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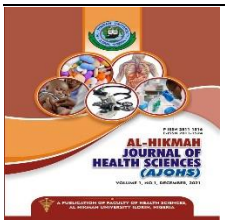

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Haemopoietic Multi-Potent Stem Cells Differentiation-Enhancing Effects of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* Leaves Extracts

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ABSTRACT

Objective: This study investigated the probable haemopoietic multi-stem cells differentiation enhancing effects of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* leaves extracts. **Materials and Methods:** Haemopoietic bone marrow stem cells from irradiated guinea pigs were harvested on day 13th post-irradiation, cultured and treated with varying concentrations (0.313-100%) of aqueous leaf extracts of each of the plant extracts. The degree of differentiation was determined by MTT assay. **Results:** The degree of differentiation was highest in *Telfairia occidentalis*, followed by *Camellia sinensis* and *Parquetina nigrescens* respectively. Combination of the three plants showed a synergistic stimulatory effect on cell differentiation. Also, strong positive correlation between the concentrations of each of the extract of the three plants and the degree of differentiation. **Conclusion:** *Camellia sinensis, Telfairia occidentalis and Parquetina nigrescens* leaves extracts have positive differentiation enhancing effects on haemopoietic multi-potent stem cells individually and when combined.

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Introduction

Ionizing radiation destroys both mature blood and hematopoietic progenitor/stem cells in the bone marrow, reducing the number of tissue-specific adult stem cells that are critical for hematopoietic repair and regeneration. While loss of mature blood cells is a key

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factor in radiation morbidity, mortality is believed to occur due to prolonged myelo-suppression from the loss of hematopoietic progenitor (HPC) and primitive hematopoietic stem cells (HSC) (Mauch, *et al.,* 1995 and Yong, *et al.,* 2014). A delicate balance between HSC self-renewal, proliferation and differentiation is required to ensure proper bone marrow HSC repopulation, progenitor cell reconstitution and mature blood cell production (Jun and Irving, 2010). Normally, HSC quiescence ensures life-long maintenance of its pool, protecting against premature exhaustion of hematopoietic potential (Cheng *et al.*, 2000 and Yong *et al.,* 2014).

In recent years, the important role of radiation-damaged bone marrow, skin and gastrointestinal tract epithelium in the severity of the acute radiation illness has come to light (Dainiak *et al.,* 2003; Turai *et al.,* 2004). The current therapeutic strategies of irradiated normal tissue consist mainly of the treatment of symptoms or bone marrow transplantation and intensive care in severe cases. New multi-targeted therapeutic approaches are necessary to inhibit further destruction of vital organs or even to prevent the onset of acute radiation syndrome. Irritation of these organ systems is critical in driving the destructive process from a circumscribed radiogenic lesion to a state of multi-organ involvement (MOI) or failure (MOF) (Turai *et al*., 2004).
Stem cells are undifferentiated cells with the ability to proliferate and produce large numbers of differentiated progeny (Fuchs and Segre, 2000). Haemopoietic stem cells are scarce with 1 in every 20 million nucleated cells in bone marrow (Lanza *et al*., 2006). Although its exact phenotype is unknown, immunologically it is $CD34^+$ $CD38^-$ and has the appearance of a small or medium-sized lymphocyte. Cell differentiation occurs from the stem cell via the committed haemopoietic progenitors which are restricted in their developmental potential (Lanza *et al*., 2006). Haemopoiesis is a process regulated by a complex network of soluble factors that stimulate the growth and differentiation of haemopoietic progenitor cells (HPC) (Metcalf, 1988). The proliferation and differentiation of HPC are influenced to a large extent by interactions among various cell types in the haemopoietic compartment and by haemopoietic cytokines produced by stromal cells and lymphocytes (Jacobsen, 1996). Bone marrow transplantation performed after myelo-ablative treatment of the recipient has been used to reconstitute haemopoiesis (Sato *et al.*, 1993).

The use of hematopoietic cytokines together with bone marrow transplantation has been shown to hasten neutrophil and platelet recovery, suggesting that appropriate combinations of these factors may be used to promote full reconstitution of the haemopoietic compartment (Knobel *et al*., 1994; Lane *et al*., 1995; Williams *et al.,* 1996). Cytokines modulate haemopoiesis by maintaining self-renewal and stimulating the proliferation and maturation of committed progenitor cells required for the continuous replacement of mature blood cells (Vijay *et al*., 2014). Various combinations of cytokines including interleukin-1 (IL-1), IL-3, IL-6, stem cell factor (SCF) and erythropoietin (EPO) have been found to support the growth of multi-potent progenitor cells in vitro. Granulocyte-colony-stimulating factor (G-CSF) and EPO are two growth factors that provide effective treatments for neutropenia and anaemia and are used to enhance peripheral blood progenitors as an alternative to bone marrow transplantation for cancer patients (Vijay *et al*., 2014).

