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EVALUATION OF THE HAEMATOLOGICAL AND BIOCHEMICAL EFFECTS OF AVERON®, A HERBAL FORMULATION, AGAINST CYCLOPHOSPHAMIDE-INDUCED IMMUNOMODULATED MALE RATS

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ABSTRACT: The study was carried out to evaluate the immunomodulatory activity and the effect on the biochemical parameters of Averon®, an herbal formulation, on cyclophosphamide induced immunosuppression in rats. Haematological and biochemical parameters were evaluated after 21 days on blood samples collected by retro-orbital puncture at sacrifice. Results showed that death occurred in the immunosuppressed groups before collection of blood by retro-orbital puncture whereas in the immunoprophylactic groups, there was a minimal dose-boosting effect of the white blood cells that was statistically significant. There was no significant statistical difference in alkaline phosphatase, total bilirubin, cholesterol, high density lipoproteins, low density lipoproteins, triglycerides and total proteins ($p \leq 0.05$). However, a statistical significant difference was seen in the alanine aminotransferase and creatinine levels ($p \leq 0.05$) and aspartate aminotransferase ($p \leq 0.001$). Averon® is thus said to be better suited as an immunoprophylactic herbal formulation than immunostimulatory with better immunoboosting effects at higher doses (>400mg/kg) in the order of 800mg/kg and above.

INTRODUCTION: The term immunomodulatory means regulation of the immune system by suppression and stimulation of the cells and organs of the immune system [1]. Cyclophosphamide (**Figure 1**) is an oxazaphospharine class of alkylating agent widely used in cancer chemotherapy [2] and used to simulate immunosuppression with a very narrow therapeutic index and undergoes a complicated process of metabolic activation and inactivation [3, 4].

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The main use of cyclophosphamide is with other chemotherapy agents in the treatment of lymphomas, some of brain cancer, leukemia, and some solid tumors [5, 6]. Previous studies has shown that biological actions of cyclophosphamide are dose-dependent [7].

At higher doses, it is associated with increased cytotoxicity and immunosuppression, while at low, continuous doses, it shows immunostimulatory and antiangiogenic properties. The suppressive effects of cyclophosphamide on the lymphoid organs, white blood cell counts and other immune functions have been well documented [8, 9, 10, 11, 12, 13]. The immunosuppressive effect of cyclo-phosphamide on humoral immunity is a major concern in the treatment of cancer patients [14].

In many developing countries, traditional and herbal medicine remains the most accessible and most commonly used form of medicare [15]. For reasons such as cost, patients in developing countries continue to rely on herbal preparations as against orthodox medicines. The herbal preparations may contain a single plant material or polyherbal, in which case, contains multiple plant materials. An example of such preparations is Averon®, indicated for immunosuppression and so used to boost immunity. Hence, the immunomodulatory activity and effect on the biochemical parameters of Averon®, an herbal formulation, on cyclophosphamide induced immunosuppression in rats.

MATERIALS AND METHOD:

Experimental animals: Male Swiss Albino rats (weights 125 ± 25g) were obtained from the Laboratory Animal Center of the College of Medicine, University of Lagos, Lagos, Nigeria. The animals were kept in well-ventilated and hygienic compartments, maintained under standard environmental conditions and fed with standard rodent pellet (Livestock Feed PLC, Lagos, Nigeria) and water *ad libitum*. Animals were housed under standard conditions of (25±5˚C), 12:12hr light and dark cycle and humidity (55±10%). The animals were cared for and used in accordance with the Institute of Laboratory Animal Research (ILAR) guidelines for care use of animals in experimental studies [16].

Test formulation and drugs: Averon® (Batch Number: AV002DG; Manufacture date: December 2011; Expiry date: December 2012; manufactured by the Pax Herbal Clinic and Research Laboratories Ltd. Benedictine Monastery, off Benin-Auchi Road, Ewu-Esan, Edo State, Nigeria) was the test product for the experiment. The formulation was labelled to contain Aloe species and acclaimed to be an immunobooster. Cyclophosphamide (Cycram®; Korea United Pharm. Inc. Chungnam, Korea) was used to induce immunosuppression. Cycram® is labelled to contain 500mg cyclophosphamide to be dissolved in 20 mls of water for injection according to the manufacturer's instructions.

