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In vitro and *in vivo* Antioxidant Effect of Some Selected Alcoholic Bitters Sold in South East Nigeria

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Abstract

There has been proliferation of herbal drinks known as alcoholic bitters. Bitters in this present study, is a beverage, often alcoholic, flavoured with herbal substances that gives it a bitter or bittersweet flavor. Unconventional traditional medicine practice which employ the use of herbs have in recent times been gaining much publicity and recognition, for their solution to ailments seemingly elusive to the system of orthodox medical practice. In this study, the *in-vitro and in-vivo* antioxidant effect of some selected alcoholic bitters (*Alomo, Orijin, Action* and *Jedi jedi*) sold in South East Nigeria was evaluated using standard methods. The results obtain showed that *Alomo* bitters and *Jedi jedi* have great potential to scavenge free radicals (EC_{50}: *Alomo*, 31.6%; *Jedi jedi, 44.09%;* BHA, 36.32%) and had no significant effect ($P<0.05$) on the antioxidant enzymes studied. *Jedi jedi* and *Alomo* bitters also showed a high reducing power capacity. They had a better electron donating ability compared to the standard ($OD_{0.5}$: *Alomo*, 7.81%; *Jedi jedi,* 6.86; BHA, 8.83%). This was attributed to their high content of polyphenols. On the other hand, *Action* bitters and *Orijin* bitters had significant effect on the antioxidant enzymes, leading to lipid peroxidation. The *in-vitro assay* of their antioxidant activity showed a very low potential to scavenge free radical (EC50: *Action, 181.52%; Orijin, 775.*04%). Their reducing power capacity was also significantly lower than the other group and the standard ($OD_{0.5}$: *Action,* 27.5%; *Orijin*, 57.88%). This could be as a result of their low concentration in some antioxidant molecules. The administration of these substances was on a daily bases during the experiment, thus regular intake of this bitters may cause oxidative stress.

Keywords

Unorthodox Traditional Practice, Herbal Drinks, Alcoholic Bitters, Antioxidant

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1. Introduction

The development of several human diseases has been linked to overproduction of reactive oxygen species (ROS). Oxidative stress occurs when there is imbalance between production and accumulation of ROS in cells and tissue and the detoxification mechanism [1]. Different cellular organs where there is high consumption of oxygen are the endogenous sources of ROS. They include endoplasmic reticulum, mitochondria and peroxisomes.

Some diseases attributed to oxidative stress includes cancer, cardiovascular, neurodegenerative, and metabolic disorders, inflammation, and aging [2]. ROS are continuously generated by tumours at increased levels that have a dual role in their development. Oxidative stress can promote tumor initiation, progression, and resistance to therapy through DNA damage, leading to the accumulation of mutations and genome instability, as well as reprogramming cell metabolism and signaling. On the contrary, elevated ROS levels can induce

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tumor cell death. This review covers the current data on the mechanisms of ROS generation and existing antioxidant systems balancing the redox state in mammalian cells that can also be related to tumors [2].

Aerobic organisms have developed antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and DT-diaphorase. SOD carries out the dismutation of O^{-2} to H_2O_2, in subsequent reactions; the H_2O_2 is catalyzed by catalase or by GPx, and converted into H_2O and O_2. SOD is a metalloenzyme and is the most important and most powerful detoxification enzyme in the cell. Depending on the type of metal ion required as a cofactor by SOD, there are several forms of the enzyme [3; 4]. CAT uses iron or manganese as a cofactor and catalyzes the degradation or reduction of hydrogen peroxide (H_2O_2) to produce water and molecular oxygen, thus completing the detoxification process initiated by SOD [5]. Millions of H_2O_2 molecules are broken down b CAT in a second. The main location of CAT is peroxisomes, and its main function is to get rid of the H_2O_2 generated during the oxidation of fatty acids. GPx is an important intracellular enzyme that breaks down H_2O_2 in water and lipid peroxides in their corresponding alcohols; this happens mainly in the mitochondria and sometimes in the cytosol. The activity of GPx depends on selenium. In humans, there are at least eight enzymes GPx, GPx1–GPx8. Among glutathione peroxidases, GPx1 is the most abundant seleno peroxidase and is present in virtually all cells. The enzyme plays an important role in inhibiting the process of lipid peroxidation and, therefore, protects cells from oxidative stress [6]. Oxidative damage of the functional proteins and the fatty acids of the cell membrane can be caused by low GPx activity. GPx, particularly GPx1, has been implicated in the development and prevention of many diseases, such as cancer and cardiovascular diseases [7]. The reduction of quinone to quinol is catalyed b DT-diaphorase and it also participates in the reduction of drugs of quinone structure [8]. DNA regulates the production of these enzymes in cells.

