Microbial Dehydrogenase Activity Reduction by Leaf Extract of Newbouldialeavis: A Marker of its Antimicrobial Activity

Anyalogbu Ernest Anayochukwu Aniemeka

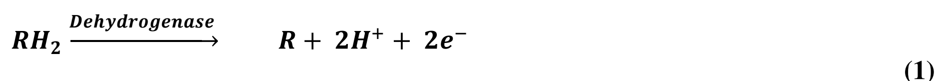
Department of Biotechnology, School of Biological Science, Federal University of Technology Owerri, Imo State, Nigeria

Abstract: Antimicrobials killor inhibit the growth of microorganisms thereby commensurately reduce the overall dehydrogenase activity of the culture. This study investigated the antimicrobial properties of the ethanolic leaf extract of the plant, *Newbouldialeavis* by evaluating the reduction in dehydrogenase activities of some pathogens exposed to it. Using approved methods, standardized cells of the isolates: *Salmonella* (*enterica*) *typhi, Staphylococcus aureus* and *Escherichia coli* were cultured and supplemented with graded concentrations of the extract and control drug. Hydrogen ions and electrons released by the dehydrogenase activities of the cultures stoichiometrically convert triphenyltetrazolium chloride to triphenyl formazan which is quantified spectrophotometrically. The leaf extract and the control drug - Gentamycin produced a dose-dependent %inhibition in dehydrogenase activities in the three pathogens giving the range: 6.25 to 93.75 and 56.41 to 90.38 in *S. typhi*, 24.59 to 70.49 and 66.47 to 91.17 in *S. aureus* and, 3.20 to 100.0 and 6.45 to 88.17 in *E. coli* respectively. The reduction by the extract was more pronounced in the two Gram negative, rod-shaped pathogens: *S. typhi* and *E. coli* with the latter being more susceptible. From this work, relative to the control drug, extract of *N. leavis* possesses antimicrobial potentials which could be exploited in the treatment of diseases in which the pathogens especially *S. typhi* and *E. coli* are implicated.

Keywords: pathogens, triphenyltetrazolium chloride, triphenylformazan, threshold inhibitory concentration.

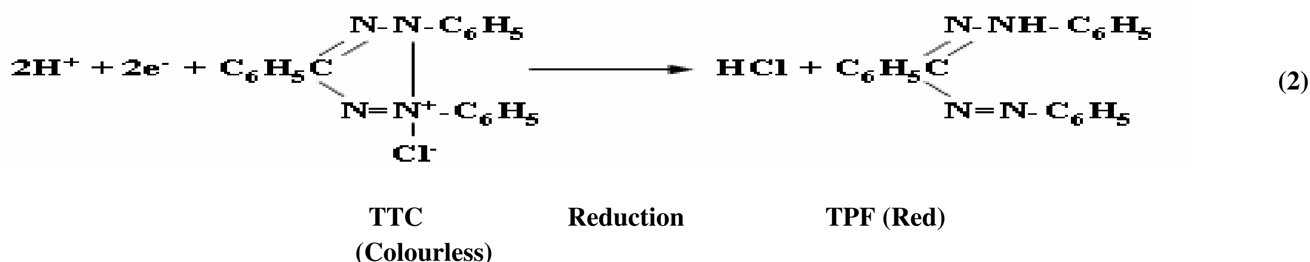
Introduction:

