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Antimicrobial action of methanol extract of *Chromolaena odorata*-Linn is logistic and exerted by Inhibition of Dehydrogenase Enzymes

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ABSTRACT:

Inhibition of total dehydrogenase enzyme activity in pathogenic gram positive and gram negative micro organisms exposed to methanol extract of *Chromolaena odorata* was used as an index for assessment of its antimicrobial activity. Assay of total dehydrogenase enzyme activity was done in the test organisms (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*) using 2,3,5-triphenyltetrazolium chloride (TTC) as an artificial electron acceptor which was reduced to the red-coloured triphenyl-formazan. Response of the bacterial isolates varied with extract concentration. Dehydrogenase activity was progressively inhibited in a logistic dose-response fashion. The gram negative *Escherichia coli* responded more markedly than *Pseudomonas aureginosa* and gram positive *Staphylococcus aureus*. Inhibitory concentrations (IC_{50}) of the methanol extract against *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa* were 208.49 µg/ml, 1361.01 µg/ml, and 903.08 µg/ml respectively. Preliminary phytochemical screening of the extract gave positive reactions for alkaloids, flavonoids, tannins, 4-hydroxybenzoic acid, and glycosides. These phytochemicals may be responsible for the observed inhibition of total dehydrogenase enzyme activity that translates to antimicrobial action in these pathogenic organisms.

Keywords:
Oxidoreductases, toxicity, enzyme inhibition, wound isolates, phytochemicals, and bacterial response.

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INTODUCTION

Selection of scientific and systematic approach for the biological evaluation of plant products based on their use in the traditional systems of medicine has continued to form the basis for an ideal approach in the development of new drugs from plants. *Chromolaena odorata* (L.) R. KING & H. ROBINSON (formerly *Eupatorium odoratum* L.), a perennial belonging to the plant family *Asteraceae* (=Compositae), is a diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures of Southern Asia and Western Africa. It is a weed of 13 crops in 23 countries and has been described as the world's worst weed (Holme *et al*., 1977). This common plant called Siam weed is known among the Igbos of the South-Eastern Nigeria as: 'Elizabeth', 'Independence leaf', 'Enugu plantation weed', or 'Awolowo weed'. *Chromolaena odorata* is used as a gargle for sore throat and cold. Traditionally, fresh leaves or a decoction of *C. odorata* have been used throughout Vietnam for many years as well as in other tropical countries for the treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis (Le, 1995). Decoctions of leaves have been used by traditional healers in the South-Eastern Nigeria for the treatment of liver diseases.

Several subclasses of flavonoids have been isolated from *C. odorata* extracts. The phenolic acids present in ethanol extract of *C.odorata* are protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids. The complex mixtures of lipophilic flavonoid aglycones present in *C.odorata* included flavanones, flavonols, flavones and chalcones. *C. odorata* also contains high concentrations of amino acids (Phang, *et al*., 2001). The use of the total dehydrogenase assay has been used as a tool in probing response of microorganisms to antibacterial agents (Alisi *et al*., 2008) and is recognized as a useful indicator of the overall measure of the intensity of microbial, metabolism (Tabatabi 1982; von Marsi and Schinner, 1991). This method is preferred over culture method for enumeration of microorganisms which can underestimate number of viable cells due to lack of homogeneity in distribution or difficulty in being readily desorbed from the substrate matrix (Oberbremer and Muller- Hurtig, 1989; Torstensson, 1997). Dehydrogenase assay is also an effective primary test for assessing the potential toxicity of metals to planktonic (Nweke, *et al*., 2006), and heterotrophic (Nweke, *et al*., 2007) bacteria. We had earlier also assessed toxicity of antimicrobial agent to pathogenic bacteria using the dehydrogenase assay (Alisi, *et al*., 2008; Nwaogu, *et al*., 2008; Nwaogu, *et al*., 2007).

Results of an earlier study showed that the extract of the leaves of *C. odorata* inhibited the growth of some bacteria (Alisi and Onyeze, 2009). Inhibition of dehydrogenase enzymes in pathogenic pure microbial cultures by methanol extracts of *C. odorata* has not been demonstrated. This work is therefore aimed at studying the inhibition of total dehydrogenase enzymes in pathogenic pure microbial cultures exposed to methanol extract of *Chromolaena odorata*.