There has been proliferation of herbal drinks known as alcoholic bitters. Bitters are beverage, often alcoholic, flavoured with herbal constituents that give it a bitter or bittersweet flavour. The generic term applies to all bitter liquors and herbal bitters. Bitters are produced from herb and root extracts, narcotic components of (primarily) tropical and in the subtropical plants and spices, They are usually dark in colour and valued for their ability to promote appetite and digestion hence their use as patent medicine and as aid in digestion and as flavouring in cocktails. Bitters are made up of numerous groups of chemical compounds extracted from the herbs and roots (medicinal plants) that have the common characteristic of a bitter taste and act to increase the vital energy centres in the body [8].

In developing countries where the greater number of the population live on less than on dollar a day, majority cannot afford the money to buy refined and well packaged alcoholic beverages, due to this, they resort to cheap alcoholic beverages. Some brands of bitters available in south east Nigeria are *Alomo*, Action, Heritage, *Orijin*, *Jedi jedi*, Ikemba among others. Nigeria, bitters are seen as a health tonic that treats a wide range of diseases [9]. Many people believe that some of these bitters treat infertility in both sexes, treat malaria, increase libido, detoxify the system, and calm an upset tummy. Many also use bitters for other reasons such as part of their weight loss regime, to regulate sugar level, prevent pregnancies, abort unwanted pregnancies, treat heartburn, and support the proper functioning of the liver and other key organs in the body [9].

Orijin Bitters has become one of the fastest growing bitters drink available in the Nigerian market. *Orijin* Bitters is made by Guinness Nigeria Plc which is owned by the multinational drinks company Diaego. The drink is made locally here in Nigeria. *Orijin* Bitters tends to differentiate itself from the competition by having a taste which is a little bit sweet as well as bitter. Ingredients listed on the label include water, alcohol; sugar and plant extract (kola nut, prune, oakwood, bitter orange peel, wormwood). *Orijin* bitters is available in 20 cl and 75 cl sizes, with an ABV (alcohol by volume) of 30%. *Orijin* Bitters can be found in shops across all the major cities in Nigeria as well as online via Konga among others.

Jedi jedi bitter cleanser is an alcoholic drink blended and bottled in Nigeria with ABV (alcohol by volume) of 33%. *Jedi jedi* is available in 125mls. *Jedi jedi* bitter cleanser consists of extracts from natural herbs for healthy and active life. Ingredients listed on the label include water, Ethyl alcohol, plant extracts and natural flavors.

Alomo bitters are authentic African herbal bitters that originate from Ghana but widely consumed in other African countries as well, especially in Nigeria. It is a Ghanaian variant of bitter that are well received in its neighbouring countries, such as Nigeria, Togo, Ivory Coast as well as Burkina Faso. It is herbal based and produced by Kasapreko. In Nigeria, there has been a surge in demand of *Alomo* bitters, seen through the presence of various *Alomo* bitter brands that heavily competes with one another. Indeed, they are also sold everywhere possible, such as in stores, markets, roadside stalls and more.

Action Bitters is a wine colored full-bodied spirit with a slight bitter-sweet and appetizing aroma. The brand claims to be for the class of consumers who desire to restore their aphrodisiac lifestyle. This brand is basically targeted at the young, trendy and inspirational men and women whom want to be on top of their game while maintaining an Active lifestyle.

The focus of this study is to evaluate the *In vitro* and *in vivo* antioxidant of some selected alcoholic bitters found in South

East Nigeria. The result of this research will help to verify and authenticate the claims made by the producers and some of the consumers.

