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EFFECT OF POLYHERBAL FORMULATION ON NEUTROPHIL FUNCTION AND TDAR IN WISTAR ALBINO RAT

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ABSTRACT

This study evaluate the effect of polyherbal formulation (PHF) on Neutrophil Function and T-cell dependent antibody response in Wistar Rat. The result shows that there is significance difference in Neutrophil Adhesion (NA) test across the seven groups ($p<0.001$). When comparing between group 6 (treated with PHF) and 7 (treated with polyherbal formulation and cyclophosphamide) there is significance difference in NA ($p=0.009$). Nitroblue tetrazolium (NBT) reduction test result shows that there is significance difference across the groups ($p < 0.001$). There is significant difference in NBT reduction between group 1 (normal control) and group 6 (PHF highest dose), group 2 (Positive control) and group 6, group 6 and group 7 ($p=0.009$), and group 3 (Negative control) and group 7 ($p=0.01$), however there is no significance difference between group 1 compared with group 7, ($p=0.033$). In T-cell dependent antibody response the study revealed that there is significance difference in concentration of both KLH IgM and IgG between the seven groups ($p<0.001$). When comparing group 1 and 6, group 3 and 7 as well as group 6 and 7 there is significance difference in concentration of KLH IgM ($p = 0.009$). Similarly there is significance difference in comparing group 1 and 6, 2 and 6, 3 and 7 as well as 6 and 7 on KLH IgG concentration ($p = 0.009$). Neutrophil function and serum concentration of IgM and IgG against KLH increase with increase dose of PHF up to 1000 mg/kg/bwt. The PHF has potential immunomodulatory activity in wistar rat.

KEYWORDS: Immunomodulation, Medicinal, Neutrophils, Plants, T-Lymphocytes.

INTRODUCTION

The use of medicinal plants for curing of several different diseases have been recognized over a long period of time.[1, 2] Traditional medicine are prepared from combinations of medicinal plants which may influence numerous molecular pathways.[3. 4] Immune system is a remarkably and sophisticated defense system within vertebrates which protect them from invading agents.[5] It is able to generate different type of cells and molecules capable of recognizing and eliminating limitless varieties of foreign and undesirable agents due to its ability to differentiate between self and non self.[6] Innate immune response is the first line of defense against foreign agent or any antigenic insult.[7] It involves the hematopoietic cells component that includes macrophages, dendritic cells, mast cells, neutrophils, etc.[8] Neutrophils play a crucial role in host defenses against bacteria, fungi, toxins etc.[9] The other line of defense is adaptive immune responses which consist of humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity which is mediated by T lymphocytes. T-cell-dependent antibody response (TDAR) is used to assess multiple immune functions. Antigens are referred to as T-dependent when B lymphocytes require T cell help in order to elicit a desired antigen-specific antibody response.[10]

Modulation of the immune system refers to any change in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response.[11] Some herbs possess immunomodulating properties that may play an important role in reducing the risk of various diseases, although the working mechanisms of some of the herbs are unclear and remain to be elucidated, they are worth further studying as newly potential therapy agents for immunomodulation.[12]

Polyherbal formulations (PHF) are collection of therapeutic entities that are formulated and prepared on the basis of the healing properties of individual ingredients with respect to the condition of sickness such herbal constituents with diverse pharmacological activities principally work together in a dynamic way to produce maximum therapeutic benefits with minimum side effects.[13] The Polyherbal formulation called "*Garjin"* locally consists of five plant materials namely: Black cutch- *Bark (Acacia polyacentha wild)*, orchid bush-Bark *(Bauhinia rufescens lam),* Gum Arabic tree-Bark *(Acacia Senegal),* Baobab-leaves *(Adensonia digitata),* Garlic-Yellow-bulb *(Allium sativum)*. It was claimed to have immunomodulatory activities by a local herbal medical practioners in Kano, Nigeria. Consequently, it is used by some people in the state. However, there is no scientific data on the *"Garjin"* polyherbal formulations that established the claims made by the herbalist. This study will provide some scientific information about the claim made by the local herbalist using neutrophils and TDAR as a model.

