SLIIT Journal of Humanities and Sciences
Vol. 5(2), 2024; Page 28-41
DOI: https://doi.org/10.4038/sjhs.v5i2.72

Received: 02 February 2024 *Accepted: 15 October 2024*

Human Primary Stem Cell Cultures as Screening Platforms for Botanical Preparations as Alternatives to Animal Models: Evaluating Cell Proliferation Stimulatory and Inhibitory Effects

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Abstract

Animals are widely used as human "models" in scientific research, yet primary human stem cell cultures provide an alternative under the 3R (Reduction, Replacement and Refinement) concept to reduce and replace animal models. This study investigated the cell stimulatory effects of selected botanical preparations on primary human stem cell cultures. In-house established primary human fibroblast (hFSC), mesenchymal (hMSC), and haematopoietic (hHSC) stem cells, derived from surgical waste, were characterized by immunophenotyping. The proliferation effects of *Carica papaya* leaf concentrate (MLCC), *Vernonia/Mallotus* (VMD), and *Ficus benghalensis* (FBD) distillates were assessed using the MTT assay. MLCC significantly stimulated proliferation in male and female hMSCs and hHSCs ($p<0.05$). FBD promoted proliferation in male hMSCs and hHSCs but inhibited female hMSCs. VMD inhibited both male and female hMSCs yet stimulated female hHSCs at 0.6%. Notably, VMD at selected concentrations exhibited approximately 1.5-fold higher cell stimulation than the positive control ($p<0.05$). These findings highlight effective botanical concentrations for cell proliferation, demonstrating the utility of primary human stem cell platforms as "models" in biomedical research. This approach aligns with the "replacement" principle of the 3R concept, promoting ethical and human-relevant alternatives to animal testing.

Keywords: Animal models, Botanical preparations, Cell proliferation stimulants, Human stem cells, 3R concept, Reduction.

Introduction

Animals play a crucial role in scientific research, particularly in biomedical research as highly specific models for humans. These models have contributed significantly to the development of life-saving treatments for both humans and animals. The 3R principle in animal research -Replacement, Reduction, and Refinement- was introduced by Russell and Burch in 1959 to promote ethical and humane research practices. "Replacement" emphasizes the use of alternative methods to substitute animal models in research, reduction contributes to the less use of animal models, whereas refinement decreases the severity of inhumane procedures (Hubrecht and Carter., 2019). One such widely adopted alternative as a replacement is primary cell cultures, which help minimize (reduce) and, in some cases, replace the need for animal models (Hussain et al., 2023).

Stem cells are diverse cell populations residing in various tissues, capable of self-renewal and differentiation into specialized cell types (Zakrzewski et al., 2019). They are classified based on their differentiation potential (potency) and the developmental stage from which they are derived. Additionally, induced pluripotent stem cells (iPSCs) are artificially generated by reprogramming mature cells to regain pluripotency (Kim et al., 2022).

Adult stem cells, such as human hematopoietic stem cells (hHSCs) and human mesenchymal stem cells (hMSCs), can be isolated from sources like bone marrow, cord blood, and other adult tissues. These cells are widely used in current treatment regimens for various diseases (Zakrzewski et al., 2019). However, their low natural abundance necessitates strategies to enhance their numbers *in vitro*, *ex vivo,* and *in vivo*. To achieve this, researchers employ pharmaceutical interventions known as stem cell stimulants.

Synthetic and recombinant stimulants, including stem cell factors, fibroblast growth factors, interleukins, transforming growth factor, platelet-derived growth factor and vascular endothelial growth factor other growth factors, are commonly used singly or in combination (Li et al., 2021). However, these agents are often toxic, cause side effects, and are costly. Consequently, pharmaceutical innovations are shifting toward phyto-stimulants, plant-derived alternatives, that are more affordable and less toxic (Udalamaththa et al., 2016).

Plant-based compounds have proven their efficacy against a plethora of disorders. Important drugs such as Colchicine, Etoposide, and Demecolcine against tumours, Ephedrine as an antihistamine agent, Glaziovine as an antidepressant, and many other drugs against a wide range of diseases have shown the importance of exploiting phyto-based bioactive compounds (Helmenstine, 2024). Drug development pipelines have been hindered by prolonged turnaround times, driven by the sheer volume of compounds that need to be screened, compounded by the lack of clear direction on optimal targets. Traditional medicine systems worldwide have noticeably helped reduce this hinderance by contributing to the knowledge of time-tested specialized botanicals (Udagama et al., 2025). As a result, main drug regulatory authorities such as the Food and Drug Administration, USA, and World

Health Organization (WHO) have set forth specific regulatory frameworks for the global recognition of these much effective botanicals (U.S. Food and Drug Administration, 2016; WHO, 2004). Screening for effective drugs based on botanical preparations, and phyto-compounds on stem cell platforms has yielded numerous promising outcomes, with reported bioactivities including neurogenesis, muscle cell differentiation, and hematopoietic cell development (Udalamaththa et al., 2016).