Preparation of test formulation and drug: Averon®, a liquid formulation, was oven-dried at controlled temperatures of 40°C, until completely dry and the residue re-constituted for experimental use with distilled water. The immunosuppressant, cyclophosphamide, was reconstituted with water for injection to obtain a working concentration of 25 mg/ml.

Acute toxicity studies: The acute toxicity study was conducted as per the OECD guidelines. 5 Male Swiss Albino rats were fasted overnight, followed by administration of a single bolus dose (2000 mg/kg) per oral of Averon® and then observed over 14days for mortality and physical/behavioural changes. The animals did not show any mortality at the dose of 2000mg/kg and hence its 1/10th dose, that is, 200 mg/kg and 1/5th dose, that is, 400 mg/kg were used as the therapeutic doses of the Averon® formulation to represent low and high doses respectively.

Immunosuppressant dose determination: A preliminary study was carried out to determine the dose of cyclophosphamide that can affect immunosuppression without causing mortality in the rats. Three groups of 5 animals each were selected. Group 1 was administered 50mg/kg intraperitoneally (i.p); Group 2 animals were administered 30mg/kg (i.p) while Group 3 was administered 10mg/kg (i.p). The animals were observed for a period of 7days and haematological parameters were checked.

Experimental design: All animals were acclimatized for 10 days and were divided into six groups, each consisting of eight animals.

- Group I (negative control group) received standard rodent pellets throughout the period of the study.
- Group II (Cyclophosphamide group) received the standard rodent pellets for a period of 21 days and on 19th, 20th and 21st days was injected with cyclophosphamide (30 mg/kg intraperitoneally).
- Group III was injected with Cyclophosphamide (30mg/kg i.p) for 3days (days 1-3) followed by per oral administration of 200 mg/kg Averon® on days 4 - 21.
- Similarly, Group IV was administered Cyclophosphamide (30mg/kg i.p) for 3days

(days 1 - 3) followed by per oral administration of 400mg/kg of Averon® on days 4 - 21.

- Groups V and VI were administered 200mg/kg and 400mg/kg per oral Averon® respectively for 21days followed by cyclophosphamide (30 mg/kg i.p) on the 19th, 20th and 21st days, 1 hour post-administration of the respective oral treatment.

Haematological investigations: Blood samples were collected from all groups on the 22nd day of the experiment by retro-orbital puncture into clean EDTA bottles and haematological parameters were analysed for red blood cells (RBC), haemoglobin (Hb%), platelets (PLT), total white blood counts (WBC) and differential leucocytes counts (DLC) using a ADVIA 2120 Haematology System (Bayer HealthCare LLC, USA).

Biochemical analysis: Blood was collected from the retro orbital plexus of each animal and transferred into a lithium heparinised tubes for biochemical analysis. The samples were then centrifuged using a centrifuge and the plasma obtained were analysed for alanine aminotransferase and aspartate aminotransferase [17], alkaline phosphatase [18], total bilirubin [19], cholesterol [20, 21], high density lipoproteins (HDL), low density lipoproteins (LDL), triglycerides (TRIG), total proteins [22] and creatinine [23].

Statistical analysis: Data were analysed with a statistical software (Graphpad Prism 5) and values were expressed as Mean ± SEM and differences between the groups were statistically determined by analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant levels were considered at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS: The results for the haematological parameters are presented in **Table 1** while those for the clinical chemistry parameters are presented in **Table 2**.

