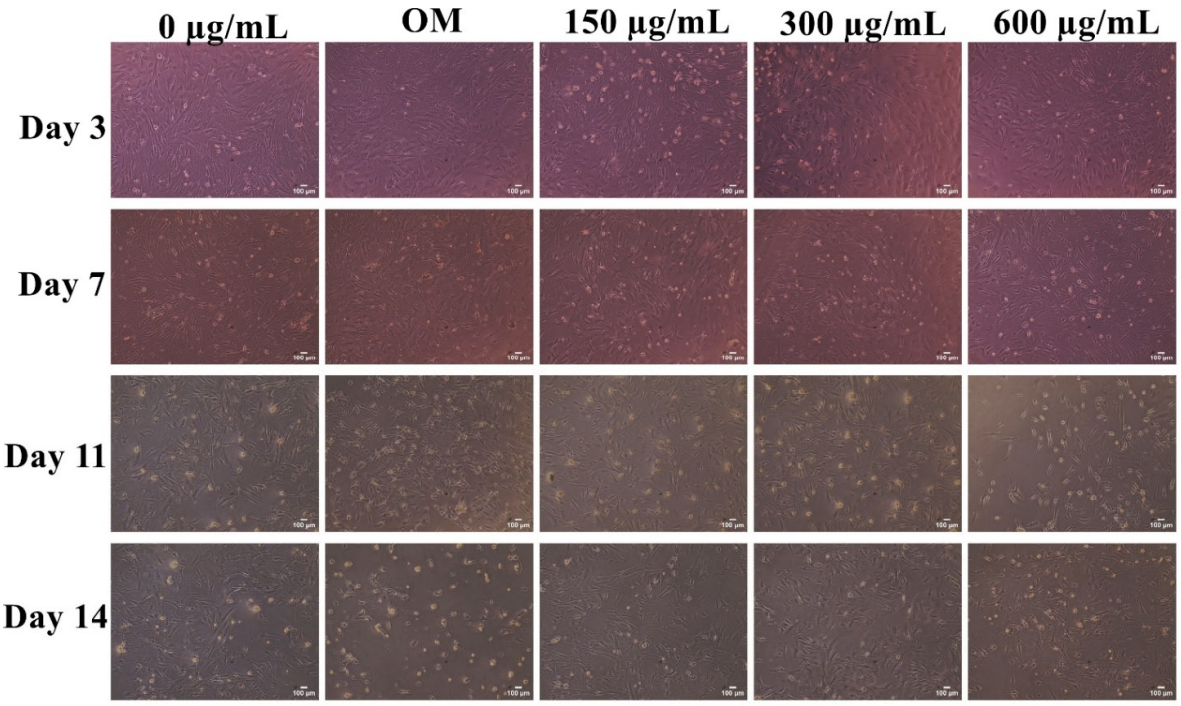

Figure 6. Morphological details of MSC-like cells during the differentiation process up to day 14. Notes: (1) horizontal rows represent morphological changes on days three to 14 in each treatment (2) vertical columns represent treatments consisting of negative control (0 µg/ml), osteogenic medium (OM), 3 treatments of GCE with 3 different concentrations of 150, 300, and 600 µg/ml. Magnification: 100X; scale bar: 100 µm.