MATERIALS AND METHODS

Plants

Fresh aerial parts of *C.odorata* were collected from Egbu and Ihiagwa in Owerri North and Owerri West local Government areas of Imo State respectively. Plant was authenticated by Professor S.E.Okeke, a plant taxonomist, of the Department of Plant Science and Biotechnology, Imo State University Owerri, Imo State. Voucher specimen is deposited in the author's laboratory.

Extract Preparation

The aerial part of *C. odorata* was shed dried at room temperature and reduced to a coarse powder in a mill (Kenwood BL357). The powder was extracted with methanol. The extract was recovered by distillation under reduced pressure at 49^{o}C in a rotary evaporator-Buchi rotavapour (Switzerland). The extracts were then dried to solid forms in vacuum desiccators, and stored in a freezer (<-4.0 ^{0}C).

Table 1: Phytochemical constituents of *Chromolaena.odorata methanol* extracts

	Alkaloids	Flavonoids	Tannins	Saponins	Glycosides	Cardiac glycosides	Steroidal aglycone	Protein	4-HBA (p-OH-benzoic acid)
Dry plant	+	+	+	+	+	+	+	+	+
MECO	+	+	+	-	+	+	+	+	+

Key: + = presence, - = absence,

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Table 1: Equations

%inhibition of DHA $y = \left[\frac{CA-TA}{CA}\right] \times 100$ ..... Eqn 1

y = %inhibition of DHA
CA = DHA of control
TA = DHA of Test

$$Y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d}$$ ..... Eqn 2

Where:
a = $Y_{(predicted)}$ at $X_{(max)}$,
b = Ymax (predicted)
c = slope parameter defining inhibition rates
d = empirical value
x = Extract concentrations (µg/ml)
y = %inhibition of DHA

$y = a + b\,[1-\exp(-((x+d\ln(2)^{1/e} - c)/d)^e)]$ ..... Eqn 3

Where: a,b,c,d and e = empirical values
x = Extract concentrations (µg/ml)
y = %inhibition of DHA

$$\Gamma\text{-Parameter} = \frac{\%INHIBITION}{100 - \%INHIBITION}$$ ..... Eqn 4

$$y = a + \frac{b}{\ln x} + \frac{c}{(\ln x)^2} + \frac{d}{(\ln x)^3} + \frac{e}{(\ln x)^4} + \frac{f}{(\ln x)^5} + \frac{g}{(\ln x)^6}$$ ..... Eqn 5

Where: a,b,c,d,e,f and g = empirical values

a	b	c	d	e	f	g
12.277894	-271.31474	2906.6846	-16437.144	51228.94	-82930.789	54093.69

x = Extract concentrations (µg/ml)
y = %inhibition of DHA

Isolation of bacterial strains and culture conditions

Pathogenic bacteria (*Pseudomonas* sp., *Staphylococcus* sp., and *Escherichia* sp. were obtained from degenerated wound. Isolates were purified on nutrient agar (Fluka) plates and characterizations were done using standard microbiological methods. Identifications to the generic level followed the schemes of Holt *et al.* (1994). The bacterial strains were grown to mid exponential phase in nutrient broth (Lab M) on a Marrienfeld rotary incubator (150 rpm) at room temperature (28 ± 2°C). The cells were harvested by centrifugation at 4000 rpm for 10 min. Harvested cells were washed twice in deionised distilled water and re-suspended in water. The re-suspended cells were standardized in a spectrophotometer to an optical density of 0.70 at 420 nm. The dry weights of the standardized cells were determined by drying volumes of cell suspension to constant weight in an

Table 2: Showing the threshold inhibitory concentrations of methanol extracts of *C.odorata* against the total dehydrogenase activity (DHA) of some wound isolates (*Escherishia.coli, Staphylococcus.aureus, and Pseudomonas.aureginosa)*

