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Combined Synergistic Effects of Aqueous Extracts of *Parquetina nigrescens, Camellia sinensis* and *Telfaria occidentalis* on Bone Marrow Haemopoietic Multipotent Stem Cells Proliferation in Irradiated Guinea Pigs

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Abstract

Cancer which is one of the most threatening human diseases is most commonly treated by chemotherapy and radiotherapy. However, these therapies are not tumor-specific. Normal tissues,

particularly the bone marrow (BM), are extremely vulnerable to *cytotoxicity* caused by these therapies. How rapidly patients recover from these treatment modalities greatly depends on the percentage of resting stem cells remaining after such treatment. Antidotes are required for the untoward side effects of these therapies. As a means to protect stem cells or help damaged stem cells to recover, the use of biological response modifiers (BRMs) has received attention. The use of fruits or vegetables has the benefits of providing a cocktail of many different *phytochemicals* with multiple actions including antioxidant and anti-inflammatory effects. Certain whole-food extracts, such as blueberry, dietary fatty acids, particularly oleic acid and *linoleic* acid have been reported recently to actively promote the proliferation of *haemopoietic* stem cells [1].

This study was done to determine the potential proliferative effect of *Parquetina nigrescens, Camellia sinensis and Telfaria occidentalis on haemopoietic multipotent* stem cells in irradiated guinea pigs bone marrow. The study shows that the plant has positive synergistic proliferative effects on *haemopoietic multipotent* stem cells. The proliferative effect correlates with the concentration of the combined extracts of the plants.

Keywords: Proliferative effect; *Parquetina nigrescens; Camellia sinensis*; *Telfaria occidentalis;* Haemopoietic; Multipotent Stem Cells; Irradiated Guinea Pigs.

1. Introduction

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 [2,3]. The current therapeutic strategies of irradiated normal tissue consist mainly of the treatment of symptoms or, in more severe cases, bone marrow transplantation and intensive care [2]. New multitargeted 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) [2]. Because tissue destruction is not only due to direct radiation-induced cell killing, other mechanisms such as activation of the immune system and subsequent auto aggressive digestion of parenchyma and tissues must be considered. These considerations could explain how the radiation-related destruction of tissue spreads and finally becomes manifested as MOI or MOF. Stem cells are undifferentiated cells with the ability to proliferate and produce a large number of differentiated progeny [4**]**. *Haemopoietic stem cell* is rare, perhaps 1 in every 20 million nucleated cells in bone marrow [5]. Although its exact phenotype is unknown, on immunological testing 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 [5]. Haemopoiesis is a process regulated by a complex network of soluble factors that stimulate the growth and differentiation of haemopoietic progenitor cells (HPC) [6]. 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 [7]. Bone marrow transplantation

performed after myeloablative treatments of the recipient has been used to reconstitute haemopoiesis [8]. 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 [9,10]. Haemopoietic stem cells (HSCs) have been investigated for many years for their utility in cancer treatments. Experimental investigations of haemopoiesis and clinical approaches to correcting its deficiencies have focused on cytokine activity. Cytokines modulate haemopoiesis by maintaining the self-renewal of stem cells and stimulating the proliferation and maturation of committed progenitor cells required for the continuous replacement of mature blood cells.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 multipotent progenitor cells in vitro. Individually, granulocyte-colony-stimulating factor (G-CSF) and EPO are growth factors for committed myeloid and erythroid progenitors, respectively. Clinically, G-CSF and EPO 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.

Green tea is a drink made from the steamed and dried leaves of the Camellia sinensis plant, a shrub native to Asia [11]. Green tea has been widely consumed in Japan, China, and other Asian nations to promote good health for at least 3,000 years [11]. 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 [11]. 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. Among these, the polyphenols constitute the most interesting group of tea leaf components and exhibit potent antioxidant activity in vitro and in vivo [12]. 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 antioxidants. Numerous studies have also demonstrated that the aqueous extract of the major tea polyphenols possesses antimutagenic, antidiabetic, antibacterial, anti-inflammatory, and hypocholesterolemic qualities [13,14].

