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PAGES	PAPERS
41-50	CD4+ T Cell Response in HIV-Positive Women Initiating Highly Active Anti-Retroviral Therapy (HAART) at General Hospital, Kabba, Kogi State, Nigeria <i>W. F. SULE and J. K. OLUMORIN</i>
51 - 54	Effect of Carbon Sources on The Extracellular Lignocellulolytic Enzymetic System of <i>Pleurotus Sajor-Caju</i> <i>Muhammad I. Massadeh, Abeer Fraij and Khalid Fandi</i>
55-56	New record of the land snail <i>Allopeas gracilis</i> (Hutton, 1834) (Gastropoda: Subulinidae) from Basrah area, Iraq <i>Murtada D. Naser</i>
57- 64	Molecular cloning and Characterization of a membrane-intrinsic (S)-2,3-di-O-digeranylglycerol phosphate synthase involved in the biosynthesis of archaeal ether-linked membrane lipids. <i>Narayan Roy, Naoki Nemoto and Akihiko Yamagishi</i>
65- 68	Exposure to Potassium Carbonate Emulsion Induced Nephro-Toxicity in Experimental Animals. <i>Akintunde J.K., Opeolu B b and Aina O.O</i>
69-76	Effect of <i>Salvia triloba</i> L. f. Extracts on Neoplastic Cell lines <i>Abdallah Ibrahim, Amin A. Aqel</i>
77-82	Toxic Effect of Dimethoate and Diazinon on the Biochemical and Hematological Parameters in Male Rabbits <i>Elias M.A.Salih</i>

CD4⁺ T Cell Response in HIV-Positive Women Initiating Highly Active Anti-Retroviral Therapy (HAART) at General Hospital, Kabba, Kogi State, Nigeria

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Abstract

المخلص

Reportedly, HIV-infected patients on HAART exhibited accelerated increase in the first three months. We therefore tested an hypothesis that there is no difference between 3 month follow-up and baseline CD4 counts of HIV-positive women initiating Stavudine, Lamivudine and Nevirapine at General Hospital, Kabba, Kogi State, Nigeria. As a prospective study, 100 consenting HIV-positive, HAART-naïve women aged 17-45 years were studied. Two blood samples were aseptically obtained by venipuncture from each woman before and after 3 months on HAART and assessed for pre- and post-therapeutic CD4 counts using automated FACSCount[®] System. Pertinent data of participants were obtained using questionnaire forms. For the purpose of comparison, the women were grouped into pre-therapeutic CD4 count of < 200; 200-349 and ≥ 350 cells/μl groups. SPSS 15.0 was used for the statistical analyses. The mean baseline and follow-up CD4 counts were respectively 320.03 cells/μl and 620.92 cells/μl (n = 100), the latter was significantly ($P = 0.001$) higher than the former. Unlike the mean follow-up CD4 count of the women in the ≥ 350 cells/μl group, mean follow-up CD4 counts of those in the < 200 and 200-349 CD4 cells/μl groups were significant ($P = 0.001$) higher than their respective mean baseline values. The 74 women who responded "adherent" to HAART had significantly ($P = 0.001$) higher mean follow-up CD4 count contrary to that of the remaining 26 "non-adherent" women who exclusively belonged to the ≥ 350 CD4 cells/μl group. The women who responded "yes" and "no" to having diabetes/hepatitis were comparable ($P = 0.62$) in their mean follow-up CD4 counts. We concluded that 75% of the HIV-positive women had significant increase in CD4 count, hence they had short-term immunologic benefit; and that rather than the pre-therapeutic CD4 count of ≥ 350 cells/μl or having diabetes/hepatitis, it was "non-adherence" to HAART that apparently accounted for lack of therapeutic benefit among the 10 women who had < 349 CD4 cells/μl after 3 months on HAART.

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Keywords: HIV-Positive Women, HAART-Naïve, Pre-Therapeutic CD4 Count, Follow-Up CD4 Count, Nigeria..

1. Introduction

In infected humans, HIV attaches to CD4 and CXCR4 surface molecules hence it preferentially infects a subset of human lymphocytes known as CD4⁺ T cells - the T helper 1 and 2 cells (Murray et al., 2000; Takahashi, 2004; NIAID, 2006). These are the cells respectively responsible for orchestrating cell-mediated and humoral immunities in humans during infections or malignancy (Cooke, 2001). During HIV infections, these cells lose their normal immunologic functions (Carcelain, 1999) followed by lysis due to productive intracellular

replication of the virus (Murray et al., 2000). The large numbers of infectious progeny virions subsequently released by the lysed cells infect other susceptible cells thereby killing more infected cells. This results in massive depletion of this subset of lymphocytes. Initially however, large numbers of new CD4⁺ T cells are produced by the HIV-infected to offset the destroyed cells which partly accounts for the long (about 5-12 years) incubation period (NIAID, 2006) of HIV disease or AIDS in the absence of antiretroviral therapy. The war of attrition induced by HIV replication continues however, until the infected body's ability to replace the massive loss of CD4⁺ T cell wanes.

The absolute count of CD4⁺ T cell (CD4 count) of healthy HIV-negative human has been variously reported to range from 500-1,696 CD4⁺ T cells/μl of whole blood (Takahashi, 2004; NIAID, 2006, Kuby, 1997, Klose et al.,

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2007), the value may be below 350 and oftentimes less than 200 cells/ μ l for HIV-positive individuals (Kuby, 1997; WHO, 2006). Such HIV victims are respectively described as immunosuppressed; and severely immunosuppressed (Anastos et al., 2004). Either of the immune statuses puts HIV-infected at increased risk for contracting opportunistic infections (OIs) which, if untreated, eventually leads to death. It follows therefore that the pivotal role of CD4⁺ T cells in fighting off infections makes their absolute number (and function) a veritable indicator of immune status for HIV and AIDS patients (Anastos et al., 2002).

Though the scourge of HIV and AIDS still ravages mankind in sub-Saharan Africa especially, the advent of highly active anti-retroviral therapy (HAART) has brought considerable succour to the affected (Gange et al., 2002; Erb et al., 2000; Bing et al., 1999) and by extension, to the care-givers and all stakeholders worldwide. The antiretroviral drugs (ARVs) comprising six different classes (FDA, 2008), selectively attack the viral enzymes (reverse transcriptase and protease) thereby preventing HIV-1 replication in infected cells while others target the viral architectural molecules to inhibit HIV entry into susceptible cells. Inhibitory effects of the ARVs pave way for the restoration of CD4⁺ T cell numbers and function in already overwhelmed HIV and AIDS victims (Carcelain, 1999; Li et al., 1998; Bucy et al., 1999; Mattapallil et al., 1999).

HAART is, however, no cure for AIDS, hence it is recommended to be taken by HIV and AIDS patients a life-time (Boschi et al., 2008; PAGAA, 2008). However, HIV-1- related morbidity and mortality have been considerably reduced by the use of HAART (Gange et al., 2002; Erb et al., 2000). Furthermore, when appropriately taken by HIV-positive pregnant women, HAART can significantly reduce mother-to-child-transmission (MTCT) of HIV-1 (Marazzi et al., 2006; Public Health Task Force, 1998). It must be noted however, that some HIV and AIDS patients on HAART show complete therapy failure while some show discrepant responses (Perin and Telenti, 1998; Barreiro et al., 1999). An adequate CD4 response for most patients on ART is defined as an increase in CD4 count in the range of 50 or 100 to 150 cells/mm³/year with an accelerated response in the first 3 months (Kaufmann et al., 2003). The accelerated increase in CD4 count within 3 months following initiation of HAART had been attributed to redistribution of CD4⁺ T cells from lymphoid tissues (Bucy et al., 1999; Carcelain et al., 1999) and proliferation of naïve CD4⁺ T cells (Pakker et al., 1998). In addition to numerical improvement, restoration of CD4⁺ T cell functions was also observed after 3 months of HAART (Li et al., 1998).

Due to shared routes of transmission, HIV and HBV infections are often found in the same individual (Konopnicki et al., 2005); though some conflicting reports abound, some studies however, have shown that the co-infection had no impact on HIV-patients on HAART regarding immune recovery (or viral suppression) (Omeland et al., 2008; Law et al., 2004; Konopnicki et al., 2005). Another clinical condition, a metabolic disorder, pertinent

to the present study is diabetes. This had also been documented as having no known impact on the effectiveness of HAART (Gallant, 2001).

On realization of the devastating effects of HIV and AIDS on medical, social and economic lives of its citizens, Nigerian government demonstrated strong commitment to fighting the scourge by implementing Africa's largest ART programme (Kombe et al., 2004), with supports from PEPFAR and other donor agencies.

The three generic ARVs offered to HIV and AIDS patients in Nigeria are Lamivudine (3TC), Stavudine (d4T) and Nevirapine (NVP). This is in accordance with WHO recommendation of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitors (NNRTI) as first-line therapy (WHO, 2006). Many authors have documented the therapeutic benefit of these drugs on people living with HIV and AIDS (PLWHAs) in different parts of the world including Nigeria (Aina et al., 2005; Badri et al., 2002; Erhabor et al., 2006; Gautam et al., 2008; Laurent et al., 2004). But before ART is offered to HIV and AIDS patients, assessment of their CD4⁺ T cells/ μ l of whole blood is essential; this is first done following laboratory confirmation of HIV infection. The CD4 count at this time is called the baseline count (pre-therapeutic count). The baseline CD4 count is useful for the determination of immunodeficiency state; to decide whether or not to initiate ART and for monitoring immunologic response of HIV patients to ART. A WHO laboratory criterion for instituting ART in a resource-limited setting, like Nigeria, is < 350 CD4 cells/ μ l (WHO, 2006). CD4 count is thereafter done regularly at 3-6 month interval as part of follow-up assessment. This is necessary to monitor response of HIV and AIDS patients to ART in order to know whether or not the therapy is effective which may necessitate modification or change in the HAART. The CD4 count can be done manually using Dynabeads[®] method or by automated technique using flow cytometry machines (Partec Cyflow[®] or BD FACSCount[®]) (Gautam et al., 2008; Erhabor et al., 2006; Nwokedi et al., 2007).

The importance of monitoring HIV and AIDS patients on ART at different centers in a resource-poor setting like Nigeria cannot be overemphasized. In addition, different studies comparing men's and women's use of HAART reported lopsidedness of use of more men than women (Lynn, 1999; Mocroft et al., 2000); reports corroborating these stated that women were underrepresented in HIV and AIDS clinical trials; less aware of their eligibility to participate in such trials, and less likely to be recruited into such studies by their health providers (Stone et al., 1997; Edelstein and Jacobson, 1999; CDC, 2006). In view of these, and the fact that there are few published studies, to the best of our knowledge, on the CD4 count response of HIV- positive, HAART-naïve women in Kogi State, Nigeria, we conducted baseline and three month follow-up CD4 count among adult HIV-positive females who initiated HAART in General Hospital, Kabba with the view to establishing benefit or lack thereof from the therapy.

2. Materials and Methods

2.1. Study Area / Population

This study was carried out between May and October, 2008 in General Hospital, Kabba, Kabba/Bunu local government area (LGA), Kogi state. The major ethnic group in kabba is the Okun (Yoruba-speaking people). Kogi state is the most centrally located state in Nigeria and bordered by nine other states. The participants were HIV-positive women attending the General Hospital.

2.2. Study design

This is a prospective cohort study. The objectives of this study were explained to the Management of General Hospital, Kabba and permission to undertake the study was subsequently granted. An officer in the hospital explained the objectives and details of the study to newly diagnosed HIV-positive, ART-naïve women attending the hospital. Altogether one hundred women verbally consented and were consecutively recruited to enroll in this study. They were interviewed and their responses documented into questionnaire forms. Each patient was given an ID number. After this, about 5 ml of blood was aseptically collected by venipuncture from each woman into K₃ EDTA BD Vacutainer® blood collection tube and the tube correspondingly labeled. Blood samples were kept at room temperature and analyzed for CD4 count within four hours of collection. After this, each woman was referred to ART section for counseling, prescription and collection of ARV drugs (i.e. Lamivudine (3TC), Stavudine (d4T) and Nevirapine (NVP)). The CD4 count so obtained was used to categorize the women into pre-therapeutic (i.e. baseline) < 200, 200-349 and ≥ 350 CD4 cells/μl groups. All the women, some of whom were pregnant, showed willingness and gave consent to start the ART regimen; they were all subsequently started on HAART since clinical guidelines recommended initiating HAART for pregnant HIV-positive (Yeni et al., 2004) and those with < 350 CD4 count/μl (DHHS, 2006). Those with OIs were appropriately referred for chemoprophylaxis. The women were informed to report in the hospital for regular medical check-ups, especially after three months for follow-up CD4 count evaluation. Five laboratory confirmed HIV-negative, apparently healthy adult females were included at follow-up as controls. The demographic data collected from the women include educational status; marital status; age; having diabetes/hepatitis; pregnancy status; presence of any infections and at follow-up, information on adherence to ART regimen. "Adherence" was defined as patient's response of compliance with ARV drug regimen.

2.3. Laboratory CD4 count

The CD4 count of each whole blood sample was prepared by adding 50 μl blood to BD reagent. Automated FACSCount® System (Becton Dickinson, USA) was used for the cell count. The CD4 count was carried out according to manufacturer's instructions.

2.4. Data analysis

The results of this study were presented with descriptive statistics. Mean values were presented together with 95% confidence interval of mean (95% CI). We used

paired and independent samples t-tests, CHI² and ANOVA to establish statistical difference or lack thereof between patients' variables. Two-tailed hypothesis was used with $P \leq 0.05$ as indicator of statistical significance, SPSS 15.0 for Windows® was used for the analyses

3. Results

One hundred (100) HIV-positive ART-naïve women initiating HAART participated in the study. While some of them were apparently healthy, some appeared physically unthrifty; however, none died during the course of the study. They had age range of 17-45 years (yrs) (mean = 31.57 yrs; 95% CI: 30.05-33.09 yrs). The women studied as control were students in tertiary institution, they had mean age of 26 yrs (n = 5; range 23-28 yrs; 95% CI: 24.25-27.75 yrs). Statistical analysis showed that the mean age of the control was significantly less ($P = 0.001$) than that of the study subjects. The women were categorized into pregnant (n = 39, mean age = 32.10 yrs) and non-pregnant (n = 61, mean age = 31.23 yrs) subsets and compared, they were statistically similar ($P = 0.59$) in mean age. Some of the HIV-positive women met the AIDS classification criterion based on their baseline CD4 count; Table 1 shows the age distribution of the women with respect to baseline CD4 count categories.

The demographic data of the HIV-positive women on HAART are as shown in Figure 1; the Figure also reveals high level (74%) of "adherence" to the ART regimen; none of the "adherent" women had decline or no-change in CD4 count at follow-up. The women comprising the 26% "non-adherent" were exclusively in the ≥ 350 cells/μl group and included 11 each with primary and secondary education and 4 having tertiary education. Other demographic/clinical data of the 26 "non-adherent" women are: married (14), unmarried single (6), divorced (4) and widow (2); "yes" to diabetes/hepatitis (16); non-pregnant (21).

In all, 74 and 26 women responded "adherence" and "non-adherence" respectively to the ART regimen. Mean CD4 counts at baseline and follow-up were 224.92 and 688.32 cells/μl respectively for the "adherent" women; the latter being significantly ($P = 0.001$) higher. Baseline CD4 counts for these women significantly ($P = 0.001$) correlated ($r = 0.87$) with their follow-up values. For the "non-adherent" women, we recorded 590.73 (350.00 – 1,112.00 cells/μl) and 429.08 cells/μl (161.00 – 940.00 cells/μl) respectively as mean baseline and follow-up CD4 counts, with the latter being significantly ($P = 0.005$) lower. We observed no significant ($P = 0.15$) correlation ($r = 0.29$) between the baseline and follow-up CD4 counts for the women in this group. Comparison of the mean follow-up CD4 counts of the "adherent" and "non-adherent" women revealed significantly ($P = 0.001$) higher value of the former. Overall, 10 women still had < 349 CD4 cells/μl at follow-up (Figure 2), 9 of whom belonged to the 26 "non-adherent" women.

Nine women in the ≥ 350 cells/μl group who responded "adherent" to ART regimen had mean follow-up CD4 count of 1,157.78 cells/μl (range: 872.00 – 1,572.00 cells/μl) which was significantly ($P = 0.001$) higher than their mean baseline cell count of 623.56 cells/μl (range: 352.00 -1,189.00 cells/μl). The mean follow-up CD4 count

of these 9 “adherent” was significantly ($P = 0.001$) higher than the corresponding mean value of the 26 “non-adherent” women. In all, 13 women showed decline in CD4 count, while 9 had no-change in CD4 count at follow-up, Table 2. These 22 women were exclusively in the ≥ 350 CD4 cells/ μl group and were among the 26 “non-adherent” women.

Overall, we observed considerable changes in the proportion of women with respect to increase in CD4 count, Figure 2. Seventy five per cent of the women had significant increase in CD4 count; this comprised the entire women in the < 200 and 201-349 CD4 cells/ μl groups (Figure 2) and 10 women from the ≥ 350 cells/ μl group. We observed that 69 (92%) of the 75 women had CD4 count of ≥ 500 cells/ μl after 3 months on HAART.