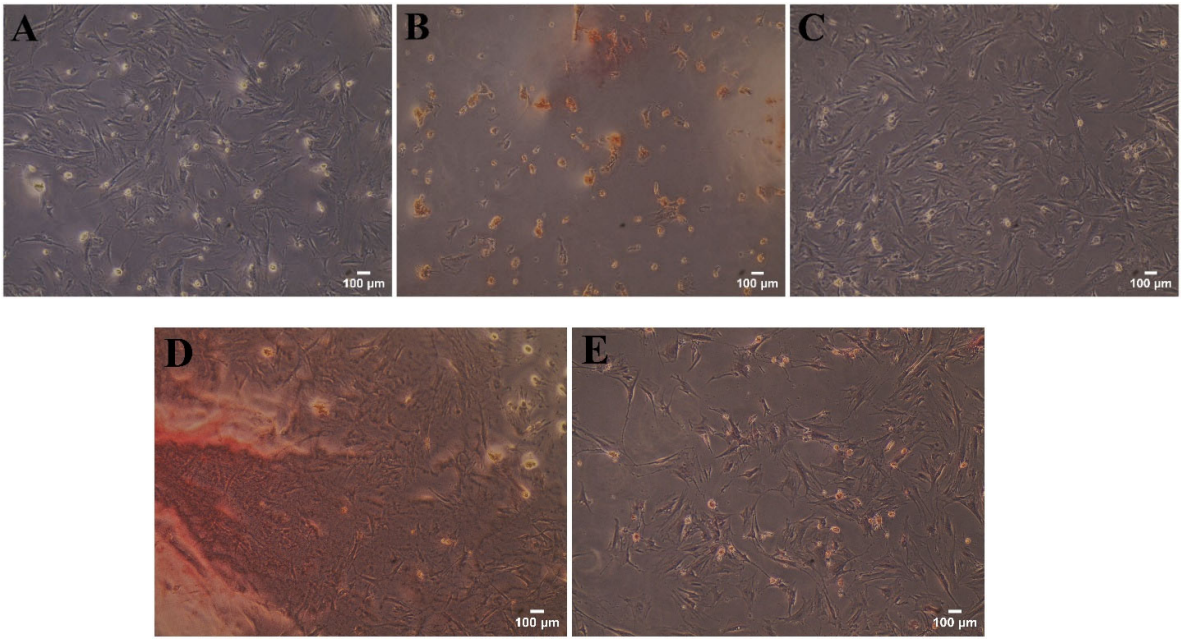

Figure 7. Cell staining using Alizarin Red S on day 14. Notes: (A) Negative control (0 µg/ml), (B) osteogenic medium (OM) is the positive control treatment, (C, D, E) GCE treatment with 3 different concentrations of 150, 300, and 600 µg/ml, respectively. Magnification: 100X; scale bar: 100 µm.

Study Limitations

This study has several limitations that should be acknowledged. First, the finding of siloxane compounds as the predominant peaks was a major issue of the phytochemical study. Siloxane compounds may represent artefacts from laboratory equipment or column bleeding during GC-MS analysis rather than actual plant metabolites. This is an initial shortcoming that future studies will need to address using other method such as Nuclear Magnetic Resonance (NMR) spectroscopy to identify the most important active compounds in *P. oblongifolia*. Second, the cytotoxic assays were performed in triplicates (n = 3). Although commonly used in preliminary screening, this can limit statistical robustness. Future studies are recommended to use a larger sample size ($n \geq 6$) to increase the reliability of statistical analysis. Finally, the differentiation study was limited to qualitative Alizarin Red S staining. Future studies should include a longer time study (21-28 days) to monitor the full progression of mineralization and quantitative assessments such as ARS quantification, alkaline phosphatase activity assays, and qPCR analysis of osteogenic markers (Runx2, osteocalcin, osteopontin) to provide more robust evidence of differentiation.

CONCLUSION

The bioactive profile of the *Premna oblongifolia* extract is primarily composed of terpenoids and fatty acids, with Phytol, Urs-12-en-28-oic acid, 3-hydroxy-, methyl ester, (3.beta.)-, and squalane identified as the most abundant compounds. This preliminary investigation indicates that the ethanol extract of *P. oblongifolia* Merr. exhibits low cytotoxic potential on MSC-like cells and demonstrates potential for maintaining cell viability. Notably, cell viability remained above acceptable levels across tested concentrations up to 1200 µg/ml, though time-dependent effects were observed. However, to completely ascertain its therapeutic promise, more comprehensive research incorporating quantitative differentiation analyses, mechanistic studies, and in vivo validation remains necessary.

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