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# *In-Vitro* Cytotoxic and Proliferative Activity of Selected Plant Extracts on Human Peripheral Blood Mononuclear Cells

**[1]Hamid, K. M., [1]Muhammad, A. I., [1]Balogun, A. O., [1]Nkom, D. M., [1]Usman, A. B., and *[2]Aliyu, M.**

*[1] Department of Immunology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto*
*[2] Department of Medical Microbiology and Parasitology, Faculty of Clinical Sciences, College of Health Sciences, Bayero University, Kano.*

## Abstract

Plant products provide a vast source of therapeutics, but not without toxicity. This study evaluated the *in vitro* cytotoxic and proliferative activity of selected plant extracts on human peripheral blood mononuclear cells (PBMCs). The PBMCs from healthy donors were exposed to varying concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) of aqueous extracts of young leaves of *Mangifera indica* (MI) and stem barks of *Commiphora kerstingii* (CK), and *Lannea acida* and *Acacia sieberiana* formulation (LAASF). Trypan blue assay was used to determine the viability of human PBMCs after isolation. Cytotoxicity and proliferation of PBMCs were determined using WST-8 assay. The total viable cell count of the isolated PBMCs in this study was 8000 x $10^4$ cells/ml while viability was 96.15%. The extract of LAASF showed the lowest percentage cytotoxicity (2.63%) at 25 µg/ml concentration, followed by CK (2.70%), then MI (7.71%). There was significant decrease in PBMCs mean absorbance scores across the different concentrations of MI ($p$=0.008), CK ($p$<0.0001) and LAASF ($p$=0.01). There was statistically significant proliferation of PBMCs for MI ($p$=0.003) and CK ($p$=0.005) compared to control. However, no significant difference was observed in LAASF on proliferation. Mean absorbance scores significantly decreased with an increase in the concentration of the extracts. The extracts have potential cytotoxicity on the PBMCs at higher concentrations. The extract of MI and CK exhibited higher cytotoxic and proliferative activity on the PBMCs than LAASF. An in-depth study to identify specific immune cells proliferated by the extracts will improve the credence of this study's findings.

**Keywords**: Herbal medicine, cytotoxicity, cell proliferation, humans, mononuclear leukocytes

## Introduction

Peripheral blood mononuclear cells (PBMCs) are immune cells with a single round nucleus. The PBMC is made up of majorly lymphocytes (T cells, B cells, and natural killer [NK] cells), followed by monocytes/macrophages and dendritic cells. These cells work together to form an indispensable army of host defenses (Hamid et al., 2021). The PBMCs are commonly isolated from whole blood using the Ficoll-Paque density centrifugation method. Other methods of PBMCs isolation include cell prepara-

*Corresponding author: +2348038468616
Email address: maliyu.med@buk.edu.ng

tion tubes (CPTs) and isolation by SepMate tubes with freshly collected blood (Grievink et al., 2016). In humans, the composition of PBMCs varies by individual; typically, lymphocytes account for 70-90%, monocytes for 10-20%, and dendritic cells account for 1-2%. Within the lymphocyte population, cell types include 70-85% $CD3^+$ T cells, 5-10% B cells, and 5-20% NK cells (Kleiveland, 2015). The PBMCs are a critical component of the immune system in defense to non-self (Pourahmad & Salimi, 2015).

For centuries, medicinal plants have been a valuable source of herbal products all over the world (Ahmad et al., 2021; Lemonnier et al., 2017). Today about four-fifths of the world's population uses herbal plant medicines (Kale et al., 2019). The identification of bioactive constituents and research on herbal plants have revealed their potential as valuable research material (Keskin et al., 2018). Plant metabolites like tannins, saponins, cardiac glycosides, flavonoids, diterpenoids and triterpenoids possess immunomodulatory function, hence possible role in treatment of diseases with deranged immune function (Renda et al., 2022). The immunomodulatory activity can be linked to proliferation of immune cells such as lymphocytes, when in contact with specific antigens, mitogens or allogenic cells and cytokines secretions (Li et al., 2022; Song et al., 2020).

