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*Full Length Research Paper*

# Screening of the fruit pulp extract of *Momordica balsamina* for anti HIV property

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**Anti-HIV properties of the fruit pulp extract of *Momordica balsamina*, a plant of the cucurbitacea family commonly used in the Northern part of Nigeria for its anti-viral efficacy in poultry, was studied *in vitro*. Peripheral blood mononuclear cells (PBMCs) were prepared from both HIV sero positive and sero negative patients and categorized into group A (sero negative), group B (PBMC co-culture), group C (PBMC co-culture containing 3.12 mg/ml of the plant extract) and group D (only the enriched culture medium). The PBMCs were cultivated for 28 days using standard methods. CD4+ counts and virus detection assay ($P^{24}$) were determined on the PBMC. Results showed that the plant extract treatment significantly ($P<0.05$) increased the CD4+ count when compared to the untreated PBMC, a contrary effect was observed on the $P^{24}$ antigen level ($P<0.05$). The implication of these findings is discussed.**

**Key words:** *Momordica balsamina*, PBMC, HIV.

## INTRODUCTION

*Momordica Balsamina* also refers to as balsam fruit or apple belongs to the family of cucurbitacea. It is a plant commonly used by local poultry farmers in Plateau State, Nigeria for general well being of birds (Mgbojikwe et al., 2002). The Balsam apple is a climber or trailer with annuals stems attaining 4-5 m length, a plant of dry savannah and clearings in secondary bush of Northern Nigeria. The fruit is orange yellow, beaked, 21/2 inches in length bursting and exposing red brown seeds (Hutchinson, 1954). This species is closely related to *Momordila charantia* (Bitter mellon) which occurs in areas of greater rainfall and whose properties and actions includes antibacterial, anti-inflammatory, anti-oxidant, antiviral, immunostilmulant, hypoglycemic among others (Bk. of Royal Bot. Gard., 1985).

This study is aimed at verifying the claims of some traditional practitioners and AIDS patients in Plateau State of Nigeria, that the agueous fruit pulp of *M. balsamina* has healing quality. We decided to investigate this claim by measuring its direct effect on HIV-1- an RNA virus belonging to the family retroviridae and is also called lymphodenopathy associated virus (LAV), and is the causative agent for AIDS. (Gallo et al., 1982)

The disease caused by this virus in human still remains a serious and major challenge to mankind in the history of human viral infection in view of its high mortality records (WHO, 1994). The only reliable option for its prevention and control is to avoid risky behaviours such as those that tend to promote exchange of blood, or body fluids containing HIV virus and/or HIV infected cells (Dimmork and Primrose, 1993).

In patients infected with HIV-1, there exists a significant and steady decline in CD4+ lymphocytes (helper/inducer)

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that correlates with progression to disease. The steady decline in CD4+ cells is related to the trophism of HIV-1 for the CD4 receptors. The percentage as well as the total numbers of CD4+ lymphocytes in the peripheral blood of patients infected with HIV is one of the parameters to monitor prognostically. During the course of HIV infection, the first proliferative dysfunction which appears is the inability of CD4+ T-cells to recognize and proliferate in response to soluble antigens such as tetanus toxoid (TT), diphtheria, or purified protein derivative and during the end stage disease, a decline in proliferative responses to polyclonal mitogens such as phytohemaglutinin (PHA), pokeweed mitogen (PWM), and concanavalin A (Con A) can be seen.

On the other hand, viral $P^{24}$ protein circulates in the blood stream either as free antigen or bound to anti-$P^{24}$ antibody in the form of immune complexes. While the level of $P^{24}$ antigen may vary from individual to individual, it is known that in many patients, this antigen can be detected relatively early in the period after exposure to HIV-1 and will often precede the process of sero conversion by several weeks. The primary role of the measurement of $P^{24}$ antigen lies in monitoring the levels of viral activity within the known infected host, such as response to the initiation of anti retroviral chemotherapy, and in serving as an independent prognostic marker of disease activity over time.

There are presently about 47 million people world wide with HIV/AIDS, the majority of which has limited access to antiretroviral drugs. Even if world wide penetration was possible, our current chemotherapeutic strategies still suffer from issues of cost, patient compliance, deleterious acute and chronic side effects, emerging single and multi drugs resistance and generalized treatment and economic issue. Even our best antiretroviral therapy (HAART), falls short of completely suppressing HIV replication. Therefore, expansion of current therapeutic options calls for an urgent need for an effective chemotherapy for the Acquired Immuno Deficiency Syndrome (AIDS), which is caused by Human Immuno Virus. This study is aimed at exploring the use of the plant, *M. balsamina* in the control of HIV infection.