MATERIALS AND METHODS

Plant Materials

Plant material was collected from Al-Mustakshif Medical Health Centre, Kano, Nigeria (RC: 1393615).The plant materials were washed under running tap water to remove the surface pollutants and the whole plants were allowed to shade-dried and made into a coarse powder which was passed through a 40-mesh sieve to get a uniform particle size. A 60 g of the powder was dissolved into 1000 ml of distil water. The solution was stirred with the use of stirrer for two hours and allowed to stay for 24 hours. It was filtered through Whatman filter paper No. 1 and were evaporated to dryness at 55°C in water bath until aqueous free solid powder (i.e. lyophilisation) was obtained. The resulting extract was subsequently labelled as aqueous extracts and preserved at 5° C in airtight bottles until further use.[14]

Experimental Animals

A total of 35 Wistar rats of 16-18 weeks old, weight between (126 g-150 g) of either sex (17 Females; 18 Males) were used in this study. The animals were purchased from the Department of Veterinary Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University (ABU), Zaria, Nigeria and kept in the animal house of Faculty of pharmaceutical sciences, Usmanu Danfodiyo University, Sokoto (UDUS), Nigeria. They were allowed to acclimatize for two weeks and fed with standard pelletized growers' feed (Vital feed, Jos, Plateau) and water *ad libitum.* The animals were maintained under the Institutional standard laboratory conditions for experimental animal.

Animal grouping and treatment

A total of 35 Wistar rats were randomly divided into seven groups and each group contains five rats. Group 1 received 10ml/kg/bwt of normal saline, Group 2 received 50mg/kg/bwt of Levamisole Hydrochloride (Guangehou Kafen Biotech Co., Ltd, China), Groups 3 received 200 mg/kg/bwt of Cyclophosphamide (Guangehou Kafen Biotech Co., Ltd, China)subcutaneously (sc)at day one, Group 4 - 6 received 250 mg/kg/bwt, 500 mg/kg/bwt and 1000 mg/kg/bwt of PHF using intragastric tube respectively, for 14 days. Group 7 received 200 mg/kg/bwt of Cyclophosphamide subcutaneously (sc) at day one and 1000 mg/kg/bwt of PHF. The animals were immunized twice (i.e. 5^{th} and 9^{th} day) with 0.1 ml normal saline containing 300µg of (KLH) Keyhole Limpet Hemocyanin (Biosearch Technologies, Inc., US) intravenously.

Blood collection and processing

On day 9, two milliliter (2 ml) of blood was collected from each rat by cardiac puncture prior to the second immunization with KLH. The blood was centrifuged at 500 g for five minutes to obtain a serum. The serum was used to measure the concentration of IgM antibodies against the KLH (i.e. primary antibody response). On day 15, four milliliter (4 ml) of blood sample was collected by cardiac puncture from each Wistar rat under mild anesthesia. Out of which, 2 ml of the blood was dispensed into heparinized container for neutrophils function assessment. The remaining 2 ml of the blood samples were transferred into clean plain tubes, allowed to clot within 30 minutes of collection then centrifuged at 500 g for five minutes to obtain neat serum and transferred into a labelled cryovials. The serum obtained was used to measure the concentration of IgG antibodies against the KLH (i.e. secondary antibody response).