Despite advancements in drug manufacturing techniques, a number of diseases continue to kill a significant number of people worldwide (Thomford et al., 2018). Scientific research on herbal remedies has triggered the development of newer, promising, and efficient bioactive drugs to treat illnesses (Usman et al., 2021). There is a misconception that herbal medicines are non-toxic (Ekor, 2014). This widely held belief is incorrect, as some therapeutic herbs have been linked to a variety of side effects, including death (Bernstein et al., 2021; Chaachouay et al., 2021). Furthermore, due to issues with standardization and dosage, insufficient identification and isolation, and a lack of clinical and toxicological evaluations of many medicinal plants, using herbal products has been difficult (Okaiyeto et al., 2021; Thomford et al., 2018). As such, experimental safety studies are required prior to developing new therapeutic agents (Ahmad et al., 2021) and subsequent development of safety profiles (Reduan et al., 2020). Herbal products have demonstrated antioxidant, antimicrobial and anti-inflammatory properties (Watafua et al., 2022).

A *Mangifera indica* L belongs to the Anacardiaceae family, richly found in Nigeria, Mexico, Brazil, South and Southeast Asia, India, Philippines, China, Thailand, Indonesia, Pakistan, and Bangladesh (Kumar et al., 2021). The *M. indica* fruit is popularly known as mango, while its local name at Sokoto is ‘mongoro’ (Keta, 2016). Mangiferin, ascorbic acid, benzophenones, flavonoids, carotenoids, phenolic acids, and tocopherols are among the many phytochemicals extracted from its leaves, which were shown to be promising in treatment of diabetes mellitus (Kulkarni & Rathod, 2018; Kumar et al., 2021), infections (Dzotam & Kuete, 2017), obesity (Ramírez et al., 2017), hyperlipidaemia, and diarrhoeal diseases (Kumar et al., 2021).

*Acacia sieberiana* DC belongs to Fabaceae family distributed in tropical and sub-tropical regions (Salisu et al., 2021), commonly known as Paperback thorn and in North-western Nigeria as ‘farar kaya’ (Keta, 2016). The *A. sieberiana* has luteolin, ellagic acid, isoferulic acid, gallic acid, kaempferol, apigenin, quercetin, glucoside dihydroacacipetalin and acacipetalin as some of its phytochemical contents and have been used traditionally for treatment of cancer, diarrhoea, and infections (Watafua et al., 2022).

*Lannea acida* A. Rich, belongs to Anacardiaceae family, richly found in sub-Saharan Africa dry savannah, commonly used for the treatment of malaria, rectal haemorrhoids, diarrhoea, malnutrition and dysentery (Olusola et al., 2020). The *L. acida* fruit is commonly called plum mango, while its local name at Sokoto is ‘faru’ (Keta, 2016). In Ghana *L. acida* is used for treatment of eye inflammation, beriberi, schistosomiasis, and haemorrhoids (Maroyi, 2018).

A *Commiphora kerstingii* Engl. belongs to Burseraceae family, growing as a tree commonly in sub-Saharan Africa widely distributed in Nigeria, Togo and Central African Republic. The *C. kerstingii* is commonly known as torchwood and locally called ‘hana gobara’ in Sokoto and neighbouring states (Keta, 2016).The *C. kerstingii*

possesses anthraquinones, alkaloids, tannins, saponins, essential oils, flavonoids, proteins, terpenoids and carbohydrates as its phytochemicals. It is used locally as arrow poison anti-dote and treatment of diabetes mellitus, cancer, measles, rheumatism and asthma (Lawrence et al., 2022).

As outlined above, all the four herbal constituents used for this study (*M. indica, C. kerstingii, L. acida, and Acacia sieberiana*) have been shown to be used locally for the treatment of cancer and inflammatory diseases, which piqued our interest in studying them together to assess their potential effect on PBMC proliferation, as a foundation for the development of therapeutic agents. Again, in most instances, *L. acida, and A. sieberiana* are mostly used together as a polyherbal formulation. The main problem associated with herbal medicine is the lack of dosage standardisation and data regarding evaluation of toxicity (Mainasara et al., 2016). Limited data exist on the effect of herbal products on immune cells. The current *in-vitro* study aims to provide baseline data on the cytotoxic and proliferative effects of the aforementioned herbal products on human PBMCs to establish their safety and potential therapeutic benefits.