## MATERIALS AND METHODS

### Preparation of *M. Balsamina*

*M. Balsamina* fruit was harvested from the bushes around the National Veterinary Research Institute, Vom, Nigeria. The ripened fruit of *M. balsamina* was collected and cut open with sharp knife. The pulp was removed and the seeds separated using a sieve of 0.25 mm. The resulting pulp was blended with a blender and dried at 80°C for 24 h in a hot air oven. The powdered fruit pulp was stored in a dessicator.

### Sample collection and preparation of PBMCs

The blood samples were collected from HIV individuals. While donor PBMCs were gotten from individuals that have been previously screened (HIV- seronegative status established) at the PLASVIREC Jos, Nigeria using the double enzyme linked immunosorbent assay techniques; the patients' PBMCs was gotten from already confirmed HIV-1 seropositive individuals using the western blot technique. Blood in either case was collected from patients, through the anticubital vein using sterile needle and syringe for each patient/donor. About 7 ml of blood samples obtained in each case were transferred into a previously lablelled clean and sterile 10 ml vacutainer bottle containing $K_3$EDTA and mixed by inversion several times. Under aseptic conditions, the plasma was removed and discarded. Phosphate buffered saline (PBS) was added to the cells and the volume made up to 10 ml at approximate ratio of 2:1. Again proper mixing was ensured by several inversion. About 4 ml of the PBS suspended cells were carefully layered on a 3 ml Ficoll-hypaque solution in a sterile centrifuge tube ensuring that the sample do not mix with the ficoll-hypaque solution. The centrifuge tubes were appropriately labeled and covered and then centrifuged at 400 x g for 30 min at 20°C.

Upper layer was drawn off using a sterile pipette tip, leaving the lymphocyte layer undisturbed at the interface. The lymphocyte layer was carefully transferred into another sterile centrifuge tube with minimum amount of the ficoll-hypaque solution. About 6 ml of PBS solution was added mixed and centrifuged at 100xg for 7 min at 20°C to wash the cells. The supernatant was removed and fresh PBS added. The above process was repeated twice.

### Experimental design

Peripheral blood mononuclear cells (PBMCs) were prepared from both HIV sero positive and seronegative patients and grouped as follows:

Group A - Sero negative PBMC with culture medium only.
Group B - Sero negative PBMC cocultured with sero positive PBMC and culture medium.
Group C - Sero negative PBMC cocultured with sero positive PBMC and treated with 3.12 mg/ml of the plant extract, all had culture medium added.
Group D - The culture medium alone.

Each of the groups was kept over a period of 28 days while changing the medium thrice weekly. The CD4+ count and $P^{24}$ count was monitored in each group. Phytochemistry and mineral element analysis of the fruit pulp extract was also carried out.

Donor PBMC's was stimulated with 0.05 ml of PHA for 72 h before being used for cell co-culture and prior to this, the cell concentration was determined using standard methods. Coculture of patient and donor PBMC was carried out in a tissue culture flask at a ratio of 1:1 and incubated at 37°C in 5% $CO_2$ using humidified incubators, with the caps slightly loosened to permit medium gas exchange. Fifty percent replacement of culture media was made three times a week and the medium that is removed was saved for virus detection assays. Cultures were supplemented with fresh PHA stimulated donor PBMC at weekly intervals.

### CD4+ count (using flow cytometry)

CD4+ count was carried out using flow cytometric method (Cyflow SL by Partec).

### Measurement of HIV-1 $P^{24}$ antigen

Measurement of virion associated $P^{24}$ levels in cell free culture supernatants was carried out using quantitative HIV-1 $P^{24}$ antigen ELISA kit system (Assay kit were purchased from Partec GmbH, Germany). Cell viability was also determined using Trypan blue dye

assay (obtained from National Veterinary Institute Vom, Nigeria) Initial cytotoxicity studies of the plant extract was also carried out. Due to limited fresh PBMCs concentrations not toxic to the vero cells was used in the experiment.

### Phytochemical screening

Phytochemical screening for resins, alkaloid, flavonoid, anthroquinine, glycosides, steroidal ring, steroids, terpenes, cardiac glycoside, saponins and carbohydrate were analysed using the powdered extract of *M. balsamina* fruit pulp.