Some plant extracts consumed locally such as *Camellia sinensis, Telfairia occidentalis and Parquetina nigrescens* were reported to have long age usage in promoting good health by correcting anaemia and treatment of malaria caused. Thus, it become imperative to establish the synergistic differentiation activities of the leave extracts of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* on haemopoietic stem cell.

Materials and Methods

Plant Collection and Identification

Camellia sinensis was obtained from government registered pharmaceutical premises in Ilorin, Nigeria while fresh samples of *Parquetina nigrescens* and *Telfairia occidentalis* plants were obtained within Ilorin metropolis. The plants were identified by carrying out macroscopical examination on plant samples as stipulated by Dalziel (1968), and confirmed and authenticated by staff in the herbarium, Department of Plant Science, University of Ilorin, Nigeria. *Parquetina Nigrescens* was given Serial Number 876 and Ledger Number 67 while *Telfairia Occidentalis* was given Serial Number 959 and Ledger Number 150. The samples were air dried at room temperature.

Aqueous extraction of Plant

Extraction was carried out as described by Olowosolu and Ibrahim (2006). Ten grams of each of plant material were macerated in pestle and mortar with 100ml distilled water. Filtrate obtained was subsequently passed through Whatman's No. 1 filter paper under aseptic conditions and the filtrate was collected in fresh sterilized glass tubes and used within 24h for the research work (Agbor *et al.,* 2001). The final concentration of 1gm/ml was obtained as

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aqueous extract which served as the stock solution for dilutions needed during the course of the work.

Experimental Animal

Twelve young male guinea-pigs weighing approximately 450 grams were obtained from the animal house, LAUTECH College of Medicine Osogbo, Osun-State, Nigeria. The animals were housed in Department of Anatomy animal house, University of Ilorin in a temperature and humidity-controlled environment maintained at 12-hour light/dark cycle. Food and water were available *ad libitum* throughout the experiment. Animals were treated according to the National and European Union Directive 2010/63/EU guidelines for handling animals used for scientific purposes

Ethical Approval

Ethical clearance and approval for the animal study was given by the University of Ilorin Ethical Review Committee (UERC) with reference number UERC/ASN/2018/1109.

Irradiation of the Guinea-Pig

The irradiation of the guinea-pigs was carried out at University College Hospital which was in accordance with the type, dose and method of irradiation as well as the after-care procedures adopted by Harris (1967). Each guinea-pig was separately irradiated under general anesthesia (intra-muscular ketamine 5mg/Kg body weight plus 1 mg Atropine). The animal was placed in a cotton-gauze bag and positioned lying on its side. Irradiation was given to each flank, the irradiation time being divided equally between each side, i.e. the animal was turned over onto its opposite side half-way through the procedure. Each animal was given 200r (2.0Gy) whole-body gamma-irradiation under general anaesthesia, using a Co^{60} therapy unit as source, at a dose rate of 98.560cGy/minute.

Post irradiation Care of the Animals

To minimize the two hazards enumerated by Harris, 1967, i.e., the danger of internal haemorrhage from minor trauma and the risk of infection, resulting from the effects of irradiation on haemopoietic tissues, each irradiated animal was kept in a separate cage and excessive handling avoided. Each animal was adequately fed and given adequate supply of water.

Bone Marrow Harvest

Bone marrow cells from guinea pigs were harvested by the method of Galvin *et al.* (1996). The animals were slaughtered by cervical dislocation and the Femurs were carefully located and removed aseptically. Adherent soft tissue and cartilage were stripped from the bones and the tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into the proximal end of the bone and flushing with phosphate buffer saline into a universal bottle containing phosphate buffer saline (PBS), 200 Units/ml heparin, Hanks balanced salt solution (HBSS), suspended with 2% fetal calf serum (FCS). The suspended marrow cells were further diluted with the diluting factor of 1 in 20 and the cells were counted to achieve a cell count of 1.0 X 10^9/L (Miroslav, 2008*).*

Microscopic Observation of Harvested Bone Marrow

The initial phase of final haematopoietic recovery at 13 days post irradiation as described by Harris, (1967) was further confirmed by cytochemical reactivities as described by Caxton-Martins (1973) using May-Grunwald's staining technique. The harvested cells stained with May-Grunwald's staining technique shows cells of high nuclear:cytoplasmic ratio, lepto chromatic nucleus, basophilic cytoplasm and presence of a nuclear hof. Cytochemically, cells were negative for myeloperoxidase, specific and non-specific esterase, leucocyte alkaline phosphatase and acid phosphatase, Sudan Black B, Periodic acid Schiff.