The traditional method of estimating microbial population in a culture medium by counting colony forming units (CFUs) on plates has been recognized as arduous and flawed due to human error (Frost *et al*., 2016). Hence, the need for a faster and more accurate method.Dehydrogenase is an intracellular enzyme found in all living microbial cells and its activity (DHA) is thus considered an indicator of overall microbial activity in a medium (Januszek*et al*., 2014; Nwanyanwu*et al*.,2017).According to Wolińska*et al*.(2016) the use of dehydrogenase activity (DHA) as an indicator of metabolic (electron transport chain) activity of microorganisms was initiated by Lenhard in1956. Wolińska and Stępniewska (2012) reported that Casida and co-researchers in1964 proposed a method for the determination of dehydrogenase activity in microorganisms. In this method, a dye such as 2, 3, 5-triphenyltetrazolium chloride (TTC) is employed as indicator of the electron transport system/respiratory activity (Januszek *et al*., 2014) involved in dehydrogenaseactivity and therefore invaluable in quantifying microbiological activity (Wolińska *et al*., 2016). Frost *et al*. (2016) showed that the use of 2,3,5-Triphenyltetrazolium chloride (TTC) technique was a viable alternative to the traditional time-consuming manual counting of CFUs. The 2,3,5-triphenyltetrazolium chloride (2-(p-Iodophenyl)-3-(pnitrophenyl)-5-phenyl tetrazolium chloride), awater soluble heterocyclic organic salt, is a colourless redox indicator which upon reduction produces water insoluble red 1,3,5-triphenyl formazan (TPF) crystals which can be quantified by the use of the UV-Visible Spectrophotometry(Junillon*et al*. 2012., Wolińska *et al*., 2016).Dehydrogenase enzyme assay provides a direct measure of the enzyme activity which is a direct indication of the active microbial biomass/population(Subhani*et al*., 2001). Dehydrogenase by its activity (oxidation of organic compounds) releases hydrogen ions and electrons (Equation 1). This stoichiometrically reduces colourlessTTC (hydrogen acceptor) to red-colouredTPF (Equation 2).



(1)

Oxidation of organic substrate



(2)

TTC (Colourless) **Reduction** **TPF (Red)**

As stated by Mahmoud and Ghaly (2006) and, Wang *et al*. (2019), one unit of dehydrogenase activity (DHA) reduces 1 µmol of TTC to 1 µmol of TPF per minute.As only live, actively growingmicroorganisms could through theirdehydrogenase activity reduce TTC to TPF (Kang *et al*., 2014, Wolińska *et al*., 2016), the reduction in the total TPF produced from TTC in the presence of an antimicrobial agent therefore indicates reduction/inhibition of microbial dehydrogenase activity. This in turn should be an indication of the reduction on the microbial populationbrought about by the antimicrobial agent vice-versa (Subhani *et al*., 2001;Ujowundu, 2017). Although extracts and active constituents of plant have been in use for treatment of diseases, as stated byReghu*et al*.(2017) and,Khameneh*et al*.(2019) the appearance of antibiotic-resistant microbes and hence, newer and more fatal diseases (Barker*et al*., 2022) have necessitated the need to discover novel antimicrobials.This need prompted this study on the antimicrobial properties of *Newbouldialeavis* leaf extract via its inhibition of dehydrogenase activities in some pathogens.*Newbouldia laevis* (*P. Beauv*) also known as Boundary tree, an angiosperm of the family *Bignoniaceae*is found wild or grown as living hedge in West tropical Africa - Senegal to Cameroon and Gabon (Hassan, *et al*. 2015). The plant is an evergreen shrub/small tree reaching height 20 m (in Eastern Nigeria) and girth 2.70 m (in S. Leone) with shiny dark green leaves and showy terminal purple flowers (Burkill, 1985).It is known in various Nigerian languages as: Ogilisi or ògírìsì (Igbo), Ogiriki (Urhobo), Íkhímì (Edo), Kontor (Tiv), Itömö (Ibibio), Akoko (Yoruba), Aduruku (Hausa) and in other African countries as lifui (Togo), Sesemasa (Ghana), Gimgid (Senegal), Kallihi (The Gambia), Canhom (Guinea), Sherbro (Sierra Leone) and Kinkin (Mali) (Bafor and Sanni, 2009; Akerele*et al*., 2011). The plant is renowned in African folk medicine for the use of its parts in the treatment of a range of diseases (Akerele *et al*., 2011; Egba*et al*., 2014). The common causes of these diseases are some pathogenic microorganisms. Among these are*Salmonella typhi, Staphylococcus aureus* and*Escherichia coli*.