Laboratory Analysis

Neutrophil function

Neutrophils Adhesion and Nitroblue tetrazolium (NBT) reduction tests were used to assess neutrophil function. For NA test, 1.5 ml of the heparinized blood collected on day 15, was used for total leukocytes count (TLC) and differential count (DLC). Initially the TLC and DLC was performed (i.e. untreated blood count) followed by incubation with 80 mg/ml of Nylon fibers (Guangehou Kafen Biotech Co., Ltd, China) for 10 min. at 37^{o}C. The incubated blood samples were again analyzed for TLC and DLC (i.e. treated blood count).[15] For NBT test, on day 15, 0.5 ml of the 2 ml heparinized blood was taken on a clean glass slide, incubated at 37°C for 30 min. It was gently washed with cold saline (should not be dried), tapped gently, and excess saline was removed. Nitroblue tetrazolium (Sigma-Aldrich, USA) was added then incubated for 30 min at 37°C, the procedure continue as describe by.[16]

Keyhole Limpet Hemocyanin antibodies

To measure the serum concentration of KLH antibodies (i.e. IgM and IgG), sandwich-ELISA technique (Sunlong Biotech Co, Ltd., China) was used. The procedure was performed according to manufacturer's instructions. The assay range for KLH-IgM is 4.5 ng/L -150 ng/L and

assay sensitivity is 1.0 ng/L. For KLH- IgG, assay range is 6 ng/L -200 ng/L and assay sensitivity is 1.0 ng/L.

Statistical Analysis

The statistical analysis was carried out using SPSS 21 Software package (IBM, USA). Test for normality was performed to ascertain normal distribution of the variables. Data was not normally distributed based on tests of normality results: Shapiro-Wilk, supported by Q-Q plot. Therefore the results obtained are presented as Median. Kruskal Wallis test was carried out to explore differences on variables across the groups. Mann Whitney test was used as post hoc test to compare some groups. The p value ≤ 0.05 was used to determine the level of statistical significance. However Bonferini adjustment was used to determine the significance level of post hoc test therefore the adjusted p-value is ≤ 0.01.

RESULTS

The results obtained from the current study revealed that there is a statistically significance difference in NA test across the seven groups (χ^2 =22.09, $p < 0.001$). Group 2(positive control) recorded a highest percentage median score (Median = 73.6%) and the least is group 3 (Negative control) (Median = 15.1%). Also this study revealed that there is a statistically significance difference in NBT reduction across the seven groups (χ^2 = 31.74, $p < 0.001$). Group 2 recorded a highest percentage median score (Median =34%) and the least was group 3 (Median = 4%) as shown in Table 1. As depicted from Table 2, Post-hoc test revealed no significant difference in the NA of group 1 (Normal control) vs. group 6(p=0.028). Similarly there is no significant difference in group 1 vs. group 7, group 2 vs. group 6 and group 3 vs. group 7 (p>0.01). However, there is significance difference in group 6 vs. group 7(p=0.009). Similarly, there is significant difference in the NBT reduction test in group 1 vs. group 6, group 2 vs. group 6, group 6 vs. group 7(p=0.009) and group 3 vs. group 7(p=0.010).

From Table 3, the study revealed that there is a statistically significance difference in concentration of KLH IgM across the seven groups (χ^2 = 32.23, $p < 0.001$). Group 2 recorded a highest concentration median score (Median =91.7 ng/L) and the least is group 3 (Median = 5.91 ng/L). Similarly there is statistically significance difference in concentration of KLH IgG antibody across the seven groups (χ^2 = 30.69, $p < 0.001$). Group 2 shows a highest concentration median score (Median =143 ng/L) compared with the other groups and the least is group 3 (Median = 10.7 ng/L). As shown in Table 4, Post-hoc test revealed that there is significant difference in the concentration of KLH IgM antibody in group 1 vs. group 6, group 1 vs. group 7 and group 6 vs. group 7 (p=0.009). So also there is significant difference in the concentration of KLH IgG antibody in group 1 vs. group 6, group 2 vs. group 6, group 3 vs. group 7 and group 6 vs. group 7(p=0.009).