Preparation of Marrow Suspension

The marrow suspension was prepared and incubated in fresh autologous serum as follows: The abdomen of the anaesthetized animal was opened up and the inferior vena-cava was exposed, incised and about 5ml of blood was collected into a centrifuge tube through a glass funnel. After clotting, the blood was centrifuged for 5 minutes at 3000rpm and the supernatant serum withdrawn with a Pasteur pipette into clean, small glass tubes. After the serum was obtained, the isolated bone marrow cells were placed into the autologous serum contained in a clean glass tube fitted with a rubber stopper. This marrow suspension was used for the cytochemical studies to further establish the relative incidence of transitional cells as enumerated by Caxton-Martins, 1973 and others (Harris, Menkin and Yoffey, 1956). Using this technique, the incidence of damaged cells in the marrow smears was kept to a low level.

Culture Media Preparation

A 1000millitres of Growth and maintenance Eagles MEM media was prepared using the method described by (Seit and Weissman, 2010). One bottle of Eagles MEM powder containing 9.4 grams was dissolved completely in 1000mls of sterile double distilled de-ionized water. The mixture was autoclaved at 121^{o}C for 15 minutes with cap slightly loose and allowed to cool to room temperature. The pH of the autoclaved

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Eagles MEM was maintained at 4.3 – 4.5. The reagents dispensed into two empty 500 ml sterile bottles are 12.5mls of 7.5% Sodium bicarbonate, 5mls of L-Glutamine, 5mls of HEPES IM, 5mls of Penicillin Streptomycin and 10mls of FBS. The autoclaved Eagles MEM was added to each of the 500 ml bottles containing the reagents to reach the mark of 500 ml on the bottle. The pH was adjusted to 7.2 – 7.4). An aliquot of 250 ml of each prepared bottle of medium was added to tissue culture tubes and label appropriately for sterility testing. The aliquots were incubated at 37ºC for 5 – 7 days, inoculate into Thioglycollate broth. The prepared media was stored at +4ºC until use.

Cell Culture Technique

At 13th day Post irradiation, the bone marrow committed cells were harvested and cultured with the extract of the plant at concentration ranges of 0.313-100%. Five microliters (5µl) of suspended guinea-pig bone marrow cells harvested were cultured in a Laminar Flow Cabinet at a concentration of 1.0 X 10^9/L in 20 µl of Growth Eagles Minimum Essential Media (MEM) and 10 µl each of the plants extracts at concentration ranges of 0.313-100% were added to enhance differentiation. Another set of 24 wells were also set alongside the test to serve as control in which no extract was added. The culture plates (48 wells) were incubated at 37ºC for 72 hours. After the treatment, the cells were prepared for 3-[4, 5-Dimethylthiazol-2-YL]-2, 5-Diphenyltetrazolium Bromide (MTT) analysis of cell differentiation.

Cell Differentiation Assay

Twenty microliters (20µl) of MTT Solution were added to each well 5 hours before the end of the treatment in the culture plate. The plates were then incubated in a CO_2 incubator for 5 hours and the culture media removed with needle and syringe. Two hundred microliters (200µl) of DMSO was added to each well with pipetting up and down to dissolve crystals. Plates were re-incubated in a CO_2 incubator for 5 minutes, transferred to a micro-plate reader and the absorbance measured at 450nm.

Data Analysis

Data were analyzed using the statistical Package for Social Sciences (SPSS 16). Comparison of mean values between the two populations was made using Student's t-test for independent variables while Pearson Correlation coefficient was used to determine the relationship among the variables. P values < 0.05 were considered significant.

Results

The individual extract of the three plants was tested for induction of haemopoietic multipotent stem cells differentiation stimulation and when compared with the positive and negative controls showed statistical significance. The potential to differentiate haemopoietic multipotent stem cells was observed to be highest with *Telfairia occidentalis.*

Table 1 below shows the mean values of the differentiating potentials of individual extract of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* at a wide range of concentrations. Two controls; positive and negative controls were used. All the extracts showed significant statistical differences at p-value of 0.000 when compared with the control using ANOVA.

