Available online at http://ajol.info/index.php/ijbcs

Int. J. Biol. Chem. Sci. 4(4): 1093-1101, August 2010

International Journal of Biological and Chemical Sciences

ISSN 1991-8631

Original Paper

http://indexmedicus.afro.who.int

Inhibition of dehydrogenase activity in bacterial isolates from palm wine by extracts of *Vernonia amygdalina*

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ABSTRACT

Inhibition of dehydrogenase activity of bacterial isolates from palm wine by leaf extracts of *Vernonia amygdalina* was investigated. The cultures were exposed to extract concentrations of 0-2500 µg/ml in a nutrient broth-glucose-TTC medium. The responses of the bacterial strains varied with extract concentration. In all the isolates, dehydrogenase activities were progressively inhibited with increasing concentration of extract. The IC_{50} ranges from 83.27 ± 4.9 to 736.82 ± 13.6 µg/ml. Total inhibitory concentrations for *E. coli*, *Lactobacillus* and *Streptococcus* species were 1984.93 ± 16.5, 2102.82 ± 22.8 and 2476.79 ± 27.7 µg/ml respectively. The total inhibitory concentrations of the remaining isolates (*Staphylococcus*, *Micrococcus* and *Bacillus* species) were beyond the extract concentrations used. The findings may be of clinical relevance and further substantiates the traditional use of extracts of *Vernonia amygdalina* to control microbial load and foaming in palm wine.

Keywords: *Vernonia amygdalina*, dehydrogenase activity, palm wine, *Elaeis guineensis Raphia hookeri, Raphia vinetera.*

INTRODUCTION

Vernonia amygdalina belong to the family Compositae and was named after William Vernon, a 17th century botanist (Keay, 1989). It is abundant in grassland throughout the tropics and warmer regions. It is well known as a source of chew stick and for its bitter taste. It is a popular leafy vegetable among the Ibos of Eastern Nigeria. Its antibacterial properties have been evaluated by Akujobi et al. (2006) and it has been shown to contain cardiac glycosides, saponins, tannins and alkaloids (Akujobi et al., 2006). Extracts of the leaves of *Vernonia amygdalina* are used among the traditional palm wine tapers in Eastern Nigeria to control the microbial load and foaming in palm wine.

Palm wine is an alcoholic beverage produced from a variety of palms such as oil palm (*Elaeis guineensis*) and raphia palm (*Raphia hookeri* and *R. vinetera*). It is drunk in the tropical and subtropical Africa (Okafor, 1978). The sap, irrespective of its origin, is usually a whitish liquid, which effervesces carbon dioxide during fermentation by microorganisms. Palm wine contains

appropriate nutrients, which can support the growth of pathogenic organisms. Among these nutrients are cis-aconic acid, thiamine, riboflavin, pyridoxine and ascorbic acid, as well as protein and simple sugars (Oyagade et al., 2004). Some microorganisms shown to be associated with palm wine are *Leuconostoc mesenteroides*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Lactobacillus plantarum*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Leuconostoc lactis*, *Saccharomyces torulopsis*, *Saccharomyces cerevisiae* and *Saccharomyces chevalieri*. (Oyagade et al., 2004).

Measurement of microbial enzyme activity has been used in the assessment of ecotoxicological impacts of environmental substrates. In this regard, dehydrogenase activity has been widely used. The dehydrogenase assay is an effective primary test for assessing the potential toxicity of metals to soil microbial activities (Aoyama and Nagumo, 1997; Chander and Brookes, 1995; Kelly and Tate, 1989; Rogers and Li, 1985), toxicity of metals to planktonic (Nweke et al., 2006) and heterotrophic (Nweke et al., 2007) bacteria from tropical river sediments. Toxicity of plant extracts to pathogenic bacteria has been assessed using the dehydrogenase assay (Nwaogu et al., 2007; Nwaogu et al., 2008; Alisi et al., 2008)

The aim of the study is to examine the inhibitory activity of dehydrogenase of bacteria isolates in palm wine as a means of control of microbial load in palm wine. This study will also expose new frontiers or improve on the current application of the plant extracts.

MATERIALS AND METHODS

Sample collection and preparation

The leaves of *Vernonia amygdalina* were collected from Nekede, Owerri-west LGA of Imo State, Nigeria. Dr F.N. Nbagwu, a plant taxonomist of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria, identified the plant.