Telfaria occidentalis, family Cucurbitaceae, is an herbal plant cultivated mostly in the West African sub-region [15]. The leave extract of the plant is used locally in the treatment of malaria and anaemia [16]. Apart from the nutritional [17], agricultural and industrial importance [18], the plant is also medicinally useful. It possesses anti-inflammatory [19], antibacterial [20], erythropoietic [21], anticholesterolemic [22] and antidiabetic [23;24] activities. The ripe fruit contains up to 13% oil. The leaves and the young shoots of the plant are frequently eaten as a potherb [25; 26; 27]. The seeds of the plant are also popular items of diet and are cooked whole and ground up into soups. The leaves also contain protein, vitamins, and flavours [25,16]. In Nigeria, the herbal preparation of the plant has been employed in the treatment of sudden attack of convulsion, malaria and anaemia [16]. 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 [28;29] has been in traditional medicine practice for centuries [30]. In Oyo State Nigeria, the leaves have been reputed for treatment of helminthiasis (intestinal worm) while the roots are reputed for use as an antirheumatic [30]. Over the years, *Parquetina nigrescens* has been used as an ingredient in the medications for insanity [31] and as an aphrodisiac in East Africa [32]. Decoction of the bark is given as a cardiactonic, while the leaf and root decoction have been recommended for the treatment of gonorrhea and menstral disorders [33]. 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 [33;34]. The leaves of the plant have been used for the treatment of wound in Africa [28,29] 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 [35], the Jubi formular was shown to restore decreased haematocrit and haemoglobin concentration in Trypanosoma brucei induced anaemia [36]. Agbor *et al.,* (2001) also investigated and confirmed the antianaemic activity of aqueous extracts of *Parquetina Nigrescens* leaf on haemorrhagic anaemia induced in rats.

This study was done to establish the synergistic proliferative activities of the leave extracts of *Parquetina nigrescens, Camellia sinensis* and *Telfaria occidentalis* on haemopoietic stem cell. It would be of great benefit to identify certain natural compounds that can promote proliferation of haemopoietic stem cells or other stem cells individually or synergistically, such that the natural compounds could be taken in the form of a supplement that would have a significant, measurable effect [1].

2. Materials and method

Plant: A packet of Camellia sinensis was obtained from One Step Pharmacy at Saw-Mail, Ilorin on 18th of April, 2009 with Cash Sales Invoice No 334 and fresh samples of *Parquetina nigrescens* and *Telfaria occidentalis* plants were obtained within Ilorin metropolis. The plants were identified by carrying out macroscopical examination on plant samples as stipulated by Dalziel (1968)[39] and confirmed and authenticated by staff in the herbarium of Department of Plant Science, University of Ilorin, Nigeria. *Parquetina Nigrescens* was given Serial Number 876 and Ledger Number 67 while *Telfaria Occidentalis* was given Serial Number 959 and Ledger Number 150. The samples were dried in an incubator at 37^{o}C (Uniscope, USA).

Extraction and Sterilization of the plant extracts

Aqueous extract

Plant extract was carried out by the method of Olowosolu and Ibrahim (2006)[37]. Ten (10) gm of each of plant material were macerated in pestle and mortar with 100ml distilled. 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 [35].

The final concentration of 1gm/ml was obtained as aqueous extract which served as the stock solution for dilutions needed during the course of the work.

Animal Source: 12 Young male guinea-pigs approximately 450g.wt obtained from the animal house, LAUTECH College of Medicine Osogbo, Osun-State, Nigeria. Animals used for experiments were housed in animal house of the Department of Anatomy, University of Ilorin in a temperature and humidity-controlled room that was maintained on a 12-hour light/dark cycle. Food and water were available *ad libitum* throughout the experiment.

Method of Irradiation of the Guinea-Pig: The type, dose and method of irradiation as well as the after-care of the irradiated guinea-pigs were all based on the procedures adopted by Harris, (1967)[38]. Each guinea-pig was separately irradiated under general anesthesia (im ketamine 5mg/kg body weight plus 1mg 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 University College Hospital, Ibadan, Nigeria at a dose rate of 98.560cGy/min.

After-Care of the Irradiated 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 animal after irradiation was kept in a separate cage and excessive handling avoided until it was due for sacrifice. 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[40]. The animals were sacrificed 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, 200units/ml heparine, Hanks balanced salt solution (HBSS), suspended with 2% fetal calf serum (FCS). The suspended marrow cell was further diluted with the diluting factor of 1 in 20 and cell counted to achieve a cell count of 1.0×10^9/L [41].