2. Methodology

2.1. Sample Collection

The alcoholic bitters, *Alomo* bitters, Acrion Bitters, *Orijin* Bitters and *Jedi jedi* were purchased from a store at Eke-Awka Market, Awka, Awka South Local Government Area, Anambra State Nigeria.

2.2. DPPH Scavenging Activity Assay

The stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) was used for the determination of free radical scavenging activity of the samples. This was assayed using the method described by [10]. An aliquot of the diluted bitters (0.3ml) each (0-100%) were mixed with 2.7ml of methanolic solution of DPPH (100µM) in test tubes. The mixture was votexed and kept in dark for 60mins. The absorbance was taken at a wavelength of 517nM using UV-VIS spectrophotometer. Butylated Hydroxy Anisole (BHA) was used as standard. The percentage scavenging activity was calculated using the formular:

$$\%RSA= [(ADPPH-As)/ADPPH]\times100$$

Where As is the absorbance of the test solution and A_{DPPH} is the absorbance of DPPH solution. The EC50 (concentration of sample at 50% RSA) was calculated from the graph of %RSA against the sample concentration.

2.3. Reducing Power Capacity Assay

The reducing power was determined according to the method of [11]. This method is based on the principle of increase in the absorbance of the reaction mixture. An aliquote of the diluted sample 2.5ml of various concentrations (0-100%) was mixed with 2.5ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20mins. 2.5ml 0f 10% Trichloroacetic acid was added and the mixture centrifuged at 1000rpm for 8min. The upper layer (5ml) was mixed with 5ml of deionised water followed by the addition of 1ml of 0.1% ferric chloride. The absorbance was measured at 700nM. The graph of absorbance at 700nM against the concentrations was plotted. Vitamin C was used as a standard antioxidant.

2.4. Animals

Thirty Wistar albino rats were purchased from Chris Farm at Mgbakwu in Awka North LGA, Anambra state and acclimatized for one week during which they were fed with standard feed and distilled water.

2.5. Experimental Design

At the end of one week, the animals were weighed and randomly divided into five groups of six animals per group. Group A is the Negative control. They were feed with standard feed and distilled water per day. Group B, C, D, and E were administered orally, 1.4ml/kg body weight of Action *bitters, Alomo bitters, Jedi jedi* and *Oriin bitters* respectively per day with standard feed. All the groups had free access to water. After 30 days, the animals in all the groups werefasted for 12 hours and sacrificed. Blood was collected by cardiac puncture using syringe and placed in a plain bottle. The blood was centrifuged at 4000rpm for 30 mins and the serum was used for the assay.

2.6. Superoxide Dismutase Activity Assay

Superoxide dismutase (SOD) activity was determined by the method of [12]. The principle is based on the ability of SOD to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480nm. The reaction mixture (3 ml) contained 2.95 ml of 0.05 M sodium carbonate buffer, pH 10.2, 0.02 ml of sample and 0.03 ml of 0.3mM adrenaline in 0.005 N HCl was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 3 min. $\sum= 4020M^{-1}\ cm^{-1}$.

2.7. Catalase Activity Determination

Catalase activity was determined according to [13]. It was assayed colorimetrically at 620nm and expressed as micromoles of H_20_2 consumed / min / mg protein at 25°C The reaction mixture (1.5ml) contained 0.1ml of tissue homogenate, 1.0ml of 0.01 phosphate, buffer (PH 7.0) and 0.4ml of 2M H_20_2. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent (5% $K_2Cr_20_2$ and glacial acetic acid were mixed in the ratio, of 1:3 respectively)

$$\sum = 40m^{-1}\ CM^{-1}$$

2.8. Malondialdehyde

Malondialdehyde (MDA) an index of Lipid peroxide reacts with thiobabarturic acid (TBA) to give a complex pink colour. This was used to assess lipid peroxidation using the method of [14]. An aliquot of the serum (0.1ml) was mixed with 0.9ml of normal saline. This was followed by the addition of 2.0ml of (1:1:1 ratio) TCA-TBA-HCL reagent. (0.37% TBA, 0.24mM HCl and 15% TCA) the mixture was boiled at 100°C for 15 min and allowed to cool. Flocculent materials were removed by centrifuging at 3000rpm for 10 min. The supernatant was removed and the absorbance read at 532nm against the blank