The mean baseline CD4 count of the 100 HIV-positive women was 320.03 cells/ μl (range 1-1,189 cells/ μl ; 95% CI: 269.67-370.29 cells/ μl); at follow-up their mean CD4 count was 620.92 cells/ μl (range 161-1,572 cells/ μl , 95% CI: 569.51-672.33 cells/ μl); with change in CD4 count ranging from decline to increase (mean = +300.89 cells/ μl , range: -735.00 to +711.00 cells/ μl ; 95% CI: 237.45-364.33 cells/ μl). However, CD4 count of the control evaluated at follow-up period gave mean value of 2,209.40 cells/ μl (range 1,886.0-2,551.0 cells/ μl ; 95% CI: 1,946.84-2,471.96 cells/ μl) which was significantly higher ($P = 0.001$) than mean follow-up CD4 count of the women on HAART. The mean CD4 count of the women at baseline, follow-up and mean change in CD4 count are as shown in Figure 3.

Four of the subjects (4.0%) had severely depleted CD4⁺ T cells of ≤ 5 CD4 cells/ μl , but at follow-up, they had mean increase of +398.25 cells/ μl (range 317.00-443.00 cells/ μl ; 95% CI: 343.26-453.24 cells/ μl).

Women in the three baseline CD4 groups were statistically comparable ($P = 0.88$) in mean age. Their CD4 counts are as shown in Figure 3. Those in the < 200 CD4 cells/ μl group had baseline range of 1-198 CD4 cells/ μl ($n = 37$, mean = 97.30 cells/ μl ; 95% CI: 76.34-118.25 cells/ μl) with 317-882 CD4 cells/ μl at follow-up (mean = 570.57 cells/ μl ; 95% CI: 529.10-612.03 cells/ μl). The 200-349 CD4 cells/ μl group had baseline range of 200-345 CD4 cells/ μl ($n = 28$, mean = 265.18 cells/ μl ; 95% CI: 246.19-284.17 cells/ μl); 401-1,336 CD4 cells/ μl at follow-

up (mean = 722.43 cells/ μl ; 95% CI: 649.96-794.90 cells/ μl). The ≥ 350 cells/ μl group had baseline range of 350-1,189 cells/ μl ($n = 35$, mean = 599.17 cells/ μl ; 95% CI: 522.73-675.61 cells/ μl), with 161-1,572 cells/ μl at follow-up (mean = 616.46 cells/ μl ; 95% CI: 479.19-753.72 cells/ μl).

The women who responded “yes” and “no” to having diabetes/hepatitis were 67 and 33 respectively. Those with “yes” to this clinical condition had mean baseline and follow-up CD4 counts of 312.49 and 611.66 cells/ μl respectively, with the latter being significantly ($P = 0.001$) higher. For those with “no” to diabetes/hepatitis, their mean follow-up CD4 count (639.73 cells/ μl) was also significantly ($P = 0.001$) higher than the mean baseline value (335.33 cells/ μl). While there was no significant ($P = 0.27$, $r = 0.14$) correlation between the baseline and follow-up CD4 count for the HIV-positive women with “yes” to diabetes/hepatitis, a significant ($P = 0.005$) correlation ($r = 0.48$) was observed for those with “no” to the clinical condition. A significant finding was that the mean follow-up CD4 counts of these two groups were comparable ($P = 0.62$).

Eleven and two of 16 “non-adherent” women with “yes” to diabetes/hepatitis respectively had decline and no change in their CD4 count at follow-up and represented the same women with decline and no change in CD4 count among the 67% (Figure 1) that responded “yes” to diabetes/hepatitis. Of the 33% with “no” to diabetes/hepatitis, only 2 and 7 respectively had decline and no change in their CD4 count at follow-up. These women were comparable in proportions ($\text{CHI}^2 = 0.80$; $\text{df} = 1$; $P = 0.37$) with respect to increase in their CD4 count at follow-up.

The pregnant subset of the HIV-positive women recorded baseline range of 31-1,112 CD4 cells/ μl ($n = 39$; mean = 255.87 cells/ μl ; 95% CI: 184.17-327.57 cells/ μl); at follow-up 181-1,216 CD4 cells/ μl (mean = 625.10 cells/ μl ; 95% CI: 558.94-691.26 cells/ μl) was recorded. The non-pregnant had a range of 1-1,189 cells/ μl as baseline ($n = 61$; mean = 361.05 cells/ μl ; 95% CI: 293.94-428.16 cells/ μl) and 161-1,572 CD4 cells/ μl (mean = 618.25 cells/ μl ; 95% CI: 544.92-691.58 cells/ μl) at follow-up, Figure 3.

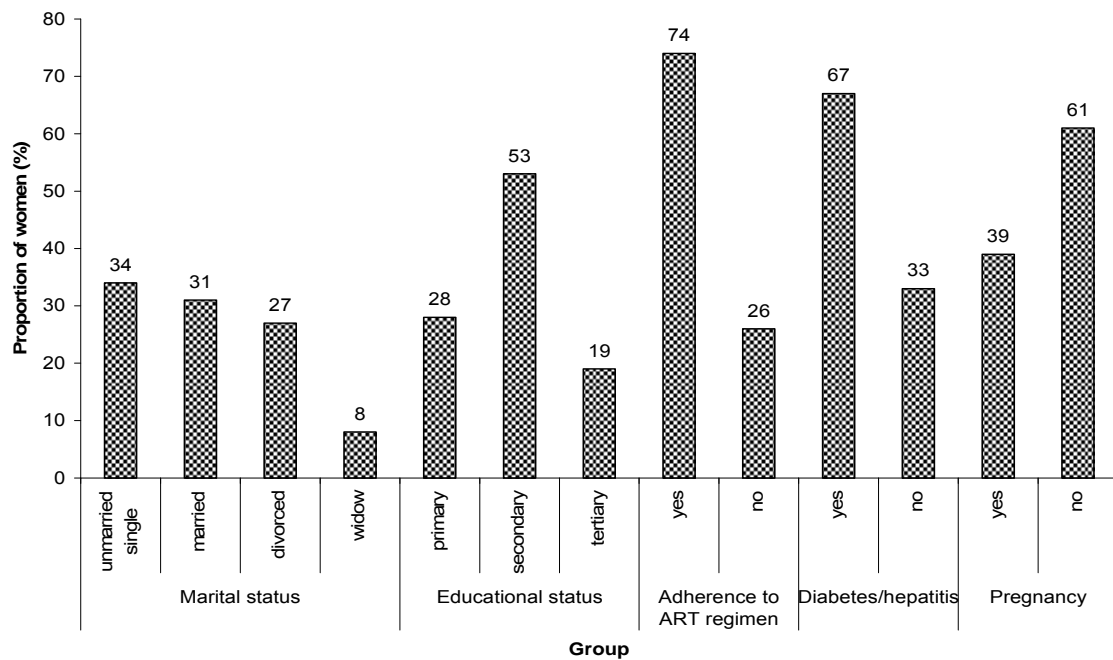


Figure 1. Demographic distribution of HIV-positive women on HAART at General Hospital, Kabba, Kogi State, Nigeria.

Table 1. Age distribution of baseline CD4 count of HIV-positive women on HAART at General Hospital, Kabba, Kogi State, Nigeria.

Age range (years)	Groups based on baseline CD4 count			
	< 200	200-349	≥ 350	Row total
17-29	18	15	15	48
30-39	11	7	13	31
40-45	8	6	7	21
Column total	37	28	35	100

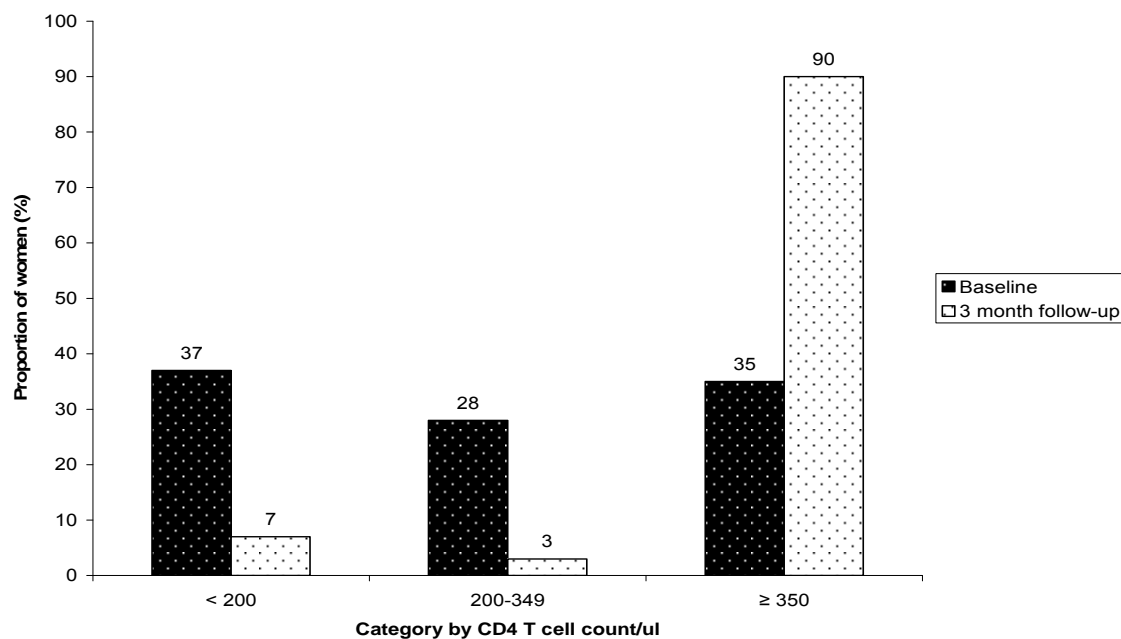


Figure 2. Proportional trend of HIV-positive women on HAART according to baseline and follow-up CD4 count categories at General Hospital, Kabba, Kogi State, Nigeria

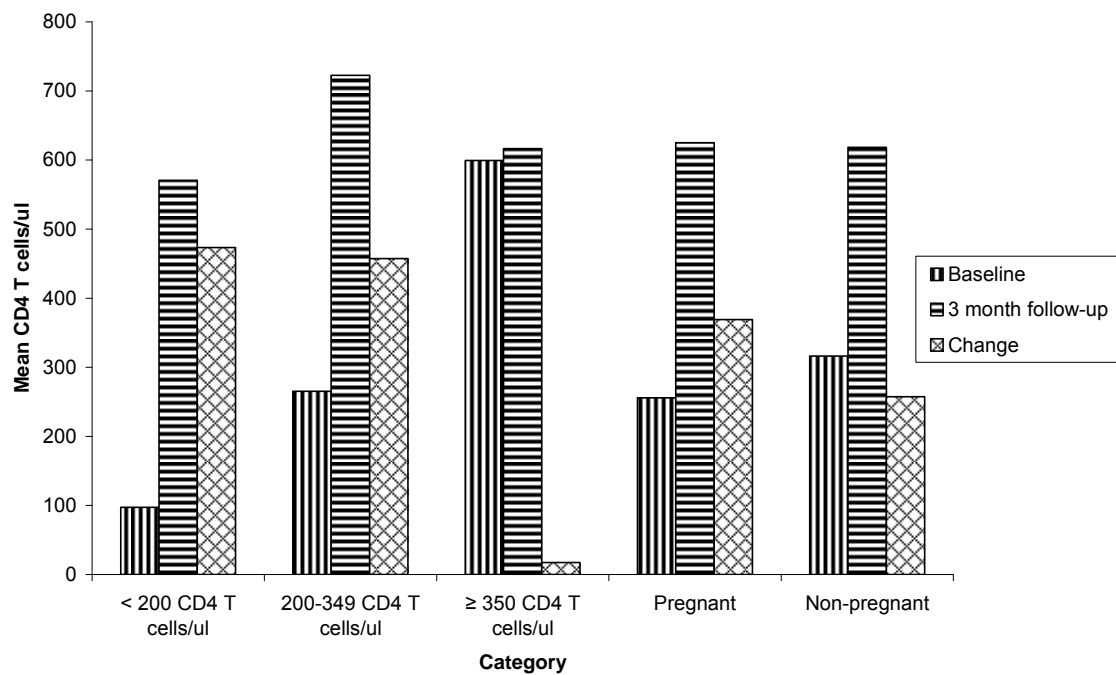


Figure 3. Mean CD4 count of HIV-positive women on HAART at General Hospital, Kabba, Kogi State, Nigeria.

Table 2. The HIV-positive women having baseline of ≥ 350 cells/ μ l with no-change or decline in CD4 count after 3 months on HAART at General Hospital, Kabba, Kogi State, Nigeria.

S/No.	Baseline	Follow-up	Change
1	867	867	0
2	423	423	0
3	946	940	-6
4	558	557	-1
5	430	430	0
6	392	392	0
7	544	544	0
8	367	367	0
9	559	559	0
10	714	714	0
11	397	397	0
12	473	470	-3
13	927	192	-735
14	694	182	-512
15	609	199	-410
16	815	200	-615
17	1,112	403	-709
18	559	193	-366
19	350	161	-189
20	450	181	-269
21	356	216	-140
22	740	193	-547

4. Discussion

This study was designed to assess the baseline and follow-up CD4 count in HIV-positive adult females initiating 3TC, d4T and NVP at General Hospital, Kabba, Kogi State, Nigeria with the view to establishing a short-term therapeutic (immunologic) benefit or lack thereof from the HAART. We observed that 37 of the women met the AIDS criteria (< 200 cells/ μ l), while 28 met the eligibility criterion for initiation of HAART according to pre-therapeutic CD4 count evaluation (WHO, 2004), Table 1.

At 3 month follow-up, 7 women still categorized as AIDS patients, 3 had 200-349 CD4 cells/ μ l with 90 women having ≥ 350 CD4 cells/ μ l (Figure 2). The 7 women that categorized as AIDS patients and 2 of the 3 that had 200-349 cells/ μ l after 3 months on HAART responded "non-adherent" to the ART regimen despite belonging to those with pre-therapeutic CD4 count of ≥ 350 cells/ μ l, Table 2.

Overall, the mean 3 month follow-up CD4 count ($n = 100$) was significantly ($P = 0.001$) higher than the mean baseline value. This indicated general immunologic benefit of the HAART to the HIV-positive women after a short-term period of 3 months. The finding that four women with pre-therapeutic AIDS-defining CD4 count of ≤ 5 cells/ μ l had mean increase of +398.25 cells/ μ l at follow-up further supported the beneficial effect of the HAART. This partly supported the findings that even advanced immune suppression can be overcome with HAART that results in CD4 counts of greater than 0.200×10^9 cells/L (Anastos, 2004) and that a CD4 count of less than 5×10^6 /L did not necessarily mean imminent death (Sabin et al., 1997). It hence implies that very low baseline CD4 count may not be as crucial to survival of HIV-positive patients on HAART as the follow-up CD4 count.

As previously reported, the statistically significant increase in the CD4 count of the women could be due to accelerated CD4⁺ T cell increase that occurs within first few months in HIV-positive patients after initiating first line ART (Bucy et al., 1999; Carcelain et al., 1999).

All the women in the < 200 cells/ μ l and 200-349 cells/ μ l groups had significantly ($P = 0.001$) higher mean CD4 count at follow-up compared to their corresponding mean baseline counts. Contrary however, was the case for the women in the ≥ 350 cells/ μ l group who had mean increase of 17.29 CD4 cells/ μ l (Figure 3) with no significant ($P = 0.80$) difference between their mean follow-up and baseline CD4 counts. The women with pre-therapeutic CD4 count of < 349 cells/ μ l therefore appeared to have more immunologic benefit than those having ≥ 350 CD4 cells/ μ l prior to HAART. Similar observations had been previously reported but in a long-term study (Erabor et al., 2006). Though the three pre-therapeutic groups of the women differed significantly ($P = 0.001$) in mean baseline CD4 counts; at follow-up, each mean CD4 counts of the < 200 cells/ μ l and 200-349 cells/ μ l groups became comparable ($P = 0.47$ and $P = 0.12$) to that of ≥ 350 cells/ μ l group. We observed that the mean follow-up CD4 count of the 200-349 cells/ μ l group was significantly ($P = 0.03$) higher than that of the < 200 cells/ μ l group. This observation indicated poor immunologic response to HAART for those HIV-women having severely depleted

CD4⁺ T cells (i.e. < 200 cells/ μ l). As regards mean change in CD4 counts, both the < 200 cells/ μ l and 200-349 cells/ μ l groups had significantly ($P = 0.001$) higher mean change in CD4 count than that of the ≥ 350 cells/ μ l group, but the < 200 cells/ μ l and 200-349 cells/ μ l groups had comparable ($P = 0.80$) mean change, Figure 3.

With reference to only the women in the < 200 cells/ μ l and 200-349 cells/ μ l groups ($n = 65$), the change in CD4⁺ T cell count ranged from 122.0 to 1,106.0 CD4 cells/ μ l with mean change of 466.37 CD4 cells/ μ l in 3 months (assuming 90 days), this gave a mean change of about 5.18 CD4 cells/ μ l per day for each HIV-positive woman.

Comparison of the pregnant and non-pregnant subsets revealed each group had significantly ($P = 0.001$) higher mean CD4 count at follow-up than the corresponding mean baseline values. We observed that, though the two groups were just statistically ($P = 0.05$) different at mean baseline CD4 counts, they however became comparable at follow-up ($P = 0.90$). These observations showed that the 2 groups had short-term immunologic benefit from the HAART. We observed that 10 and 7 non-pregnant women respectively experience decline and no-change in CD4 count at follow-up, while only 3 each had corresponding experiences among the pregnant. Furthermore, all these 23 women were a subset of the 26 who responded "non-adherence" to ART who also belonged to the ≥ 350 cells/ μ l group. With these observations, the effect(s) of pregnancy on the change in CD4 count could not be clearly established as the non-pregnant had greater numbers of women with decline and no-change in CD4 count compared to the pregnant. A reason, for this could be the greater number of non-pregnant women in this study. A study with equal proportion of both subsets might reveal otherwise.