The fresh leaves were dried for four days. The dried leaves were ground into powder form using mechanical grinder. To 100 g of the leave powder in a conical flask were added 200 ml of 95% ethanol. This was covered, shaken every 30 min for 6 h and then allowed to stand for five days. The solution was subsequently shaken and filtered using Whatmann number 1 filter paper. The filtrate was evaporated to dryness using a rotary evaporator (Model type 349/2, Corning ltd). The extract was stored at 4 ^{o}C.

Fresh raphia palm wine were collected from palm wine tapers in Imo State using sterile containers and transported on ice to the laboratory. They were analyzed within 1 h of collection. Bacterial isolates were obtained from the palm wine using pour plate method after 10-fold serial dilution of the palm wine sample. The isolates were identified using their morphological and biochemical characteristics (Ogbulie et al., 2007). The bacterial isolates were *Lactobacillus* sp., *Micrococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Staphylococcus* sp. and *Esherichia coli*.

The bacterial isolates were grown to mid exponential phase in nutrient broth (Lab M) on a rotary incubator (150 rpm) at room temperature (28 ± 2 ^{o}C). The cells were harvested by centrifugation at 6000 rpm for 8 min and washed thrice in distilled water. The washed cells were re-suspended in distilled water and the turbidity adjusted to an optical density of 0.85 at 500 nm. An aliquot of 0.3 ml of the cell suspension was used as inoculum in the dehydrogenase activity assay. The dry weight of the cells was determined by drying a 10 ml aliquot of cell suspension in a pre-weighed crucible to constant weight in an oven at 110 ^{o}C.

Antimicrobial activity evaluation

The dehydrogenase assay method as described by Alisi et al. (2008) and Nweke et al. (2007) was adopted for this study. The

dehydrogenase activity (DHA) was determined using 2,3,5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor, which was reduced to the red-coloured triphenylformazan (TPF). The assay was done in 4 ml volumes of nutrient broth-glucose–TTC medium supplemented with varying concentrations (0-2500 µg/ml) of the leaf extract in separate screw-capped test tubes. About 0.3 ml volume of the bacterial suspension was inoculated into triplicate glass tubes containing 2.5 ml of phosphate-buffered (pH 6.8) nutrient broth-glucose medium supplemented with varying concentrations of the plant extract solution. They were incubated in a rotary incubator (150 rpm) at room temperature (28 ± 2 ^{o}C) for 30 min. Thereafter, 1 ml of 0.4% (w/v) TTC in deionized water was added to each tube to obtain final extract concentrations of 0, 20, 50, 100, 200, 400, 800, 1400, 2000 and 2500 µg/ml in different test tubes. The control consisted of the isolates and the medium without *Vernonia amygdalina* extract. The reaction mixture was further incubated statically at room temperature (28 ± 2 °C) for 16 h. The triphenylformazan produced was extracted in 4 ml amyl alcohol and determined spectrophotometrically at 500 nm. The amount of formazan produced was determined from a dose-response curve [0-200 µg/ml TPF (Sigma) in amyl alcohol]. Dehydrogenase activity was expressed as mg of TPF formed per mg dry weight of cell biomass per hour. Inhibition of dehydrogenase activity in the isolates by *Vernonia amygdalina* extract was calculated relative to the control. The percentages of inhibition of each of the test organisms were linearized against the concentrations of the extracts using gamma parameter (r) [r = % Inhibition / (100 - % inhibition)] (Kim et al., 1994). The toxicity threshold concentrations (IC_{50}) were then determined from the linear regression plots. The total inhibitory concentrations (IC_{100}) were estimated from the linear regression of log transformation plots of the dose-response data.

Statistical analysis

Data obtained from this study were analyzed using two-way analysis of variance (ANOVA) and values for $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Six different pathogenic bacterial species were isolated from palm wine. They are *E. coli*, *Staphylococcus* sp., *Bacillus* sp., *Lactobacillus* sp., *Streptococcus* sp. and *Micrococcus* sp. Some of these bacterial species (*E. coli*, *Lactobacillus* and *Staphylococcus*) were reported by Oyagade et al. (2004) to be associated with palm wine. However, *Streptococcus*, *Bacillus* and *Micrococcus* species may be contaminants from the vessels used for the palm wine. This also proves that palm wine has enough nutrients to support the growth of pathogenic microorganisms (Oyagade et al., 2004).