Salmonella (*enterica*) *typhi*, a Gram negative rod-shaped, flagellated facultative anaerobic obligate bacterium of the family *Enterobacteriaceae* is the causative agent for typhoid fever. Globally, on annual basis, close to 21 million people have typhoid fever related cases while about 161,000 deaths are recorded (Butt*et al*.,2022).*Staphylococcus aureus*, a Firmicutes, on the other hand is a round-shaped facultative bacterium that exhibit positive responses to Gram staining and, catalase and nitrate reduction tests. The organism is commonly found commensal in the upper respiratory tract and on the skin but could become an opportunistic pathogen causing a range of diseases via pus-forming or toxin-mediated mechanisms (Ondusko and Nolt, 2018).

Escherichia coli,also of the family *Enterobacteriaceae*and rod-shaped, is a Gram- and oxidase- negative bacterium. Scientific world is now having a rethink about the organism being just an innocuous normal intestinal microflora of endotherms that is widely used in recombinant DNA technology as a cloning host. Croxen *et al*. (2013) attributed much sicknesses and deaths to pathogenic variants of *E. coli*. According to Cortes-Penfield*et al*.(2017);Delpech*et al*.(2018), there is global riseof multi-resistant isolates of *E. coli* with

capacity for Extended Spectrum Beta-Lactamase (ESBL) productionwhich alarminglyprecipitated increased antimicrobial resistance for some drugs.

In this work, the reduction in the dehydrogenase enzyme activity in these pathogens in the presence of *Newbouldialeavis* leaf extract was assayed as a marker of the extract'santimicrobial activity.

Materials and Methods

Chemicals and Reagents. Analytical grade chemicals and reagents were used and include: Ethanol, Amyl alcohol, Nutrient agar, 2,3,5-triphenyltetrazolium chloride (TTC), 1,3,5-triphenyl formazan (TPF), phosphate buffer (pH 6.8) from FlukaChemie GmbH, Industriestrasse 25, Buchs Switzerland. And, nutrient broth (CM0001B) (ThermoFisher Scientific Inc., 168 3rd Avenue, MA. USA).

Sample collection and preparation:

Wholesome fresh leaves of *Newbouldialeavis* were collected from the plant at Ojoto community, Idemili South Local Government Area of Anambra State, Nigeria. The leaves were rinsed with distilled water, shookvigorously to remove most of the water and dried to constant weight under shade. The dried leaves were pulverized in a mill (Kenwood BL357), extracted using 72% ethanoland the extract concentrated using a rotary evaporator as described by Ujowundu (2017) with slight alteration.*Escherichia coli* and *Staphylococcus aureus* and, *Salmonella typhi*were sourced from vaginal swab and stool respectively. The isolates were purified on nutrient agar (Fluka)plates, characterized and identified to generic levels via standard microbiological procedures involving Gram staining and an array of biochemical tests (Nwanyanwu*et al*. 2017; Ujowundu 2017;Anyalogbu*et al*., 2021).Inoculum used was prepared as described by Akujobi*et al*.(2010) with slight modification. The isolates were grown to mid exponential phase in nutrient broth on a rotary incubator (150 rpm) at room temperature (28 ± 2 ^{0}C), harvested by centrifugation at 6000 rpm for 8 min and then washed twice with de-ionized water. The washed cells were re-suspended in de-ionized water and then standardized in a spectrophotometer to an optical density of 0.70 at 420 nm. The dry weight of the standardized cells was obtained by drying a 2.50 ml aliquot to constant weight in an oven set at 110^{0}C. A 0.3 ml aliquot of the standardized cells was used as inoculum in the dehydrogenase enzyme / Antimicrobial activity assay.

Dehydrogenase enzyme / Antimicrobial activity assay.

