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## Proliferative Effect of Aqueous Extracts of *Parquetina Nigrescens* on Haemopoietic Multipotent Stem Cells in Irradiated Guinea Pigs

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### ABSTRACT

Patients suffer a decrease in haemopoietic stem cells as a consequence of disease, radiation or chemotherapy. Radiotherapy and chemotherapy are common therapeutic modalities for cancer, leukemia, and lymphoma. Unfortunately, these therapies are not tumor-specific. Normal tissues, particularly the bone marrow (BM), are extremely vulnerable to cytotoxicity caused by these therapies. Antidotes are required for the untoward side effects of these therapies. Although a lot of potential better treatments are currently being developed, few research studies have investigated the proliferative effect of plant extracts which may modulate stem cells self-renewal and differentiation. However, in recent years there has been an upsurge of interest on the effects of various dietary insufficiencies on haemopoietic and immune responses (Sanberg *et al.*, 2006). Other investigators have recently reported that dietary fatty acids, particularly oleic acid and linoleic acid, actively promote the proliferation of haemopoietic stem cells (Sanberg *et al.*, 2006) as well as modulate the self-renewal of intestinal epithelial cells. This study was done to determine the potential proliferative effect of *Parquetina nigrescens* on haemopoietic multipotent stem cells in irradiated guinea pigs bone marrow. The study shows that the plant has positive proliferative effects on haemopoietic multipotent stem cells. The proliferative effect correlates with the concentration of the *P. nigrescens*.

**Keywords:** Proliferative effect, *Parquetina nigrescens*, Haemopoietic, Multipotent Stem Cells, Irradiated Guinea Pigs.

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### INTRODUCTION

Stem cells are undifferentiated cells with the ability to proliferate and produce a large number of differentiated progeny (Fuchs and Segre, 2000). Bone marrow is the main source of haemopoietic stem cells (HSCs) and Mesenchymal stem cell. Haemopoiesis is a process regulated by a complex network of soluble factors that stimulate the growth and differentiation of haemopoietic progenitor cells (HPC) (Metcalf D, 1988). HPC have two major characteristics: self-renewal ability and the capacity to differentiate into all lineages of haemopoietic cells (Ogawa, 1993). 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).

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.

In vitro, 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. 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. However, such treatments are costly, and are not without certain risks.

*Parquetina nigrescens* (periplocaceae) a shrub found in equatorial West Africa (Irvine, 1961; Mabberly, 1987) has been in traditional medicine practice for centuries (Adeyemo, 1994). The parts of the plant used for traditional medicine include the leaves, roots and the latex (Gill, 1992). *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.

This study was done to establish the proliferative activities of the leave extracts of *P. nigrescens* on haemopoietic stem cell. It would be of great benefit to identify certain natural compounds that can promote proliferation of hematopoietic stem cells or other stem cells, synergistically, such that the natural compounds could be taken in the form of a supplement that would have a significant, measurable effect (Sanberg *et al.*, 2006).

## MATERIALS AND METHOD

### Plant Materials

Fresh sample of the plant was obtained within Ilorin metropolis. The plant was identified by carrying out macroscopical examination on plant samples stipulated by Dalziel (1968) 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. The sample was dried in an incubator at 37°C (Uniscop, USA).

### Plant Extracts

The leaves were cut into pieces and prepared for extraction as described by (Dina *et al.*, 2001). 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. Plant extract was carried out by the method of Olowosolu and Ibrahim (2006). Plant material was macerated in pestle and mortar with 100ml distilled water at room temperature and then filtered using muslin cloth. 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).

### 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, Unilorin 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). 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<sup>60</sup> 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. 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  $1.0 \times 10^9/L$  (Miroslav, 2008).

### Microscopic observation of harvested Bone-Marrow

Harris, 1960; has given a report of the haematopoietic 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), as the initial phase of final haematopoietic recovery which was further confirmed by cytochemical reactivities as described by Caxton-Martins (1973) using May-Grunwald's staining technique.