	Inhibitory Concentrations Against Wound Isolates MECO(µg/ml)				
	IC_{20}	**IC_{50}**	**IC_{70}**	**IC_{80}**	**IC_{100}**
Escherishia coli	19.15	208.49	*	4736.25	9755.00
Staphylococcus aureus	111.11	1361.01	*	ND	ND
Pseudomonas aureginosa	206.27	903.08	1843.57	2605.12	5473.75

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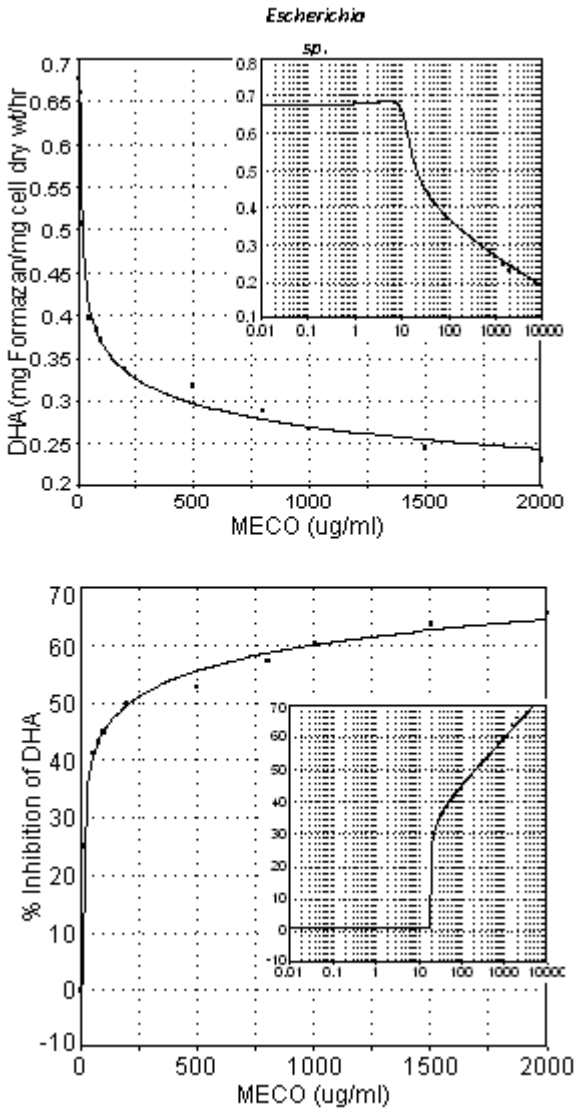

Figure 1a: Inhibition of dehydrogenase activity (mg Formazan/mg cell dry weight/hr) in pathogenic (wound isolates) bacteria (*Escherichia.sp*) by ethanol extract of *Chromolaena odorata*- showing dehydrogenase activity, percentage inhibition and log %inhibition with graded concentrations of methanol extract of *C.odorata*.

oven at 110°C. These standardized cell suspensions were used as inoculums in the dehydrogenase activity assay.

Screen Test for TTC reduction (Dehydrogenase activity)

On a colony of each bacterial isolate growing on nutrient agar, one drop of 1:1 mixture of aqueous solution of TTC (0.4 %w/v) and glucose (2 & w/v) was placed. The plates were incubated at room temperature for 10 minutes. Production of red coloured formazan was suggestive of TTC reduction.

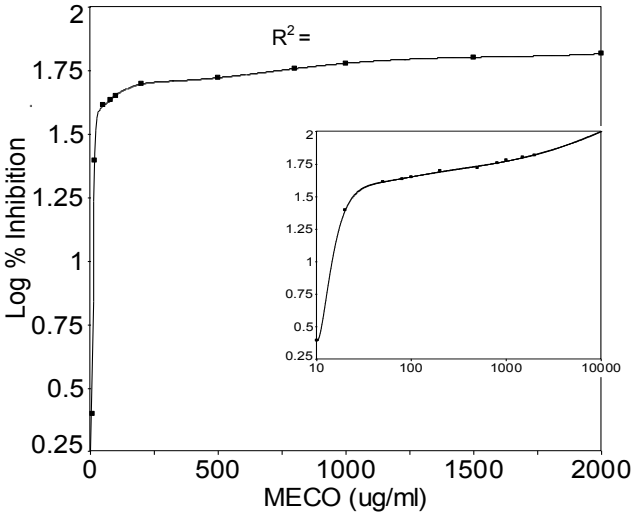

Figure 1b: Plot of Log percentage inhibition of DHA against graded concentration of methanol extract of *chromolaena odorata*