The method described byAkujobi*et al*.(2010) was used (with slight modification) in this assay. Dehydrogenase enzyme activity reduction was quantified using 2,3,5- triphenyltetrazolium chloride (TTC) as artificial electron acceptor. TTC, a water soluble colourless organic salt, was stoichiometrically reduced to red water-insoluble 1,3,5-triphenyl formazan (TPF) crystals by accepting electrons produced by the activity of the enzyme in viable cells (Equations 1 & 2). In this assay reaction mixture was first prepared.A 2.5 ml of phosphate-buffered (pH 6.8) nutrient broth-glucose medium supplemented with a concentration of the extract solution was placed in a screw-capped test tube and inoculated with 0.3 ml of the standardized cells. The culture was incubated in a rotary incubator (set at 150 rpm and 28 ± 2 0C) for 30 min. Then 1.0 ml of 0.4% (w/v) TTC in deionized water was added to the test tube to obtain the final extract concentration. This was done in triplicate with all the concentrations of the extract (0, 25, 50, 100, 250, 500, 1000, and 2000 µg/ml). One set, not supplemented with the extract solution, was prepared and served as control. The mixture was then allowed to stand, at room temperature (28±2^{0}C), for 16 h. Subsequently, the TTC reduced to TPF (during the static incubation period) was extracted with 4 ml of amyl alcohol and the absorbance read in a spectrophotometer set at 500 nm (λmax).A standard dose–response (absorbance) curve (0 - 24 µg/ml TPF in amyl alcohol, y = 0.487x, R2 = 0.997) was prepared from a stock solution produced by dissolving 0.03 g triphenyl formazan in 500 ml amyl alcohol. Via the absorbance, the amount of TPF in the test was extrapolated from a standard curve. The

amount (in milligrams) of TPF formed per mg dry weight of cell biomass in an hour represents the dehydrogenase activity.Microbial Dehydrogenase Activity reduction/inhibition by the leaf extract of *Newbouldialeavis* was calculated relative to the control as follows: % Inhibition of DHA activity = 100 – [(DHA of test /DHA of control) x 100].

For every organism assayed, percentage inhibitions (responses)were linearized (linear regression) against the extracts concentrations (doses)using gamma parameter (ɼ) equation (Nweke*et al*., 2006).

$$ɼ = \frac{\%\text{Inhibition}}{100 - \%\text{Inhibition}}$$

Inhibitory concentration of extracts required to reduce 50% of the dehydrogenase activity, IC50, were thenestimated using the fitted line, i.e., Y = a*X + b, IC50 = (0.5 - b)/a.The total inhibition concentrations (IC100), were estimated using ED50plus V1.0 software (Anuthara and Matthew, 2022).

Statistical Analysis.

All analyses were done in triplicate determinations. Means and standard deviations of the data generated were obtained using Descriptive Statistics in statistical package for social sciences (SPSS) version-20.

Results:

Three pathogenic bacterial species: *Salmonella* (*enterica*) *typhi*(Gram negative and rod-shaped), *Staphylococcus aureus*(Gram positive and round-shaped), and *Escherichia coli*(Gram negative and rod-shaped) were studied for the effect of the leaf extracts of the plant,*Newbouldialeavis*on their dehydrogenase enzyme activity. The results show that in the presence of the extracts, dehydrogenase activities in the organisms were diversely reduced. As shown in Figures: 1, 2 and 3, relative to a control drug- Gentamycin, the reduction/inhibition were more pronounced in the two Gram negative and rod-shaped pathogens: *Salmonella* (*enterica*) *typhi* and *Escherichia coli* with the latter being more vulnerable. From Table:1, the inhibitory concentration of extracts required to reduce the dehydrogenase activity in the organisms by 50%, IC50, calculated from the linear regression plot (Fig.:4) and the total inhibition concentrations (IC100) ranged (µg/ml) between 113.39±0.04 and 153.73±0.02, and 1523.91±0.85 and 1701.41±0.14 respectively.