### Preparation of Bone Marrow Suspension

Fresh autologous serum was used to prepare the marrow suspension. 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 pipette 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. A similar technique was used by Harris, Menkin and Yoffey, 1956. 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:-

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

- Autoclave at 121°C for 15 minutes with cap slightly loose
- Allow to cool to room temperature
- Check the pH of the autoclaved Eagles MEM (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.
- Dispense the reagents below into the empty sterile 500ml bottles respectively.

| 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      |

- Add the autoclaved Eagles MEM to each of the 500ml bottles containing the reagents above to reach the mark of 500ml on the bottle.
- Check the pH (7.2 – 7.4)
- Make an aliquot of each prepared bottle of medium into tissue culture tubes and label appropriately for sterility testing.
- Incubate the aliquot at 36°C for 5 – 7 days, inoculate into Thioglycollate broth.
- Store the prepared media at +4°C. The prepared media should only be aliquot into 250ml sterile bottles after the sterility testing result is out. (WHO, 2004)

### Cell Culture Technique

At 13<sup>th</sup> day Post irradiation the bone marrow cells were harvested and cultured with the extract of the plant at concentration ranges of 0.313-100%.

5µl of suspended guinea-pig bone marrow cells harvested were cultured in a Laminar Flow Cabinet at a concentration of  $1.0 \times 10^9/L$  in 20µl of Growth Eagles Minimum Essential Media (MEM) and 10µl of the plant extracts was added to enhance proliferation. Another set of 24 Wells were also set alongside with the test to serve as control however, extract of the *P.negrescens* was not added to enhance the proliferation. The culture plates (48 wells) were incubated at 37°C 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 proliferation.

### Cell Proliferation Assay

Five hours before the end of the treatment in the culture plate, 20µl of MTT Solution were added to each well.

The plates were then incubated in a CO<sub>2</sub> 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 re-incubated in a CO<sub>2</sub> incubator for 5minutes, transferred to microplate reader and the absorbance measured at 550nm (S).

## RESULTS AND DISCUSSION

**Table 1:** A comparison of means of proliferative effects of *P.nigrescens* extract and control .

| EXTRACT                    | CONCENTRATION VARIATION(0.313-100% ) | MEAN±STD DEV. OF PROLIFERATION | t-test         |
|----------------------------|--------------------------------------|--------------------------------|----------------|
| PARQUETINA NIGRESCENS (PN) | 24                                   | 2.43±1.10                      | P-VALUE =0.001 |
| CONTROL                    | 24                                   | 0.93±0.01                      |                |

Table 1 above shows the mean value of the proliferative potentials of *P. nigrescens*. The extract shows significant statistical difference in proliferation at p-value of 0.001 when compared with the control using t-test.

**Table 2:** Correlation between concentrations of the extracts of the *P. nigrescens* and degree of proliferation.

| Parameters     | <i>P. nigrescens</i> |
|----------------|----------------------|
| Mean±sd        | 2.4303±1.10          |
| Calculated 'r' | 0.964                |
| P-value        | .001                 |

Table 2 above shows a strong positive correlation between concentration of the extracts of *P. nigrescens* and the degree of proliferation of the cultured guinea-pig bone marrow haemopoietic stem cell harvested 13<sup>th</sup> Day post-irradiation.

## DISCUSSION

The present study investigates the potentials proliferative effects of *P. nigrescens* on haemopoietic multipotent stem. The present study demonstrates induction of proliferation of stem and progenitor cells by using plant's extract at a wide range of concentrations.

The results obtained from this study shows that there was a significant statistical difference in proliferation of the stem cells with mean value of 2.43±1.10 for the test and 0.93±0.01 for the control at p-value of 0.001(Table 1). There was also strong positive correlation between the concentration of the extracts and the degree of proliferation of the haemopoietic stem cells at 'r' value of 0.964 and p-value of 0.001(Table 2). In other words, the degree of proliferation is directly proportional to the concentration of the extracts. This finding was similar to the one reported by Sanberg et al (2006) 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.

Agbor et al (2001) reported the erythropoietic potential of *Parquetina nigrescens*. *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 results of the present study reflect the justification of local use of these plants to treat anaemia and equally suggest that administration of the extracts of the plant at the doses considered may cause or induce proliferation of haemopoietic multipotent stem cell at even the lowest dose and increases as concentration increases. The active components involved and the mechanism of action would require further elucidation.

## CONCLUSION

This study confirms the usefulness of the extract of the *P. nigrescens* in restoring normal haemopoiesis as an alternative to both bone marrow transplantation and administration of cytokines for cancer patients, immunosuppressed patients and other haemopoietic syndromes as a consequence of disease, radiation, chemotherapy and stress.

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