The six organisms, when subjected to dehydrogenase assay, showed that they were able to reduce TTC to the red formazan at variable rates. The dehydrogenase activities of the isolates showed that *E. coli* with 1.127 ± 0.032 mg of formazan formed per mg cell dry weight per hour had the least dehydrogenase activity, followed by *Lactobacillus* (1.255 ± 0.042 mg formazan/mg cell dry weight/h). The highest dehydrogenase activity was obtained with *Bacillus* sp. (2.316 ± 0.51 mg formazan/mg cell dry weight/h) (Table 1). This is at variance with the work of Nweke et al. (2006) in which the gram-negative bacteria had higher rate of dehydrogenase activity than the gram-positive ones. These variations may be due to differences in bacterial physiology, including cell wall components or dehydrogenase systems, since different microorganisms have been reported to have different dehydrogenase systems (Praveen-Kumar, 2003). However, the present study is in consonance with reports of earlier studies

(Nweke et al., 2007; Nwaogu et al., 2007; Nwaogu et al., 2008; Alisi et al., 2008).

The effect of the different concentrations of the extracts on the bacterial isolates with respect to the dehydrogenase activity is shown in Figure 1. The response of the bacterial dehydrogenase activities to the extracts is concentration-dependent and varies among the organisms. Dehydrogenase activities were inhibited progressively with increase in concentrations of the extracts in all the bacterial species. However, *E. coli* was most sensitive to the deleterious effect of the extracts followed by *Lactobacillus*, while *Staphylococcus* was more tolerant than the other isolates as depicted by the threshold inhibitory concentration of the extracts against the test organisms (Table 2). The dehydrogenase activity correlated with extracts concentrations with R^2 values greater than 0.88 ($0.8833 \leq R^2 \leq 0.9801$) in all the bacterial isolates (Figure 2).

Table 1: Dehydrogenase activities in the control tests.

Test Organism	Dehydrogenase Activity (mg formazan/mg cell dry weight/h)
E. coli	1.127 ± 0.032
Micrococcus sp	1.734 ± 0.026
Staphylococcus sp	2.132 ± 0.12
Streptococcus sp	1.624 ± 0.053
Bacillus sp	2.316 ± 0.51
Lactobacillus sp	1.255 ± 0.042

TABLE 2: Threshold inhibitory concentrations of *V.amygdalina* extract against the bacterial isolates.

Test organisms	Inhibitory Concentrations (µg/ml)	
	IC_{50}	IC_{100}
Streptococcus sp	324.38 ± 7.2	2476.79 ± 27.7
E. coli	83.27 ± 4.9	1984.93 ± 16.5
Micrococcus sp	423.00 ± 10.1	ND
Bacillus sp	736.82 ± 13.6	ND
Lactobacillus sp	214.93 ± 6.3	2102.82 ± 22.8
Staphylococcus sp	431.41 ± 9.7	ND

ND = Not determined (above 2500 µg/ml)