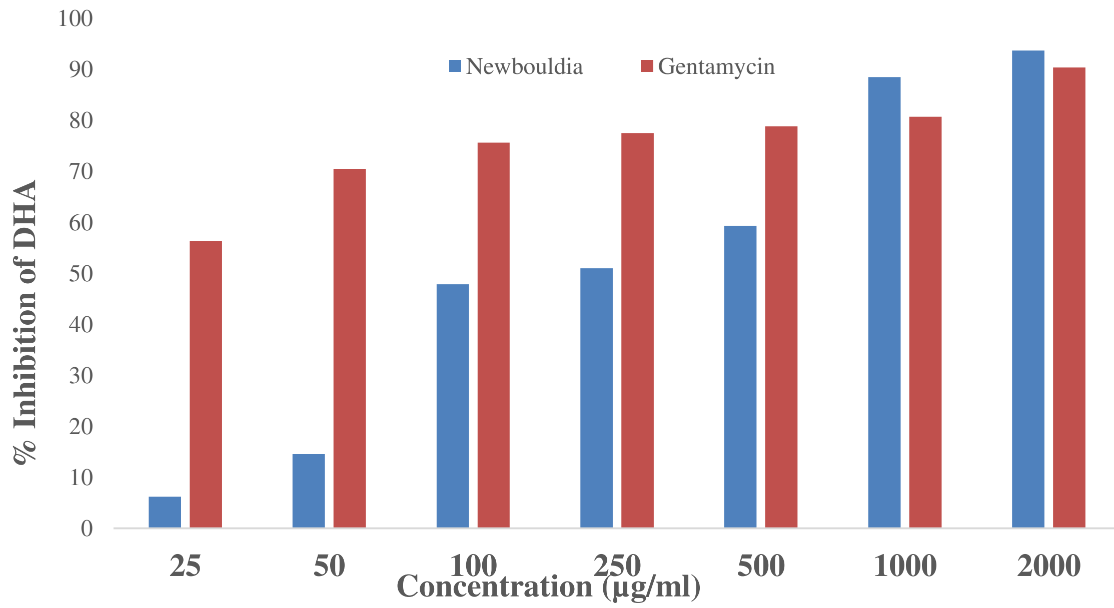


Fig. 1: Percentage inhibition of DHA in S. typhi by N. leavis leaf extract and a control drug - Gentamycin

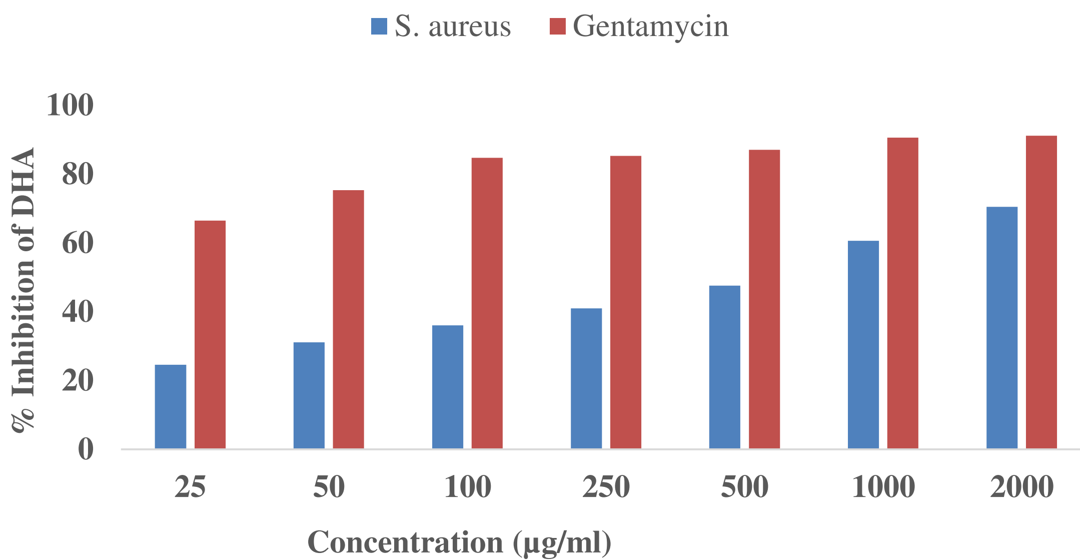


Fig. 2: Percentage inhibition of DHA in *S. aureus* by *N. leavis* leaf extract and a control drug - Gentamycin