Malondialdehyde (in µM) was calculated using the molar extinction coefficient for using the molar extinction coefficient for MDA – TBA complex of $1.56x105M^{-1}cm^{-1}$

2.9. Determination of Glutathione Peroxidase Activity

This was determined by the method of Beutter and Kelly as adapted by [15]. Hydrogen peroxide (H_2O_2) is reduced by oxidizing reduced glutathione (GSH) to form GSSG. The reaction mixture contained 1mL of 0.3M phosphate buffer (pH 7.4), 0.3mL of 10mM (GSH), 0.3mL of 15mM H_2O_2 and 1.37mL distilled water. An aliquote, (0.1mL) of the serum was added to the mixture in the cuvette, shaken and absorbance was read at 340nm. Molar extinction co-efficient of 1.622 x $10^{-3}M^{-1}cm^{-1}$ was used to calculate enzyme activity which was expressed in unit mg protein.

2.10. Data Analysis

Data obtained from the study were analyzed using the Statistical Package for Social Sciences software for windows version 17 (SPSS Inc., Chicago, Illinois, USA). Statistical analysis of the results obtained were performed by using analysis of variance (ANOVA) Tests to determine if significant difference exists between the mean of the test and control groups. The limit of significance was set at $p<0.05$.

3. Results and Discussion

3.1. DPPH Free Radical Scavenging Activity of Alcoholic Bitters

The results of the DPPH radical scavenging activity of the alcoholic bitters and the standard antioxidants (BHA) is presented in Figure 1. All the *bitters* showed varying activities against the DPPH radical. *Alomo* bitters exhibited the highest activity against the free radical (EC_{50}: *Alomo*, 31.6%;). EC_{50} is a term used to express the amount of the substance that lead to 50% reduction in the concentration of the free radicals in solution. The lower the EC_{50}, the better the activity of the substance against free radicals. This was followed by *Jedi jedi* (EC_{50}: *Jedi jedi, 44.09%*). There was no significant difference in the activity of *Alomo* and *Jedi jedi* when compared to the standard antioxidant (EC_{50}: BHA, 36.32%; $P<0.05$). Action bitters and *Orijin* bitters had a low activity against the free radical (EC_{50}: *Action, 181.52%; Orijin, 775.*04%). Their activity was significantly lower than that of *Alomo, Jedi jedi* and BHA. Anti oxidant properties of herbal mixtures have been attributed to their polyphenolic contents [16]. Phenolic compounds are generally characterised by at least one aromatic group. They are synthesized mainly from cinamic acid which is formed from phenylalanine by the Action of phenylalanine ammonia lyase (Pal). *Alomo* bitter is an alcoholic herbal formulation that contains different herbs; *Capparis erythrocarpus Khaya ivorensis*, *Lecaniodiscus cupanioides, Dialium guineense*. These plants are rich in polyphenols and other phytochemicals. Thus the observed high activity in *Alomo* bitter and *Jedi jedi* may be attributed to their phytochemical constituents.