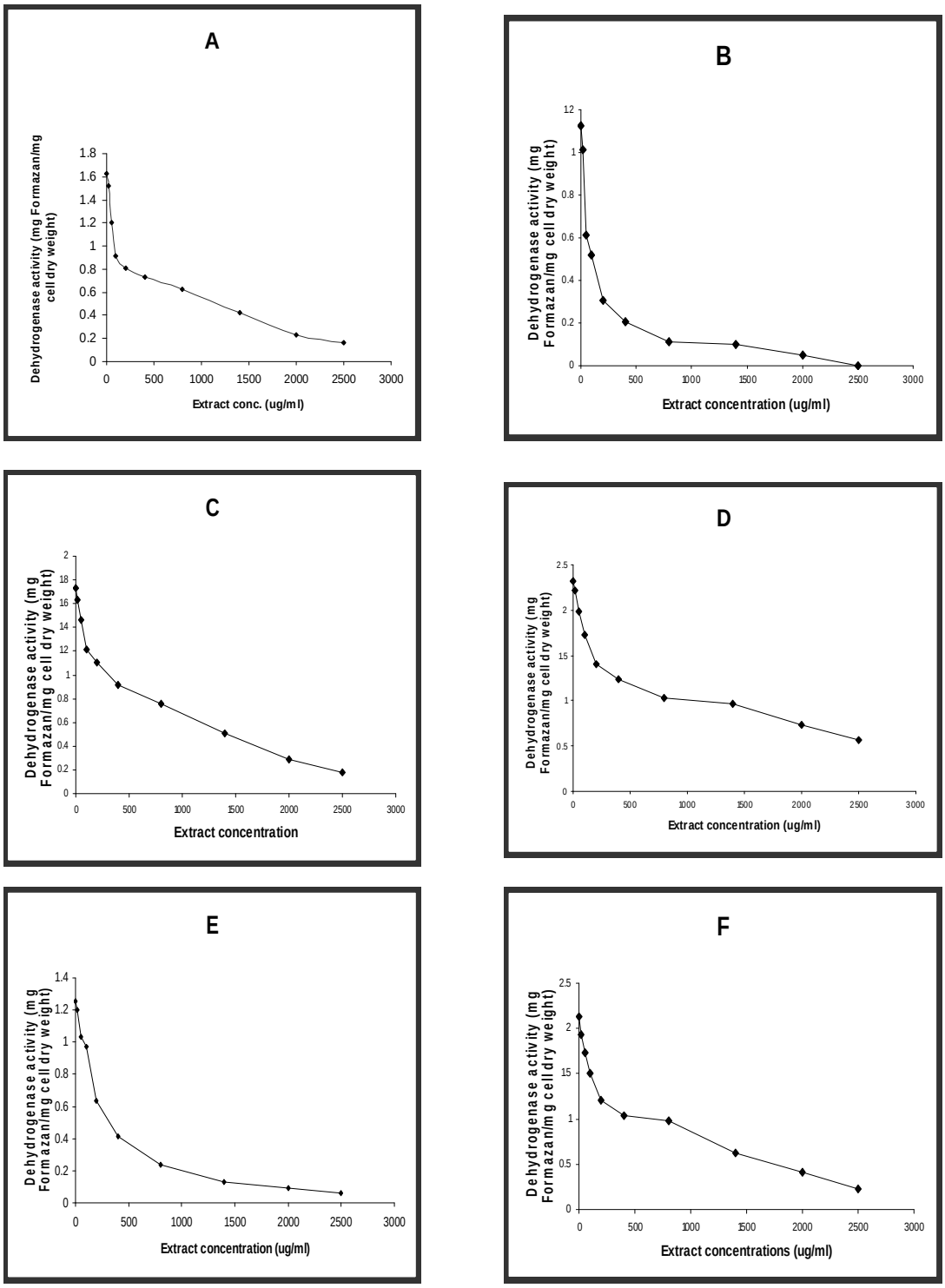

Figure 1: Dehydrogenase activity of (A) *Streptococcus* sp., (B) *E. coli*, (C) *Micrococcus* sp., (D) *Bacillus* sp., (E) *Lactobacillus* sp., (F) *Staphylococcus* sp. in response to various concentrations of *Vernonia amygdalina.*