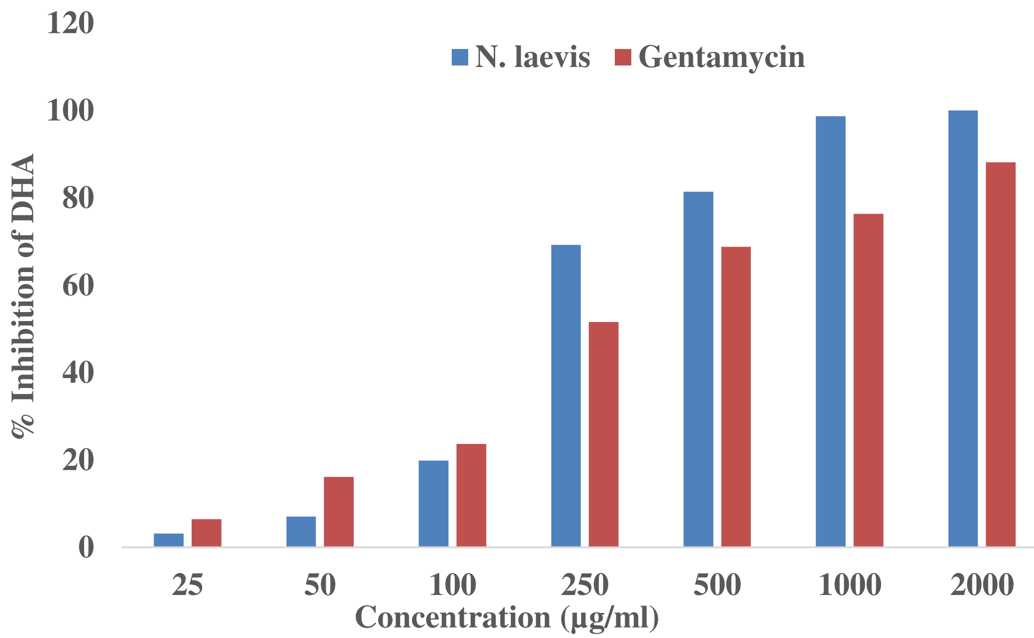


Fig. 3:Inhibition of DHA in E. coli by N. leavis leaf extract and a control drug - Gentamycin

Table 1: Threshold inhibitory concentration of *Newbouldialeavis* leaves extract against the test organisms

Pathogenic organisms	*Newbouldialeavis* leaves extracts (µg/ml)	
	IC50	IC100
Salmonella typhi	123.07±0.03	1615.29±0.42
Staphylococcus aureus	153.73±0.02	1701.41±0.14
Escherichia coli	113.39±0.04	1523.91±0.85