Sri Lanka, one of 36 global biodiversity hotspots, and home to a time-tested traditional medicine system, offers numerous insights into the potential of botanical preparations for stem cell therapy (Udagama et al., 2025). Previous research conducted by our team reported that the mature leaf juice of the Sri Lankan wild-type cultivar of *Carica papaya* exhibits significant blood and immune cell-enhancing activity *in vivo*, along with a notable *ex vivo* proliferation capacity of murine bone marrow cells (Jayasinghe et al., 2017). This leaf juice has been traditionally used in South Asian medicine to increase platelet counts in patients affected by dengue fever. Additionally, *Ficus benghalensis* has demonstrated important bioactivities in modern research, while its botanical preparations have been traditionally used to treat dysentery, diarrhea, skin disorders, inflammation, and diabetes (Murugesu et al., 2021). In contrast, *Vernonia zeylanica* and *Mallotus repandus* have not been used together as a botanical preparation. However, both plants have been individually investigated for their bioactivities in recent research platforms (Petchprayoon et al., 2024; Mendis et al., 2019; Rukshala et al., 2021).

Animal models have long been widely used to study the effects of botanicals, as they offer valuable insights into a drug's impact on the entire organism, and its various systems. However, many compounds deemed effective and non-toxic in these models have failed in clinical trials, highlighting the need for more relevant screening platforms that closely mimic human physiology (Robles-Loaiza et al., 2022). In response, research groups are increasingly turning to primary human adult stem cell models in the early stages of drug screening to better replicate human-specific biological responses.

This study aimed to investigate cell proliferation stimulatory effects of selected botanical preparations from the mentioned plants on primary human stem cell cultures. Utilizing such an inexpensive screening platform derived from biological waste, this approach could serve as a novel alternative to animal models in the search for effective natural human stem cell stimulants, a key factor in tissue regeneration and repair (Udalamaththa et al., 2021).

Materials and Methods

Ethical approval

Ethical approval for the studies was granted by the Ethics Review Committee, Faculty of Medicine, University of Colombo (ERC/15/133), and the Ethics Review Committee of the Lady Ridgeway Hospital for Children, Colombo 8 (ERC-LRH/DA/01/2016), Sri Lanka.

Preparation of botanical extracts

Mature leaves of *Carica papaya* of the Sri Lankan wild type cultivar were collected

from plants maintained without insecticides from a home garden at Borelasgamuwa, Colombo district (longitudes 6.8412° N; latitudes 79.9025° E). The mature leaf juice of *Carica papaya* Sri Lankan wild type variety (MLCC) was prepared essentially following the procedure described by Gammulle et al. (2012). The aqueous distillate of *Vernonia/ Mallotus* (VMD) was prepared using fresh mature leaves, collected as practiced in Sri Lankan Traditional Medicine from two individual plants from a home garden in Gampaha (longitude- 80◦ 0′ 51.7176″ E, latitude- 7◦ 5′ 14.3160″ N), according to the method described by Ratnayake et al (2024).

The aqueous distillate of *Ficus benghalensis* was prepared using fresh leaflets and aerial roots collected from a home garden in Gampaha (longitude- 80◦ 0′ 51.7176″ E, latitude- 7◦ 5′ 14.3160″ N). It was prepared according to the traditional medicine practices by homogenizing 250g of fresh leaflets and 250g of fresh aerial prop roots in 1500 ml of distilled water for 10 min, and filtering through eight layers of muslin cloth. The resultant filtrate was distilled using a Liebig condenser following the standard distillation process.

All three leaf materials were collected in the months of May-June of the respective year. All botanical preparations were sterilized using 0.45 µm syringe filters prior to use in cell culture protocols.

Collection of human biological samples from surgical waste

Prior approval was obtained from the Director, de Soysa Maternity hospital, Colombo 08 to collect human cord blood and cord tissue samples. Samples were collected into sterile containers with transport medium with informed consent from voluntary participants, who underwent elective C-section for term delivery. Human neonatal prepuce (foreskin) samples obtained from routine circumcision surgeries at Lady Ridgeway Hospital were collected into sterile containers with transport medium following informed consent.

All collected samples were transported to the cell culture facility in the Combinatorial Research Laboratory at the University of Colombo and were processed immediately.

Establishment and characterisation of primary human stem cell lines

Explant method was used to isolate human mesenchymal stem cells from umbilical cord tissue samples, and human dermal fibroblast stem cells from neonatal prepuce samples according to methods described by Venugopal et al. (2011), and Vangipuram et al. (2013), respectively. Cell surface markers for characterisation of stem cell lines were selected according to the ISCT guidelines (Dominici et al., 2006). Immunophenotyping was carried out according to the manufacturer's instructions (BD stemflow human Mesenchymal Stem Cell [hMSC] analysis kit, BD Biosciences, USA). Trilineage differentiation was carried out according to previously described methods by Venugopal and co-workers (2011).