Table 1: Effects of Individual Plant extract 13th Day Post Irradiation

Protocol	Culture Well	Mean ± Std Deviation	*p*-value
Telfairia occidentalis (TO) treated culture plates	24	3.54±0.30	0.001
Camellia sinensis (GT) treated culture plates	24	3.03±0.62	
Parquetina nigrescens (PN) treated culture plates	24	2.88±0.72	
Non-extract treated culture plates	24	0.18±0.01	
GM-CSF treated culture plates	24	0.88±0.05	

The values are expressed as mean ± SEM, **Sig.** p<0.05

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Table 2: Effects of Combined Plants extracts on 13th Day Post Irradiation.

Protocol	Culture Well	Mean ± Std Deviation	*P*-Value
Telfairia occidentalis (TO) plus *Camellia sinensis* (GT) treated culture plates	24	2.85±1.04	0.001
Parquetina nigrescens (PN) plus *Camellia sinensis* (GT) treated culture plates	24	1.61±0.70	
Combined GT/PN/TO treated culture plates	24	1.89±1.15	
Non-extract treated culture plates	24	0.18±0.01	
GM-CSF treated culture plates	24	0.88±0.05	

The values were expressed as mean ± SEM **Sig.** P < 0.05

Table 2 showed the combined extracts of the three plants when compared with the positive and negative controls showed statistical significance. The potential to differentiate haemopoietic multipotent stem cells synergistically was observed to be highest when *Telfairia occidentalis* was combined with *Camellia sinensis.*

Table 3 below shows a correlation between the individual and combined extracts and the degree of differentiation of the cultured Guinea-pig bone marrow haemopoietic stem cell harvested 13th Day post-irradiation. All the extracts of the plants showed a significant statistical difference at p-value of 0.000 using Pearson's correlation.

Table 3: Relationship between Plant extracts and degree of differentiation

Protocol	Culture Well	Mean ± Std Deviation	Calculated 'r' (Pearson's)	P-Value
Telfairia occidentalis (TO) treated culture plates	24	3.5850±0.01	0.769	0.001
Camellia sinensis (GT) treated culture plates	24	2.9130±0.68	0.846	0.001
Parquetina nigrescens (PN) treated culture plates	24	2.7475±0.90	0.845	0.001
Combined GT/PN/TO treated culture plates	24	1.9359±1.14	0.819	0.001

The values were expressed as mean ± SEM **Sig.** P < 0.05

Discussion

This study demonstrates induction of differentiation of haemopoietic multipotent stem cells and progenitor cells by *Parquetina nigrescens*, *Camellia sinensis* and *Telfairia occidentalis* extracts at a wide range of concentrations.

Camellia sinensis is a green tea made from the steamed and dried leaves of the *Camellia sinensis* plant, a shrub native to Asia. Green tea has been widely consumed in Japan, China and other Asian nations to promote good health for at least 3,000 years (Costa *et al.,* 2002). Tea, a leaf extract of the plant *Camellia sinensis*, is the second most consumed beverage in the world, with an estimated 18-20 billion cups consumed daily and an estimated average consumption of 1 L/person/day in the United Kingdom (Costa *et al.,* 2002). The chemical composition of green tea is complex: polyphenols, alkaloids (caffeine, theophylline, and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, fluoride, minerals and trace elements, and other undefined compounds. The polyphenols constitute the most interesting group of tea leaf components and exhibit potent anti-oxidant activity in vitro and in vivo (Wu *et al.,* 2002). Tea has been considered a medicine and a healthful beverage since ancient times, but recently it has received a great deal of attention because tea polyphenols are strong anti-oxidants. The anti-mutagenic, anti-diabetic, anti-bacterial, anti-inflammatory, and hypocholesterolemic qualities of the aqueous extract of the major tea polyphenols have also been demonstrated (Feng *et al.,* 2001; Kondo *et al.,* 2002).