We recorded a composite observation that the women who responded "adherence" had significantly higher mean follow-up CD4 count compared to their baseline values unlike the significant decline among the "non-adherent" and significantly higher mean follow-up CD4 count of the "adherent" compared to that of the "non-adherent". These pointed to the positive role played by "adherence" to HAART on the CD4⁺ T cell response after 3 months. Furthermore, we observed that 9 "adherent" women belonging to ≥ 350 cells/ μ l group had significant increase in their mean CD4 count at follow-up (data not shown) and that this was significantly higher than the corresponding value of the 26 "non-adherent", this underscored the immunologic benefit of the HAART to these 9 "adherent" HIV-positive women vis-à-vis their pre-therapeutic CD4 count that was ≥ 350 cells/ μ l. It implied therefore that rather than pre-therapeutic value of ≥ 350 CD4 cells/ μ l, it was "non-adherence" to ART regimen that was apparently responsible for the non-beneficiary effect of the HAART to those who still had < 349 cells/ μ l after the 3 months.

We recorded that all the 26 "non-adherent" HIV-positive women had pre-therapeutic CD4 count of ≥ 350 cells/ μ l and that the majority of women with decline and no-change in CD4 count after 3 months also belonged to this CD4 count category (Table 2); we therefore suggested that having CD4 count above the HAART eligibility criterion probably predisposed the women to "care-free" attitude regarding compliance with the HAART regimen.

Unlike the study of Erhabor et al. (2006) who though studied 53 males and 47 females (aged: 18-56 yrs) in University of Port-Harcourt Teaching Hospital, Nigeria, and observed higher mean increase in CD4 count among the < 200 cells/ μ l group than that of the 200-350 cells/ μ l group; we observed the reverse in this study (Figure 3). The same study by Erhabor et al. (2006) concluded that there was no long-term advantage in initiating HAART at a pre-therapeutic CD4 count of > 350 cells/ μ l; we could not clearly compare the therapeutic benefit experienced by the 9 “adherent” women having \geq 350 cells/ μ l with those studied by Erhabor et al. (2006) because of the short-term period of our own study (3 months) and smaller sample size.

The mean CD4 count of 2,209.40 cells/ μ l recorded for the 5 controls was higher than the 818 cells/ μ l reported for 89 HIV-seronegative female miners (mean age: 37.5 yrs) in Jos, Nigeria (Aina et al., 2005) and the mean CD4 count of 1,295 cells/ μ l for 100 healthy controls (age 18-38 yrs) in a similar study in Kano, Nigeria (Nwokedi et al., 2007). The mean baseline CD4 count of 320.03 cells/ μ l observed here was slightly higher than mean baseline count (302 cells/ μ l) for 500 males and females HIV-positive patients reported by Nwokedi et al. (2007) and clearly higher than corresponding value of 255 cells/ μ l for 37 male and female HIV-patients studied by Mirabeau et al. (2005). The latter reported mean follow-up of 284 cells/ μ l at week 12 (3 months) for the same HIV-patients; Gautam et al. (2008) also recorded 278 cells/ μ l as the mean CD4 count after 3 months on HAART for 43 drug-naïve AIDS patients in new Delhi, India. These 2 mean values were both lower than the follow-up CD4 count of 620.92 cells/ μ l observed in this study.

We observed that both the women who responded “yes” and “no” to having diabetes/hepatitis had mean follow-up CD4 counts that were significantly ($P = 0.001$) higher than their respective mean baseline values, this reflected the two groups equally benefited from the therapy. Their statistically comparable ($P = 0.62$) mean follow-up CD4 counts apparently pointed to no observable effect of the clinical conditions on the CD4⁺ T cell response of the HIV-positive women after 3 months on HAART. This was in support of some previous studies that diabetes and hepatitis had no impact on HAART effectiveness (Omeland et al., 2008; Law et al., 2004; Konopnicki et al., 2005; Gallant, 2001).

We concluded that the HAART was more immunologically beneficial over the 3-month period to the HIV-positive women with pre-therapeutic CD4 count of \leq 349 cells/ μ l than those with \geq 350 cells/ μ l. And that, in all, 75% of the women had short-term therapeutic benefit as shown by their appreciable CD4 count increase. But rather than the pre-therapeutic CD4 count of \geq 350 cells/ μ l or having diabetes/hepatitis, it was “non-adherence” to HAART regimen that apparently accounted for lack of therapeutic benefit among the 10 women who had < 200 (AIDS-defining) and 200-349 CD4 cells/ μ l, as well as, for those in the \geq 350 cells/ μ l group with decline or no-change in CD4 count after 3 months on HAART. The benefit of the therapy or lack thereof to those with \geq 350 cells/ μ l at baseline needs to be re-assessed among larger number of HIV-positive women initiating 3TC, d4T and NVP. Adherence to ART regimen needs to be continually

emphasized to intending users of HAART to increase the chance of benefiting from the therapy and to forestall possible emergence of ARV-resistant strains of HIV.

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Effect of Carbon Sources on The Extracellular Lignocellulolytic Enzymetic System of *Pleurotus Sajor-Caju*

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Abstract

The edible fungus *Pleurotus sajor-caju* was investigated for its ability to grow on different carbon sources and to produce various ligninolytic and cellulolytic enzymes such as laccase, lignin peroxidase, manganese peroxidase, xylanase and cellulase. The production pattern of these extracellular enzymes was studied during the growth of this fungus in shake cultures for a period of 20 days. The presence of complex polysaccharides containing substrates such as olive mill wastewater (OMW) and wheat straw in the growth medium were found to be favorable for the production of the above extracellular enzymes. Simple carbon sources were rapidly utilized and consumed by the fungus which lead to high biomass formation with low levels of enzymes activity. On the other hand, complex carbon sources exhibited a similar growth rates and a better induction process for more enzyme biosynthesis and production. The maximum enzymatic activities were obtained between 2 and 14 days of culture growth.

المخلص

تم دراسة امكانية انماء الفطر الصالح للاكل من نوع *Pleurotus sajor-caju* على انواع مختلفة من المصادر الكربونية لانتاج انزيمات متخصصة بتحطيم مادتي السيليلوز واللقتين. كما دراسة عملية انتاج هذه الانزيمات اثناء نمو الفطر في اوساط مخفوقة بشكل متواصل لمدة 20 يوم. اظهرت النتائج ان الاوساط المحتوية على سكريات معقدة مثل المياة العادمة للزيتون ودقيق قشر القمح كانت الافضل لانتاج الانزيمات المختلفة. ان استخدام السكريات البسيطة ادى الى استهلاكها السريع من قبل الفطر الذي ادى بالنهاية لانتاج كمية اكبر من الكتلة الحيوية و كميات اقل من الانزيمات. بالمقابل ان استخدام المواد الكربونية المعقدة اظهر نموا مشابها وعملية تحفيز افضل لانتاج الانزيمات. ان اعلى فعالية للانزيمات كانت قد سجلت اثناء الفترة ما بين 2-14 يوما من نمو الفطر.

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Keywords: *P. sajor-caju*, lignocellulolytic enzymes, carbon source, shake culture.

1. Introduction

A large fraction of the carbon fixed by photosynthesis each year is deposited as lignocellulose that forms the structural frame work of higher plants and would therefore, forms the renewable resource since in a balanced system the biomass removed for substrate usage can be replaced by replanting and reforestation (Batt, 1991). As population increases, traditional agriculture will be hard-pressed to meet the demand for food and more efficient use of all the carbohydrates available are required (Mitchell *et al.*, 2000). With the currently available agricultural waste materials there would be sufficient biomass as feedstock to processes for fuel and food production in biotechnological processes. Biodegradation of these wastes into useful products is an example of biotechnological process that not only uses lignocellulosics

as energy feed stock but is also associated in pollution abatement (Wan Mohtar *et al.*, 2003).

lignocellulosic materials are degraded by a few number of microorganisms. White rot fungi are considered to be the most promising group of microorganisms that degrade this complex structure (Lechner and Paptinutti, 2006). *Pleurotus sajor caju* has been extensively studied because of its powerful ligninolytic enzymes production (Massadeh and Modallal, 2008; Hameed *et al.* 2005). Generally white rot fungi are so important because they produce extracellular polyphenol oxidases particularly lignin peroxidases, manganese peroxidases and laccases which are highly effective in degrading lignin (Revankar and Lele, 2006). Studies demonstrate that under certain conditions laccase and manganese peroxidases are able to oxidize both the phenolic and non-phenolic substrates (Cabaleiro *et al.*, 2006). These enzymes are synthesized during primary and secondary metabolism in response to nitrogen, carbon or sulphur limitation (Cabaleiro and Couto, 2007). Several environmental conditions, such as high oxygen tension, culture age and medium composition,

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were also reported to affect the profiles of its isoenzymes and the level of its activity (Dosoretz *et al.*, 2007). In the present study, *P. sajor-caju* has been selected to be used as the source for lignin-degrading enzymes and the fermentation behavior for the enzyme production by this fungus was examined. This paper describes our findings on the influence of various carbon sources on the lignin degrading enzymes production by *P. sajor-caju*.

2. Materials and Methods

2.1. Maintenance of *P. sajor-caju* Culture

A subculture of *P. sajor-caju* was obtained from Plant Pathology and Mycology Research Laboratory at the Faculty of Agriculture/Jordan University of Science and Technology/ Jordan. The fungus was maintained on potato dextrose agar (PDA) Petri plates (9 cm in diameter) and stored at 4 °C till use.

2.2. Medium composition and culture conditions

Shake flask cultures of *P. sajor-caju* were performed at room temperature 28 °C (± 2 °C) with continuous agitation at 150 rpm in 250 ml Erlenmeyer flask containing 50 ml medium. The medium employed for fungal growth and metabolism consisted of (g l⁻¹): (NH₄)₂SO₄, 1.4; KH₂PO₄, 2; CaCl₂, 0.3; MgSO₄, 0.3; FeSO₄.7H₂O, 0.005; MnSO₄.7H₂O, 0.0016; ZnSO₄.7H₂O, 0.0014; CoCl₂, 0.002; protease peptone, 0.75 and Tween 80, 1; with a final pH of 5.5 (Sternberg, 1976). The carbon sources listed in Table 1 were used at a final concentration of 1% (w/v) except for the olive mill wastewater (OMW) that was used at a final concentration of 5% v/v. Olive mill Wastewater used in this study was collected from a three-phase centrifugal olive mill located around Al-Hashimiyah area (Zarqa, Jordan). Wheat straw was chopped and then milled to 1-2 mm particle size. The flasks were then inoculated by a mycelium plug (radius of 10 mm and 2 mm in thickness) cut at the advancing edge of *P. sajor-caju* grown on the solid cultures media. Samples from duplicate flasks were taken periodically, centrifuged at 8000 × g for 20 min at 4°C. The clear supernatant was used for determination of enzyme activity.

2.3. Analytical methods

Laccase activity was determined at pH 5 by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm for 2 min. 100 µl sample was added to 890 µl of a solution containing 50 mM sodium malonate and 1 mM 2,6-DMP (pH 4.5) in a 1 ml cuvette. The enzyme activity was expressed as units/ml, where 1 unit was defined as 1 mmol of substrate oxidized per minute (Munoz *et al.*, 1997). Lignin peroxidase (LiP) activity was assayed using a method described by Tien and Kirk (1984). The assay mixture contained 2 mM veratryl alcohol, 0.4 Mm H₂O₂ in 50 mM sodium tartrate buffer (pH 6.8) and 0.2 ml of filtered supernatant. Veratryl alcohol oxidation was followed at 310 nm. Manganese peroxidase activity (MnP) was determined according to Giardina *et al.* (2000). The assay mixture contained 0.5 mM MnSO₄, 0.5 mM H₂O₂ in 50mM sodium malonate buffer (pH 4.5) and 0.2 ml of crude enzyme preparation. All enzyme activities were expressed in Unit/L. The activity of the extracellular

cellulase (endoglucanase) was assayed as described by Mandels *et al.* (1976). Xylanase enzyme activity was determined according to the method of Miller *et al.* (1959). The Fungal growth was determined by measuring the dry weight content of mycelia collected at the end of each experiment after media filtration. Dissolved protein concentration was estimated by using Lowry method (Lowry *et al.* 1951).

3. Results and Discussion

Table 1 shows the effect of various carbon sources on the growth and the production of laccase, LiP, and MnP. Based on their structure, the carbon sources were divided into five groups. The fungus grew rapidly in the medium containing monosaccharides, disaccharides, polysaccharides and complex polysaccharides. Nevertheless, low or no growth was observed in the culture supplemented with phenolic compounds which subsequently resulted in lower enzyme production. Stoilova *et al.* (2006) reported that, phenolic groups lowered the growth of different types of microorganisms. Nevertheless, some phenolic compounds stimulated the production of lignin degrading enzymes.

Table 1: Lignin peroxidase (LiP), Manganese peroxidase (MnP), and Laccase enzymes production by *P. sajor-caju* when grown on various carbon sources.

Carbon sources (1% w/v)	Growth (g/l)	LiP (U/l)	MnP (U/l)	Laccase (U/l)
Monosaccharides				
Glucose	4.1	65	81	ND*
Xylose	3.7	23	29	ND
Disaccharides				
Lactose	2.8	ND	ND	ND
Sucrose	3.1	22	11	ND
Maltose	3.4	26	18	ND
Polysaccharides				
α-cellulose	2.4	31	28	10
Carboxymethyl cellulose	2.5	33	31	18
Soluble starch	2.5	12	21	7
Xylan	2.6	10	18	ND
Lignin indulin	3.1	160	155	120
Complex Polysaccharides				
Wheat straw	4.1	145	158	112
OMW**	3.9	165	170	131
Phenolic compounds				
Phenol	1.1	12	9	21
2,6 Dimethoxyphenol	1.3	20	9	24
Dichlorophenol	ND	ND	ND	20
Dinitrophenol	0.8	8	ND	10
Syringic acid	1.4	22	18	31

* not detected. ** OMW was used at a percentage of 5%.

After 10 days of incubation, the growth of *P. sajor-caju* was similar when utilizing monosaccharides, disaccharides and polysaccharides as a carbon source. However, better growth was observed in the medium supplemented with complex polysaccharides such as OMW or wheat straw. Eventhough fungal growth was satisfactory, monosaccharides and disaccharides showed no significant effect on the biosynthesis of enzymes. The rate of utilization of complex polysaccharides by the fungus was constant, eliminating the feedback inhibition by the products or other intermediates on the fungal growth. The growth and the activity of LiP and MnP were almost similar using either wheat straw or OMW as substrates. However, the production of laccase enzyme was found to be higher in a culture containing OMW than in culture containing wheat straw as a carbon source. Massadeh and Modallal (2008) claimed that OMW was a suitable substrate for the growth of *P.sajor-caju* and its contents induced laccase enzyme production which in turn reduced phenolic compounds present in the wastewater. Nevertheless, it was observed that LiP and MnP were produced by *P. sajor-caju* in the growth medium containing glucose although lignin was not present in the culture medium. However, in the medium containing lignin, significant amounts of LiP, MnP and laccase were produced. The existence of a liginolytic enzyme system that is synthesized irrespective of the presence of lignin or lingo-cellulose suggests that the liginolytic system of *P. sajor-caju* may be relatively non-specific.

The time course profile of LiP, MnP, and laccase production was examined in the culture medium containing glucose, lignin, and OMW as substrates (Figure 1). As indicated in this figure, LiP was produced significantly during the first stages of fungal growth on whatever substrate. LiP activity was detected initially on the second day of incubation and increased rapidly to reach a maximum activity after 4 days of incubation. MnP was detected from the first day of incubation and increased slowly, achieving maximum production after 7 days of incubation. At this stage, the production of LiP was declining while laccase enzyme production started a bit later after 7 days of incubation. The maximum laccase activity was observed in all cultures after 12 days of incubation except for glucose supplemented cultures where it was not detected. This result is in agreement with the findings of Tsioulpas *et al.* (2002) and Massadeh and Modallal (2008) where they reported late production of laccase enzyme by *Pleurotus* sp. This result is further supported by the data of fungal growth where it was observed that the *P. sajor-caju* entered stationary phase after 13 days of incubation (except for glucose supplemented cultures) which coincided with the maximum activity of laccase enzyme. These results suggest that the production of these enzymes was necessary for fungal growth survival along its growth phases. When glucose was used as the carbon source, it allowed higher growth rate compared to other substrates, but it ceased early (after 8 days of incubation). Laccase enzyme was not detected in this culture indicating that *P. sajor-caju* did not need this enzyme for its survival.