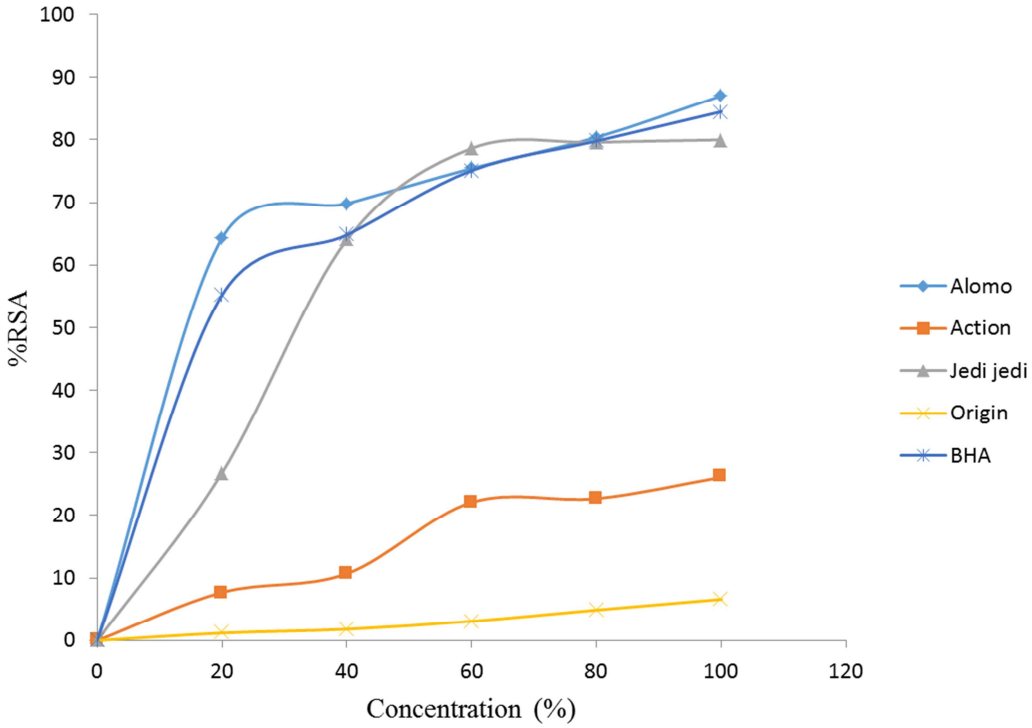

Figure 1. A graph showing the free radical scavenging activities of the alcoholic bitters compared with that of a standard antioxidant, BHA. EC50: *Alomo*, 31.6%; *Action, 181.52%; Jedi jedi, 44.09%; Orijin, 775.*04%; BHA, 36.32%.

3.2. Ferric Reducing Power Capacity of the Alcoholic Bitters

The reducing potential of the bitters was evaluated by determining their ability to reduce Fe^{3+} ion because the reducing power reflects the ability of the bioactive compounds in the sample to donate electron [10]. The results for ferric reducing activity of the *bitters* compared to BHA used as standards are reported in Figure 2. These bitters exhibited dose dependent reducing power potential which shows that they are capable of donating electron and this increased with an increase in concentration when compared to the standards BHA. *Jedi jedi* bitter had the highest ($OD_{0.5}$: *Jedi jedi,* 6.86) electron donating ability as seen in the green curve in Figure 1. $OD_{0.5}$ is used to express the amount of the substance that gave absorbance of 0.500. The lower the $OD_{0.5}$, the higher the electron donating ability of the substance [17]. *Alomo* also showed a high electron donating ability *Alomo*, 7.81%). Both *Alomo* and *Jedi jedi* showed a better reducing power capacity when compared to the standard (OD0.*5* BHA, 8.83%)

This result suggests the presence of reductants in the *bitters* extracts. The increase is as a result of reduction of Fe^{3+} to Fe^{2+}. There was a significant difference between Action and *Orijin* bitters and the control ($p< 0.05$) both of the two bitters had a low reducing power capacity ($OD_{0.5}$: *Action,* 27.5%; *Orijin*, 57.88%)

The existence of reductones in the alcoholic bitters are the keys to its reducing power, and these exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom [17].