Values are means ± standard deviations of triplicate determinations

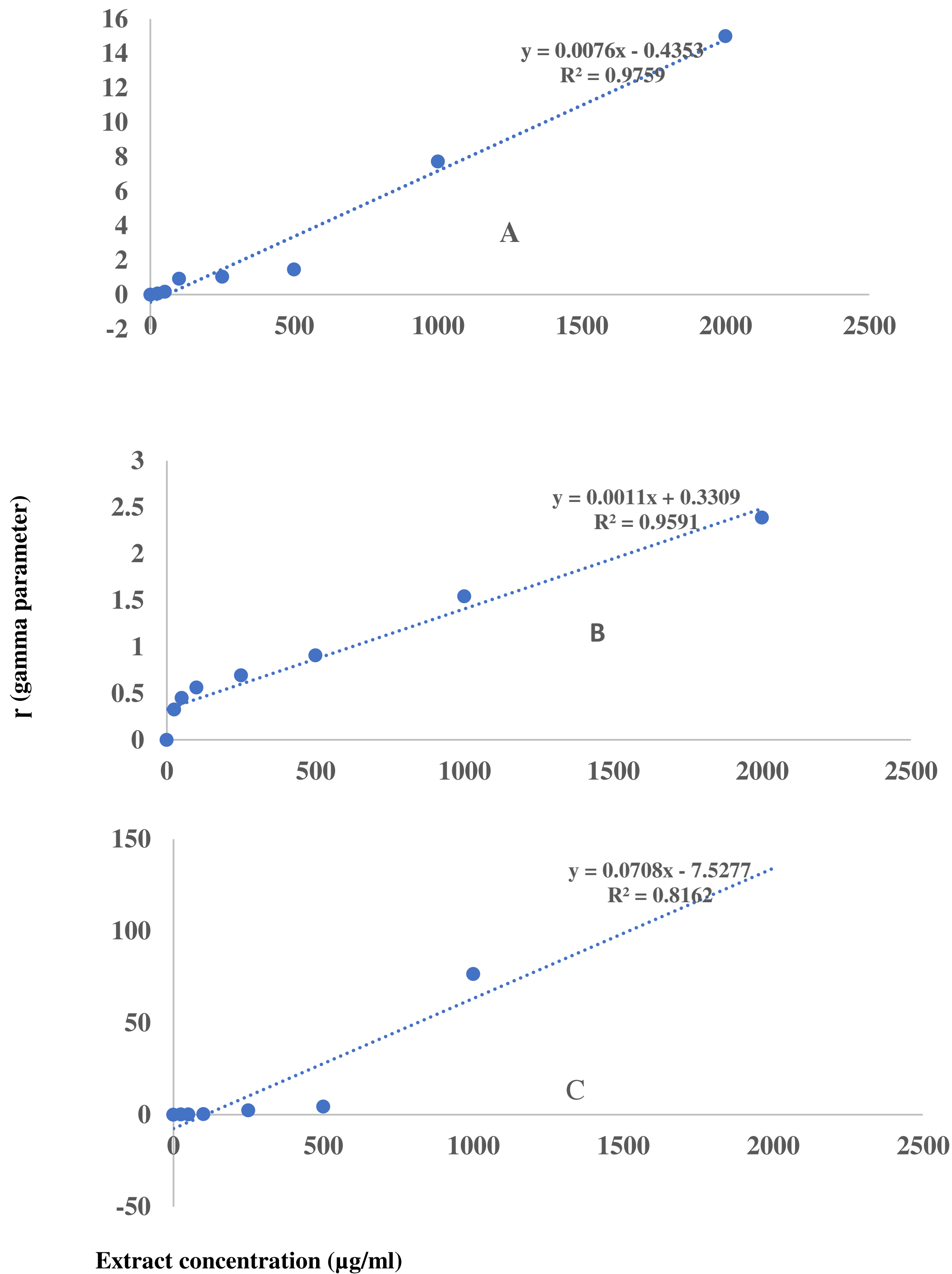


Figure 4: Linear regression of the gamma parameter (ɼ) values obtained from the mean (n=3)percentage inhibition data of leaf extract of *Newbouldialeavis* against: *Salmonella typhi* (A), *Staphylococcus aureus* (B) and *Escherichia coli* (C).

Discussion

Dehydrogenases are intracellular enzymes found in all living microbial cells. Thus, determination of dehydrogenase activity (DHA) in a medium provides a direct measure of the microbial activity which is a direct indication of the active microbial biomass/population in that medium (Subhani *et al*., 2001; Nwanyanwu*et al*.,

2017). Antimicrobials, on the other hand are agents that kill or inhibit the growth of microorganisms (Burnett-Boothroyd and McCarthy, 2011). Change in the dehydrogenase activity of a culture in the presence of an antimicrobial therefore represents the interference of the antimicrobial with the microbial population. Dehydrogenase enzymes oxidize organic compounds releasing hydrogen ions and electrons which stoichiometrically reduce water soluble, colourless2,3,5- triphenyltetrazolium chloride (TTC) to red-coloured water-insoluble 1,3,5-triphenyl formazan(TPF). In this research work, the changes in the dehydrogenase activities in three microbial cultures: *Salmonella (enterica) typhi*, *Staphylococcus aureus* and *Escherichia coli*resulting from the presence of graded doses of ethanolic leaf extracts of the plant,*Newbouldialeavis* and a control drug - Gentamycin were quantified by measuring the colour intensity of the TPF produced.