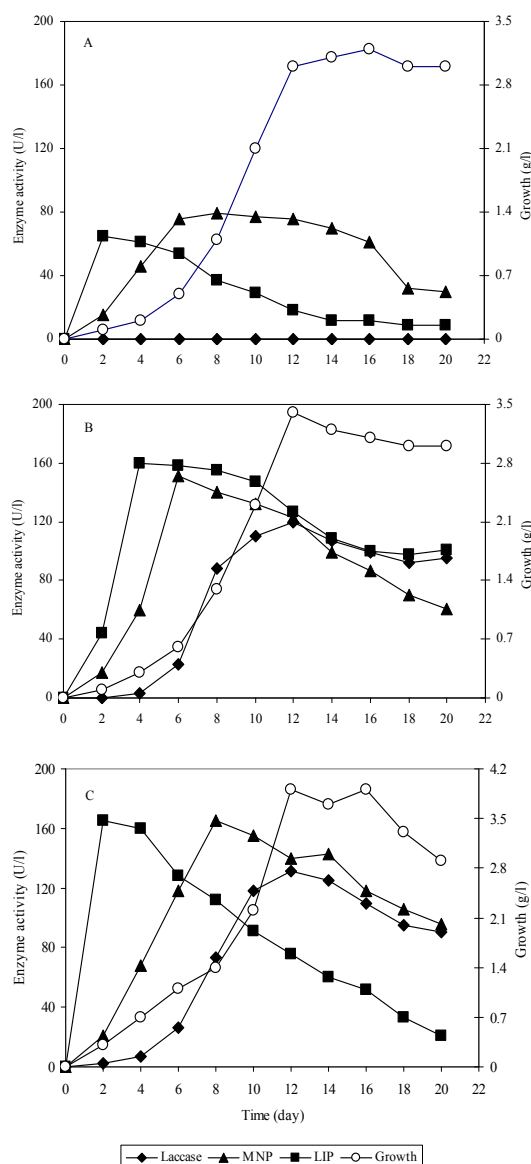


Figure 1: Extracellular ligninolytic enzymes production during the growth of *P. sajor-caju* on (A) glucose, (B) Lignin, and (C) OMW.

The cultures of *P. sajor-caju* grown on wheat straw and OMW as substrates were further analyzed for other extracellular enzymes (Table 2). Cellulase and xylanase enzymes were detected significantly in both cultures employing wheat straw or OMW as substrates which could be due to the high content of cellulosic and hemicellulosic material present in these substrates. Thus, the use of lignin indulin as the substrate was only necessary to enhance the production of LiP, MnP, and laccase while cellulase and xylanase were not detected. These results indicated that the lignin-degrading enzymes are inducible enzymes, and their production is dependent on the varying concentration of the accompanying materials within the lignocellulosic substrates. Therefore, it was clearly shown that the activity of the lignocellulolytic enzymes varied significantly depending upon the proportion of lignocellulosic materials present in wheat straw, OMW, and lignin indulin.

In conclusion, the production of lignin-degrading enzymes by *P. sajor-caju* depends upon the available substrate (inducer) in the production medium, although it is shown that there were no substrate specificities for enzyme production except the presence of the right inducible carbon source. Complex lignocellulosic substrates such as wheat straw and OMW exhibited similar growth rate, which subsequently resulted in a high production of extracellular enzymes.

Table 2: Effect of complex carbon sources on cellulase and xylanase enzymes production by *P. sajor-caju*.

Carbon source	Dissolved protein (mg/ml)	Xylanase (U/ml)	Cellulase (U/ml)
Lignin	0.6	0.1	ND*
Xylan	0.8	9.1	0.1
α -Cellulose	1.1	2.3	2.7
Wheat Straw	1.3	9.3	1.8
OMW	1.1	9.8	2.2

* not detected.

Acknowledgments

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New record of the land snail *Allopeas gracilis* (Hutton, 1834) (Gastropoda: Subulinidae) from Basrah area, Iraq

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Abstract

Allopeas gracilis is recorded for the first time in southern Iraq. It was collected from two localities (Abu-Al Khaseeb region and Hareer region) from Basrah city.

الخلاصة

ثمان وعشرون عينة من القوقع الارضي *Allopeas gracilis* تم جمعها من منطقة ابو الخصب ومنطقة حرير، مدينة البصرة خلال شهر نيسان 2008 واذار 2008 على التوالي. يعتبر هذا التسجيل الثالث من القواقع الارضية من مدينة البصرة بعد *Monacha obstructa* (Pfeiffer, 1842) و *Xeropicta mesopotamica* (Mousson, 1874). ويعتبر النوع *Allopeas gracilis* من الانواع الواسعة الانتشار في شبه الجزيرة العربية.

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Keywords: *Allopeas Gracilis*; Abu-Alkhaseeb; Hareer Region, Basrah City.

1. Introduction

Little is known about the land snails of Iraq. Most of our present knowledge is based on old literature (Pallary, 1939; Germain, 1921; Biggs, 1959; Najim, 1959). As far as southern Iraq is concerned, very limited studies addressed the land snails of this area, however, recent studies recorded additional two species to Basrah area (Abdul-Sahib 2005; Al-Khafaji, 2009). Neubert (1998) presented an outstanding monograph on the freshwater and land snails of the Arabian Peninsula, where 70 species of land snails recorded.

Allopeas gracilis is a neotropical species with a wide distribution in the Indopacific area. This species was perhaps introduced to the Arabian Peninsula through human activities and now known from different parts of Arabian Peninsula including Saudi Arabia, Yemen and Oman (Neubert, 1998).

The present study reports *Allopeas gracilis* from Basrah area for the first time, and adds a new record for the land snails of Iraq.

2. Materials and methods

Twenty eight specimens of the land snail *Allopeas gracilis* were collected from Hareer region (30°34'43.52"N

47°44'3.93"E) and Abu-Al Khaseeb region (30°28'17.51"N 47°53'39.59"E) during March 2008 and April 2008, respectively. Specimens were deposited in the Senckenberg Museum, Frankfurt am Main, Germany and (Naturhistorisches Museum der Burgergemeinde Bern, Switzerland). Measurements of shells, height, width, aperture height and aperture width.

3. Results and Discussion

3.1. Shell description

Conical elongated slender opaque shell (Fig. 1). The shell consists of 6 whorls, maximum length 7.7 mm and 3.0 mm width. The protoconch is dome-shaped and smooth within the first whorl, but sutural crenulation starts with the second protoconch whorl. The teleconch whorls are evenly rounded with a deep suture which is crenulated by minute papillae. The surface of the whorls is covered by fine and dense axial striae which are curved suprasuturally. The aperture is oval and lacks any dentition. The columella is straight and somewhat thickened. The umbilicus is closed.

3.2. Measurements (n=8)

Shell height (X=7.17, SD=0.426), shell width (X=2.78, SD=0.164), aperture height (X=2.11, SD=0.339), aperture width (X=1.037, SD=0.176)

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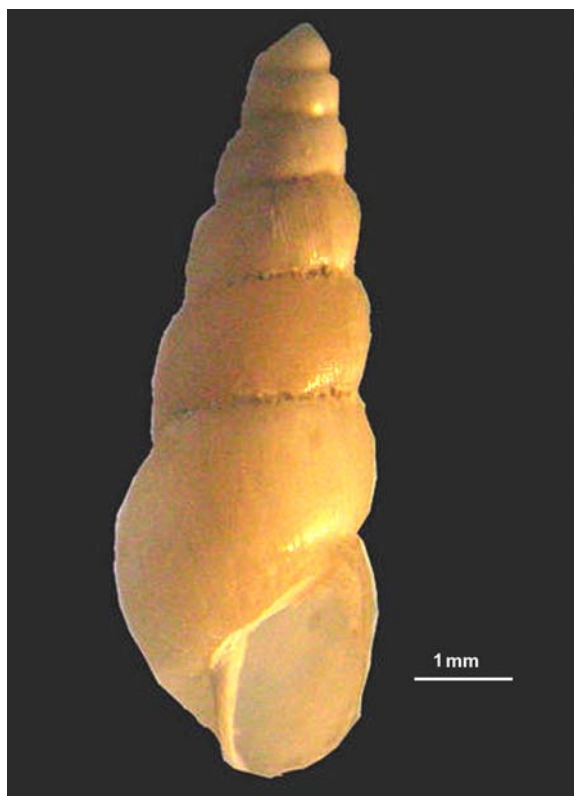


Fig. 1 *Allopeas gracilis*

4. Distribution and habitats

Allopeas gracilis is the third new record of the land snails in Basrah, after *Monacha abstructa* (Abdul-Sahib, 2005) and *Xeropicta mesopotamica* (Al-Khafaji, 2009). *Allopeas gracilis* is collected from Basrah city, Abu-AlKhaseeb region and Hareer region both regions are rural which lie at the Shatt Al- Arab river, agricultural nature, many different types of vegetables are grown there in wide distance areas, irrigated by the water of Shatt Al- Arab.

Both living and Shells of *Allopeas gracilis* were collected directly on the soil of the farms and the grasses herbous plants, closely to the Shatt Al- Arab . In the Hareer region *Allopeas gracilis* is associated with the landsnail *Xeropicta mesopotamica* . Neubert (1998) listed this species from Saudi Arabia , Yemen and Oman from different habitats.

5. Conclusion

The finding of *A. gracilis* is not surprising to our region since the species is widely distributed in the Arabian Peninsula, and due to the fact that this species can easily distributed by human activities. One more reason, the climate in the Arabian Peninsula is similar to southern Iraq, where as humidity dominates Basrah area. The present record adds to the land snail fauna of Iraq.

Acknowledgements

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Molecular cloning and Characterization of a membrane-intrinsic (S)-2,3-di-O-digeranylgeranyl glyceryl phosphate synthase involved in the biosynthesis of archaeal ether-linked membrane lipids.

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Abstract

The second step in the biosynthesis of the core membrane diether lipids in archaea is synthesis of digeranylgeranyl glyceryl phosphate from geranylgeranyl glyceryl phosphate and geranylgeranyl pyrophosphate. The reaction is catalyzed by (S)-2,3-di-O-geranylgeranyl glyceryl phosphate synthase (DGGGP synthase). The gene encoding the DGGGP synthase was cloned from *Methanocaldococcus jannaschii* (MJ 0279) and expressed in the cells of *Escherichia coli* C41 (DE3). The membrane protein was then solubilized by 2% n-Octyl- β -D-glucopyranoside and purified to homogeneity by a combination of heat treatment, DEAE-Sepharose, Resource Q and Hydroxyapatite column chromatography. The native polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis of purified DGGGPS gave a single band at 30 kDa. The optimum temperature and pH of the purified enzyme was 70°C and pH 6.0, respectively. The enzyme requires Mg^{2+} for optimal activity, while EDTA inhibits the activity. Other characteristics, including substrate specificity, salt effects and detergents effects were determined.

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Keywords: Archaea, isoprenoid, 2,3-di-O-geranylgeranyl glyceryl phosphate synthase, *Methanocaldococcus jannaschii*, *Escherichia coli* C41 (DE3).

1. Introduction

The core structures of membrane lipids of archaea have some unique properties that permit archaea to be distinguished from the others, i.e., bacteria and eukaryotes. The structures of archaeal membrane lipids were analyzed and determined in methanogens, halophiles, and thermophiles (Gambacorta et al, 1995). Investigation of the unique archaeal membrane-lipid biosynthesis may provide a clue to the early evolution of life. Koga et al, (1998) have reported that sn-glycerol-1-phosphate dehydrogenase, which determines the enantiomeric specificity of ether lipids, does not show similarity to the eubacterial enzyme, sn-glycerol-3-phosphate dehydrogenase, which is responsible for enantiomeric ester lipid synthesis. They suggested that G-1-P dehydrogenase and G-3-P dehydrogenase originated from different ancestral enzymes and this difference is responsible for the

divergent evolution of archaea and bacteria (Koga et al, 1998). The structures of membrane lipids have some interesting and remarkable properties and the archaeal diether membrane lipids are homologues of glycerolipids in other organisms but they differ. The hydrocarbon moieties of the archaeal lipids are fully reduced C_{20} or C_{25} prenyl groups, whereas the ordinary glycerolipids contain linear acyl groups. The alkyl groups are attached to glycerol via an ether bond in archaeal lipids, while glycerol and the acyl chains are ester-bonded in the bacterial and eukaryotic glycerolipids. The complete polar lipid composition of *Thermoplasma acidophilum* HO-62 was determined by Shimada et al, (2001). Kon et al, (2002) also described the ether biosynthesis pathway in the thermoacidophilic archaeon. Recently the biosynthetic pathway of ether lipids in archaea has been analyzed and postulated by several researchers (Koga and Morii, 2006; Koga and Morii, 2005; Nishihara and Koga 1995). Ether bond formation proceeds in two steps (Fig. 1): geranylgeranyl glyceryl phosphate (GGGP) synthase (GGGPS) catalyzes the reaction between glycerol-1-phosphate (G-1-P) and geranylgeranyl pyrophosphate (GGPP) forming GGGP (Chen et al, 1993;

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after electrophoresis, digested with *Nde*I and *Bam* HI and then ligated into the *Nde*I-*Bam*HI sites of the pET-21c.

2.4. Expression and Purification of recombinant enzyme

Escherichia coli C41 (DE3) was transformed with plasmid and cultivated in 1 liter of M9YG broth supplemented with ampicillin. When the A_{660} of the culture reached 0.6, 1 mM IPTG was added to the culture media for gene expression. After an additional 5h cultivation, the cells were harvested and disrupted by sonication. The homogenate was centrifuged at 20,000Xg for 15 min and precipitate was collected. n-Octyl- β -D-glucopyranoside at a final concentration of 2% was added to the precipitate to solubilize the recombinant protein. The homogenate was centrifuged again at 20,000Xg for 15 min, and the supernatant was recovered as a crude extract. The supernatant after heat treatment at 70°C for 20 min was applied to a DEAE-sepharose column (diameter 1.5, height 5cm) which was equilibrated with 20 mM Hepes buffer pH 7.0 containing 1% n-Octyl- β -D-glucopyranoside. The enzyme was eluted with stepwise gradient 300 mM NaCl concentration. The active fraction, eluted at 0.3 M NaCl, was dialyzed against 20 mM Hepes buffer pH 7.0 and loaded onto a Resource Q column (Amersham-Pharmacia Biotech) equilibrated with same buffer. The active fractions were eluted with a 0-0.3M NaCl gradient. The purification process was repeated with a Resource column at pH 8.0. The active fractions were eluted with a 0-0.2M NaCl gradient. Finally the active fraction from Resource Q column was dialyzed against 5mM sodium phosphate buffer (pH 6.8) and loaded onto a hydroxyapatite column (BIO-RAD). The adsorbed proteins were eluted with a 5-500 mM linear gradient of sodium phosphate buffer (pH 6.8) at a flow rate of 1 ml/min. The DGGGP protein, eluted with 200 mM sodium phosphate buffer was collected and stored at 4°C. Protein concentration was determined with a BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Protein fractions were analyzed by SDS-PAGE. Proteins bands were stained with Coomassie Brilliant Blue R-250.

2.5. Enzyme assay and product analysis.

The reaction mixture 100 μ l containing 100 mM Hepes buffer, pH 7.0, 5 mM $MgCl_2$, 0.2 mM G-1, 3-P, 0.14 μ M radio labeled GGPP and 1 μ g GGGP synthase was incubated at 55°C for 20 min to produce radio labeled GGPP, which is the substrate for the next reaction. Then 1mM unlabeled GGPP and 1 μ g DGGGP synthase was added and incubated at 55°C for 10 min. The DGGGP formed was incubated with alkaline phosphatase (500 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$) at 37°C for 1h. The labeled products were extracted with 300 μ l methanol, 150 μ l chloroform, and 150 μ l pentane. The lower phase was collected and dried under a stream of nitrogen gas. The residue was dissolved in a small amount of chloroform-methanol (2:1, v/v) and analyzed by reversed-phase TLC using a percolated plate, LKC-18F, developed with acetone/H₂O (9/1). Radioactive spots of the products of the TLC plate were detected by autoradiography by X-ray film with Enhance (NEN life Science Products, Boston, Mass.). Each spot was scraped off the plate, the contents were extracted and the activity was estimated on a liquid

scintillation counter LSC-1000 (Aloka, Tokyo, Japan) with scintillation cocktail Scintisol AL-1 (Dojindo, Kumamoto, Japan).

2.6. Effect of temperature and pH.

The effect of temperature on DGGGP synthase activity of the purified enzyme was measured at different temperature 40, 50, 60, 70, 80, 90 and 100°C under optimal reaction conditions in 100 mM hepes buffer pH 7.0, 5 mM $MgCl_2$, 2 mM G 1, 3-P, 1 mM unlabeled GGPP and 0.14 μ M level GGPP at 55°C. DGGGP synthase of the purified enzyme was measured at different pH 4.0-9.0 under the same optimal reaction condition.

2.7. Effect of metal ions and detergents

The effects of various metal ions and detergents were tested on the activity of purified enzyme at reaction conditions in 100 mM Hepes buffer pH 7.0, 5 mM $MgCl_2$, 2 mM G 1, 3-P, 1 mM unlabeled GGPP and 0.14 μ M level GGPP at 55°C. The enzyme was incubated with 100 mM of metal ions, such as Na^+ , K^+ and NH_4^+ and 5 mM metal ions Mg^{++} , Ca^{++} , Mn^{++} and Zn^{++} , for 20 min under standard conditions. Activity was measured as described above. The relative enzyme activity was calculated.