## Materials and Methods

### Plant Collection and Identification

Fresh young leaves of *Mangifera Indica* (MI) were collected at Wamakko Local Government (LG), Sokoto, stem bark of *Commiphora kerstingii* (CK) were collected at Wudil LG, Kano, and stem barks of *Lannea acida* and *Acacia sieberiana* used for the formulation (LAASF) were collected at Dange/Shuni LG, Sokoto. The plant materials were identified by Dr. M. E. Halilu, at the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. The assigned voucher numbers were MI (UDUS/PCG/ANAC/0001), CK (PCG/UDUS/BURS/0002), *Lannea acida* (PCG/UDUS/ANAC/0004) and *Acacia sieberiana* (PCG/UDUS/FABA/0001).

### Plant Preparation and Extraction

Fresh plant materials were washed thoroughly with tap water and rinsed with distilled water. The plant materials were then air-dried at room temperature for 14 days. After drying, they were ground using pestle and mortar and then passed through a sieve to obtain a fine powder which was then weighed using a weighing balance (TXB 4224, Shimadzu, USA). Exactly 120 g of each dried powder was dissolved in 1.5 L of distilled water and the preparation was left to soak for 24 h at room temperature. The preparations were then filtered using Whatman No. 1 filter paper. The resultant filtrates were concentrated to dryness using a hot oven at 60$^{o}$C under reduced pressure (Gadanya & Muhammad, 2018). The yield of each extract was MI (20 g), CK (25 g), and LAASF (28 g). The percentage yield of each extract was 16.6%, 20.8% and 23.3% for MI, CK and LAASF respectively

### Blood Collection

Three millilitres (3 ml) of whole blood was collected using Monovette vacutainer system from apparently healthy volunteer that consented to participate in the study. The sample collected was transferred into a labelled lithium heparin tube, mixed properly and used for isolation of human PBMCs (Hamid et al., 2021).

### Ethical Statement

This study was approved by State Health Research Ethics Committee under the Ministry of Health, Sokoto State. The approval letter contained the following reference numbers: MI (SHHREC/016)2021), CK (SKHREC/017)2021) and LAASF (SKHREC/018)2021). The research was carried out in line with the Helsinki declaration (Rickham, 1964).

### Isolation of PBMCs

The PBMCs were isolated using Histopaque-1077 (Sigma-Aldrich® Co. UK) as described in Hamid et al. (2021). Briefly, three millilitres (3 ml) of Histopaque-1077 was added to a 15 ml centrifuge tube and brought to room temperature. Three millilitres (3 ml) of whole blood were layered carefully onto the Histopaque-1077, and centrifuged at 400 x *g* for 30 min at room temperature. After centrifugation, the tubes were

carefully removed and the upper layer was aspirated with a Pasteur pipette to within 0.5 cm of the opaque interface containing mononuclear cells. The opaque interface was carefully transferred into a clean centrifuge tube with a Pasteur pipette. The cells were washed by adding 10 ml of isotonic phosphate-buffered saline (PBS) solution, and mixed gently and then centrifuged at 300 x *g* for 10 min. The supernatant fluid was aspirated and discarded. The cells were resuspended with 5 ml of isotonic PBS solution; it was then mixed gently and centrifuged again at 300 x *g* for 10 min. This was repeated once, after that, the supernatant fluid was discarded. The cells were resuspended in 2 ml of RPMI-1640 (Beijing Solarbio Science and Technology Co. Ltd, China), treated with 10% heat-inactivated foetal bovine serum (FBS), and immediately used.