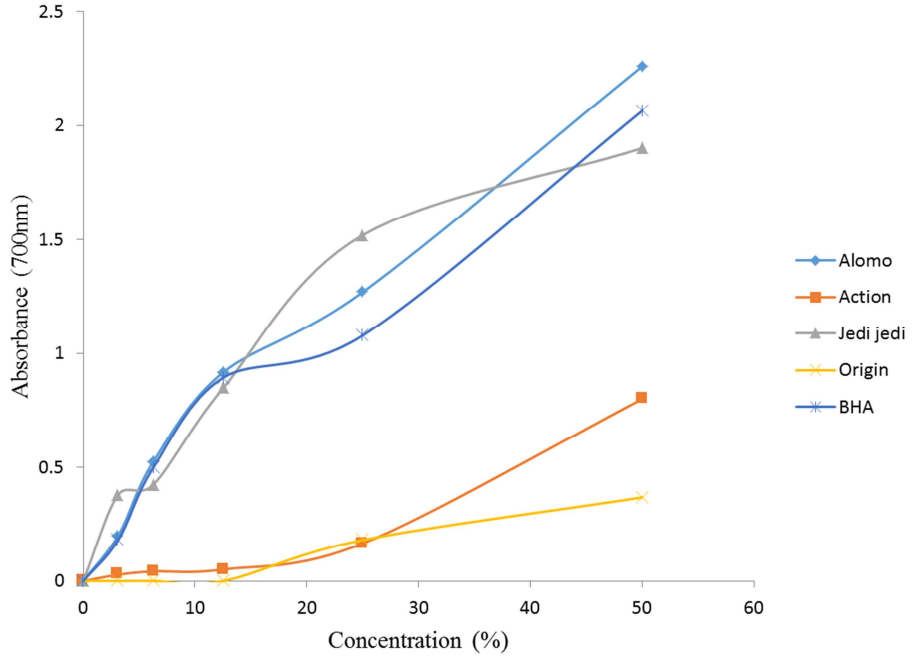

Figure 2. A graph showing the ferric reducing potential of the *Action* bitter, *Alomo* bitter, *Jedi jedi Orijin* bitters compared with that of BHA, a standard antioxidant. $OD_{0.5}$: *Alomo*, 7.81%; *Jedi jedi,* 6.86; *Action,* 27.5%; *Orijin*, 57.88% and BHA, 8.83%.

3.3. Effect of the Alcoholic Bitters on Antioxidant Enzymes

SOD, CAT and glutathione Peroxidase (GPx.) activity of the experimental subjects were assayed to know the effect of the administration of the alcoholic bitters on the antioxidant enzyme activity. The antioxidant system of the body defends the reactive oxygen species produced in the body. Superoxide dismutases (SOD) help the body to remove the superoxide radicals by converting it to hydrogen peroxide (H_2O_2). The conversion of hydrogen peroxide to water and oxygen is catalyzed by catalases. Glutathione peroxidises are also involved in the removal of H_2O_2. If the production of free radicals increases beyond a certain level in the body of the organism, the defensive enzyme systems fail and the condition leads to oxidative stress [15]. The result of the effect of the alcoholic bitters on enzymatic antioxidant system is presented in Figure 3. The SOD and Catalase activities of the groups that received *Orijin* and *Action* bitters were significantly higher than those of other groups and the control ($P<0.05$).

According to [18], metabolism of ethanol leads to alcohol-induced oxidative stress. In their study, an intake of ethanol caused depletion in reduced glutathione (GSH) level and decrease in antioxidant activity. It also led to an elevated level of Malondialdehyde (MDA), an index of lipid peroxidation. From the Figure 4, it was observed that there is a rise in the level of MDA in the group that received *orijin* and *action bitters.* This indicates that the intake of these bitters

caused oxidative stress. This could be due to their low antioxidant activity *in vitro*. These samples may contain lower amount of antioxidant phytochemicals compared to *Alomo* bitters and *Jedi Jedi.*

A previous study by [19] indicated that the presence of reactive oxygen species (ROS) in the system triggers the release of antioxidant enzyme which helps in scavenging the species that initiate peroxidation, breaking the autoxidative chain reaction, quenching O_2^-, and preventing the formation of peroxides. Thus, *Orijin* and Action bitters may have induced oxidative stress which led to the rise in the antioxidant enzyme activities. There was no significant difference between the control group and the groups that received *Alomo* bitter and *Jedi jedi* respectively (P>0.05). They do so by being oxidizing themselves. Antioxidants are often reducing agents such as, thiols, ascorbic acid or polyphenols [20].