3. Results and Discussion

3.1. Cloning, Expression and purification of DGGGP synthase

DDGGP synthase gene from *S. solfataries* (Morii et al, 2000) was used as a key sequence and DNA data bases were searched. We found candidate gene MJ 0276 as DGGGP Synthase in *Methanocaldococcus jannaschii* gene sequence. Primers were designed for ORF MJ 0276. PCR was done using these primers and the *Methanocaldococcus jannaschii* genomic DNA as the template. A DNA fragment with the expected length about 900 bp was amplified and cloned in a pCR T7/CT-TOPO vector. The cloned gene was subcloned into high-expression vector pET-21c. Sequence analysis of the subcloned fragment confirmed the full ORF MJ 0276 in the vector and the plasmid was named pMJ 0276. The recombinant DGGGP synthase was expressed in the cells of *E. coli* C41 (DE 3) harboring the pMJ 0276 plasmid. The *E. coli* cells harboring the plasmid were grown until mid-log phase and gene expression was induced by IPTG at 37°C. Fig. 2 shows the product of the DGGGOH by DGGGP synthase in the TLC plate after induction of *Escherichia coli* extract. Poulter and Zhang (1993) showed that the activity of DGGGPS is in membrane portion. For solubilization and purification, the membrane protein requires detergents for extraction from the membrane. By adding 2% n-Octyl- β -D-glucopyranoside or N,N-Dimethyldodecyl-amine N-oxide (LDAO) to the precipitate, we succeeded in solubilizing the enzyme, which enabled us to purify the enzyme. After solubilization of the protein, the extract was heated at 70°C for 30 min to remove *Escherichia coli* proteins. We then used DEAE-Sepharose, Resource Q ion-exchange column chromatography for further purification of the enzyme. Finally hydroxyapatite column was used to purify the enzyme described details in Materials and methods section. The SDS-PAGE showed the single band of protein of molecular mass 30 kDa (Fig 3), was similar

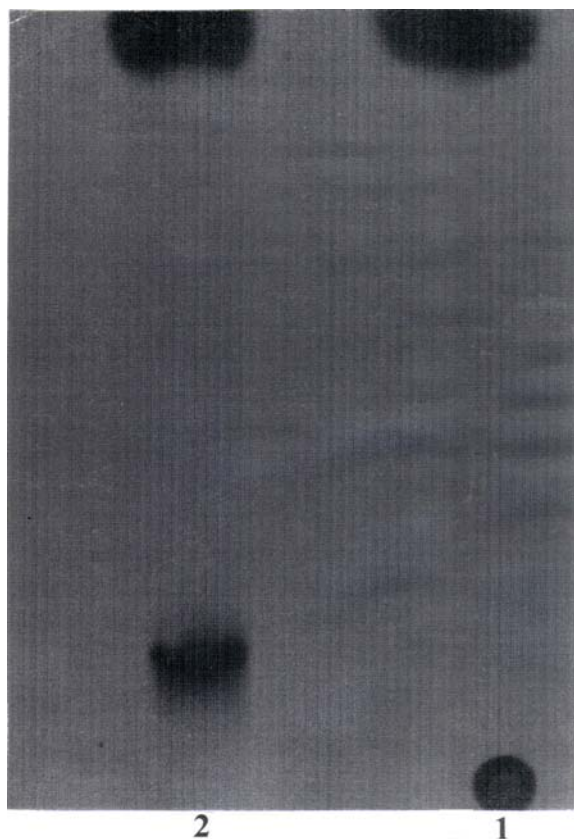


Fig. 2. TLC plate showing the product of the DGGGP synthase reaction of *E. coli* cell extract. Lane 1 represents reaction without DGGGP synthase and Lane 2 represents with DGGGP synthase.

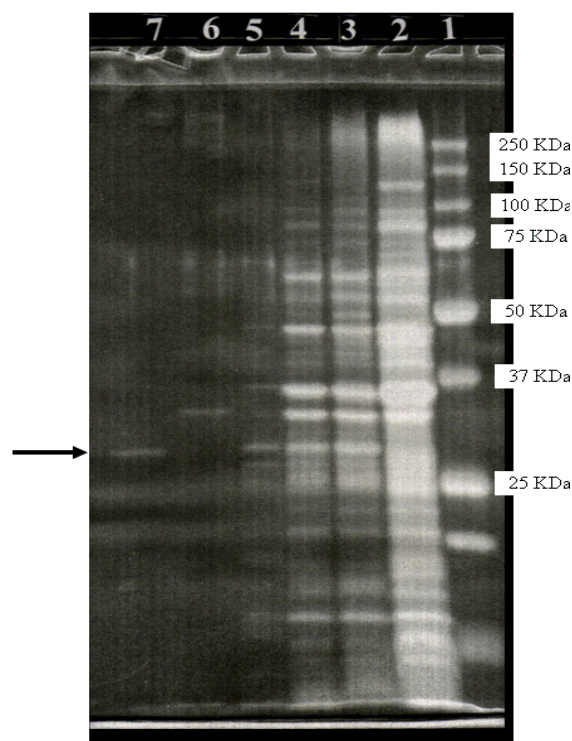


Fig. 3. SDS-PAGE analysis of samples from the purification steps of DGGGP synthase from *Methanocaldococcus jannaschii*. Lane 1, protein standard marker; 2, After sonication; 3, After heat treatment; 4, After DEAE-sepharose column; 5, After Resource Q pH 7.0; 6, After Resource Q pH 8.0; 7, After Hydroxyapatite. Arrow indicates the DGGGP synthase.

to that calculated from its amino acid sequence (31.7 kDa). The purification process is summarized in Table-1. The specific activity at the final step of purification was 75 nmol/min/mg and purification fold is 750. Recently, the DGGGP synthase has been cloned and purified from archaea *Sulfolobus solfataricus* and reported (Hemmi et al, 2004) but they did not mention the specific activities and purification yield due to instability of the activities of the enzyme.

3.2. General properties of DGGGP synthase

3.2.1. Optimum temperature and pH:

Maximal activity of DGGGP synthase was seen around 70°C (Fig. 4a) at temperatures in the normal range of *Methanocaldococcus jannaschii* growth temperature. At 90°C the DGGGP synthase lost about 80% activity and at 100°C almost no activity was detected. Purified DGGGP synthase was active over a wide range of pH between pH 5.0 to pH 7.0 and the optimum pH for the enzyme reaction was shown to be around 6.0 (Fig. 4b). The optimum pH value is similar to the optimum pH value of purified DGGGP synthase from *Sulfolobus solfataricus* reported recently (Hemmi et al, 2004).

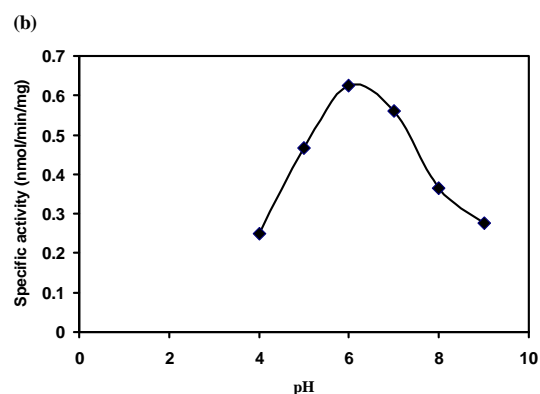


Fig. 4. (a) Temperature and (b) pH dependence of DGGGP synthase.

3.2.2. Effect of salts and metal ions:

The enzyme activity of DGGGP was significantly decreased when 5 mM Mg^{2+} was replaced with an equivalent concentration of EDTA, which indicates the requirement of a divalent metal ion for activity. The optimum concentration of Mg^{2+} was 2.5-5 mM and the enzymatic activity was slightly inhibited by higher concentrations of Mg^{2+} (Fig. 5). The metal ion could be replaced by 5 mM Ca^{2+} , although the enzyme activity fell by about 30% by Ca^{2+} , and NH_4^+ , whereas enzyme activity fell by 91.8% by 5mM Mn^{2+} and 91% by 5 mM Zn^{2+} but 100 mM Na^+ and K^+ did not affect the enzymatic activity of DGGGP synthase. The effects of different metal ions on purified DGGGP synthase were shown in Table 2. The enzyme activity of the purified DGGGP synthase was measured under optimal reaction conditions in 100 mM HEPES buffer pH 7.0, 5 mM $MgCl_2$, 2mM G 1, 3-P, 1 mM unlabel GGPP and 0.14 μ M level GGPP at 55°C. We also used sodium phosphate buffer instead of HEPES buffer and we did not see any change of the activity.

Table 1: Purification table of DGGGP synthase from *Methanocaldococcus jannaschii*

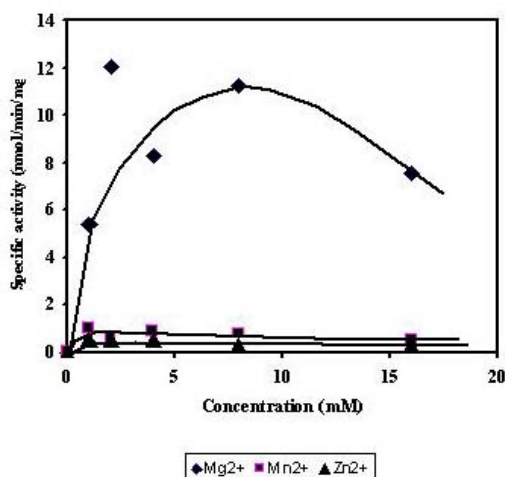
Steps	Volume (ml)	Protein (mg)	Activity (nmol./min)	Recovery (%)	Specific activity (nmol./min/mg)	Purification (fold)
Sonication	24	1000	100	100	0.100	1
Detergent	8	185	80	80	0.43	4.3
Heat treatment	7	79.4	75	75	0.94	9.4
DEAE-sepharose	7	50	64	64	1.28	12.8
Resource Q (pH 7.0)	5	2.25	47	47	20.9	20.9
Resource Q (pH 8.0)	5	0.6	40	40	66.7	667
Hydroxy-apatite	2	0.4	30	30	75	750

Table 2: Effects of metal ions on purified DGGGP synthase activity

Metal ion	Concentration (mM)	Relative activity (%)
MgCl ₂	5	100
CaCl ₂	5	71.2
MnCl ₂	5	8.2
ZnCl ₂	5	9.0
EDTA	5	7.3
NaCl	100	90.2
KCl	100	109.7
NH ₄ Cl	100	70.7

Table 3: Effects of detergents and substrate on purified DGGGP synthase activity.

Detergents or substrate	Relative activity (%)
n-Octyl-β-D-glucopyranoside 1%	100
N,N-dimethyldodecyl-amino N-oxide (LDAO) 1%	101.5
Triton X-100 1%	91.4
Chaps 1%	69.5
Nonidet P-40 1%	39.0
Deoxycholate 1%	36.7
G-1, 3-P	100
G-3-P	28.7

Fig. 5. Mg²⁺ dependence of DGGGP synthase. Reactions were carried out at 45°C

3.2.3. Effect of detergent:

To check the effect of detergents on DGGGP synthase, we used the detergents 1% Chaps, 1% Triton X-100, 1% Nonidet P-40, 1% Deoxycholate, 1% n-Octyl-β-D-glucopyranoside and 1% N, N-dimethyldodecyl-amino N-oxide (LDAO) described detailed in Material and Methods section. The DGGGP synthase activity was not changed when n-Octyl-β-D-glucopyranoside and LDAO was used. But the activity was decreased about 10% by Triton X-100, 30% by Chaps, and 61% by Nonidet P-40 was used (Table 3).

3.2.4. Substrate specificity of the enzyme

We used the substrate G-1, 3-P and GGPP to the reaction mixture for the enzymatic activity of DGGGP synthase. When the substrate G-1, 3-P was replaced with G-3-P, the enzymatic activity was decreased 71.3% (Fig. 6). The residual activity 28.7% can be attributed to the impurity of G-3-P (95% purity) and mixture of G-1, 2-P. Also when we did not use GGPP synthase enzyme in the 1st step reaction, the DGGGP synthase activity was almost completely lost. Similar result was obtained when we did not use GGPP in the reaction mixture. Thus DGGGP synthase is specific for G-1-P and GGPP. Specificity for G-1-P as a substrate has also been reported or GGPP synthases from our laboratory study (Yamagishi et al, 2003) and *M. thermotrophicum* (Chen et al, 1993) and *Halobacterium halobium* (Poulter and Zhang, 1993).

3.2.5. Molecular mass

The purified DGGGP synthase gave single peaks of absorbance at 280 nm corresponding to 29.6 kDa, on the gel filtration using a Superdex 200 HR column (GE Healthcare Bio-sciences Corp, Piscataway, NJ, USA; Fig. 7). This result indicated that the DGGGP synthase was a monomeric protein.

4. Conclusion

In summary, we have successfully purified DGGGP synthase from a cell free extract of the archaeon *Methanocaldococcus jannaschii*. The MJ 0996 gene of the

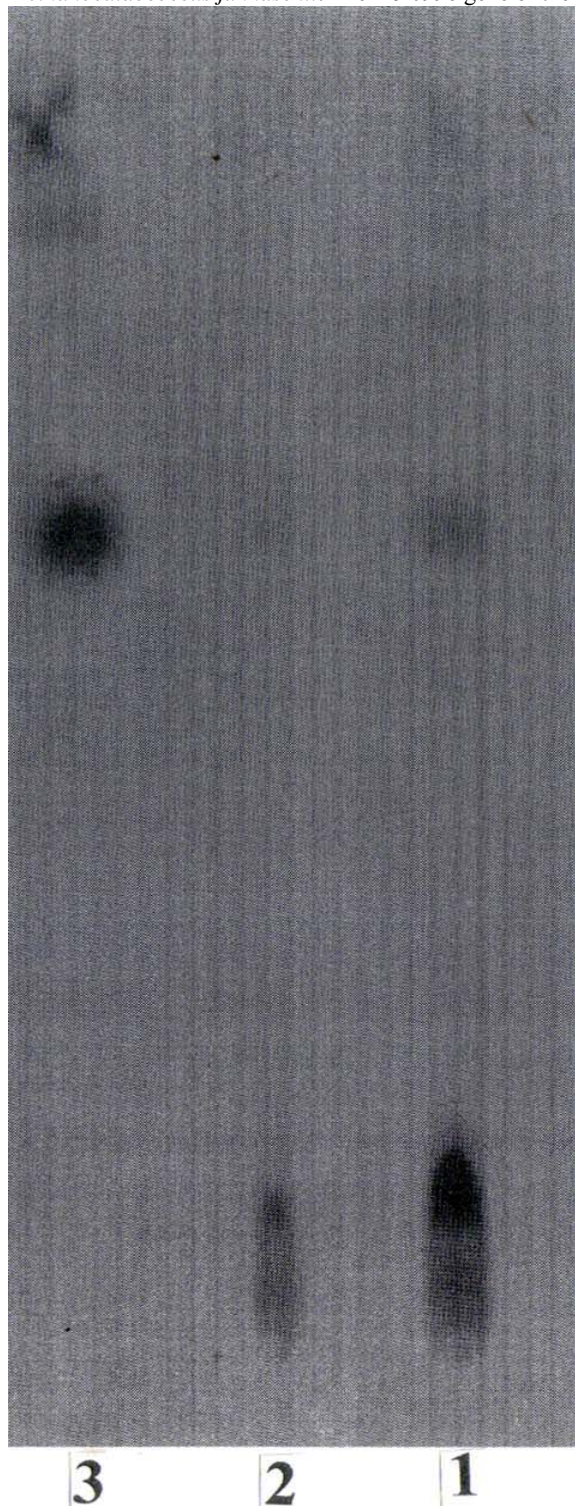


Fig. 6. TLC plate showing the product of the DGGGP synthase reaction of G-1, 3-P and G-3-P. Lane 1— G-1, 3-P; Lane 2— G-3-P; Lane 3— no glycerol phosphate.

Methanocaldococcus jannaschii genome can be functionally assigned as DGGGP synthase based on the N-terminal amino acid sequence of the purified enzyme.

Furthermore in this study, we cloned the gene encodes DGGGP synthase from genomic DNAs of *Methanocaldococcus jannaschii* and expressed in the cell of *Escherichia coli* C41 (DE3). The membrane protein DGGGP synthase after solubilized was purified by a combination of heat treatment and four chromatographic steps. The yield of the membrane protein is about 30%. So, it is possible to get enough protein by large culture using jar fermentor. It should be interesting to study the crystallize structure of the membrane protein and structure of the membrane protein DGGGP synthase and this study is underway in our laboratory.

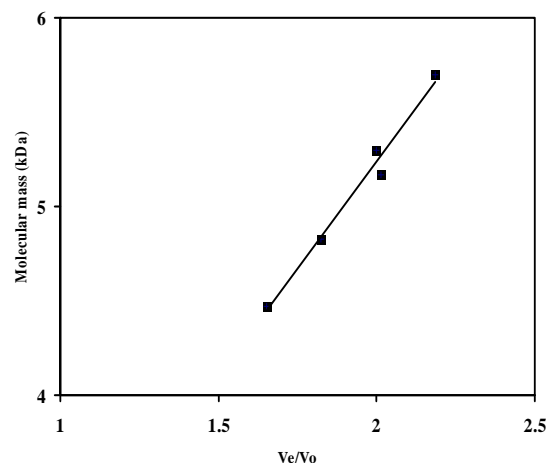


Fig. 7. Determination of the molecular mass of DGGGP synthase by gel filtration. Marker proteins were apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 Kda), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The relative retention volume of DGGGP synthase is indicated by an arrow.

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Exposure to Potassium Carbonate Emulsion Induced Nephro-Toxicity in Experimental Animals.