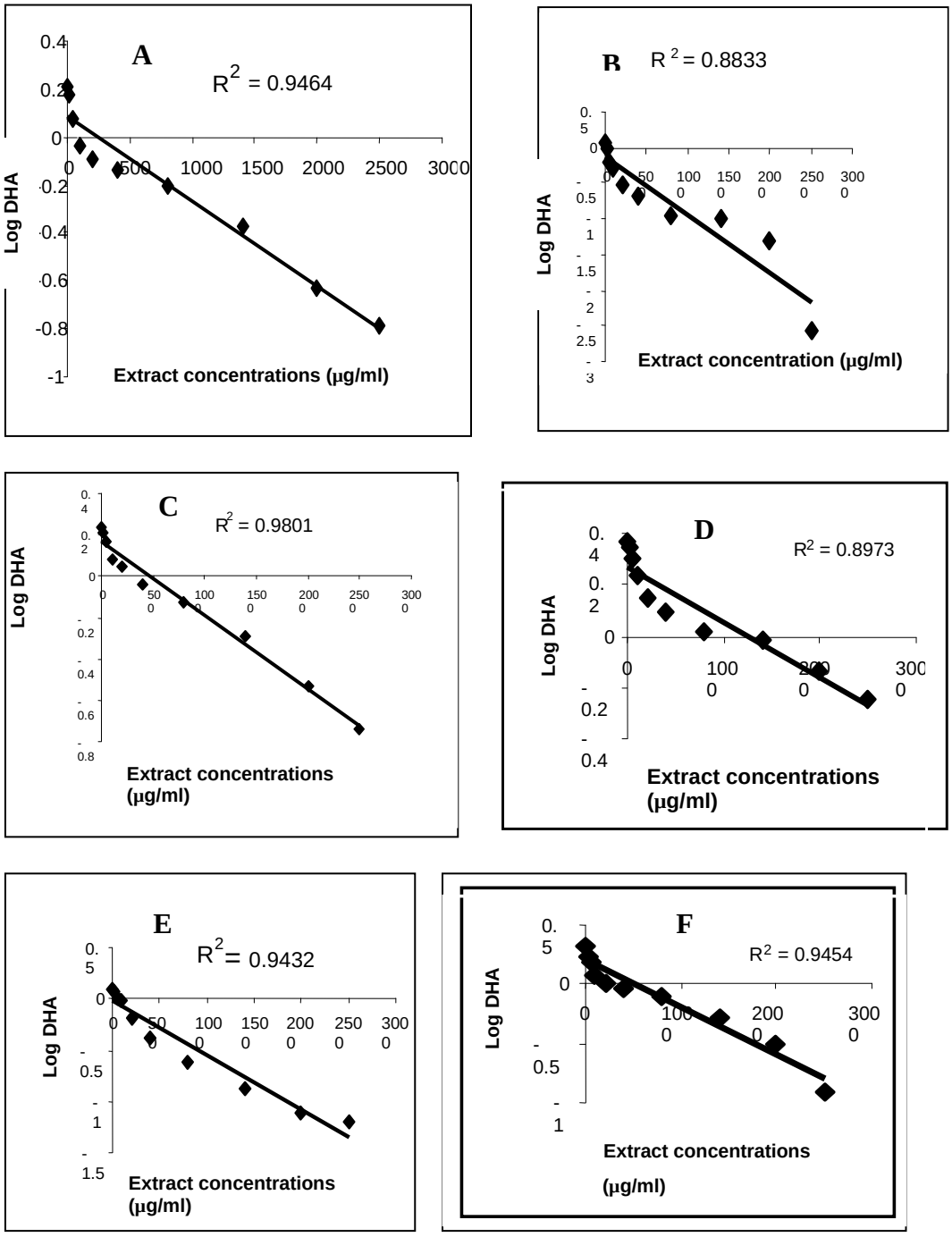

Figure 2: The log transformed plot of dehydrogenase activity of (A) *Streptococcus* sp., (B) *E. coli, (*C) *Micrococcus* sp., (D) *Bacillus* sp., (E) *Lactobacillus* sp., (F) *Staphylococcus* sp. in response to various concentrations of *Vernonia amygdalina*.