Cord blood samples were processed according to the protocol recommended by EasySep™ Human Cord Blood CD34 Positive Selection Kit II, and hHSCs were cultured, maintained, sub-cultured, and cryopreserved according to protocols used by Broxmeyer and Colleagues (2003).

MTT assay

Stem cell proliferative stimulatory activity of botanical preparations was evaluated using the MTT assay. Briefly, hMSCs, and hFSCs in passage no 03, were plated into ninety-six well plates (Corning, USA) at a seeding density of 5000 cells/well, whereas hHSCs were plated at 15,000 cells/well, and incubated at 37 °C in a CO_2 incubator for 24 hours in the standard complete cell culture medium. Next, the cells were treated in triplicates, for 48 hours with appropriate percentages of botanical preparations (the percentage range was selected after few trials with wide range of percentages), where the crude juice/distillate were diluted in distilled water and incubated under standard cell culture conditions. The positive control for hMSCs and hFSCs was the synthetic growth factor - β fibroblast growth factor (bFGF at 4 ng/µl, Sigma-Aldrich, USA), while the Synthetic Cytokine Cocktail (Stem cell factor; 50 ng/ml, Thrombopoietin; 10 ng/ml, Flt3L; 50 ng/ml, Interleukin 6; 10 ng/ml) was for the hHSCs. The positive and normal (sans treatment) controls were maintained in culture medium. MTT assay was carried out according to Bellagamba and co-workers (2016). Optical density (OD) measurements were obtained using a microplate reader (BIO-RAD, Model 680,USA) using a 540 nm filter. OD values for each percentage of botanical preparation were compared with normal and positive control OD values to calculate the percentage of cell proliferation or inhibition (proliferation stimulatory or inhibitory effects) as follows:

$$\text{Percentage of proliferation} = \text{inhibition} = \frac{(\text{Test OD} - \text{Control OD})}{\text{Control OD}} * 100$$

Statistical analysis

Statistical analyses were performed using SPSS 29.0 (IBM, USA). All *ex vivo* experiments were performed in triplicate and presented as mean ± SD. Results were generated from three independent experiments and statistically compared using one-way ANOVA. Tukey's post hoc test was performed for multiple comparisons. Significance was set at $p<0.05$.

Results and Discussion

Effects of botanical preparations on hMSCs

Figures 1, 2 and 3 illustrate the effects of three different botanical preparations on male and female hMSCs. Significant proliferative stimulatory effects were observed at 0.2% and 0.6% concentrations of MLCC for female and male hMSCs, respectively ($p <0.05$) (Figure 1). No significant differences were found between the proliferative effects of MLCC and the positive control, bFGF FBD (Figure 2) demonstrated significant proliferative effects on male hMSCs at concentrations of 0.05%, 0.2%, 0.4%, 0.8%, and 1%, while showing significant inhibitory effects on female hMSCs at all tested concentrations ($p<0.05$). The proliferative effects of FBD were similar to the synthetic stimulant, bFGF ($p>0.05$). In contrast, VMD (Fig. 3) exhibited significant inhibitory effects on both male hMSCs (at 0.6% and 1.2%) and female hMSCs (0.1% – 0.8%) ($p<0.05$).

Figure 1.

Proliferative stimulatory effects of mature leaf juice of the Sri Lankan wild-type cultivar of Carica papaya (MLCC) on male and female human mesenchymal stem cells (hMSCs). PC: Positive Control. Significant proliferative/ inhibitory percentages are denoted *(p<0.05).