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Abstract

The study investigated the possible toxic effects of potassium carbonate emulsion on some biomarkers of tissue damage in rabbits. Exposure of rabbits to potassium carbonate (K_2CO_3) emulsion at 50mg/L and 100mg/L via oral drinking for 14 consecutive days caused significant increase in the creatinine and uric acid at 100mg/L by 48.6% and 126.3% respectively. Also, potassium carbonate (K_2CO_3) emulsion significantly increased serum blood urea nitrogen (BUN) at 50mg/L and 100mg/L concentration. The results however suggested that potassium carbonate emulsion exposure via oral drinking could precipitate kidney damage.

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Keywords: Potassium Carbonate Emulsion, Nephro-Toxicity, Serum Metabolites.

1. Introduction

Potassium is an essential dietary mineral and electrolyte. Normal body function depends on tight regulation of potassium concentrations both inside and outside of cells (Peterson, 1997). A limited number of enzymes require the presence of potassium for their activity. The activation of sodium, potassium-ATPase requires the presence of sodium and potassium. The presence of potassium is also required for the activity of pyruvate kinase, an important enzyme in carbohydrate metabolism (Sheng, 2000). Severe hypokalemia may result in muscular paralysis or abnormal heart rhythms (cardiac arrhythmias) that can be fatal (Sheng, 2000; Food and Nutrition Board, 2004). The richest sources of potassium are fruits and vegetables. People who eat large amounts of fruits and vegetables have a high potassium intake (8-11 grams/day) (Sheng, 2000; Food and Nutrition Board, 2004). A recent dietary survey in the U.S. indicated that the average dietary potassium intake is about 2,300 mg/day for adult women and 3,100 mg/day for adult men (Hajjar et al., 2001). The use of potent potassium supplements in potassium deficiency requires close monitoring of serum potassium concentrations. Potassium supplements are available as a number of different salts, including potassium chloride, citrate, gluconate, bicarbonate, aspartate and orotate (Hendler & Rorvik, 2001). Abnormally elevated serum potassium

concentrations are referred to as hyperkalemia. Hyperkalemia occurs when potassium intake exceeds the capacity of the kidneys to eliminate it. The most serious complication of hyperkalemia is the development of an abnormal heart rhythm (cardiac arrhythmia), which can lead to cardiac arrest (Mandal, 1997). The present study was undertaken to elucidate toxicity associated with exposure to potassium carbonate emulsion.

2. Materials and methods

Twelve California rabbits (males) weighing 1.05kg – 1.65kg were purchased from the animal house of the college of animal Science University of Agriculture, Abeokuta, Ogun State, Nigeria, housed in cages and exposed to 12hr light/ dark photoperiod cycle. The animals were left to acclimatize for two weeks prior to the administration of the emulsion. They had free access to food and water *ad libitum*.

2.1. Exposure study

The experimental animals were divided into three groups of four. Groups I and II received 50ppm and 100ppm of potassium carbonate (K_2CO_3) emulsion respectively for two weeks and group III was administered with physiological saline for the same period.

2.2. Blood Sample Collection

The animals were sacrificed by cervical dislocation after a 12hour fasting period. In all cases, blood samples

were collected by cardiac puncture into clean dry centrifuge tubes and allowed to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 minutes at 3000g using a bench centrifuge. Plasma aliquots were collected and preserved at 70°C for biochemical assays.

2.3. Clinical chemistry

Serum total protein, Albumin and potassium ion (K^+) as well as biomarkers of nephro-toxicity such as urea, creatinine and uric acid. The total protein was determined by Lowry et al (1951). Albumin concentrations were measured by the stigma bromocresol purple (BCP) (Beale and Croft, 1961). Serum K^+ was determined by flame emission spectrophotometer using UNICAL 1300. The estimation of urea was measured by the method of Coloumbe and Farreaus (Hervey, 1953). Creatinine was determined by the method of Eichhorn et al (1961). Uric acid was estimated by the method of Henry using commercial kit (Randox).

2.4. Statistical Analysis

Treated groups were compared to control group by student's t- test using Microsoft excel. All data were expressed as mean \pm S.D (n = 4). A value of $P < 0.05$ was considered to indicate a significant difference.

3. Results

The result of effects of potassium carbonate emulsion on plasma albumin, plasma protein and blood K^+ ion are presented in Table 1. Treatment with the emulsion of carbonate resulted in significant increase in serum potassium ion (K^+) and albumin.

Potassium carbonate emulsion in rabbits caused significant increase in total albumin in concentration dependent manner when compared with the control. The levels of total protein decreased but not in concentration dependent manner when compared with the control. Table 2 shows the result potassium carbonate emulsion on uric acid, creatinine and urea. The emulsion significantly increased uric acid, creatinine and urea by 126.3%, 48.6% and 458.8% respectively at highest concentration (100mg/L) when compared with the control ($P < 0.05$). As shown in table 2.0, the results of urea, creatinine and uric acid showed that oral administration of the potassium carbonate emulsion significantly increased at concentration 100mg/L by 458.8%, 48.6% and 126.3% respectively when compared with the control.

Table 1: The effects of potassium carbonate emulsion on plasma proteins and electrolyte.

Parameter	Control	50mg/L	100mg/L
Albumin	3.64 \pm 0.03	4.07 \pm 0.32* (11.8)**	4.95 \pm 0.75* (36.0)**
Total Protein	9.73 \pm 0.08	8.31 \pm 1.16 (14.6)**	8.39 \pm 0.94* (13.8)**
Blood K^+ ion	0.78 \pm 0.01	2.99 \pm 1.84* (283.3)**	2.34 \pm 2.06* (200.0)**

*Values differ significantly from control ($P < 0.05$).

**Percentage change compared with the control.

Table 2: The effects of potassium carbonate emulsion on the kidney.

	Control	50mg/L	100mg/L
Urea	0.80 \pm 0.00	2.83 \pm 0.25*(253.8)**	4.47 \pm 0.85*(458.8)**
Creatinine	0.70 \pm 0.09	0.97 \pm 0.37(38.6)**	1.04 \pm 0.16*(48.6)**
Uric acid	0.80 \pm 0.21	1.51 \pm 0.73(88.8)**	1.81 \pm 0.21*(126.3)**

* Values differ significantly from control ($P < 0.05$)

**Percentage change compared with the control.

4. Discussions

Civilization and technological advancement had created a great harm to human health through the introduction of food additives as chemical preservatives, flavours, seasonings, modification of food texture, nutritional quality or colorants (Timothy and Peckham, 1978). Similarly, there have been increasing efforts in agricultural activities particularly in preservation of farm products which is as a result of the ever-increasing human population as well as food losses to pests and disease both on field and in storage (Sheng, 2000; Food and Nutrition Board, 2004). Some of these activities and chemical

preservatives have resulted in food poisoning and unsafe for human consumption with significant public health implications while some are carcinogenic inducers e.g. potassium bromate (Hajjar et al., 2001; Hendler & Norvik, 2001; Mandal, 1997).

In the present study rabbits were orally exposed to different concentrations of potassium carbonate emulsion. Early works on the study of potassium carbonate emulsion had established that preservatives are dose-dependent. It was observed that sodium and potassium salts of benzoic acid have been found to cause no deleterious effect when used in small quantity (Morris et al., 1986; Milks, 1991). Also, potassium carbonate before meals promotes the

secretion of gastric juice but large doses neutralized the free hydrochloric acid (HCL) in the stomach and render the chyme neutral or alkaline, this interferes with the secretions from the pancreas, liver and intestines and hindered digestion (Mikkelsen, 1984). It was investigated that potassium ion causes depression of the central peripheral nervous system by depressing the reflexes and paralyses by higher concentration (Welt et al., 1960; Perkins, 1984; Weisburg, 1999). It was also noted that salt poisoning causes progressive muscular weakness, inflammation of the gut, dark colour liver, inability to stand, convulsion, excessive thirst and eventually death with haemorrhage and severe congestion in gastro intestinal (GIT) tract, liver, muscle and kidney (Gordon and Andrew, 1966; Shahr and John, 1991).

In acute or chronic renal failure, the use of potassium-sparing diuretics and insufficient aldosterone secretion (hypoaldosteronism) may result in the accumulation of excess potassium due to decreased urinary potassium excretion. This suggests that the emulsion of the carbonate might precipitate kidney damage. The significant decrease in protein synthesis as recorded in the study indicated possible damage(s) to liver however reducing protein synthesis. The rabbits treated with 100ppm of the emulsion showed symptoms such as withdrawal from food, excessive thirst, drowsiness (weakness, reduced irritability, and polyuria, an indication of potassium acute toxicity (Mandal, 1997; Liu et al., 2000).

In conclusion, this study shows that high exposure to emulsion of potassium carbonate (K_2CO_3) may precipitate nephro-toxicity and induce liver damage. This then suggests a potential risk to humans that may come in contact with this supplement since liver and kidney are the major sites of chemical and drug metabolism.

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Effect of *Salvia triloba* L. f. Extracts on Neoplastic Cell lines

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Abstract

The search for novel anticancer drugs continues. This work includes a preliminary study of the effect of two crude extracts of Greek or Mediterranean sage (*Salvia triloba* L. f.) on three malignant human cell lines and one malignant murine cell line.

A boiling water extract and a methanolic extract were prepared from dried leaves of *S. triloba*. Yields of extraction were 9.8 and 22.4%, respectively. Tested cell lines included human larynx epidermoid carcinoma (HEp-2), human rhabdomyosarcoma (RD), human glioblastoma multiforme (AMGM5) and murine mammary adenocarcinoma (AMN3).

Both extracts exhibited time-dependent, cell specific inhibitory effects on HEp-2, RD and AMN3 malignant cell lines. AMGM5 cell line was resistant to the effects of both extract as inhibition could only be recorded after 72 hrs of exposure to the highest extracts concentrations, 625 and 1250 µg/ml. In addition, growth of HEp-2, RD and AMN3 cells under treatment with either extract was biphasic during the first 48 hrs of treatment as cells were stimulated at lower concentrations and inhibited at higher concentrations.

It seems likely that extracts of *Salvia triloba* may act at various therapeutic levels, especially in cancer treatment, but further research is required to evaluate the practical values of therapeutic application.

المخلص

البحث عن أدوية مبتكرة ضد السرطان يستمر. وفي هذا البحث قمنا بدراسة للمستخلصات الخام من ميرمية البحر الأبيض المتوسط أو الميرمية اليونانية (*Salvia triloba* L.F.) على أربعة أنماط خلوية خبيثة.

جهزت مستخلصات الماء المغلي و الميثانول من الأوراق المجففة للميرمية. وقد كانت كميته الإستخلاص 9.8 و 22.4 %، على التوالي. تضمنت الأنماط الخلوية المُجرّبة أربعة أنماط:

human larynx epidermoid carcinoma (HEp-2), human rhabdomyosarcoma (RD), human glioblastoma multiforme (AMGM5) and murine mammary adenocarcinoma (AMN3). طريقه عمل كلا المستخلصين كان معتمدا على الوقت، ومعدل التأثير التثبيطي الخلوي المتخصص على الأنماط الخلوية الخبيثة HEp-2, RD, AMN3, كان واضحا. بينما النمط الخلوي AMGM5 كان مقاوما للتأثير التثبيطي الخلوي لكلا المستخلصين ولكن كان هناك نوع من التثبيط الخلوي بعد التعرض للمستخلصات لمدة 72 ساعة وبتراكيز عالية بلغت 625 و 1250 µg / ml. بالإضافة لذلك كان تكاثر الخلايا HEp-2, RD, AMN3 تحت تأثير كلا المستخلصين ثنائي الطور حيث حفزت الخلايا بالتراكيز القليلة في أول 48 ساعة وكانت مثبطة في التراكيز العالية.

يبدو أن مستخلصات نبتة الميرمية تقيد في الخاصية العلاجية الطبيعية، خصوصا في علاج السرطان، ولكن البحث المستفيض مطلوب لتحديد القيمة العملية للتطبيق العلاجي.

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Keywords: *Salvia triloba*, cytotoxicity, cell line, biphasic effect.

1. Introduction

Natural products have long been a fertile source of cure for cancer, which is projected to become the major cause of death in this century (Mukherjee *et al.*, 2001). Of these natural products, plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasoning, beverages, cosmetics, dyes and medicine. Most of this therapy

involves the use of plant extracts or their active components (Craig, 1999).

Crude plant extracts from *Dioscorea membranacea* (Thailand) (Itharat *et al.*, 2004), *Platycodon grandiflorum* A. DC. (Korea) (Lee *et al.*, 2004), and *Entanda abyssinica* (Tanzania) (Kamuhabwa *et al.*, 2000) have shown significant cytotoxic effects against malignant cell lines *in vitro*. Mediterranean Sage (*Salvia triloba* L. f.) is the most popular medicinal herb in Palestine, Jordan and Lebanon (Abu-Rmaileh & Afifi, 2000; Ali-Shtayeh *et al.*, 2000; Gali-Muhtasib & Affara, 2000). Twenty species of *Salvia* grow wild in Jordan (Oran & Al-Eisawi, 1998). The leaves of the plant are boiled and used as a herbal tea for treatment of bloating, gastric disorders, abdominal pain,

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oral infections, gum and tooth pains, headaches, cough, influenza and cold, feminine sterility, skin disorders, nervous conditions, asthma, rheumatism and diabetes (Oran & Al-Eisawi, 1998; Abu-Rmaileh & Afifi, 2000; Ali-Shtayeh *et al.*, 2000; Perry *et al.*, 2003; Salah & Jager, 2005)

The aqueous and oil extracts of sage have been shown to possess antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Kamatou *et al.*, 2007; Kamatou *et al.*, 2008; Kamatou *et al.*, 2010). *Salvia* is the largest and the most important genus of the family Lamiaceae. Plants belonging to this genus show high diversity in their secondary metabolites as well as in pharmacological effects. Several species of *salvia* are included in many pharmacopeias. They are used for alimentary, pharmacological and cosmetic purposes (Kamatou *et al.*, 2005; Perry *et al.*, 2003).

In this study, an aqueous and a methanolic extract were prepared from the dried leaves of *Salvia triloba* L. f. and tested for cytotoxicity against four types of neoplastic cell lines. Both extracts showed cytotoxic effects against Hep-2, RD and AMN3 cell lines, while AMGM5 cells showed resistance to both extracts.

2. Materials and Methods

2.1. Cell Lines

Human larynx epidermoid carcinoma (HEp-2), human rhabdomyosarcoma (RD), human cerebral glioblastoma multiforme (AMGM5) and murine mammary adenocarcinoma (AMN3) cell lines were all kindly provided by the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), Baghdad, Iraq and were maintained at 37°C.

2.2. Reagents

Cell lines were grown on RPMI-1640 medium (Sigma, USA) with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.

2.3. Plant Material

Fresh *Salvia triloba* L. f. plant was obtained from local market in Jordan. Representative specimens were taken to the Hashemite University Herbarium, the Hashemite University, Zarqa, Jordan, where they were identified as *Salvia triloba* L. f. of the family Labiatae (Lamiaceae). Plant leaves were separated and placed in the shade inside a well-ventilated room. Dried leaves were ground to a fine powder using a coffee grinder. No sieving was applied and the powder was put in air-tight polyethylene bags and placed in the freezer at -20°C till use.

2.4. Preparation of Crude Extracts

Aqueous extract was prepared by adding boiling distilled water to 50 gm of plant material. The mixture was left to cool down at room temperature. The methanolic extract was prepared using absolute methanol. Both extracts were filtered through Muslin, Whatman No. 1 filter paper and dried at 37°C. Dried powder was kept in screw-cap bottles at -20°C till use. Serial dilutions of each extract in RPMI-1640 medium were used for treating the cell lines with final concentrations of 1250 µg/ml, 625 µg/ml, 312.5 µg/ml, 156.25 µg/ml, 78.125 µg/ml and 39 µg/ml.

2.5. Cytotoxicity Assay

The protocol adopted for cytotoxicity assay using MTT (Sigma, USA) was that of Betancur-Galvis *et al.* (2002). The cells were plated in 96-well flat-bottomed plate. After adhesion, extract dilutions were added to the appropriate wells and the plates were incubated for 24, 48 or 72 hrs at 37°C, 5-10% CO₂ in a humidified environment. Untreated cells were used as controls. The supernatants were removed from the wells of the microtitration plate at the end of each exposure period. Then 28 µl of MTT (2 mg/ml) solution in phosphate buffered saline (PBS) was added to each of the wells in the microtitration plate. The plate was covered with self-adhesive film and incubated for 1.5 hrs. at 37°C. At the end of this incubation period 130 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The plates were placed on a shaker for 15 min and the absorbency was read at 492 nm (OD₄₉₂) using a multi-well spectrophotometer (Organon Teknika, Austria).

3. Results

3.1. Effect on HEp-2 Cell Line:

The aqueous extract showed a time-dependent effect on viability of HEp-2 cells. Viability decreased with time reaching its lowest after 72 hrs of treatment with all concentrations used (Fig. 1.a). The lowest percentage of cell viability resulted from treatment with 1250 µg/ml for 72 hrs and reached 34.55%. The 50% growth inhibitory concentration (GI₅₀) was 1054.6872 µg/ml after 72 hrs of treatment. The highest three concentrations of the methanolic extract, 312.5, 625, and 1250 µg/ml, showed both time- and concentration-dependent effects (Fig. 1.b). Cell viability reached as low as 34.9, 28, and 18.7% after treatment with 1250 µg/ml for 24, 48, and 72 hrs, respectively. In addition, treatment with 625, 312.5 and with 39 µg/ml for 72 hrs caused significant reduction in cell viability, which reached 31, 55.1, and 57.9%, respectively. GI₅₀ was 1095.5 and 377.625 µg/ml following 48 and 72 hrs of treatment, respectively.