Table 1: Effect of aqueous extracts of PHF on Neutrophils function in wistar rats

Group (n=5)	Dose (kg/bwt)	Neutrophil Adhesion (%)	NBT reduction (%)
		Median	Median
1(NS)	10ml	18.3	10
2 (LEV)	50 mg	73.6	34
3 (CPY)	200 mg	15.1	4
4 (PHF)	250 mg	30.5	14
5 (PHF)	500 mg	37.3	16
6 (PHF)	1000 mg	49.5	21
7 (PHF+ CPY)	1000 mg +200 mg	18.5	6
Kruskal Wallis Test		**χ^2 =22.09, p < 0.001**	**χ^2 = 31.74, p<0.001**

***NS**: Normal saline; **CPY**: Cyclophosphamide; **LEV**: Levamisole hydrochloride; **PHF**: Polyherbal formulation; **NBT**: Nitroblue tetrazolium; Significant p-value is ≤0.05*

Table 2: Comparism between groups on the effect of aqueous extracts of PHF on Neutrophils function in wistar rats.

Group (n=5)	Neutrophil Adhesion	NBT reduction
	p-value	p-value
1 vs. 6	0.028	0.009
1 vs. 7	0.117	0.033
2 vs. 6	0.251	0.009
3 vs. 7	0.754	0.010
6 vs. 7	0.009	0.009

*Post Hoc test (Mann Whitney U), **NBT:** Nitroblue tetrazolium, Significant p-value is ≤0.01 (with Bonferini adjustment)*

Table 3: Effect of aqueous extracts of PHF on TDAR against KLH antigen in wistar rats.

Group (n=5)	Dose (kg/bwt)	KLH IgM(ng/L)	KLH IgG(ng/L)
		Median	Median
1 (NS)	10ml	45.5	38.3
2 (LEV)	50 mg	91.7	143
3 (CPY)	200 mg	5.91	10.7
4 (PHF)	250 mg	51.7	38.3
5 (PHF)	500 mg	62.4	51.5
6 (PHF)	1000 mg	91.6	70.5
7 (PHF+ CPY)	1000 mg + 200 mg	11.7	20.6
Kruskal Wallis Test		**χ^2 =32.23, p< 0.001**	**χ^2 =30.69, p < 0.001**

***NS**: Normal saline, **CPY**: Cyclophosphamide; **LEV**: Levamisole hydrochloride; **PHF**: Polyherbal formulation; **KLH**: Keyhole Limpet Hemocyanin; Significant p-value is ≤0.05*

Table 4: Comparism between groups on the effect of aqueous extracts of PHF on TDAR against KLH antigen in wistar rats.

Group (n=5)	KLH IgM	KLH IgG
	p-value	p-value
1 vs. 6	0.009	0.009
1 vs. 7	0.016	0.028
2 vs. 6	0.602	0.009
3 vs. 7	0.009	0.009
4 vs. 7	0.009	0.009

*Post Hoc test (Mann Whitney U), **KLH**: Keyhole Limpet Hemocyanin, Significant p-value is ≤0.01 (with Bonferini adjustment)*

DISCUSSION

Polyherbal formulations are abundantly gaining popularity globally as compared to allopathic medicine for the treatment of different types of ailments[17], both immunostimulation and immunosuppressant needed to be put in check and balance depending on the interest in treatment regimen in order to regulate the normal immunological function.[18]

The result showed significant increase in NA as well as NBT reduction with increase dose of PHF across the treatment groups (p< 0.001). Neutrophil adhesion is significantly higher in group 6 (49.5 %) when compared with group 7 (18.5%) (p = 0.009). Our finding is consistent with the study of Bagwan and colleagues which reported that ayurvedic polyherbal formulations and Levamisole causes increase in NA.[18] It may be suggested that the herbal formulation has some effect on NA in group 6 but failed to neutralize the suppression effect of cyclophosphamide in group 7. The failure may be attributed to the dose used in this study the highest dose is 1000 mg and probably may not be enough to give the desired prospect. Indeed some formulations have the ability to show significant immunomodulatory response in higher dose.[19] Some plant constituents tend to affect NA. For instance Flavonoids, Polyphenol and Vitamin had been reported to enhance NA,[20] although a recent study reported contrary.[21]