**Trypan Blue assay**

The PBMCs isolated were counted using trypan blue assay as described in Hamid et al. (2021). Briefly, ten microliters (10 µl) of 0.4% Trypan Blue (Sigma-Aldrich® Co. United Kingdom) solution (w/v) and 10 µl of the PBMCs suspension were transferred into a cryovials tube (dilution factor = 2) and mixed, it was then allowed to stand for maximum of 5 min. While the cover-slip was in place, the pipette was used to transfer 10 µl of Trypan blue-PBMCs suspension mixture to both chambers of the haemocytometer. Both the viable and non-viable cells were counted using a light microscope. Non-viable cells were stained blue, whereas viable ones remain colourless. The percentage of viable cells was calculated.

**Cytotoxicity Test**

Different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) of the MI, CK, and LAASF were used to determine the cytotoxicity on the PBMCs using WST-8 cell proliferation assay (Beijing Solarbio Science and Technology Co. Ltd. China). The procedure was carried out according to manufacturers' instructions. The cells (100 µl/well) were seeded in a 48-well plate with RPMI-1640 medium at a concentration of 1 x $10^5$ cells/well and incubated at 37°C and 5% $CO_2$ in a humidified atmosphere incubator for 24 h. The cells in the 48-well plate were then treated with different concentrations (10 µl/well) of the extracts (25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) and incubated at 37°C and 5% $CO_2$ in a humidified atmosphere incubator for 4 h. After the incubation period, 10 µl/well of WST-8 reagent was added to the treated cells and then incubated for 1 h at 37°C and 5% $CO_2$ in a humidified atmosphere incubator. Peripheral blood mononuclear cells suspended in RPMI-1640 were used as a negative control, RPMI-1640 and FBS were used as a media background control, PBS only was used as normal control, and an empty well as blank. All treatments were performed in duplicate. The absorbance was read using ELISA microplate reader at 450 nm and percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{100 \times (\text{Control} - \text{Sample})}{\text{Control}}$$

**Proliferation Assay**

The procedure was performed according to the manufacturers' instructions. Peripheral blood mononuclear cells suspension (100 $\mu$l) was dispensed into a 48-well microliter plates at a density of 1 x $10^5$ cells/well in RPMI-1640 at 37ºC in 5% $CO_2$ humidified incubator for 24 h. Phytohaemagglutinin (PHA: 10 µg/ml; Sigma-Aldrich® Co. United Kingdom) was added to each of the PBMCs cultures with 10 $\mu$l of the extracts at $IC_{50}$ concentration (25 µg/ml) incubated for 4 h at 37°C and 5% $CO_2$ in a humidified atmosphere incubator. Thereafter, 10 µl/well of WST-8 cell proliferation assay reagent was added and then incubated for 1 h at 37°C and 5% $CO_2$ in a humidified atmosphere incubator. The PBMCs suspended in RPMI-1640 were used as a negative control, RPMI-1640 and FBS were used as a media background control, PBS only was used as normal control, and an empty well as blank. PBMCs not treated with the extract, RPMI-1640, FBS, and PBS were considered controls. All treatments were performed in duplicate. The absorbance was read using an ELISA microplate reader at 450 nm. The amount of absorbance is proportional to cell number (proliferation).

**Statistical Analysis**

The results obtained were entered into SPSS version 21 (IBM, USA) for analysis. Effect of the

extracts on PBMCs and its proliferation were expressed as means and standard deviations (SD), whereas cytotoxicity was expressed in percentages. One-way between-groups analysis of variance (ANOVA) with a post-hoc test (Bonferroni) was carried out to compare between groups. An independent sample t-test was carried out to compare the mean absorbance of control and treatment on PBMCs proliferation. A *p*-value less than 0.05 is considered statistically significant.

## Results

### PBMCs Cell Count and Percentage Viability

The total viable cell count of the PBMCs was 8000 x $10^4$ cells/ml, while the total non-viable cell count of the PBMCs was 320 x $10^4$ cells/ml. The percentage viability of the PBMCs was 96.15%.