Microscopic Observation of Harvested Bone Marrow

Harris, 1960; has given a report of the haemopoietic events occurring in guinea-pig bone-marrow following sub-lethal whole body gamma irradiation at 13days after irradiation. This stage was described by Harris, (1967)[38], as the initial phase of final haematopoietic recovery which was further

confirmed by cytochemical reactivities as described by Caxton-Martins (1973)[42] using May-Grunwald's staining technique.

Preparation of Marrow Suspension

The marrow suspension was prepared from fresh autologous serum. The abdomen of the anaesthetized animal was opened up and the inferior vena-cava was exposed and incised and about 5ml of blood collected in a centrifuge tube through a glass funnel. After clotting, the blood was centrifuged for 10 min. at 3000rpm and the supernatant serum withdrawn with a Pasteur pippette into clean, small glass tubes. While the serum was being obtained, the isolated bone marrow cell was 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[42]. A similar technique was used by Harris, Menkin and Yoffey, 1956[43]. Using this technique, the incidence of damaged cells in the marrow smears was kept to a low level.

May-Grunwald Staining Technique.

- Air dry films were fixed by immersing in a jar of methanol for 20-25min.
- Transfer to a staining jar containing May-Grunwald's stain freshly diluted with an equal volume of buffered water for 15min.
- Transfer directly without washing to a jar containing Giemsa's stain freshly diluted with 9 volumes of buffered water, PH 6.8 for 10-15min.
- Transfer the slide to a jar containing buffered water PH 6.8, rapidly wash in 3-4 changes of water and finally allow to stand undisturbed in water for 2- 5min for differentiation to take place.
- Stand the slide upright to dry and report using X100 objective.
- Report following the conventional method of reporting bone marrow smear.

Culture Media Preparation: Eagles MEM is mainly used. To prepare 1000ml of Growth and maintenance Eagles MEM media, the procedures involved include:-

1 bottle of Eagles MEM powder containing 9.4g was dissolved completely in 1000ml sterile double distilled de-ionized water

- This was autoclaved at 121^{o}C for 15 minutes with cap slightly loose and allow to cool to room temperature.
- The pH of the autoclaved Eagles MEM was ensured to be between (4.3 – 4.5)
- Label 2 empty sterile 500ml bottles indicating growth or maintenance medium, the date the medium is prepared and the expiration date, batch number as well as the initials of the personnel who prepares it.

- The reagents below were dispensed into the empty sterile 500ml bottles respectively as shown in the table 1

Table 1. **Culture Media Preparation**

REAGENTS	GM (ml)	MM (ml)
7.5% Sodium bicarbonate	7.5	12.5
L-Glutamine	5	5
HEPES IM	5	5
Penicillin Streptomycin	5	5
FBS	**50**	**10**

- The autoclaved Eagles MEM was added to each of the 500ml bottles containing the reagents above to reach the mark of 500ml on the bottle.
- The pH was ensured to be between (7.2 – 7.4)
- An aliquot of each prepared bottle of medium was added to tissue culture tubes and label appropriately for sterility testing.
- The aliquot the aliquot was incubated at 36^{o}C for 5 – 7 days, inoculate into Thioglycollate broth.

The prepared media was stored at +4^{o}C. The prepared media was aliquoted into 250ml sterile bottles after the sterility testing result is out. [44]

Cell Culture Technique

5µl of suspended guinea-pig bone marrow cells harvested 13th Day Post irradiation were cultured in a Laminal Flow Cabinet at a concentration of 1.0 X 10^{9}/L in 20µl of Growth Eagles Minimum Essential Media (MEM) and 10µl of individual extracts of the plants were added to enhance proliferation. For combined extracts, the cell suspension volume and the Growth media were increased proportionately. In other words, when two of the extracts were combined, the cell suspension was increased to 10µl and Growth media increased to 40µl. The culture plates (24 wells; for each of the extracts) were incubated at 37◦C for 72 hours. After the treatment, the cells were prepared for MTT analysis of cell proliferation.

Cell Proliferation Assay

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 5hr and the culture media removed with needle and syringe. 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 microplate reader and the absorbance measured at 550nM (S).

3. Result

Table 2 A Comparison of Mean±Sd of Proliferative effects of Individual and Combined extracts 13th Day Post Irradiation at concentration dependent manner.