**Test for resins:** To 0.5 g of fresh *M. balsamina* fruit extract 5 ml of boiling ethanol was added. This was filtered through Whatman No.1 filter paper and the filtrate diluted with 4 ml of 1% aqueous HCl. The formation of resinous precipitate indicated the presence of resins.

**Test for alkaloids:** 0.5 g of *M. balsamina* fruit pulp was added to 5 ml of 1% aqueous HC1 on a steam bath. This was filtered and 1 ml of the filtrate treated with a few drops of Draggendorf's reagent and a second 1 ml portion treated similarly with Wagner's reagent. The formation of precipitates was an indication of the presence of alkaloids (Trease and Evans, 1989).

**Test for tannin:** 0.5 g of *M. balsama* fruit was stirred with 10 ml of distilled water. This was filtered and a few milliliters of 5% ferric chloride added to the filtrate. A deep green colouration showed the presence of tannin. A second portion of the filtrate was treated with a few milliliters of iodine solution. A faint bluish colouration confirmed the presence of tannin (Trease and Evans, 1989).

**Test for saponin:** 0.5 g of *M. balsamina* fruit was shaken with water in a test tube. Frothing which persist on warming was taken as evidence for the presence of saponin (Wall et al., 1952, 1954).

**Test for glycosides:** 0.5 g of *M. balsamina* fruit extract was stirred with 10 ml of boiling distilled water. This was filtered and 2 ml of the filtrate hydrolyzed with a few drops of concentrated HC1 and the solution rendered alkaline with a few drops of ammonia solution. 5 drops of this solution was added to 2 ml of Bennedicts qualitative reagent and boiled. A reddish–brown precipitate showed the presence of glycosides (Trease and Evans, 1989).

**Test for flavonoids:** 0.5 g of the *M. balsamina* fruit pulp extract was dissolved in 2 ml dilute NaOH solution. A few drops of concentrated $H_2SO_4$ were then added. The presence of flavonoid was indicated when the solution became colourless.

**Borntrager's test for anthraquinone:** 0.5 g of *M. balsamina* was taken into a dry test tube and 5 ml of chloroform was added and shaken for 5 min. The extract was filtered, and the filtrate shaken with an equal volume of 100% ammonia solution. A pink, violet or red colour in the ammoniacal layer (Lower layer) indicated the presence of free anthraquinones (Trease and Evans, 1978).

**Keller Killiani test for cardiac glycoside:** 0.5 g of *M. balsamina* was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underlayed with 1 ml of concentrated sulphuric acid. A brown ring obtained at the interphase indicated the presence of a deoxy sugar typical of cardenolides (Trease and Evans, 1978).

**Salkwoski test for steroidal ring:** 0.5 g of *M. balsamina* was dissolved in 2 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface indicated the presence of steroidal ring (Sofowora, 1982).

**Lieberman's test for steroidal terpenes:** 0.5 g of *M. balsamina* was dissolved in 2 ml of chloroform and 1 ml of acetic anhydride was added, then 2 drops of concentrated $H_2SO_4$ was added. A pink colour which changes to bluish green on standing indicated the presence of steroidal terpenes (Yen, 1971).

**Test for carbohydrates:** 0.5 g of *M. balsamina* was heated with 1 ml of concentrated sulphuric acid. Blackening and effervescence occurred indicating the presence of carbohydrate.

### Elemental analysis

0.2 g of the powdered *M. blasamia* fruit pulp was weighed into a porcelain crucible and ashed in a murfle furnace pre-heated to $600^oC$ for 4 h. The crucible was then transferred directly to a dessicator and allowed to cool. The weight of the ash was noted. The ash was treated with a few milliliters of 5 N HCl and a few drops of concentrated $HNO_3$ and boiled. This was cooled, filtered and the filtrate made up to 50 ml in a standard volumetric flask with de-ionized water. This solution was used for the determination of cations – $Ca^{2+}$,$Pb^{2+}$, $2n^{2+}$, $Fe^{2+}$,$Mn^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Se^{2+}$, $Na^+$ and $K^+$ using atomic absorption spectrophotometer (AAS) as described by AOAC (1975).

### Statistical analysis

The data obtained from the CD4+ counts and $P^{24}$ antigen readings were statistically analysed using two-way analysis of variance (ANOVA) (Robert and Rolif, 1981) in order to ascertain statistical significance.