3.2. Effect on RD Cell Line

Cell viability reached its lowest percentage after 72 hrs of exposure to all concentration of the aqueous extract (Fig. 1.c). Viability decreased to as low as 9.36, 22.1, 53.2, 54.7, 57.8, and 34.66% at 1250, 625, 312.5, 156.25, 78.125, and 39 µg/ml, respectively. Under treatment with the aqueous extract, growth of RD cells was found to be biphasic (nonmonotonic) (Fig. 1.c). The aqueous extract stimulated cell growth over a range of 39-625 µg/ml. In contrast to its growth-promoting effects at lower concentrations, the aqueous extract inhibited cell growth at the highest concentration, 1250 µg/ml. However, this biphasic effect diminished with time of exposure and all concentrations showed inhibitory effects on RD cells during the 72-hour exposure period. This resulted in 50% least inhibitory concentration (LC₅₀) of 63.9219 µg/ml, while GI₅₀ was 580.375 µg/ml after 48 hrs and 342.75 µg/ml after 72 hrs of exposure.

The methanolic extract stimulated growth of RD cells during the first two days of incubation over a range of 39-312.5 µg/ml. In contrast to this growth-stimulating effect

at lower concentrations, the methanolic extract inhibited cell growth at the highest two concentrations, 1250 and 625 $\mu\text{g/ml}$. However, the time-dependent effect seemed to prevail over the growth-stimulatory effect after 72 hrs of exposure, resulting in inhibition of cell growth (Fig. 1.d). LC_{50} was 48.8282 $\mu\text{g/ml}$. After 48 and 72 hrs of exposure, GI_{50} was 592.125 and 590.2813 $\mu\text{g/ml}$, respectively.

3.3. Effect on AMGM5 Cell Line

Fig. 1.e. demonstrates that significant reduction in growth of AMGM5 cells of the aqueous extract was at the highest two concentrations after the longest incubation period: After 72 hrs of treatment, the concentrations 1250 and 625 $\mu\text{g/ml}$ inhibited cell growth by 31.8 and 44%, respectively. Noteworthy, however, is that viability did not go beneath the 50% barrier under any concentration or incubation period. As a result, GI_{50} was >1250 $\mu\text{g/ml}$. Also, only the highest two concentrations of the methanolic extract, 1250 and 625 $\mu\text{g/ml}$, caused significant inhibition of growth of AMGM5 cells after 72 hrs (Fig. 1.f). However, growth percentages of AMGM5 cells even under those two concentrations were still higher than 50%. GI_{50} was >1250 $\mu\text{g/ml}$.

3.4. Effect on AMN3 Cell Line

The aqueous extract had a time-dependent effect on viability of AMN3 cells. Cell viability decreased with incubation time and significant reduction in viability was recorded on day two of exposure (Fig 1.g). The concentrations 312.5, 625 and 1250 $\mu\text{g/ml}$ caused significant reduction in cell viability after 48 hrs of incubation with AMN3 cells as cell viability reached 39, 31, and 36%, respectively. Therefore, percentages of inhibition were 61, 69, and 64%, respectively. Aqueous extract concentrations 39, 78.125, 625 and 1250 $\mu\text{g/ml}$ caused significant reduction in viability after 72 hrs of exposure by lowering cell viability to 49.3, 46.57, 19.76 and 20.88%, respectively. This reflected percentages of inhibition of 50.7, 40, 80.24 and 79.12%, respectively. The aqueous extract had a biphasic effect on growth of AMN3 cells: After 24 hrs of incubation, lower concentrations significantly increased percentage of growth, while the highest concentration, 1250 $\mu\text{g/ml}$, inhibited cell growth giving 34% inhibition. Extended treatments with the different concentrations of the aqueous extract for 48 and 72 hrs diminished the biphasic effect of the extract, and the time-dependent inhibitory effect was more profound. As a result, the lower concentrations had insignificant effect on cell growth after 48 hrs of treatment, while the higher concentrations (>156.25 $\mu\text{g/ml}$) showed significant inhibitory effects. After 72 hrs of treatment, most concentrations significantly inhibited cell growth. LC_{50} of the aqueous extract was 42.6133 $\mu\text{g/ml}$. GI_{50} of the extract was 578.125 $\mu\text{g/ml}$ after 48 hrs and became as low as 289.0625 $\mu\text{g/ml}$ after 72 hrs of treatment.

Incubation of AMN3 cells for 24 hrs with the three lowest concentrations of the methanolic extract: 39, 78.125 and 156.25 $\mu\text{g/ml}$ caused significant concentration-dependent increases in cell proliferation thus reaching 120, 150, and 167%, respectively (Fig. 1.h). However, this stimulatory effect became insignificant at the concentration 312.5 $\mu\text{g/ml}$ and even inhibitory at the highest two concentrations 625 and 1250 $\mu\text{g/ml}$ as cell

viability went down to 39 and 40%, respectively. The second and third days of treatment with lower concentrations: 39, 78.125, 156.25 and 312.5 $\mu\text{g/ml}$ had no significant effect on cell viability. However, the highest two concentrations, 625 and 1250 $\mu\text{g/ml}$, significantly decreased cell viability during the second and third day of treatment. On the second day of treatment with 625 and 1250 $\mu\text{g/ml}$, cell viability was 35 and 34% and reflecting percentages of inhibition was 65 and 66%, respectively. On the last day of treatment, viability was 54 and 17.3%, respectively, representing 46 and 82.7% inhibition. A look at Fig. 1.h. explains these figures since a clear biphasic (nonmonotonic) effect is evident. The methanolic extract of *S. triloba* had a growth-stimulating effect over the concentrations 39-312.5 $\mu\text{g/ml}$. In contrast, the higher concentrations (>312.5 $\mu\text{g/ml}$) had inhibitory activity on the growth of AMN3 cells. GI_{50} was 542.7813 and 541.6875 $\mu\text{g/ml}$ after 48 and 72 hrs of treatment, respectively.

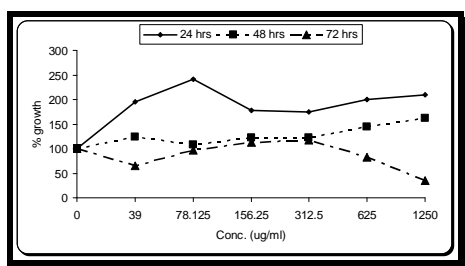
4. Discussion

4.1. Effect on Human Larynx Epidermoid Carcinoma (HEp-2) Cell Line:

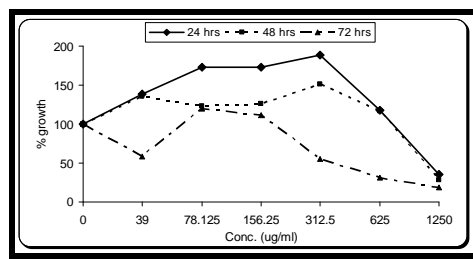
Plants contain several different families of natural products among which are compounds with weak estrogenic or antiestrogenic activity towards mammals. These compounds, termed phytoestrogens, include certain isoflavonoids, flavonoids, stilbens, and lignans (Dixon, 2004). Guo *et al.* (2004) studied the effect of daidzein, one of the most common phytoestrogens, on human nonhormone-dependent cervical cancer cells, HeLa *in vitro*. At most concentrations and incubation times, cancer cells were arrested at G_0/G_1 phase and a time-dependent manner was found. The suppressive effects of daidzein were by means of alteration in cell cycle, apoptosis, and inhibition of telomerase activity.

An increase in cell viability was recorded for HEp-2 on the first and second days of exposure to the aqueous and the methanolic extracts. However, the effect was lower on the second and became inhibitory on the third day of exposure to result in the reduction of cell viability by the end of the 72nd hour of exposure. Li and his colleagues (2002) reported that ursolic acid (UA) and oleanolic acid (OA), which are triterpene acids distributed widely in plants all over the world, had an inhibitory effect on human colon carcinoma cell line HCT15. Proliferation assays showed that proliferation of UA- and OA-treated cells slightly increased at 24 hrs and significantly decreased at 48 hrs and 60 hrs. Cell cycle analysis showed that treated cells gradually accumulated in G_0/G_1 phase, with a concomitant decrease of cell population in S period and no detectable apoptotic fraction. Therefore, it was concluded that UA and OA had significant anti-tumor activity with the possible mechanism of action of inhibiting tumor cell proliferation through cell-cycle arrest.

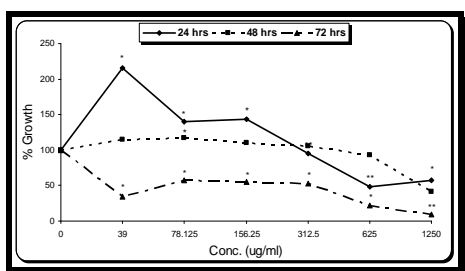
In his work on the effect of black tea polyphenols and terpenoids and green tea terpenoids, Sa'eed (2004) recorded a slight increase in density of HEp-2 cells at low concentrations on the third day of exposure, while higher concentrations decreased cell density. LeBail *et al.* (1998) showed that flavonoids at low concentrations significantly



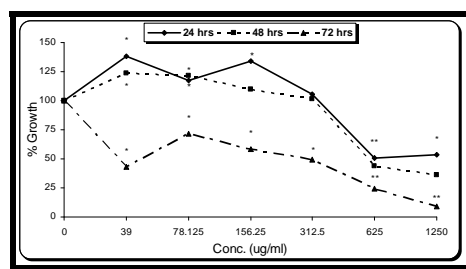
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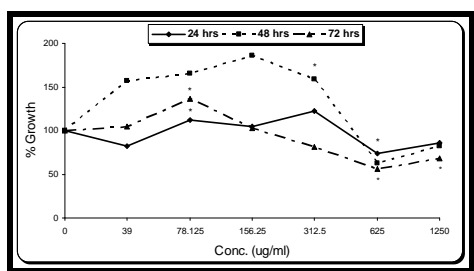
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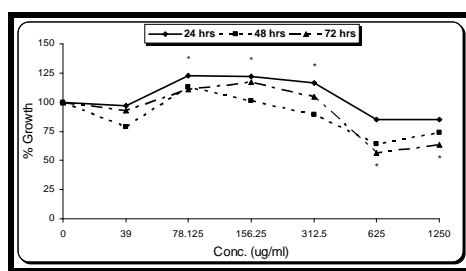
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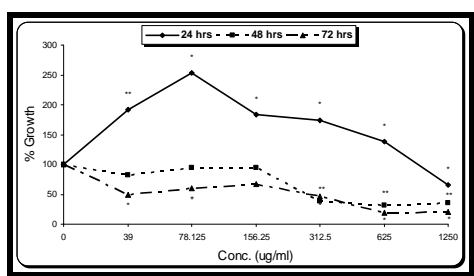
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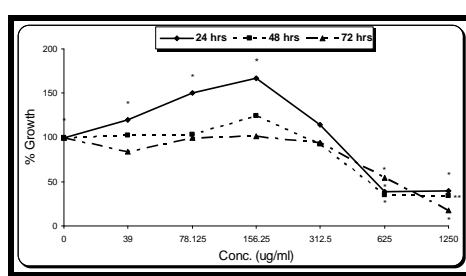
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f.



g.



h.

Figure1. Effect of *S. triloba* extracts on viability of cell lines: (a) effects of the aqueous extract on HEP-2; (b) effects of the methanolic extract on HEP-2; (c) effect of the aqueous extract on RD; (d) effect of the methanolic extract on RD; (e) effect of the aqueous extract on AMGM5; (f) effect of the methanolic extract on AMGM5; (g) effect of the aqueous extract on AMN3; (h) effect of the methanolic extract on AMN3.

enhanced the proliferation of human breast cancer cells MCF-7. The response was dose-dependent. In contrast, they reduced MCF-7 cell proliferation at high concentrations.

4.2. Effect on Rhabdomyosarcoma (RD) Cell Line:

Both the aqueous and methanolic extracts of *S. triloba* had biphasic (nonmonotonic) effects on Rhabdomyosarcoma (RD) cells *in vitro* (i.e. hormesis).

Compared with inhibition of HEP-2 cell, RD inhibition by both the aqueous and methanolic extracts of *S. triloba* was higher. This reflected the sensitivity of RD cells compared with HEP-2 cells. Al Hilli (2004) showed in his study on the effect of crude extracts of *Cyperus rotundus* L. that RD cells were more sensitive to each of the hexane, aqueous, and ethanolic extracts compared with HEP-2 cells. Highest inhibition was recorded after 72 hrs of treatment with any of the extracts. Exposure of HEP-2 and RD cells to green and black tea terpenoids and polyphenols also revealed that RD cells were more sensitive than HEP-2 cells (Sa'eed, 2004). Among HEP-2, RD-228 and Vero cells tested for long-term survival studies, RD-228 cell line was more sensitive to the total alkaloid fraction of the methanolic extracts of *Solanum pseudocapsicum* (Vijayan *et al.*, 2004).

4.3. Effect on AMGM5 Cell Line:

AMGM5 cells showed little sensitivity to the aqueous and methanolic extracts of *S. triloba* compared with HEP-2 and RD cells ($GI_{50} > 1250 \mu\text{g/ml}$). Only higher concentrations (625-1250 $\mu\text{g/ml}$) were able to exhibit inhibitory effects towards AMGM5 cells after 72 hrs of incubation, while lower concentrations caused insignificant or significant stimulatory effects.

Glioblastoma is usually rapidly fatal (Stupp *et al.*, 2005). Glioblastomas are the most common and aggressive type of malignant glioma. They are characterized by rapidly dividing cells, invasion into normal brain, and a high degree of vascularity. At present, there is no effective treatment for glioblastoma (Ouafik *et al.*, 2002). Tamoxifen has been shown to have cytotoxic activity against glioma cells at high doses. Tamoxifen caused dose-dependent growth inhibition in human glioma cell lines U87MG, U373MG and U138MG. However, a slight increase in cell growth was recorded at low concentrations of tamoxifen ($\leq 10 \mu\text{M}$) (Hui *et al.*, 2004).

The current standard of care for newly diagnosed glioblastoma is surgical resection to the extent feasible, followed by adjuvant radiotherapy (Stupp *et al.*, 2005). Oil extract of *Salvia triloba* and higher concentration may play role in inhibition of this neoplastic cell line.

4.4. Effect on AMN3 Cell Line:

Growth of AMN3 cells due to treatment with the aqueous and methanolic extracts of *S. triloba* was biphasic (nonmonotonic). However, the highest concentration, 1250 $\mu\text{g/ml}$, of both extracts showed time- and dose-dependent inhibitory effects. AMN3 cells were more sensitive to the aqueous extract than to the methanolic extract (LC_{50} of the aqueous extract was 42.6133 $\mu\text{g/ml}$ and GI_{50} of the aqueous extract was 289.0625 $\mu\text{g/ml}$ after 72 hrs, while GI_{50} of the methanolic extract was 542.6875 $\mu\text{g/ml}$ after 72 hrs of treatment). In addition, AMN3 cells were more

sensitive to crude extracts of *S. triloba* than HEP-2, RD, and AMGM5 cells.

Induction in absorbance of AMN3 cells was reported after 24 hrs of exposure to low concentrations of hexane extract of *Cyperus rotundus* L. (Al Hilli, 2004), and due to exposure to low concentrations of black tea polyphenols for 72 hrs (Sa'eed, 2004). AMN3 cells were more sensitive to the aqueous extract of *C. rotundus* L. than to the hexane or ethanolic extracts (Al Hilli, 2004). In addition, AMN3 cells were more sensitive than HEP-2 and RD cells to the crude extracts of *C. rotundus* L. (Al Hilli, 2004) and green tea polyphenols and terpenoids (Sa'eed, 2004).

Phytoestrogens are a chemically diverse group of compounds made by plants. Although estrogens stimulate the growth of many breast tumors, there is a negative correlation between the incidence of breast cancer and the phytoestrogens-rich diet of certain Asian populations. To begin resolve this paradox, the estrogenic properties of genistein and quercetin, two flavonoid phytoestrogens particularly abundant in soybeans were analyzed. At minimal effective concentrations, both flavonoids stimulated the proliferation of MCF-7 (estrogen-dependent) and MCF-7SH (estrogen-independent) cell lines of human breast cancer. At high concentrations, such as those reached with a soy-rich diet, genistein and quercetin were strong cytotoxic agents that even killed estrogen receptor-independent HeLa cells. Therefore, it was concluded that the mode of action of phytoestrogens and the balance between being risk or chemopreventive factors for breast cancer might depend on the dietary load.

Tamir *et al.* (2000) studied the estrogenic properties glabridin, the major isoflavan in licorice (*Glycyrrhiza glabra* L.) root. The effect of increasing concentrations of glabridin on the growth of three breast tumor cells (T-47D, MCF-7, and MDA-MB-468) was biphasic. Glabridin showed an estrogen receptor-dependent, growth-promoting effect at low concentrations (10 nM-10 μM) and estrogen receptor-independent antiproliferative activity at concentrations of $> 15 \mu\text{M}$.