The results showed that the leaf extract and control drug – Gentamycin produced a dose-dependent reduction/inhibition of the dehydrogenase enzyme activities in the three pathogenic organisms assayed increasing as the doses were increased.This, according to Subhani *et al*., (2001)and Ujowundu (2017) could have resulted from reduction in the number of viable cells which attests to the antimicrobial efficacy of the control drug and indicates the antimicrobial properties of the extract. The percentage reduction by the extract and Gentamycin ranged from; 6.25 to 93.75 and 56.41 to 90.38 in *Salmonella (enterica) typhi* (Fig. 1), 24.59 to 70.49 and 66.47 to 91.17 in *Staphylococcus aureus* (Fig.2), and 3.20 to 100.0 and 6.45 to 88.17 in *Escherichia coli* (Fig. 3) respectively. It is evident from this that *Salmonella* (*enterica*) *typhi*and *Escherichia coli*, Gram negative pathogens, were more susceptible to the extract (with the latter being more pronounced) than *Staphylococcus aureus*(Gram positive). This was supported by the result of the threshold inhibitory concentration of extracts required to reduce 50% of the dehydrogenase activity (IC50), and the total inhibition concentrations (IC100), against the test organisms (Table 1). *Escherichia coli* had the lowest values (in µg/ml) for IC50 and IC100 (113.39±0.04 and 1523.91±0.85), followed by *Salmonella typhi* (123.07±0.03 and 1615.29±0.42) and then, *Staphylococcus aureus* (153.73±0.02 and 1701.41±0.14) respectively. This is of great importance considering the fact that Gram-negative bacteria with their semipermeable outer membrane barrier (Barker *et al*., 2022) are known to be more resistant to antimicrobials than Gram-positive bacteria and contribute immensely in morbidity and mortality worldwide (Breijyeh*et al*., 2020). The higher susceptibility of *E. coli* relative to *S. aureus* was earlier reported by Akujobi*et al*. (2010) working with extract of *Ocimumgratissimum*.Ujowundu (2017) also reported via DHA assay, high susceptibility of *S. typhi* and *E. coli*, to the extract of *Combretum dolichopentalum.* The observeddisparity in susceptibility could be attributed to differences in cell wall components or dehydrogenase systems (Praveen and Tarafdar, 2003;Akujobi*et al*., 2010;Khameneh*et al*., 2019).

The effect of the extract and the control drug were dose-dependent in the three pathogenic organisms assayed increasing as the doses were increased. The high R^2values (>0.81) observed with the organisms in the gamma parameter regression model (Fig. 4) indicated a good linearization of the dose-response data emphasizing that the concentration of the extract was a strong factor in determining the effect on the dehydrogenase activity. Analysis of the result however showed that the extract was more effective in inhibiting the enzyme activity in *Escherichia coli*than the control drug from 250µg/ml concentration and in *Salmonella* (*enterica*) *typhi*from 1000µg/ml concentration, while the control drug had higher inhibition at all concentrations in *S. aureus* (Figures:1-3).

Conclusion:

Dehydrogenase enzyme activity (DHA) which is a measure of the active microbial population was reduced by the *Newbouldialeavis* ethanolic leaf extract in a dose-dependent manner at varying levels of sensitivity in the three pathogenic organisms assayed. Same was also observed with an established antimicrobial – Gentamycin. It could therefore be concluded from this work that ethanolic leaf extract of*Newbouldialeavis* possesses antimicrobial potentials which with further studies could be exploited in the treatment of diseases in which the pathogens; *Salmonella (enterica) typhi*, *Staphylococcus aureus* and *Escherichia coli* are implicated.

Conflicts of Interest: The author declares no conflict of interest.

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