## RESULTS

### CD4+ counts

There was a steady and significant rise in mean CD4+ counts of positive control (group A) and cocultures of groups that were treated with the plant extract (group C) within the first two weeks $P(<0.05)$. While those that the plant extract was not added to (group B), showed a decline in the mean CD4+ counts. However, a persistent decline in the mean CD4+ counts was observed in all the groups in the third and fourth weeks probably due to prolonged culturing of primary cells *in vitro* (Figure 1)

### Phytochemicals and mineral elements

The phytochemicals present in the dried powdered extract of the fruit pulp include resins, tannins, alkaloids, flavonoids, saponin, glycosides, steroidal ring and terpenes while the mineral elements include: $Mg^{2+}$, $Fe^{2+}$, $Na^+$ and $K^+$ Se and Pb were not detected (Tables 1 and 2).

## DISCUSSION

The level of mean CD4+ counts observed in the untreated cocultures (group B) seemed low when com-

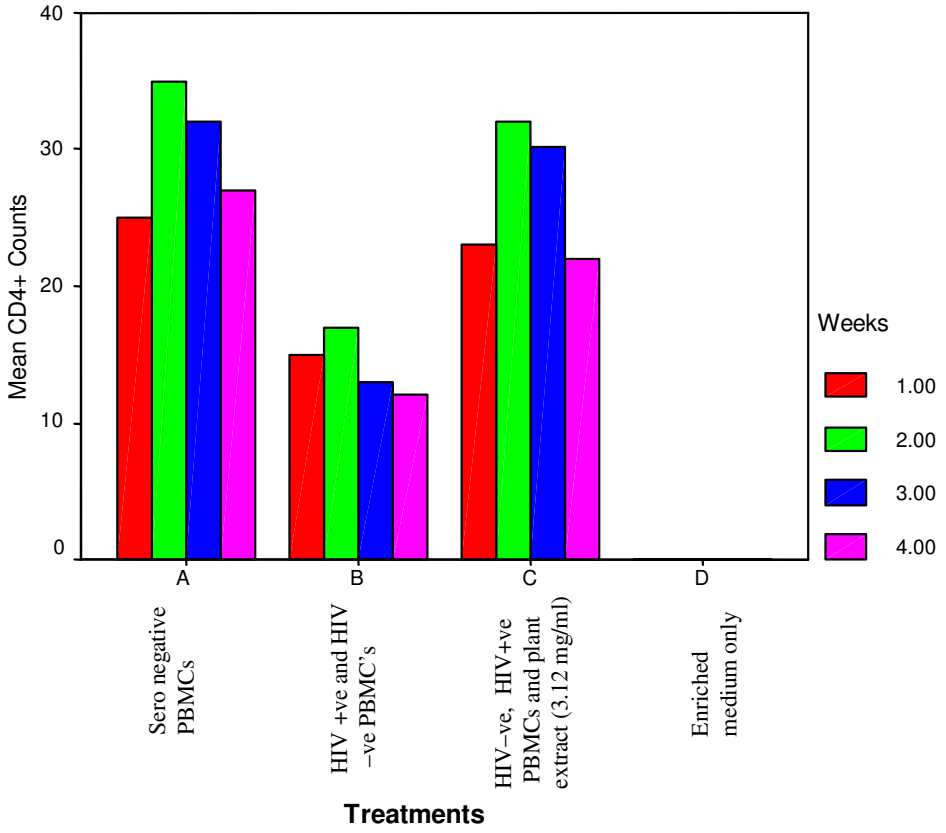


**Figure 1.** Weekly mean cd4+ counts for various treatments $P^{24}$ count. The mean $P^{24}$ counts of the plant extract-treated coculture (group C) when compared to non treated coculture (group B) were lower ($P < 0.05$). On the other hand no $P^{24}$ Antigen was detected in the control groups that had only PBMCs for seronegative donors or culture medium alone (groups A and D) as expected (Figure 2).

**Table 1.** Phytochemicals in the fruit of *Momordica blasamina*.

| COMPOUND | PRESENT |
|---|---|
| Resins | + |
| Alkaloids | + |
| Tannin | + |
| Flavonoids | + |
| Saponin | + + |
| Anthraquinone | - |
| Glycosides | + |
| Carbohydrate | + + |
| Steroidal ring | + + |
| Steroids and terpenes | + |
| Cardiac Glycosides | + |

\+ = Trace.
+ + = Present in appreciable quantity.
\- = Absent.