Studies of the effect of dietary phytoestrogen biochanin A on proliferation of MCF-7 cells also showed that biochanin A exhibited biphasic regulation on MCF-7 cells. At concentration of less than 10 $\mu\text{g/ml}$, cells responded to biochanin A by increasing cell growth and de novo DNA synthesis. The addition of biochanin A at concentrations of greater than 30 $\mu\text{g/ml}$ significantly inhibited cell growth and DNA synthesis in a dose-dependent fashion resulting in an IC_{50} value of 40 $\mu\text{g/ml}$ (Hsu *et al.*, 1999).

Sukardiman and his colleagues (2000) found that the flavonoid pinostrobin inhibited DNA topoisomerase I activity resulting in the cleavage of DNA and thus cytotoxicity towards cell culture of human mammary carcinoma. Panaro *et al.* (1999) observed that flavone acetic acid (FAA), a synthetic flavonoid, mediated G_2/M cell cycle arrest in NMU (mammary carcinoma cells of the rat). Morphological cytogenetic analysis demonstrated a colcemid-like effect of FAA on cytokinesis by causing accumulation of condensed C-metaphases, which are caused by colcemid and named accordingly.

Conolly and Lutz (2004) tried to promote a scientific discussion of the concept termed "hormesis" due to the fact

that for reactions of a complex biological system to a toxicant, nonmonotonic (biphasic) dose-effect relationships can be observed showing a decrease at low dose followed by an increase at high dose, or *vice versa*. Calabrese and Baldwin (1998) suggested that chemical hormesis was a reproducible and relatively common biological phenomenon.

5. Conclusion:

Salvia triloba has cytotoxic effect against some cancer cell lines. It seems likely that these plants may act at various therapeutic levels, but further studies are required to evaluate the practical values of therapeutic application.

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Toxic Effect of Dimethoate and Diazinon on the Biochemical and Hematological Parameters in Male Rabbits

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Abstract

Dimethoate and Diazinon are two of widely used organophosphorus insecticides in agriculture. The irrational use of Dimethoate and Diazinon in Yemen play a crucial role in the occurrence of many diseases affecting plants, animals and man. The present work was conducted to investigate the alterations in biochemical and hematological factors in male rabbits after orally administration a single dose of 1/4 LD₅₀ of Dimethoate and Diazinon for 20 days. 30 Male Rabbits weighting 1500-1700g., were divided into 3 groups with 10 animals in each, first group served as control animals, they received 5 ml. of corn oil, while animals in second group received 1/4 LD₅₀ of Dimethoate, animals in third group received 1/4 of LD₅₀ of Diazinon. The concept of this study was to evaluate the hepatotoxic, and nephrotoxic effects of Dimethoate and Diazinon, therefore, the followings Biochemical parameters in serum were studied: aminotransferases (ALT and AST), alkaline phosphatase (ALP), total proteins, albumin, uric Acid, creatinin, and blood glucose. The followings hematological parameters were studied in blood: red blood cells (RBC), hemoglobin (Hb), and erythrocytes sedimentation rate (ESR). The Biochemical analysis showed that the levels of the ALT and AST as well as ALP, uric acid, creatinin, and blood glucose in the serum of treated rabbits significantly ($P < 0.01$) increased compared to control animals, whereas either, total protein, and albumin, significantly decreased ($P < 0.01$). Hematological factors were reduced in the treated groups.

المخلص

يعتبر المبيدان الحشريان الديمثوات والديازينون من أكثر المبيدات الحشرية ذات التركيب الفسفوري العضوي استخداماً في الزراعة. ويلعب الاستخدام العشوائي لهذين المبيدين دوراً كبيراً في إصابة النباتات والحيوانات وال الإنسان بالعديد من الأمراض في اليمن. أجريت هذه الدراسة لمعرفة التأثير السمي الذي يحدثه التعاطي عن طريق الفم لمادته ربع الجرعة المميتة لهذين المبيدين على الدلائل الدموية والكيموحيوية في دم ذكور الأرانب. ولهذا الغرض تم استخدام 30 أرنباً تراوحت أوزانهم بين 1500-1700 جرام ، قسمت إلى ثلاث مجموعات تحتوي كل مجموعة على 10 حيوانات. عوملت المجموعة الأولى على أنها مجموعة ضابطة وأعطيت جرعة يومية مقدارها 5 مل من زيت الذرة لمدة عشرون يوماً وأعطيت المجموعة الثانية ربع الجرعة المميتة من الديمثوات مرة في اليوم لمدة عشرون يوماً ، وأعطيت المجموعة الثالثة ربع الجرعة المميتة من مبيد الديازينون مرة في اليوم لمدة عشرون يوماً. وكان الهدف الأساس للدراسة هو معرفة الأثر السمي لهذا المبيد على خلايا الكبد والكلى ، وعلى وفي نهاية التجربة فحصت الدلائل الكيموحيوية التالية في مصل الأرانب: الأنزيمات الناقلة للأمين (الالانين والاسبرتيت) ، وإنزيم الفوسفاتاز القلوي ، والبروتين الكلي ، واليومي ، وحمض اليوريك ، والكرياتينين وسكر الدم. كما تم فحص المؤشرات الدموية التالية : عدد خلايا الدم الحمراء ، والهيموجلوبين ومعدل ترسيب خلايا الدم الحمراء. أشارت التحاليل الكيموحيوية إلى ارتفاع ذو معنوية عالية في معدل الأنزيمات الناقلة للأمين وكذا إنزيم الفوسفاتاز القلوي ، اليوريك ، والكرياتينين ، وسكر الدم ، بينما انخفض معدل كلا من البروتين الكلي واليومي في مصل الحيوانات التي تعاطت المبيدات مقارنة بحيوانات المجموعة الضابطة. المؤشرات الدموية (خلايا الدم الحمراء والهيموجلوبين) انخفضت معدلاتها في دم الحيوانات التي تعاطت المبيدات مقارنة بحيوانات المجموعة الضابطة ، بينما ارتفع معدل ترسيب خلايا الدم الحمراء بمعنوية عالية.

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Keywords: Dimethoate, Diazinon, Hepatotoxicity, Nephrotoxicity..

1. Introduction

The control of insect pests relies heavily on the use of synthetic insecticides. But, their widespread use has led to some serious problems including toxic residues on grass and toxicity to non-target organisms such as mammals, birds and fishes (Zettler and Cuperus 1990; White 1995; and Riebeiro *et al.*, 2003).

The pollution of the environment plays a crucial role in the occurrence of many diseases affecting plants, animals and man. One of the main factors causing pollution of the environment is the irrational use of organophosphorus insecticides (Al-Haj *et al.*, 2005).

Many alterations have been observed in organs of animals due to the organophosphorus insecticides (Betrosian *et al.*, 1995; and Senanayake 1998), specially CNS, (Desi *et al.*, 1998; and Lengyl *et al.*, 2005), liver (Gomes *et al.*, 1999), and kidney (Kossmann *et al.*, 1997).

Dimethoate is an organophosphorus insecticide widely used in agriculture (Sharma *et al.*, 2005), Diazinon, also is an organophosphorus insecticide extensively used in

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agriculture (Alahyary *et al.*, 2008). Both Dimethoate and Diazinon, are two of the most widely and irrationally used insecticides in agriculture in Yemen. (Al-Haj *et al.*, 2005).

Dimethoate is an insecticide with anticholinesterase mode of action (De-Bleecker *et al.*, 1993; and Dongren *et al.*, 1999).

Begum and Vijavaraghaven (1995) observed that, the exposure of Dimethoate to the fresh water fish *claries batrachus* reduced the carbohydrates and proteins metabolism, and affect the aminotransferase activity in the liver. An increase in blood glucose in experimental rats was reported after Dimethoate orally administration period of 2 months in dose 21mg/kg. (Hagar and Fahmy 2009). An increase in lactate dehydrogenase, serum transaminase, and a decrease in the serum total protein, albumin, and globulin was observed in experimental rats after Dimethoate orally administration in dose 75mg/kg (Attia and Nasr 2009). Diazinon is an organophosphorus insecticide with anticholinesterase mode of action (Alahyary *et al.*, 2008).

Mild structural and functional change in liver as well as in tests of experimental mice was observed after a single intraperitoneal administration of Diazinon (Dikshith *et al.*, 1975).

Matin *et al* (1990) showed that the administration of Diazinon to experimental rats resulted in carbohydrate metabolism changes that were abolished by adrenalectomy, suggesting a possible involvement the adrenals in the induced changes in Diazinon-treated animals. The exposure of zebra fish to the Diazinon for up to 168 hours, a significantly reduced DNA, RNA and the total protein in the liver (Ansari and Kumar 1988).

Jyostana *et al* (2003), observed a significant biochemical and hematological alterations due to the exposure to the various pesticides. Significant damage in the hepatic cells and glucose metabolism in liver was observed as the result of Diazinon administration (Fatima *et al.*, 2006).

At the last 5 years in Yemen, we have noted a critical increase in number of people suffering from various liver and kidney diseases, as well as diabetic mellitus. Therefore, the purpose of the present study was to evaluate the hepatotoxic and nephrotoxic effects of the Dimethoate and Diazinon on male rabbits, also their effects on some blood factors and blood glucose level will be determined.

2. Materials and Methods:

2.1. Chemicals:

All chemicals used in this experiment were obtained from Sigma, USA, including Dimethoate and Diazinon.

2.2. Animals treatment and blood collection:

Thirty healthy male rabbits (1500-1700g) were divided into 2 treated groups and control, as follows:

-Control group: 10 animals treated with a single daily dose of 5ml. corn oil orally period of 20 days.

-Dimethoate group: 10 animals treated with a single daily dose of 1/4 of LD₅₀ of Dimethoate (20mg/kg) in 5ml. corn oil orally period of 20 days.

-Diazinon group: 10 animals treated with a single daily dose of 1/4 of LD₅₀ of Diazinon (25mg/kg) in 5ml. corn oil orally period of 20 days.

All animals were maintained in standard environmental conditions and kept a standard commercial diet with water *ad libitum*.

All experiment was administrated in the Animal Physiology Laboratory, Department of Biology, Faculty of Science and Education, Aden University.

After 20 days the animals were fasted over night for 12h. Then they were sacrificed, the blood was immediately collected. Blood samples were divided in two parts, one was maintained in EDTA bulb and plain tube for assay of blood factors, other was centrifuged, and serum was discarded and kept at - 21 ° C for the biochemical testes.

2.3. Alanine- aminotransferase (ALT) and Aspartate- aminotransferase (AST) Assay:

The estimation was carried out according to the method originally developed by (Reitman and Frankel 1957).

2.4. Alkaline phosphatase Assay:

ALP was determined using a colorimetric method as described by (Kind and King 1954).

2.5. Total Protein Assay:

The total protein was determined by Biuret method explained by (Tietz 1976)

2.6. Albumin Assay:

Serum albumin was determined according to the method of (Doumas *et al.*, 1971).

2.7. Glucose Assay:

Glucose was determined according to method of (Trinder *et al.*, 1969).

2.8. Creatinine and Uric acid Assay:

Creatinine and uric acid was estimated according to method explained by (Houot 1985).

2.9. R.B.C., Hb and E.S.R. Assay:

The R.B.C. count, Hb level and E.S.R. time, were determined using method described by (Sood 1990).

2.10. Statistical analysis:

The statistical analysis was performed by SPSS; continuous data are expressed as mean \pm S.E. Data were compared using one - way ANOVA. P value <0.01 was considered to be statistically significant.

3. Results:

Data in table 1 show that the treatment with 1/4 of LD₅₀ of Dimethoate and Diazinon resulted in a statistically high significant increase in the level of alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) in the serum of both treated groups, as compared to the control, this increase was higher in the Diazinon treated rabbits.

As shown in the table 1 the level of alkaline phosphatase (ALP) in the serum of rabbits treated with Dimethoate and Diazinon statistically high significant

increased compared to control rabbits, this increase was higher in Diazinon treated rabbits.

Table1: The biochemical parameters after 20 days of orally administration of Dimethoate in dose 20mg/kg. and Diazinon in dose 25mg/kg.

Biochemical	Treatments		
Parameters	Control	Dimethoate	Diazinon
AST	35.37±3.7	85.47±2.32**	93.31±2.11**
IU/L			
ALT	44.50±2.70	168.27±4.86**	176.41±6.75**
IU/L			
ALP	65.52±2.3	160.25±3.7**	174.41±4.8**
IU/L			
T.Protein	7.67±0.12	5.16±0.13*	5.17±0.17*
g/dL			
Albumin	3.58±0.40	2.01±0.19*	2.11±0.22*
g/dL			
Glucose	108.83±6.91	210.17±4.31**	245.20±5.40**
mg/dL			
Uric acid	4.02±0.74	7.85±1.34*	7.65±1.75*
mg/dL			
Creatinine	0.98±0.21	3.03±1.16*	3.9
mg/dL			

Values are expressed as means of 10 animals ± S.E.* Significance;** High significance at (P<0.01) vs. control.

Table2: The hematological parameters after 20 days of orally administration of Dimethoate in dose 20mg/kg. and diazinon in dose 25mg/kg.

Hematological	Treatments		
Parameters	Control	Dimethoate	Diazinon
R.B.C.	4.6±0.05	2.9±0.10*	2.6±0.25*
Mill/cu.mm			
Hb.	12.6±1.01	8.7±0.23*	8.1±0.34*
g/dL			
E.S.R.	5.0±1.04	13.0±1.09**	12.0±0.85**
Mm/hr			

Values are expressed as mean of 10 animals ± S.E. * Significance ** High significance at (P<0.01) vs. control

Total protein and albumin levels significantly decreased in the serum of treated with Dimethoate and Diazinon rabbits, as compared to control.

Blood sugar level highly significant increased in the serum of Dimethoate and Diazinon treated rabbits, as compared to control, the increase in blood glucose was higher in the serum of Diazinon treated rabbits than in the Dimethoate treated group.

Uric acid and creatinine levels significantly increased in the serum of Dimethoate and Diazinon treated rabbits, compared to control.

Results in table 2 showed, that the R.B.C. count and Hb level significantly decreased in the blood of Dimethoate and Diazinon treated rabbits compared to control.

The erythrocytes sedimentation rate highly significant increased in the blood of Dimethoate and Diazinon treated rabbits as compared to control.

4. Discussion:

The noticed increase in the levels of aminotransferase (ALT and AST) and the level of ALP as well as the decrease in the levels of total protein and albumin in the serum, are the major diagnostic symptoms of liver diseases (Chatterjea and Shinde 2005).

The decrease in the serum albumin may also indicate to the renal inability keeps it in; therefore it excreted with urine (Albumiurea) (Vasilenko and Grebenev 1990). The increase in the uric acid and creatinine in the serum are the major symptoms of glomerular filtration damage (Chatterjea and Shinde 2005).

Blood glucose increasing in many diseases such as Diabetes mellitus, and damage of the hepatic glycogenesis pathway (Guyton and Hall 2006).

Our results clearly showed the hepatotoxic and nephrotoxic effects of Dimethoate and Diazinon. The orally administration of 1/4 of LD₅₀ of Dimethoate and Diazinon for 20 days seriously affected the hepatocytes and renal functions, and may also the pancreas β-cells function. Our results are in agreement with (Ansari and Kumar 1988), who found that, Diazinon reduced the total protein level in Zebrafish, (Matin *et al.*, 1990), who observed that Diazinon administration to rats reduced the carbohydrate and protein metabolism, (Begum and Vijavaraghaven 1995), who showed that Dimethoate inhibited the carbohydrates and proteins metabolism, and affected the aminotransferase activity in rats, (Fatima *et al.*, 2006), who indicated an increase in blood glucose level in Diazinon administrated rats, (Attia and Nasr 2009), who noticed increase in serum aminotransferase, alkaline phosphatase and decrease in total protein and albumin in rats serum after orally administration of Dimethoate, (Hagar and Fahmy 2009), who showed that, Dimethoate orally administration resulted the increase in blood glucose level, and (Kossmann *et al.*, 1997), who assured the nephrotoxic effect of pesticides.

The results of this study showed that, the hematological parameters RBC and Hb were significantly decreased in Dimethoate and Diazinon treated rabbits when the erythrocytes sedimentation rate was highly significant increased as compared to control. The effect of organophosphorus pesticides on the Hb of several workers has been studied by (Bhatnagar 1980; and Ray 1992). The decrease in the Hb along with the decrease in the RBC might be due to the effect of pesticides on blood forming organ (bone marrow and liver), and inhibition of many steps of heme biosynthesis in rabbits, as the result of pesticides exposure (Ray 1992). The poisoning by

pesticide residues leads to the development of anemia due to interference of Hb biosynthesis and shortening of the life span of circulating erythrocytes (Betrosian 1995; and Jyotsana *et al.*, 2003). The increase of E.S.R. indicates to inflammation caused by organophosphorus pesticides (Elias and Saif 2009). Our finding is in agreement with (Jyotsana *et al.*, 2003), that showed that pesticides decrease R.B.C. and Hb levels, and (Elias and Saif 2009), who noticed the reduce of R.B.C., Hb, and increase in erythrocytes sedimentation rate in rabbits exposure to orally dose of 10mg/kg. of the organophosphorus pesticide Methidathion.

The above mentioned effects of organophosphorus pesticides could be due to their ability to form free radicals (Hazarika *et al.*, 2003; and vidyasagar *et al.*, 2004). The results of our previous work on Methidathion showed that the using of antioxidants vitamins A, C, and E, reduces the toxicity of Methidathion (Elias and Saif 2009). This fact may ensure the hypothesis of the ability of organophosphorus pesticides to form free radicals, which have been implicated as playing a role in the etiology of many alterations (Halliwell and Gutteridge 1995).

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