### Percentage Cytotoxicity of the Different Extracts on PBMCs

The percentage of cytotoxicity tends to increase with an increase in extract concentration. The extract with lowest percentage cytotoxicity was LAASF (2.63%) at 25 µg/ml, while the highest was MI (19.89%) at 200 µg/ml. At 25µg/ml, the extracts have the following percentage cytotoxicity LAASF (2.63%), CK (2.70%) and MI (7.71%). Therefore, the minimum concentration with the lowest cytotoxicity was 25 µg/ml for all the extracts and this is equivalent to minimum inhibitory concentration ($IC_{50}$) (Figure 1).

### Comparison of Different Extract Concentrations on Human PBMCs

As depicted in Figure 2, there was significant decrease in human PBMCs mean absorbance scores with an increase in concentrations of the extract; MI extract (F = 9.570, $p$=0.008), CK extract (F= 55.527, $p$<0.0001), and LAASF extract (F =8.224, $p$=0.01). When compared with control, CK extract show significant decrease in the mean absorbance scores at 100 µg/ml ($p$=0.03) and 200 µg/ml ($p$=0.004) concentrations. So also, CK extract show significant decrease in the mean absorbance scores when 50 µg/ml concentration was compared with 100 µg/ml ($p$<0.0001) and 200 µg/ml ($p$=0.03). However, MI and LAASF extracts did not show any significant statistical difference in the mean absorbance scores with that of control at any concentration ($p$>0.05). The mean absorbance scores of the PBMCs tends to decrease with an increase in concentrations of the extract.

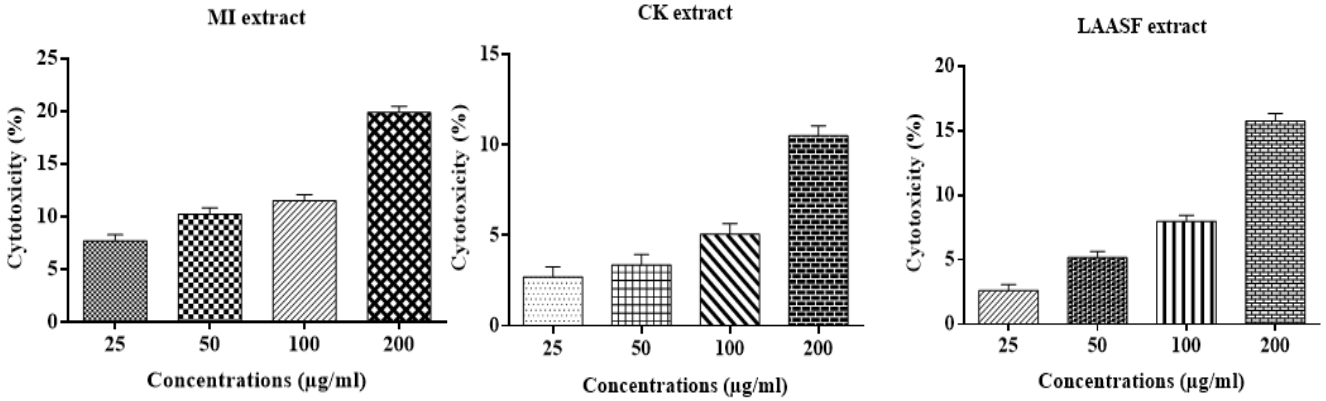


**Figure 1:** Percentage cytotoxicity of different concentrations of the extract on PBMCs. MI: aqueous extracts of young leaves of *Mangifera indica,* CK: aqueous extracts of stem barks of *Commiphora kerstingii,* LAASF: aqueous extracts of stem barks of *Lannea acid*a and *Acacia sieberiana* formulation

**Effect of Extracts on Proliferation of Human PBMCs**

From Figure 3, the higher the mean absorbance the higher the proliferation. There was significant proliferation of PBMCs treated with MI ($p$=0.003) and CK ($p$=0.005) extracts when compared with PBMCs proliferation in control. However, no statistical significant proliferation of PBMCs treated with LAASF extract when compared with the PBMCs proliferation in control ($p$=0.11).