Determination of Antimicrobial Potentials of *C. odorata* extracts by total dehydrogenase activity (DHA) assay

Total dehydrogenase assay method as described by Alisi *et al*. (2008) was employed Briefly, total dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride (TTC) (BDH England) as the artificial electron acceptor, which was reduced to the red-colored triphenyl-formazan (TPF). The assay was done in 4 ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations (0 – 2000 µg/ml) of extract in separate 20 ml screw-capped test tubes. Portions (0.3 ml) of the bacterial suspensions were inoculated into triplicate glass tubes containing 2.5 ml of phosphate-buffered (pH 6.8) nutrient broth-glucose medium amended with *Chromolaena odorata* extract and pre-incubated on a rotary incubator (150 rpm) at room temperature (28 ± 2 °C) for 30 min. Thereafter, 0.1 ml of 1 % (w/v) TTC in deionised distilled water was added to each tube to obtain final extract concentrations of 0-2000 µg/ml in different test tubes. The final concentrations of nutrient broth, glucose and TTC in the medium were 2, 2 and 0.25 mg/ml, respectively. The controls consisted of the isolates and the media without *Chromolaena odorata* extract. The reaction mixtures were further incubated statically at room temperature (28 ± 2 °C) for 8.0 h. The TPF produced were extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 500 nm (λmax). The