TABLE 1: EFFECT OF AVERON® ON THE HAEMATOLOGICAL PARAMETERS IN CYCLOPHOSPHAMIDE-INDUCED MYELOSUPPRESSION IN RATS

Parameter	I Control	II (CYP30 mg/kg)	III (CYP +AV 200mg/kg)	IV CYP+AV 400mg/kg	V AV200mg/kg + CYP	VI AV400mg/kg + CYP
PCV (%)	40.7 ± 2.08	35.3 ± 0.58	‡	‡	32.5 ± 2.30*	33.7 ± 2.37*
Hb (g%)	12.1 ± 0.68	10.6 ± 0.48			10.1 ± 0.77	10.4± 0.79
WBC 10^9/L)	9.23 ± 1.03	0.98 ± 0.22***			0.65 ± 0.15***	1.03 ± 0.19***
PLT (10^9/L)	650.3 ± 57.9	617 ± 51.4			614 ± 47.8	521.2 ± 45.5
RBC (10^{12}/L)	6.57 ± 0.22	5.84 ± 0.13			5.58 ± 0.39	5.76 ± 0.41
MCV (fl)	61.9 ± 1.33	60.6 ± 1.24			58.35 ± 0.65*	58.7 ± 0.64*
MCH (pg /cell)	18.3 ± 0.45	18.1 ± 0.45			18.0 ± 0.19	18.0 ± 0.18
MCHC (g/dl))	29.5 ± 0.32	30.1 ± 1.06			31.12 ± 0.10	30.8 ± 0.26
MPV (fl)	7.68 ± 0.20	7.45 ± 0.21			6.73 ± 0.31*	7.45 ± 0.27

‡ = Death occurred before collection of blood by retro-orbital puncture. Values are expressed as Mean ± SEM, One Way ANOVA followed by Dunnet's Test, * = P<0.05; ** = p<0.01; *** = p<0.001. Treated groups are compared with Control group. PCV = Packed Cell Volume; Hb = Haemoglobin; WBC = White Blood Cells; PLT = Platelets; RBC = Red Blood Cells; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration; MPV = Mean Platelet Volume

Groups III and IV**,** referred to as immunosuppressed groups, experienced 100% mortality before the end of the experimental period. This was due to severe myelosuppression by cyclophosphamide that Averon® could not counter. A dose-dependent increase was seen in the haematological parameters in the groups V and VI except in the platelet counts. There was no significant statistical difference ($p<0.05$) in the packed cell volume (PCV) of the cyclo-phosphamide-treated group. A significant decrease however was seen in the immunoprophylactic groups. There was also no significant statistical difference ($p<0.05$) in the haemoglobin, platelet counts and red blood cells (RBC) levels in all groups during the period of study. A marked significant statistical decrease ($p<0.001$) in the mean values of the white blood count (WBC) was observed in all the treated groups when compared to the negative control group.

TABLE 2: EFFECT OF AVERON® ON THE CLINICAL CHEMISTRY PARAMETERS IN CYCLOPHOSPHAMIDE-INDUCED MYELOSUPPRESSION IN RATS

Parameter	I Control	II (CYP 30 mg/kg)	III (CYP + AV 200mg/kg)	IV (CYP + AV 400mg/kg)	V AV200mg/kg + CYP)	VI (AV 400mg/kg + CYP)
ALP (iu/L)	117.8±5.70	90.5 ± 8.92	‡	‡	105.5 ± 9.60	109.2 ± 6.03
ALT(iu/L)	59.5 ± 1.66	35.8 ± 6.84			35.5 ± 3.03*	38.8 ± 8.05*
AST(iu/L)	26.00±1.96	15.3± 2.59***			12.83± 1.25***	13.5± 1.26***
TB (mg/dL)	0.64 ± 0.07	0.74 ± 0.06			0.79 ± 0.04	0.72 ± 0.04
DB (mg/dL)	0.28 ± 0.03	0.34 ± 0.03			0.38 ± 0.03*	0.33 ± 0.02
Chol (mg/dL)	95.69±10.3	84.1 ± 6.48			101.8 ± 8.99	96.9 ± 8.96
HDL (mg/dL)	32.32±4.51	34.5 ± 3.12			26.8 ± 0.77	26.7 ± 2.12
LDL (mg/dL)	52.54±7.89	47.5 ± 5.51			67.1 ± 9.14	63.2 ± 7.38
TRIG (mg/dL)	75.91±5.81	78.15 ± 10.6			72.3 ± 5.53	65.5 ± 3.00
T. Prot (mg/dL)	5.83 ± 0.10	6.08 ± 0.34			5.55 ± 0.12	5.63 ± 0.08
Creat (mg/dL)	0.88 ± 0.09	0.63 ± 0.13			0.52 ± 0.07**	0.58 ± 0.03*