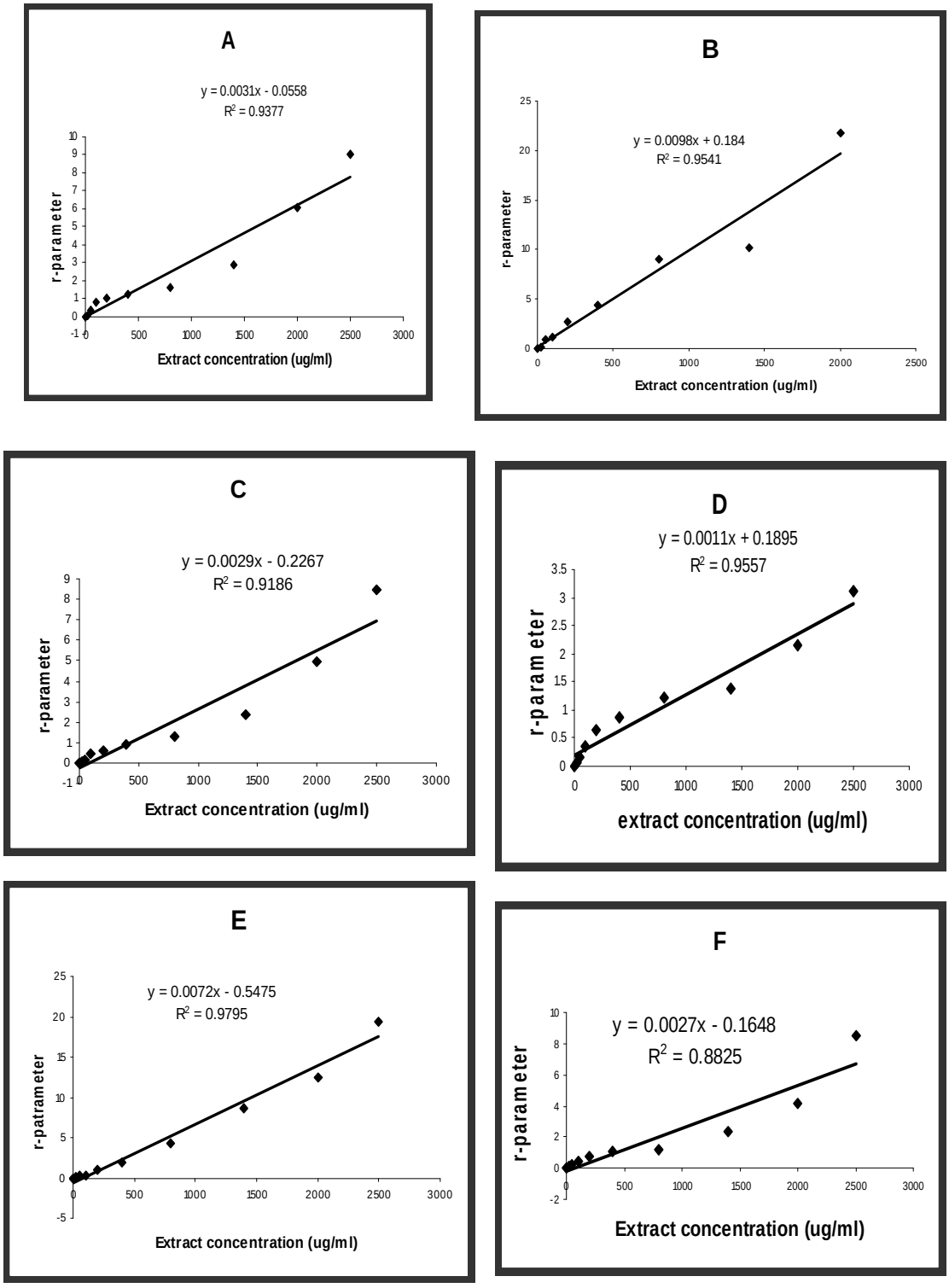

Figure 3: Gamma (Γ) parameter values of (A) *Streptococcus* sp., (B) *E. coli*, (C) *Micrococcus* sp., (D) *Bacillus* sp. , (E) *Lactobacillus* sp., (F) *Staphylococcus* sp. in response to various concentrations of *Vernonia amygdalina.*

The high R^2 values (> 0.88) observed with all the bacterial isolates indicated that extract concentration was a strong determinant of the dehydrogenase activity. This indicated that increase in the concentration of the extract would seriously affect the carbon metabolism and respiratory activities in these bacterial isolates. This finding corroborates the reports of Osadebe and Ukwueze (2004) who found that various plant extracts inhibit the growth of some hospital bacterial isolates.

The gamma parameter gave a good linearization of the dose-response data with the R^2 values greater than 0.88 ($0.8825 \leq R^2 \leq 0.9795$) in all the bacterial isolates. The gamma parameter models had higher R^2 values than the % inhibition plots (results not shown) and hence the linear regression models were used to assess the threshold inhibitory concentration of the extracts on the organisms. The 2-way analysis of variance shows that the dehydrogenase activity varied significantly ($P < 0.05$) with bacterial strain and extract concentration.

In conclusion, the extract of *Vernonia amygdalina* inhibited the dehydrogenase activity of *E. coli*, *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* sp., *Lactobacillus* sp. and *Micrococcus* sp. The inhibitory action may be due to the presence of cardiac glycosides, saponnins, tannins and/or alkaloids reported in our earlier study (Akujobi et al., 2006). This study further justifies the use of leaf extracts of *Vernonia amygdalina* in the control of microbial load and foaming in palm wine among the traditional palm wine tapers in Eastern Nigeria.

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