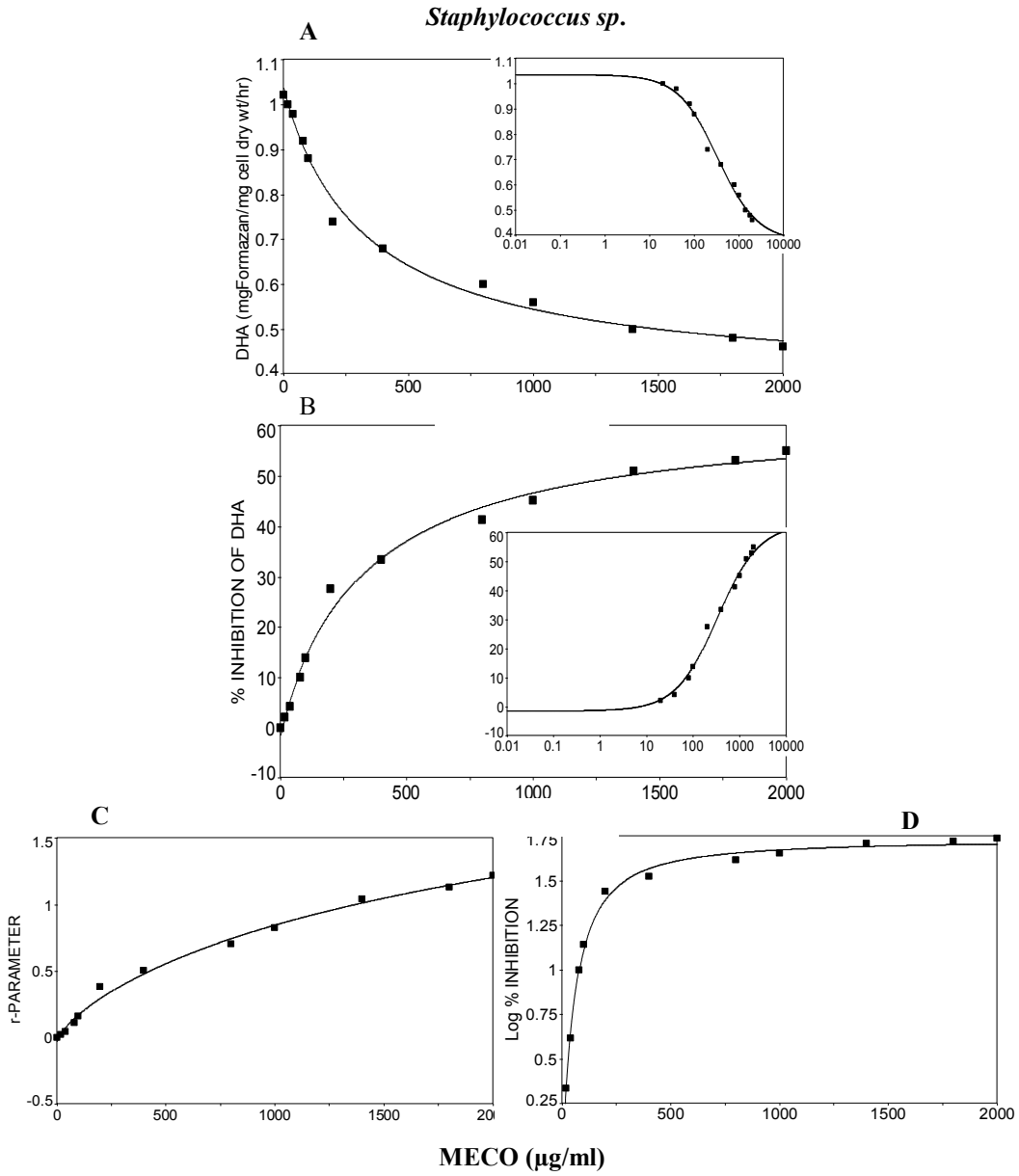

Fig.2 : Inhibition of dehydrogenase activity (mg Formazan/mg cell dry weight/hr) in Pathogenic (wound isolates) bacteria(*Staphylococcus. sp.*) by methanol extract of *Chromolaena odorata*. Plots (A,B,C,and D) show dehydrogenase activity, percentage inhibition of DHA, gamma parameters and log %inhibition of DHA on y-axis respectively against graded concentrations of extract on x-axis.

amount of formazan produced was determined from a standard dose-response curve [0 - 20 µg/ml TPF (Sigma) in amyl alcohol; y = 0.0487x; R^2 = 0.9977]. Dehydrogenase activity (DHA) was expressed as milligrams of TPF formed per mg dry weight of cell biomass per hour.

Data Analysis

Percentage Inhibition of dehydrogenase activity by MECO was calculated relative to the control as shown in equation (1) (table 1). The inhibition data generated are fitted into the model equation (2) which is a logistic dose response equation. The parameters were estimated by iterative minimization of least squares using Levenberg-marquardt algorithm (Table curve 2D systat USA) Marquardt (1964). The data of % inhibition fitted into equation (2) were used to evaluate the toxicity thresholds IC_5, IC_{20}, IC_{50}, IC_{70}, IC_{80}, IC_{100} which are the concentrations of the extracts that inhibited 5%, 20%, 50% 70%, 80%