**Discussion**

In the current study, the viability of PBMCs decreased as the concentration of all extracts studied increased (MI, CK, and LAASF). This suggests that the higher the concentration of extract, the greater the cytotoxicity on the PBMCs: lesser viability. In comparison, most of the lower concentrations of the extracts had a very low cytotoxic effect on the PBMCs, which could be attributed to the low dosage of the extracts, which may contain a lower amount of cytotoxic phytochemicals and thus do not significantly impair cell viability. This finding is consistent with previous research that found a dose-dependent decrease in PBMC viability when exposed to *Cassia occidentalis* (Hamid et al., 2021), *Vitellaria paradoxa* (Kalgo et al., 2020), *Annona squamosa, Datura metel,* and *Mentha piperita* (Sudeep et al., 2017) plants extracts.

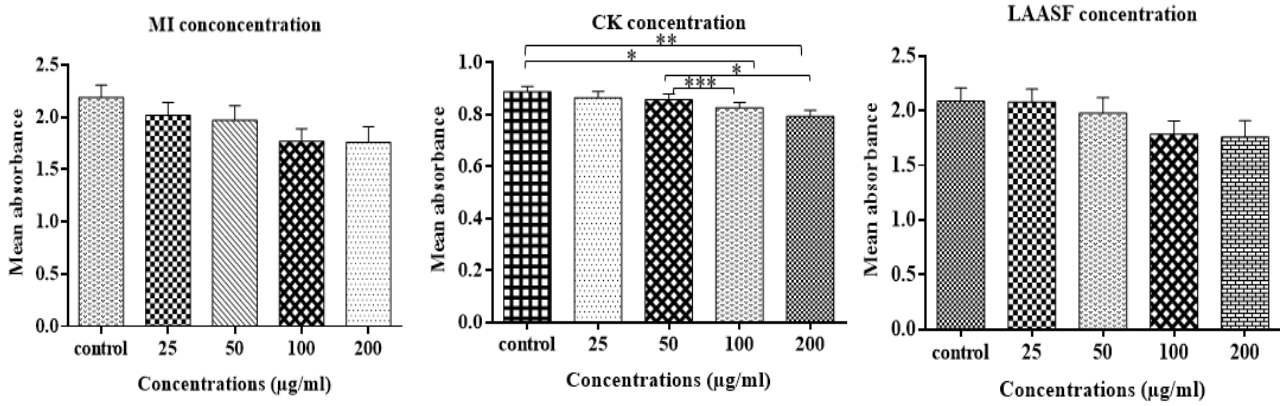


**Figure 2: Effects of different concentrations of the extracts on PBMCs. MI: aqueous extracts of young leaves of *Mangifera indica,* CK: aqueous extracts of stem barks of *Commiphora kerstingii,* LAASF: aqueous extracts of stem barks of *Lannea acid*a and *Acacia sieberiana* formulation. $^{*}p$=0.03, $^{**}p$=0.004, $^{***}p$ <0.0001**