**Table 2.** Mineral elements in *Momordica Balsamina* fruit pulp.

| Elements | Concentration (mg/g) |
|---|---|
| Calcium | 1.0947 |
| Lead | ND |
| Selenium | ND |
| Zinc | 0.3722 |
| Iron | 1.1610 |
| Manganese | 0.0264 |
| Copper | 0.0100 |
| Magnesium | 4.4973 |
| Sodium | 0.465225 |
| Potassium | 44.6300 |

ND = Not detected.to proliterate.

pared to that of treated cocultures (group C). The data presented in Figure 1 indicate that, in all likelihood, *M. balsamina* interfere with an early event of the virus repli-cative cycle in lymphocytes coculture, presumably, virus absorption; thus enabling the PHA stimulated cells Full protective activity was achieved when the compound was present only during a 2 h virus adsorption period (data not shown). No effect was seen when the comp-ound was incubated on the cells for 24 h prior to virus infection. This may likely suggest that it does not exert its anti HIV activity indirectly, for example via induction of interferon;

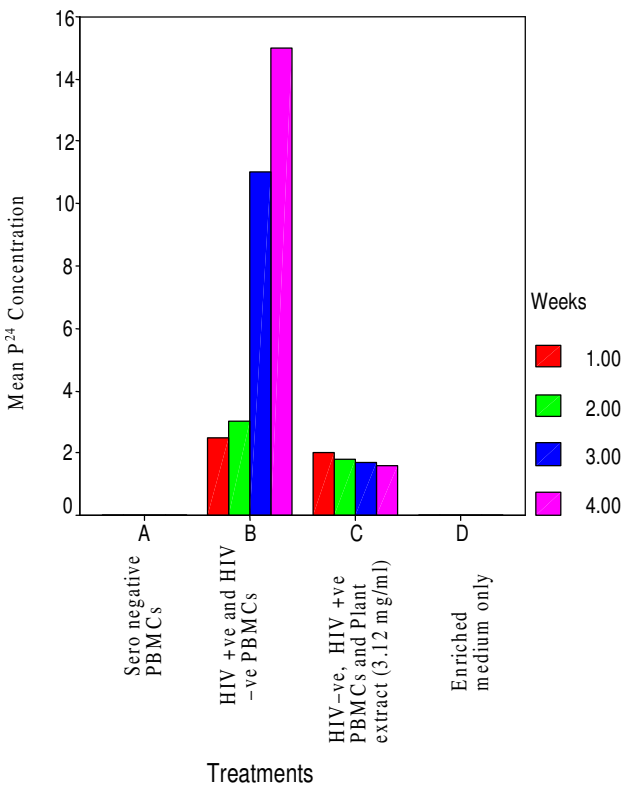


**Figure 2.** Weekly mean $P^{24}$ counts for various treatment groups. Mean CD4+ count for various treatment groups by mean $p^{24}$ concentration. For the control groups (groups A and D), $P^{24}$ levels remains 0.00 pg/ml and their CD4+ counts stood at 31 and 0.00 respectively. The $P^{24}$ concentration for non treated coculture (group B) showed a significant rise ($P<0.05$) over a period of four weeks as against that of the treated coculture (group C). Conversely there was a significant rise in the mean CD4+ count of group C ($P<0.05$) as against that of B (Figure 3).

nor does it neutralize the virus directly as established by the experiment in which the $P^{24}$ antigen was still been detected of the treated group even though in decreased concentration over the period of four weeks (Figure 2).

The effect of *M. balsamina* on the virus adsorption process needs to be further assessed possibly using more sensitive techniques. It is however, interesting to note that there appear to be a seemingly steady decline of the mean CD4+ count at the third and fourth weeks for both treated and untreated cocultures including the positive control. This is in line with the findings of Zagury and his colleagues (1986) and Papadopulus- Eleospulos (1982). They had reported that T4 lymphocytes from normal donors infected by HTLV-III *in vitro*, as well as HTLV-III infected primary T4 cells from AIDS patients, have been difficult to maintain in culture for longer than two weeks., PHA stimulation and high number of cells per milliliter of culture medium accounts for this (Zagury et al., 1986). Also substantial evidence exist, that oxidizing agents, including all mitogenic (activating) agents, can induce reversible cellular-changes, cellular activation, malignant transformation, mitogen unresponsive cells or cellular death including death by apoptosis. And that the ultimate outcome depends on the concentration of the agents, its rate of application, the initial state of the cells and the cellular milieu (Papadopulos-Eteospulos, 1982). It is therefore likely, that the presence of oxidizing agents such as the phytohaemaglutinin (PHA) and even those used in the culture medium must have been responsible for inducing cellular death by apoptosis.