The result on NBT reduction show that the formulation had significantly improve reduction ability of neutrophils in group 6 (21%) compared with normal control group 1 (10%) (p = 0.009). Similarly the formulation tends to antagonize the effect of cyclophosphamide on reduction ability of NBT by neutrophil for instance this was observed when group 3 (4%) was compared with group 7 (6%) (p = 0.01), furthermore no difference was observed between group 1(normal control) and 7. This is in line with the report that some formulations were able to neutralize the effect of immunosuppressive drugs on reducing ability of NBT by Neutrophils.[22] This finding suggest PHF may have some constituent that can antagonize the effect of Cyclophosphamide because some plant constituents such as steroid and alkaloid prevents myelosuppression induced by Cyclophosphamide and other immunosuppressive drugs.[23] In addition, Steroid alone had been reported to enhance NBT reduction.[24] Furthermore, several previous studies indicated that Tannins, Saponnin and phenol have the ability to stimulate phagocytic cells.[24, 25, 26, 27]

In this study we show that serum concentration of KLH-specific IgM and IgG significantly increase with increase in dose of the PHF across the treatment groups (p < 0.001). When comparing the serum concentration of IgM group 6 (91.6 ng/L) have higher concentration than group 1 (45.6 ng/L) (p = 0.009). This pattern was also observed when comparing the serum concentration of IgG between group 6 and 1 (p = 0.009). These findings suggest there is potential role play by the PHF in TDAR against the KLH antigen which leads to increase production of specific antibodies. In addition the treatment with PHF generally improves the immune response against the KLH antigen in the wistar rats. This also highlight that a single immunization with KLH induced an IgM-predominant response, with the IgG response being induced to a lesser extent, but increased upon secondary sensitization.[28] Some plants constituent which are also found in the PHF plays some significant role in modulation humoral immune response towards an antigen. For instance, Alkaloids have been reported to increases immunobioactivities thus enhanced the circulating antibody.[29] Flavonoids enhances the body

production of Immunoglobulin.[30] Previous studies reported that Vitamins, Glycosides and Terpenoids can enhance the production of antibody for healthy immune system.[31, 32] Phytophenolic compound had been reported to up-regulate IgM- and IgG-mediated humoral immune response.[33] Our finding is in conformity with the study of Kawai and co-workers which reported antibody response to KLH antigen which produce anti-KLH IgM as well as anti-KLH IgG after treatment with plant extract.[28] KLH acted in an immunomodulatory role too, enhancing primary and secondary antibody responses, but suppressing non-specific cellular immunity in a non-antigen-specific fashion.[34]

The formulation tends to compete with standard immunostimulant such as Levamisole, on the other hand it has antagonistic effect on standard immunosuppressor like Cyclophosphamide. For instance no significance difference was observed in serum concentration of IgM between group 2 (positive control: 91.7 ng/L) and group 6 which administered with highest dose of the PHF 1000 mg/kg/bwt (91.6 ng/L) ($p = 0.602$). However significance difference was reported between group 3 (negative control: 5.91 ng/L) and group 7 which administered with 200 mg of Cyclophosphamide and 1000 mg/kg/bwt of PHF (11.7 ng/L) ($p = 0.009$). Immunoglobulin G also follow the same pattern as that of IgM. Certainly, some formulations have the efficiency to exert immunological responses that approach that of pharmaceuticals drugs.[35]

CONCLUSION

This study revealed that the PHF has effect on NA, NBT reduction and TDAR of the Wistar rats. The PHF exert most of its effect at 1000 mg as such there is increase in NA, NBT reduction and TDAR activities in the Wistar rats with increase in the dose of the PHF. In some instances it tends to compete with immunostimulating drug or antagonize the effect of immunosuppressor drug. We suggest increase in PHF dose in future research may yield desired result. However the PHF has potential immunomodulatory activity in wistar rat thus could be useful in improving immune responses.

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