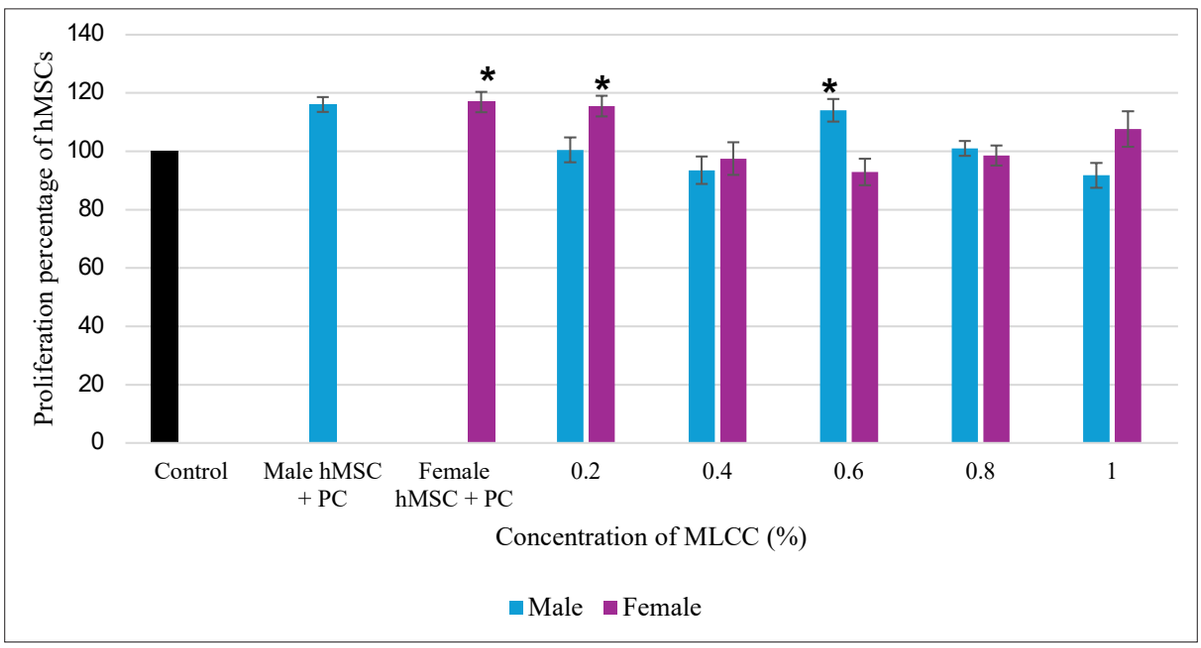


Figure 2.

Proliferative stimulatory effects of Ficus benghalensis distillate (FBD) on male and female human mesenchymal stem cells (hMSCs). PC: Positive Control. Significant proliferative stimulatory percentages are denoted *(p<0.05); Significant proliferative stimulatory percentages are denoted *(p<0.05).

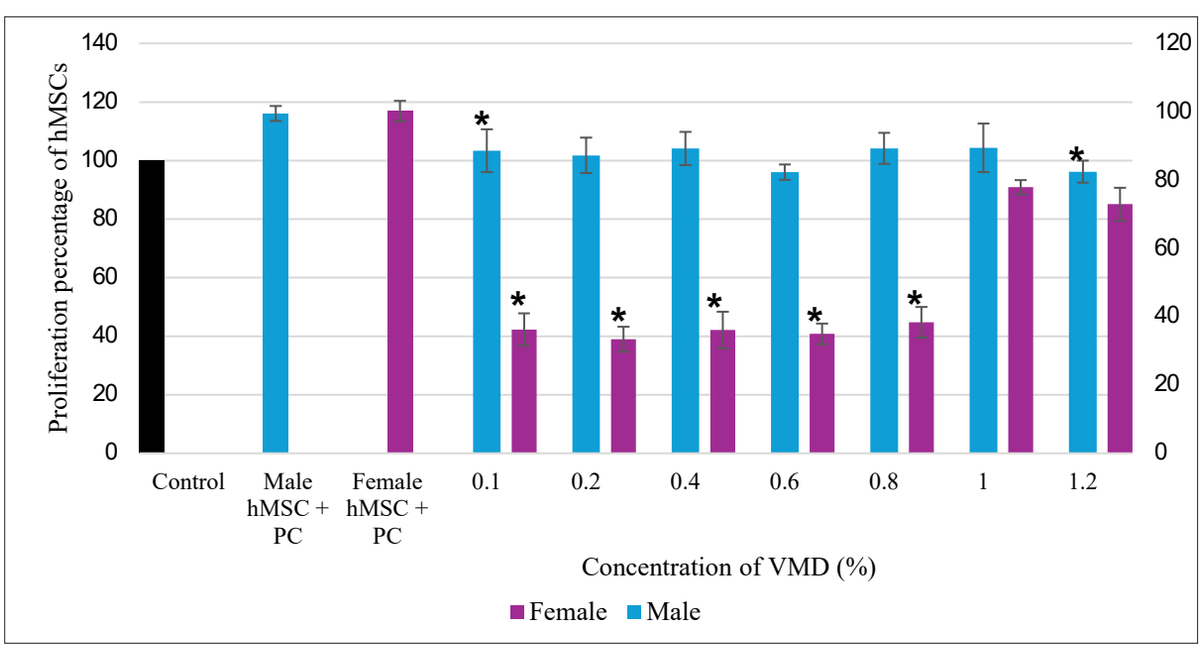
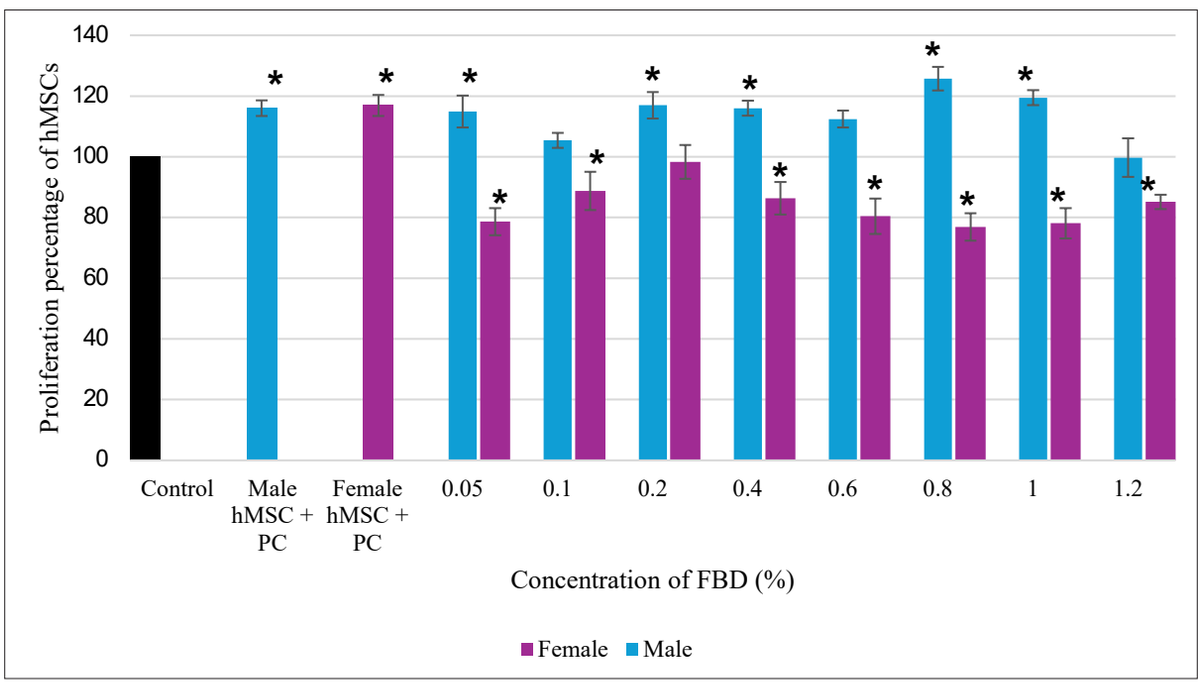