The significantly ($P<0.05$) observed inverse relationship between CD4 counts and $P^{24}$ concentration agrees with the work of Gallo (1990). It is possible that sequel to the virucidal effect of *M. balsamina* on the infected cells in group C (treated coculture) there was less abnormalities in the expression of IL-2 and IL-2 receptor as did occurred in the untreated coculture (group B) thus leading to increase proliferation of CD4+ T-cells in the latter as against the former. Hence, the remarkable ability of the plant extract to protect the cells from been destroyed by the virus *in vitro* is interesting (Tables 3).

Tannins have been reported to complex with proteins and inactivate microbial adhesion enzymes, cell envelope, transport protein, etc (Haslam, 1996). They also complex with polysaccharides (Ya et al., 2988). At least two studies have shown tannins to be inhibitory to viral reverse transcriptase (Kaul et al., 1985; Nonaka et al., 1990). Alkaloidal glycosides isolated from *Solanum khasianum* have been reported to have antiviral activity (Moore and Pizza, 1992).

Generally, phytochemicals extracted with water such as fabatin and various lectins are more commonly effective as inhibitors of viral adsorption. The role of minerals in HIV/AIDS has long been established (Figures 3). Zinc, calcium and magnesium are known to serve as coenzymes in many enzymes activities. In addition, zinc has been shown to be essential in maintaining the immune system function and HIV infected individuals are particularly susceptible to zinc deficiency (Wellinghansen et al., 2000).

In view of our study and research on the above findings on *M. balsamina* as a potent inhibitor of HIV-1 replication *in vitro*, the research on fruit pulp extract should be further pursued for its potential in the prophylaxis and therapy of retrovirus infections in humans.

**Table 3.** Cytotoxic effect of *Momordica balsamina* on vero cells.

| Concentration (mg/m) | Cells disruption |
|---|---|
| 25.00 | +++ |
| 12.50 | ++ |
| 6.25 | + |
| 3.12 | - |
| 1.56 | - |

+++ = Highly Toxic
++ = Very Toxic
+ = Toxic
- = Not Toxic

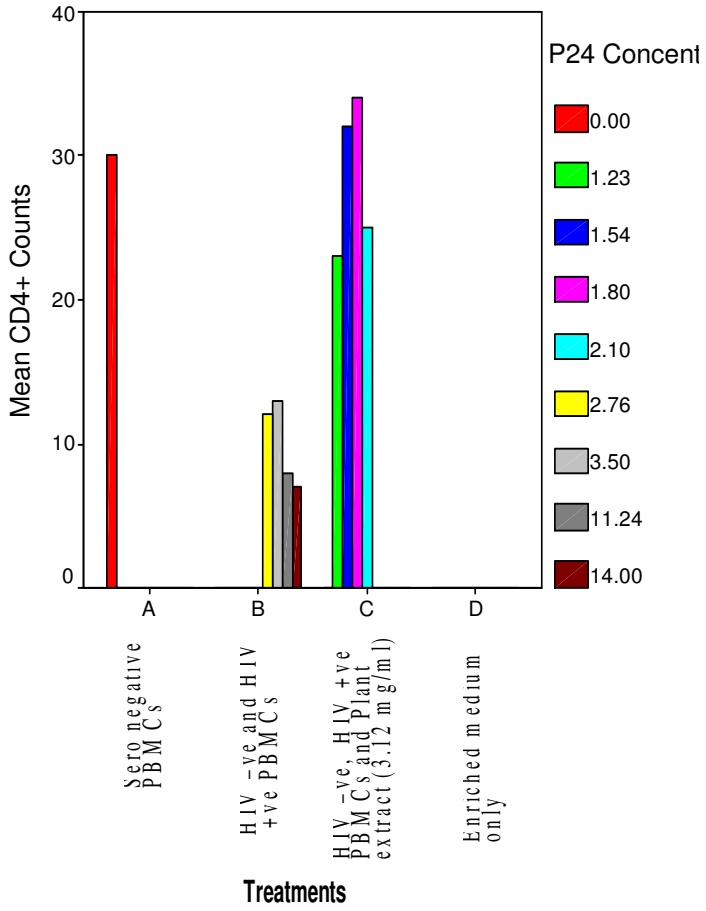


**Figure 3.** Mean CD4+ counts for various treatments groups by mean $P^{24}$ concentration.

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