Telfairia occidentalis of the family Cucurbitaceae, is an herbal plant cultivated mostly in the West African sub-region (Burkett, 1968). The leaf extract of the plant is used locally in the treatment of malaria and anaemia (Gbile, 1986). Apart from the nutritional (Okoli, *et al.,* 1983), agricultural and industrial importance (Akoroda, 1990), the plant is also medicinally useful. It possesses anti-inflammatory (Oluwole, 2003), anti-bacterial (Odoemena, 1995), erythropoietic (Ajayi *et al.,* 2000), anticholesterolemic (Eseyin *et al.,* 2005a) and anti-diabetic (Eseyin *et al.,* 2000; Eseyin *et al.,* 2005b) activities. The leaves and the young shoots of the plant are frequently eaten as a potherb (Tindall, 1968; Okigbo, 1977; Okoli and Mgbeogu, 1983). The seeds of the plant are also popular items of diet and are cooked whole and ground

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up into soups. The leaves also contain protein, vitamins and flavors (Tindal, 1968; Gbile, 1986). In Nigeria, the herbal preparation of the plant has been employed in the treatment of sudden attack of convulsion, malaria and anaemia (Gbile, 1986). Despite its widespread usage as food and medication, information on the biological activity of the plant is very scanty.

Parquetina nigrescens (periplocaceae), a shrub found in equitorial West Africa (Irvine, 1961; Mabberly, 1987) and has been in traditional medicine practice for centuries (Adeyemo, 1994). In Oyo State Nigeria, the leaves have been reputed for treatment of helminthiasis (intestinal worm) while the roots are reputed for use as an anti-rheumatic (Adeyemo, 1994). Over the years, *Parquetina nigrescens* has been used as an ingredient in the medications for insanity (Iwu, 1993) and as an aphrodisiac in East Africa (Kokwaro, 1976). Decoction of the bark is given as a cardiactonic, while the leaf and root decoction have been recommended for the treatment of gonorrhea and menstrual disorders (Gill, 1992). While the whole plant is used to stupefy fish in Ghana and Liberia, the leaves and latex are used for the treatment of rickets, diarrhoea, skin lesions and tropical skin diseases (Gill, 1992; Oliver, 1960). The leaves of the plant have been used for the treatment of wound in Africa (Irvin, 1961. Mabberly, 1987) and have sympathomimetic effects. *Parquetina nigrescens* is also a constituent of a commercial herbal preparation (Jubi formular) in Nigeria used in the treatment of anaemia in man (Agbor *et al.,* 2001), the Jubi formular was shown to restore decreased haematocrit and haemoglobin concentration in *Trypanosoma brucei* induced anaemia (Erah *et al*., 2003). Agbor *et al.* (2001) also investigated and confirmed the antianaemic activity of aqueous extracts of *Parquetina Nigrescens* leaf on haemorrhagic anaemia induced in rats.

The harvested bone marrow cells 13th day Post irradiation were cultured with the individual and different combinations of the extracts at concentration ranges. Individually, *Parquetina nigrescens*, *Camellia sinensis* and *Telfairia occidentalis* extracts showed mean differentiation values of 3.54±0.30, 3.03±0.62 and 2.88±0.72, while the non-extract treated and GM-CSF treated as controls showed 0.18±0.01 and 0.88±0.05 respectively (Table 1). When combined for synergistic effects, *Telfairia occidentalis* (TO) plus *Camellia sinensis* (GT), *Parquetina nigrescens* (PN) plus *Camellia sinensis* (GT) and Combined GT/PN/TO showed mean differentiation values of 2.85±1.04, 1.61±0.70 and 1.89±1.15 respectively (Table 2). However, synergistic differentiation inhibitory effect of the three extracts was observed when the three extracts were combined. This finding is in discordance with the study of Sanberg *et al.* (2006) who reported that certain whole food extracts were found to result in a greater percentage of proliferation and differentiation when combined than observed with the individual extracts and compounds.

When individual and combined extracts of the plants were tested at varying concentrations in order to establish the possible correlation between the varying concentrations and degree of differentiation, the degree of differentiation was found to be directly proportional to the concentration of the extracts. The results of the study confirmed that administration of the extracts of the plants induced differentiation of haemopoietic multi-potent stem cells at even the lowest dose and increases as concentration increases.

Conclusion

The administration of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* aqueous extracts induce differentiation of haemopoietic multi-potent stem cells at even the lowest dose and increases as concentration increases establishing the usefulness of the extracts in restoring normal haemopoiesis as possible alternative to bone marrow transplantation or administration of cytokines following radiation, chemotherapy or stress.

Limitation to the study

The pattern of differentiation as it relates to the deflection of the differentiation was not determined. The research was majorly to determine the overall differentiation but not to determine which cell line most favoured

Recommendation

i. The active components of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis* involved in the differentiation of haemopoietic multi-potent stem cell and the mechanism of action would require further evaluation.

ii. This study should be considered as hypothesis awaiting confirmation from longitudinal studies from multicentres

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Conflict of Interest

No conflict of interest declared

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