Figure 3.

Proliferative stimulatory effects of Vernonia/ Mallotus distillate (VMD) on male and female human mesenchymal stem cells (hMSCs). PC: Positive Control. Significant proliferative stimulatory percentages are denoted *(p<0.05); Significant proliferative stimulatory percentages are denoted *(p<0.05).



Effects of botanical preparations on hHSCs

As depicted in Figure 4, MLCC showed significant proliferative effects on male and female hHSCs at all tested concentrations, with the exception of male hHSCs at 0.2 and 0.4 % ($p<0.05$). Significant proliferative effects of FBD (Fig. 5) and VMD (Fig. 6) were evidenced on male hHSCs at all tested concentrations, with no significant impact on female hHSCs, but showing significant proliferative effects on female hHSCs at only 0.6% concentration ($p<0.05$). The significant proliferative effects of these three botanicals on hHSCs were not significantly different from the positive controls used.

Figure 4.

Proliferative stimulatory effects of mature leaf juice of the Sri Lankan wild-type cultivar of Carica papaya (MLCC) on male and female human haematopoietic stem cells (hHSCs). PC: Positive Control. Significant proliferative percentages are marked with *(p<0.05).

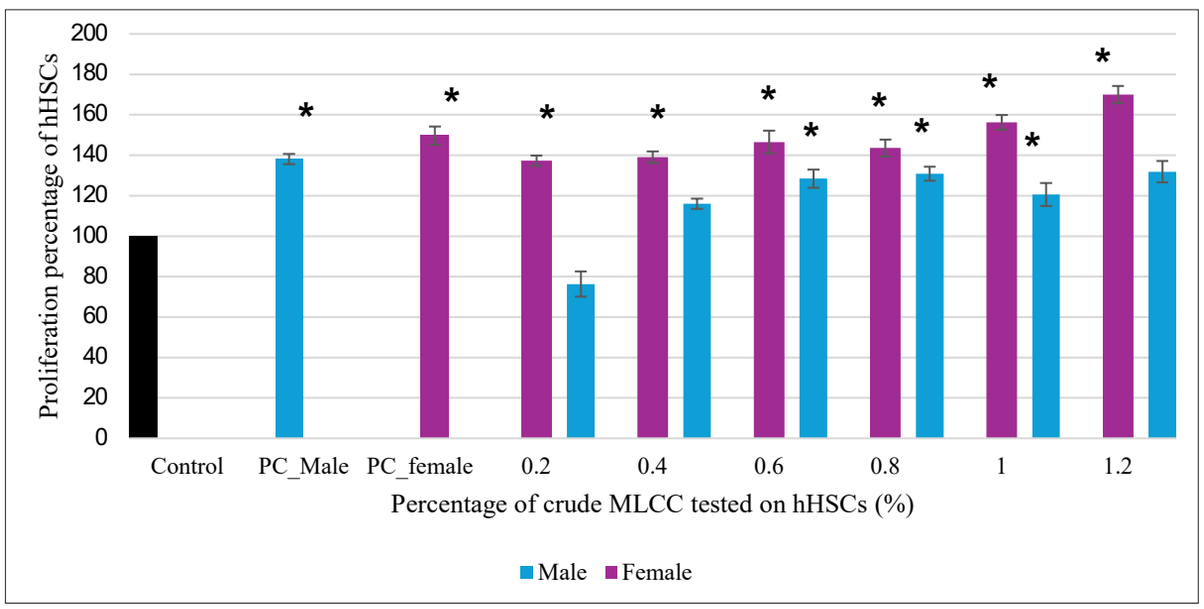


Figure 5.

Proliferative stimulatory effects of Ficus benghalensis distillate (FBD)on male and female human haematopoietic stem cells (hHSCs). PC: Positive Control. Significant proliferative percentages are marked with *(p<0.05).

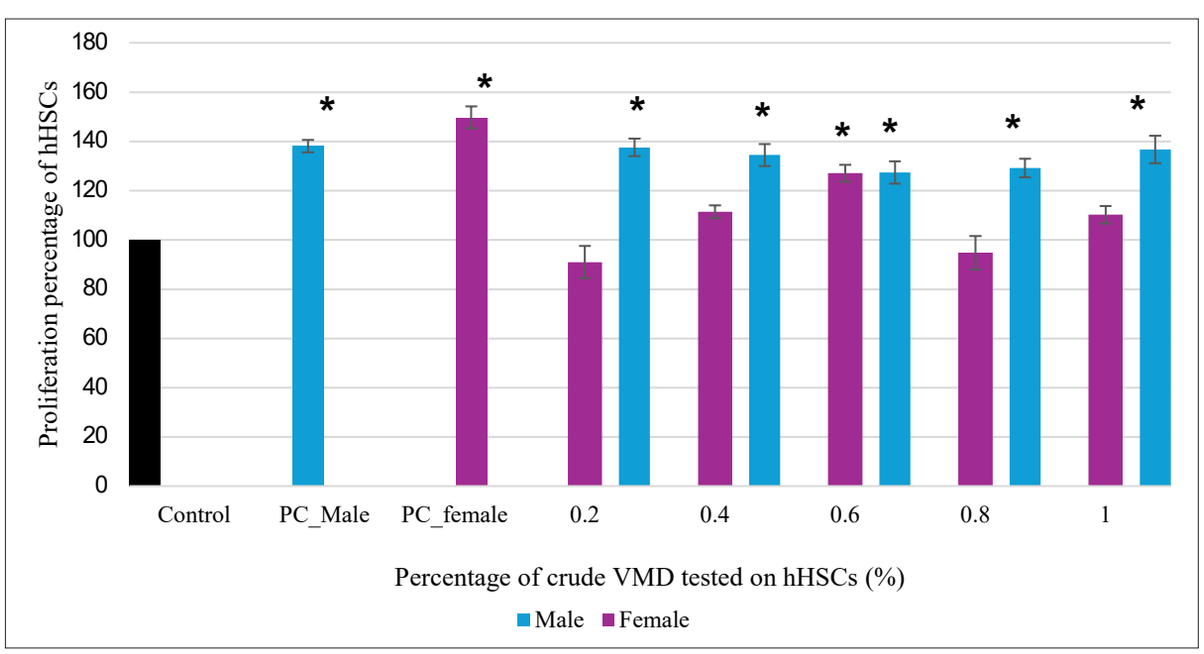
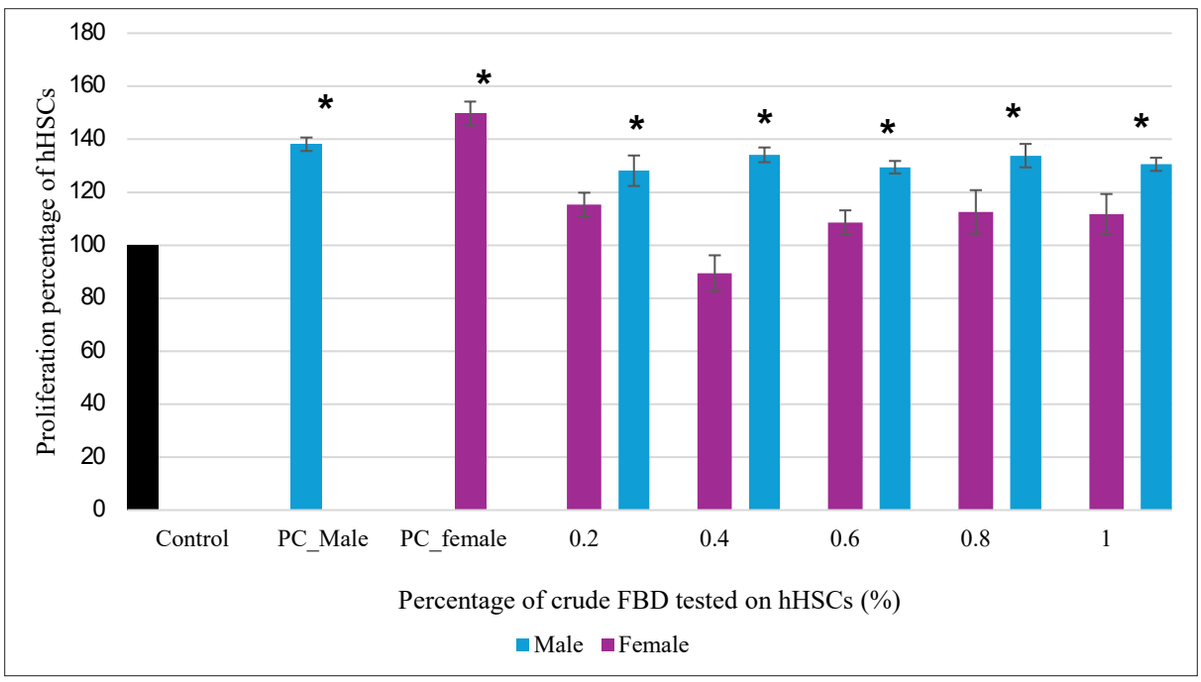