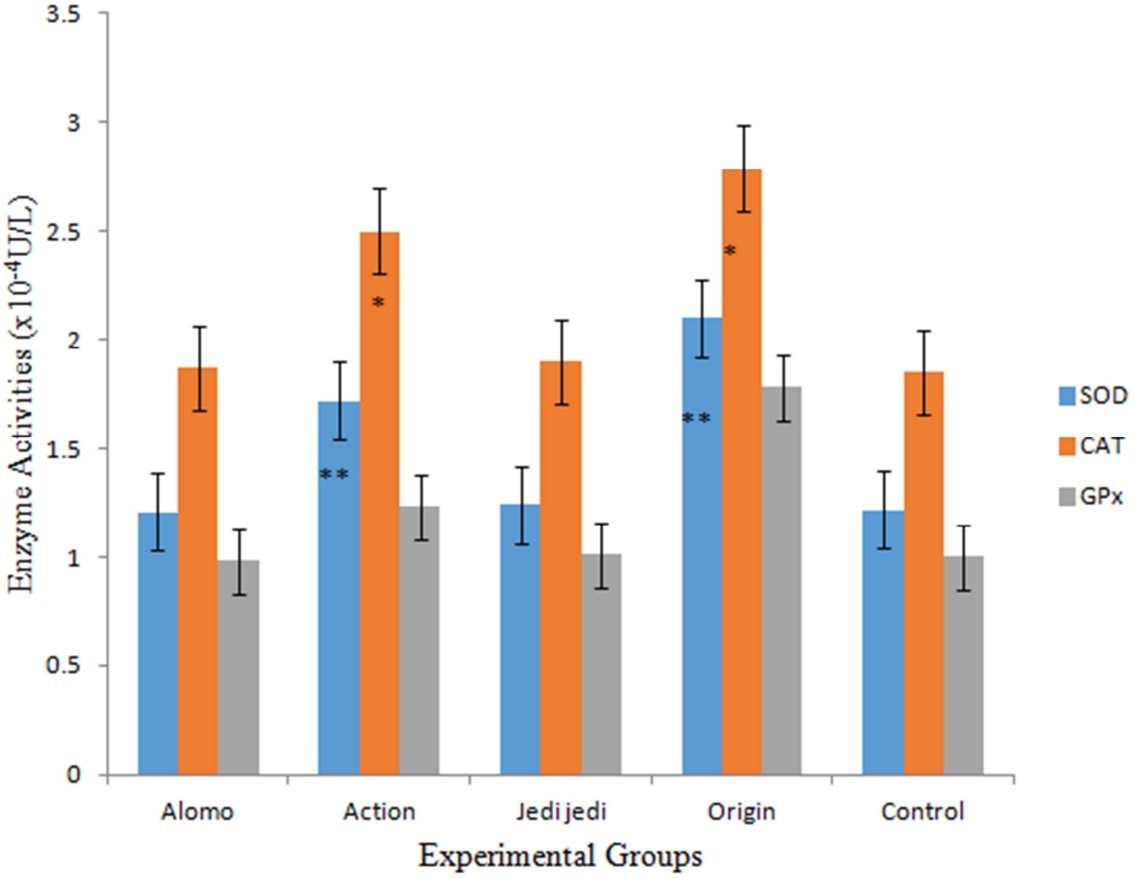

Figure 3. A bar chart showing the effect of *Action* bitter, *Alomo* bitter, *Jedi jedi* and *Orijin* bitters on the *In vivo* antioxidant enzyme activity with the control group.

*Significantly difference from the groups
** Significantly difference from the groups

3.4. Effect of the Alcoholic Bitters on Malondialdehyde (MDA) Level

Ageing and other degenerative diseases have been said to be caused by Lipid peroxidation of cellular membrane structures arising from the free radical activity. Inhibition of the initiation or progression of oxidative chain reaction can prevented or hinder these diseases. Synthetic and natural antioxidants have been reported to handle these effects by terminating their catalytic metal ions [21]. MDA; the end product of lipid peroxidation is considered to be a significant marker of the oxidative process in body cells. In Figure 4 the alcoholic bitters showed varying degree of effects on the MDA level of the groups that received them. There was a significant increase in the level of MDA in the groups that received *orijin* bitters and *Action* bitters compared to the other group and the control (P<0.05). This may be due to alcohol induced oxidative stress [22]. In the other hand, there was no significant difference in the MDA level of the control and groups that received *Alomo* bitters and *Jedi jedi*. Both *Alomo* and *Jedi jedi* bitters are herbal alcoholic beverages just like the *orijin* and *Action* bitters, thus they also have the tendency to induce oxidative stress. The insignificant change in the MDA level may be due to their bioactive compound components which may be capable of scavenging free radicals, thereby inhibiting oxidative stress.