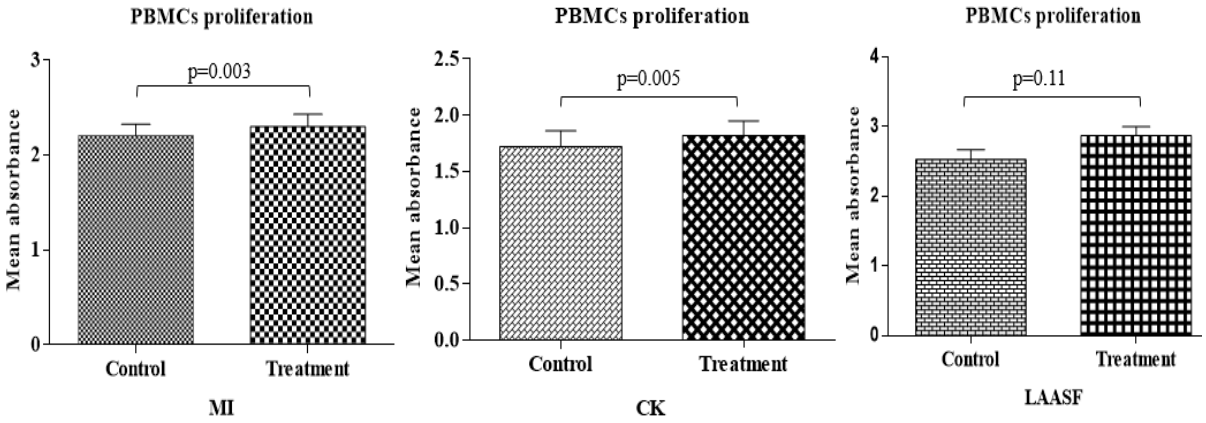


**Figure 3: Effect of different extracts on human PBMCs proliferation. The PBMCs were treated with 25 µg/ml ($IC_{50}$) concentrations of the different extracts. MI: aqueous extracts of young leaves of *Mangifera indica,* CK: aqueous extracts of stem barks of *Commiphora kerstingii,* LAASF: aqueous extracts of stem barks of *Lannea acid*a and *Acacia sieberiana* formulation.**

At the lowest concentration tested (25 g/ml), LAASF had the least cytotoxic effect on PBMCs, while MI had the highest cytotoxicity at the highest concentration tested (200 g/ml). The lowest extract concentrations showed the least cytotoxicity for all extracts. The aforementioned observation could be attributed to LAASF having fewer toxic bioactive compounds than MI, with CK being intermediate. As a result, MI may play an important role in the treatment of cancer and proliferative disorders where cytotoxicity is required, whereas LAASF demonstrated safety at lower concentrations.

Lower concentrations of MI and CK extracts induced significant proliferation of PBMCs, suggesting that in conditions of marked cytopaenia, MI and CK extracts may have a role in inducing cellular proliferation. The observed immune cells proliferation could be attributed to the presence of stimulatory bioactive compounds in the extracts. Mangiferin in the MI was shown to induce B-cell proliferation (García et al., 2003). While extracts of pomegranate peel (Okonogi et al., 2007), methanol extract of *Calophyllum brasiliense*, *Ipomoea pes-caprae,* and *Mataybaelaeagnoides* (Putri et al., 2018) were reported to induce proliferation of PBMCs. However, LAASF had no effect on PBMC proliferation. Of note, some studies reported inhibition of lymphocyte proliferation when exposed to leaf extracts of *Annona squamosa* (Sudeep et al., 2017) and *Albizia gummifera* (Makgatho et al., 2015). However, Ibrahim et al. (2016), reported that the lowest concentration of methanolic extract of leaves of CK showed the highest percentage of inhibition. Similarly, Asadi and co-workers reported inhibition of PBMCs proliferation by *Echinops lasiolepsis* extract at concentrations: 0.1, 1 and 200 µg/ml (Asadi et al., 2014). Toxicity varies from plant to plant and can inhibit cell survival (Sudeep et al., 2017). Typically, herbal extracts are a salad of phytochemicals with synergistic, allosteric, and antagonistic effects. Hence, at certain concentrations, an active substance may lose its efficacy or affect the pharmacodynamics of another constituent (Mazumdar et al., 2022). These differences in composition could be explained by the concept of chemotype phenomenon, geographical origins, and methods of extraction or detection used. An in-depth research is required to determine the precise mechanisms of these phenomena of interest by understanding the interaction of different phytochemicals at differing concentrations (Ahomadegbe et al., 2018).

## Conclusion

It can be deduced from this study that *Mangifera indica, Commiphora kerstingii,* and *Lannea acida* and *Acacia sieberiana* formulation extracts are less toxic to PBMCs at lower concentrations. However, *Mangifera indica* is the most toxic at the highest concentration of the four, and as such, this property can be used in cytotoxic therapy. At low concentrations, extracts of *Mangifera indica* and *Commiphora kerstingii* stimulate PBMC proliferation, which is desirable in cytopaenic conditions. It is therefore recommended to identify the specific immune cells that respond to the individual extracts in each case.

## Conflict of Interest

The authors declare no conflict of interest

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