Figure 6.

Proliferative stimulatory effects of Vernonia/ Mallotus distillate (VMD) on male and female human haematopoietic stem cells (hHSCs). PC: Positive Control. Significant proliferative percentages are marked with *(p<0.05).

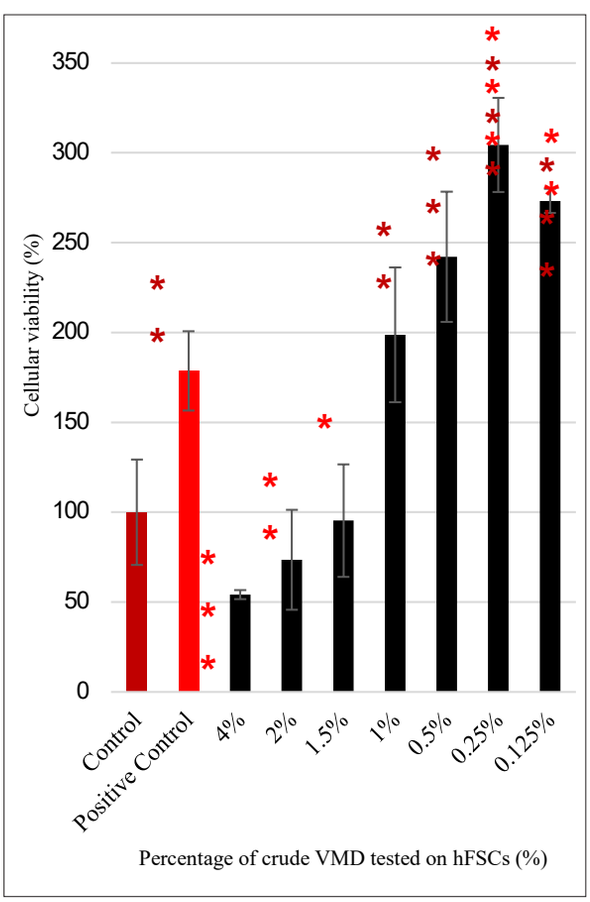


Effects of VMD on hFSCs

VMD concentrations of 0.5%, 0.25%, and 0.125% with % cellular proliferation of 242.1%, 304.3%, and 273.23%, respectively, exhibited approximately 1.4, 1.7, and 1.5-fold higher % cell proliferation stimulation than the positive control ($p < 0.01$) (Fig. 7).

Figure 7.

Effect of Vernonia/Mallotus distillate (VMD) on human fibroblast stem cell (hHFSC) proliferation. Significant proliferative stimulatory activity compared to the control is indicated by maroon star marks, while significant inhibitory or proliferative activity compared to the positive control is represented by red star marks (*p < 0.05, **p< 0.01, ***p< 0.001).