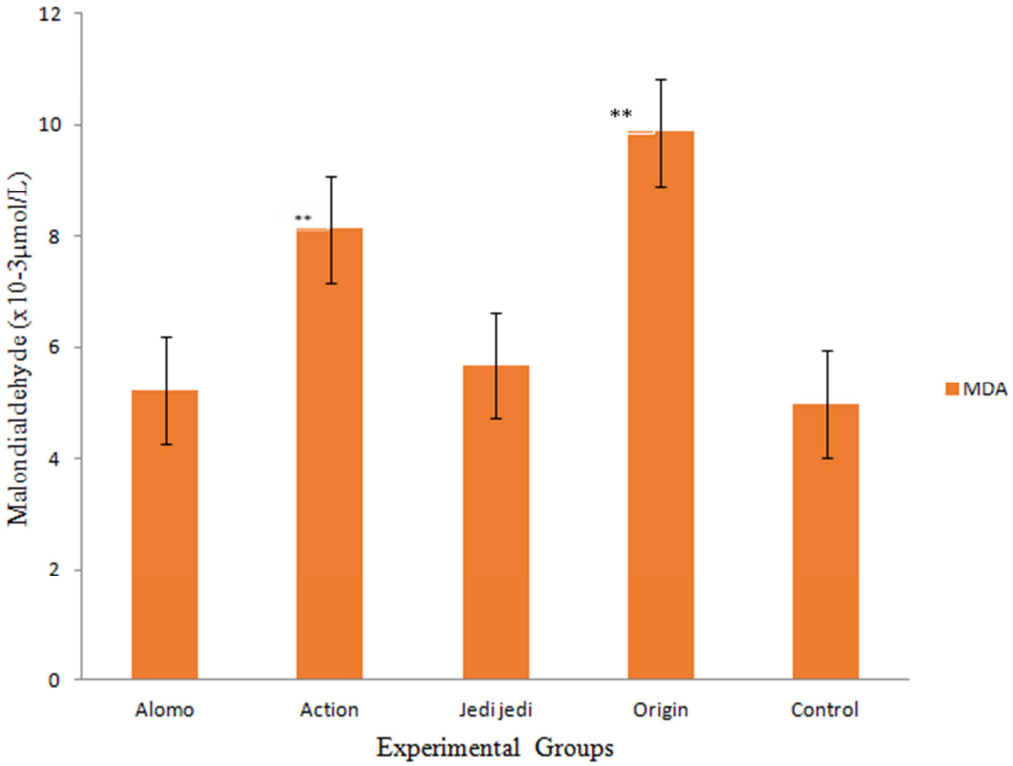

Figure 4. A bar chart showing the effect of *Action* bitter, *Alomo* bitter, *Jedi jedi* and *Orijin* bitters on the Malondialdehyde (MDA) level compared with that of the control group.

** Significantly difference from the groups

4. Conclusion

The results obtained from this study show that *Alomo bitter* and *Jedi jedi* cleanser have great potentials. The *in vitro* and *in-vivo* antioxidant activity could be attributed to the high content of polyphenols in the herbs that formed part of the ingredients. The high tendency of phenolic compounds to chelate metal ions is responsible for their antioxidant actions. They possess hydroxyl and carboxyl groups which are able to bind particularly iron and copper, thus it could offer protection against lipid peroxidation and its associated diseases. The increase in oxidative stress exhibited by *Orijin* and Action bitters may be due to alcohol-induced oxidative stress which was beyond their antioxidant capability. The administration of these substances was on a daily bases during the experiment, thus regular intake of this bitters may cause oxidative stress.

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