PLANTS EXTRACTS	CONCENTRATION VARIATION(100% - 0.313)	MEAN±STD DEV. OF PROLIFERATION	ANOVA
***Camellia sinensis* (GT)**	**24**	**2.93±1.01**	
***Parquetina nigrescens* (PN)**	**24**	**2.43±1.10**	
***Telfaria occidentalis* (TO)**	**24**	**2.96±0.93**	
Combined GT/PN/TO	**24**	**1.11±0.66**	**p-value=0.000**
CONTROL WELLS WITHOUT EXTRACT SHOWED PROLIFERATIVE VALUE OF 0.93			

Table 2 above showed the means value of the proliferative potentials of individual and combined extracts of green tea, *parquetina nigrescens* and *telfaria occidentalis* at a wide range of concentrations. All the extracts showed significant statistical differences at p-value of 0.000 when compared with the control using ANOVA.

Table 3: Correlation between concentrations of the extracts of the plants and degree of proliferation.

	GT	**PN**	**TO**	**GT/PN**	**GT/TO**	**GT/PN/TO**
MEAN±STD.DEV	2.9341±**1.00**	2.4303±1.10	2.9470±0.94	**1.**4750±**1.26**	1.7425±1.30	1.0577±0.70
CALCULATED 'r'	**0.812**	**0.964**	**0.835**	**0.956**	**0.982**	**0.980**
P-VALUE	. 000	. 000	. 000	. 000	. 000	. 000

Table 3 above showed a correlation between the individual and combined extracts and the degree of proliferation 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 correlation.

4. Discussion

This study investigates the potential proliferative effects of the Parquetina nigrescens, Camellia sinensis and Telfaria occidentalis on haemopoietic multipotent stem cells. The study demonstrates induction of proliferation of stem and progenitor cells by using plant's extract at a wide range of concentrations. The harvested bone marrow cells 13th day Post irradiation were cultured with the individual and different combinations of the extracts at concentration ranges of 0.313-100% determined to promote the proliferation of the haemopoietic stem cells , which was represented by

table five above. The results obtained on the correlation study between the concentration and the degree of proliferation the extracts of the plants showed a significant positive correlation at p-value of 0.000. In other words, the degree of proliferation is directly proportional to the concentration of the extracts. This finding coincided with the finding reported by Sanberg et al (2006)[2] on certain whole food extracts, such as Blueberry (BB), Green Tea (GT), and specific compounds, including Catechin (CH), Carnosine (Ca), and Vitamin D3 (D3) that were found to increase cell proliferation of human bone marrow cells in a dose dependent manner. A comparison of the mean proliferative effects of the individual and combined extracts at the concentration considered showed significant statistical differences at p-value of 0.000 using ANOVA. (Table 2). However, when the extracts of the plants were used individually *Telfaria Occidentalis* showed the highest proliferation at mean value of 2.96±0.93, followed by Green tea at mean value of 2.93±1.01 while *Parquetina Nigrescens* showed the least proliferation potentials at mean value of 2.43±1.10. A combination of the three extracts showed a mean proliferative value of 1.11±0.66. Synergistic proliferative inhibitory effect of the three extracts was observed when the three extracts were combined. This finding was different from the finding reported by Sanberg et al, 2006[1]. In his own finding, he reported that certain whole food extracts, such as Blueberry (BB), Green Tea (GT), and specific compounds, including Catechin (CH), Carnosine (Ca), and Vitamin D3 (D3), were found to result in a greater percentage of proliferation than observed with the individual extracts and compounds. The difference may probably due to different extracts of the plants used in his study compared with this study.

The results of the study confirms that administration of the extracts of the plant at the doses considered may cause or induce proliferation of haemopoietic multipotent stem cells at even the lowest dose and increases as concentration increases. The active components involved and the mechanism of action would require further elucidation.

5. Conclusion

The study confirms the usefulness of the extract of *Parquetina nigrescens, Camellia sinensis* and *Telfaria occidentalis* in restoring normal haemopoiesis when administered individual as against when combined. This lend credence to the local use of the plants and from this study, the plants are useful as an alternative to both bone marrow transplantation and administration of cytokines for cancer patients, immune-suppressed patients and other haemopoietic syndromes as a consequence of disease, radiation, chemotherapy and stress.

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