‡ = Death occurred before collection of blood by retro-orbital puncture. Values are expressed as Mean ± SEM, One Way ANOVA followed by Dunnet's Test, * = P<0.05; ** = p<0.01; *** = p<0.001. Treated groups are compared with Control group. ALP = Alkaline Phosphatase; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase; TB = Total Bilirubin; DB = Direct Bilirubin; CHOL = Cholesterol; HDL = High Density Lipoproteins; LDL = Low Density Lipoproteins; TRIG = Triglycerides; T. Prot = Total Proteins; Creat = Creatinine

A significant statistical decrease (P<0.05) in the alanine aminotransferase (ALT) levels in the 200 mg/kg and 400 mg/kg immunoprophylactic group was observed when compared to the control group indicating a hepato-protective function of Averon. An ALT decrease was also seen in cyclophosphamide-treated group but the decrease was not significant statistically. Results show no significant statistical difference in the total bilirubin, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides and total protein among the groups when compared to the control group.

DISCUSSION: The immunomodulatory activity of Averon®, an alcoholic extract of Aloe *spp,* was explored by evaluating their effects on cyclophosphamide induced immunosuppression and immunostimulation in rats at two doses of 200 mg/kg and 400 mg/kg per oral evidenced by the haematological parameters and the accompanying effect on the clinical chemistry of the animals. This study was carried out running two models of immunomodulation concurrently immuno-suppression and immunoprophylaxis to evaluate the immunomodulatory profile of Averon® in terms of immunostimulatory and immuno-prophylactic activity respectively.

A dose-dependent increase was seen in the WBC between groups V and VI. This is suggestive of the possible increase in white blood cells level if a higher dose of the herbal formulation were administered and for a longer duration. Our work supports previous studies that acute stress may enhance immune response whereas chronic stress may suppress the immune system [24, 25, 26]. The stress in this instance, is induced by the immunosuppressant, cyclophosphamide. Immune activation is an effective, as well as protective approach against emerging infectious diseases [27].

Bone marrow is the site of continued proliferation and turnover of blood cells and also a source of cells involved in immune activity. A pronounced degree of cell proliferation renders bone marrow a sensitive agent, particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of bone marrow to regenerate new blood cells will result in thrombocytopenia and leucopoenia [28].

Our results also show a significant decrease in the mean platelet volume (MPV) in the 200 mg/kg Averon group predictive of low platelet production. This in effect agrees with the low countering effect of Averon® on the toxicity of cyclophosphamide on the bone marrow. The immunomodulatory activity of drugs is known to vary with the dose level and most of the immunosuppressants show immunostimulation at lower dilutions [29, 30].

Administration of low doses of cyclophosphamide, an immunosuppressant, to volunteers with advanced malignancies has been observed to

enhance the lymphokine activated killer cell activity induced by co-administration of interleukin-2 [31]. Similar immunostimulating properties have been found to be associated with other immunosuppressants such as glucocorticoids and 6-thioguanine [29].

Biochemical markers play a vital role in accurate diagnosis and also for assessing risk and adopting therapy that improves clinical outcome. These parameters can tell on the liver health, kidney and other physiological milieu. Mortality in groups III and IV cannot be attributed to starvation but most likely due to a marked depression of the immunity levels affected by the cyclophosphamide 30mg/kg.

Several researches have shown that elevated liver enzymes can be caused by many different factors, including a high-fat, high-protein diet, weight gain, changing exercise levels, alcohol consumption, disease, including liver disease, and other physiological and environmental causes. Statistical analysis shows no significant statistical difference in the alkaline phosphatase (ALP) indicating no biliary stasis nor liver metastasis among the groups when compared to the control. Both ALT and AST levels can also test for liver damage.