Animal models are commonly used as primary screening platforms in preclinical studies to assess the efficacy and toxicity of potential drug candidates. However, in many countries, they are overutilized during the early stages of drug screening. With the introduction of the 3R (Replacement, Reduction, and Refinement) concept in laboratory animal sciences, alternative and more effective screening platforms have been proposed to minimize the use of animal models in initial screening studies.

Primary cell lines are a proven alternative to replace animal models in initial screening studies. In a study done by our lab group, notable *ex vivo* proliferation capacity of murine bone marrow cells was reported (Jayasinghe et al., 2017). Among these, primary human stem cell lines have shown promising potential in predicting positive outcomes in later stages of drug development. Adult human stem cells, such as hematopoietic stem cells, mesenchymal stem cells, and fibroblast stem cells, are widely used due to their minimal ethical concerns and the high availability of biological waste sources from postpartum and surgical procedures (Udalamaththa et al., 2016).

This study aimed to validate the use of primary human stem cell lines derived from biological waste materials, such as cord blood, cord tissue, and circumcision surgical waste, as an alternative to animal models in the initial screening of potential botanical preparations for stem cell proliferation stimulatory activity.

As demonstrated by the above results, the selected botanical preparations demonstrated significant proliferative stimulatory activities on hMSCs, hHSCs and hFSCs. However, the results indicated that the significance of the activity varied based on the concentration of the botanical extract, the gender of the stem cells, the stem cell type, and the specific botanical extract used. These findings align with previous reports, which demonstrated that variations in the effects of botanical preparations can be attributed to differences in phytochemical concentrations and the selective genetic influence of sex chromosome-related genes (Campesi et al., 2019).

Furthermore, the beneficial synergistic effects of botanical preparations have been well established by Caesar and Cech (2019), highlighting pharmacodynamic synergism through multitarget interactions, modulation of drug transport, enhancement of permeation and bioavailability, reduction of adverse effects, and the targeting of disease-resistant mechanisms. These synergistic effects may explain the superior stimulatory activity of botanical preparations compared to current synthetic stimulants on the selected stem cell lines. The significant findings from whole preparations emphasize the need for the scientific community to explore non-refined, simple alternatives rather than isolating individual compounds for bioactivity screening (Mehta et al., 2021; Oloya et al., 2022). If commercialized, the low production costs of these botanical preparations could substantially reduce the market price of stimulants, fostering pharmaceutical competition and increasing accessibility for patient populations in the developing world.

As a progressive step forward, stimulants can be tested in organoid cultures designed for tissue regeneration. Additionally, clinical trials may be initiated to stimulate in vivo

stem cell populations in patients requiring cell therapy. Primary cell cultures also play a crucial role in investigating the mechanisms of these drugs through various methods, such as qRT-PCR and single-cell RNA analysis. These gene expression studies pave the way for reliable and effective outcomes while ensuring low turnaround times in drug development pipelines (Van et al., 2023).

Preliminary *in vitro*/ *ex vivo* studies are crucial for exploring the potential of Sri Lankan traditional medicine, focusing on specific therapeutic targets. Sri Lanka has a wealth of time-tested effective botanical preparations proven over many generations. However, the absence of scientific validation and the reluctance of traditional practitioners to share their formulas with outsiders-due to the belief that these well-established remedies need no further proof-has impeded their integration into research and the global market as viable drug candidates (Udalamaththa et al., 2021). WHO has acknowledged the therapeutic benefits of botanical preparations and the contributions of traditional medicine practitioners. A global regulatory framework would enhance the long-term impact of these applications, ensuring their proper validation and wider acceptance (WHO, 2004).

The direct use of animal models for preliminary testing of botanical extracts would considerably increase the number of animals involved, extend the duration of experimentation, and introduce a more complex workflow (Dandri et al., 2016). Additionally, variability in the effects of the extracts could arise due to differences between animal species. While human stem cell platforms present a potential alternative, challenges such as the high costs of equipment and cell culture reagents, contamination risks, and the shortage of skilled *vivo* cellular platforms used for screening botanical preparations may not fully replicate the systemic effects observed in in vivo animal models, limiting their ability to reflect real-world outcomes.

Yet, a globally recognized framework for stem cell research platforms and the standardization of botanical preparations would greatly benefit the drug development pipeline by significantly reducing turnaround times.

Conclusions

This study presents a compelling case for the use of primary human mesenchymal, haematopoietic and skin fibroblast stem cell cultures as alternatives to animal models in scientific research, particularly in evaluating the cell-stimulatory effects of botanical preparations. The findings highlight the differential responses of human mesenchymal and haematopoietic stem cells to various plant extracts, underscoring the importance of sex-specific analyses in such studies. By demonstrating the efficacy of botanical compounds in modulating cell proliferation, this research contributes to the refinement and replacement aspects of the 3R principle, paving the way for more ethical and human-relevant biomedical research models.

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