A marked statistical significant decrease ($p<0.001$) in AST levels was also observed in all treatment groups. This is suggestive of the stabilising effect of Averon on the liver function enzymes. The AST, found in the liver, heart, skeletal muscle, kidneys, brain and red blood cells, is commonly measured as a marker for liver health. Medical research has shown that low levels of AST are normally found in the blood and when body tissue or an organ such as the heart or liver is diseased or damaged, additional AST is released into the bloodstream. The (AST/ALT) is sometimes a useful prognostic parameter especially in alcoholic liver disease [32].

The AST/ALT ratios of the groups were I (0.44), II (0.43), V (0.36) and VI (0.35). As Averon® is an alcoholic formulation of Aloe spp, it is important to put into consideration the effect of alcohol on the liver. Since the AST/ ALT ratio of the groups are <1, this ratio maybe less useful in this study in distinguishing the effect of alcoholic on the liver from other causes of hepatocellular challenges. The amount of AST in the blood is directly related to the extent of the tissue damage.

Monitoring of the liver health can be done while subjects are on immunoprophylactic administration of Averon® herbal formulation. Hence, Averon® can be recommended to patients with elevated liver enzymes as a result of liver diseases such as hepatitis and cirrhosis or on medications affecting the liver such as the statins.

The prior administration of Averon® on the subjects gave a poorer outlook on the lipid profile parameters compared with the positive and negative controls. An elevated level of cholesterol was observed in the group boosted with 200mg/kg Averon® prior to cyclophosphamide compared to other groups. As it is evidenced from the results of the study, cyclophosphamide, 30mg per kg body weight showed cholesterol and LDL-lowering effects comparable to the negative control group with values (95.69 ± 10.32) mg/dL and other treatment groups.

Previous studies confirmed that serum bilirubin concentrations were inversely related to the severity of coronary artery disease and that this inverse association was independent of sex [33]. Similar inverse relationship has been related to cardiovascular disease [34]. The relatively high values of the total and direct bilirubin concentrations in the Averon® treated groups suggest that the lowered incidence of cardiovascular challenges specifically at doses of 200 mg per kg body weight.

The detection and diagnosis of acute kidney injury currently require the use of conventional markers of kidney function, specifically, serum creatinine and urea levels and, less frequently, other urinary tests. Creatinine is an amino acid as a waste product of creatine, an important energy storage substance in muscle metabolism. Creatinine is an anhydride of creatine and is not used in the body. The Serum creatinine level is a more reliable indicator of renal function than the blood urea nitrogen (BUN) [35].

Serum creatinine concentration increases in the presence of impaired renal function. The risk of having an herbal-drug interaction is based on a variety of factors and not solely based on the pharmacologic and pharmacokinetic characteristics of the herbal.

One important factor that increases the likelihood of having an herbal-drug interaction is concomitant use of an herbal with drugs that have a narrow therapeutic index. Such drugs include digoxin, antiepileptic drugs, antineoplastic agents, immuno-suppressants, and warfarin [36].

CONCLUSION: Our study showed that the herbal formulation, Averon®, containing *Aloe spp* is better suited as an immunoprophylactic formulation than an immunostimulatory formulation. The immunostimulatory or immunoboosting effect is markedly observed when the immune system is weakly suppressed, when a low dose of the immunosuppressant is administered or with a higher dose of the Aloe formulation. When administered as an immunoprophylactic formulation, a statistically significant increase in white blood cells and possibly other haematological parameters will be observed with an increased dose in an order greater than 400mg/kg and above.

The lowered blood levels of AST and ALT suggest the hepato-protective efficacy of Averon in individuals or subjects who take medicines that can cause liver damage. Also, the elevated levels of bilirubin are predictive of cardio-protective tendencies of Averon as against the cholesterol-increasing effect. However, the available evidence is not adequate to allow for its use in clinical practice. This study poses as a strong lead for comprehensive, systematic and multi-disciplinary evaluation of the haematological and biochemical claims of this and related aloe-containing products.

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