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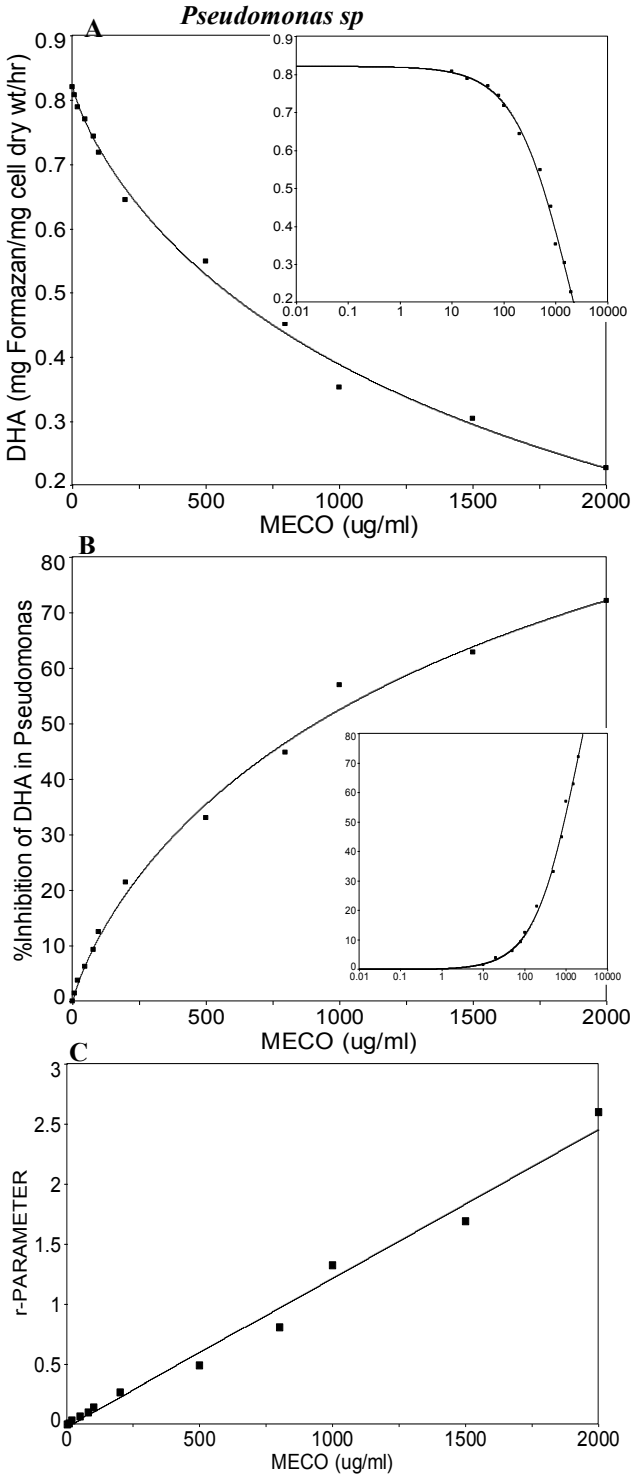

Fig.3: Inhibition of dehydrogenase activity (mg Formazan/mg cell dry weight/hr) in Pathogenic (wound isolates) bacteria(*Pseudomonas sp.*.) by methanol extract of *Chromolaena odorata*. Plots (A,B,and C) show dehydrogenase activity, percentage inhibition of DHA and gamma parameters on y-axis respectively against graded concentrations of extract on x-axis.

and 100% of controls respectively. Where IC_{100} was not determinable (ND) by fitting Eqn (1) into the model (Eqn 2), Equation (1) was transformed to their natural logarithms. Log y was plotted against x. x values at y = 2 were taken as IC_{100}. Data that did not fit into logistic dose response model Equation 2 were fitted into the WEIBULLCUM model (Eqn 3). IC_{50} were obtained from Ŕ-parameter plot where data gave high R^2-value with Eqn 4. Data whose IC_{100} was non-determinable using Equation 2 and 3, were fitted into an inverse Log(x) polynomial equation (Eqn 5). By solving for x in Eqn 5, IC_{100} (the concentration at which MECO will exert total inhibition against the tested organism) was calculated.

RESULTS AND DISCUSSION

The plant *C.odorata* was found to contain Flavonoids, Tannins, Saponins, Glycosides, Steroidal aglycones, Alkaloids and 4-hydroxybenzoic acid. The methanol extract however did not show a positive reaction for saponins (Table 1). These phytochemicals have been found to have medicinal properties and health promoting effects (Raza and John, 2007; Salah *et al.,* 1995; Del-Rio *et al*., 1997; Okwu, 2004; Liu, 2004). The use of *C.odorata* in ethno-medical practice may be due to the medicinal effects of these phytochemicals.

Methanol extract of *C.odorata* inhibited dehydrogenase activity in the organisms in a logistic dose dependent manner. Inhibition of dehydrogenase activity in *Staphylococcus aureus, and Pseudomonas aerugenosa* followed a logistic dose response abcd model (Eqn 1) while E.coli, followed weilbullcum abcde model Eqn (3) and (Eqn 5).

Threshold inhibitory concentrations of the extracts (Table 2) showed that *Pseudomonas auregenosa* responded gradually but steadily. At lower concentrations, the extracts exerted stronger inhibitory effect on the dehydrogenase activity of *Escherishia coli* and *Staphylococcus aureus* than *Pseudomonas auregenosa.* The rate of inhibition of total dehydrogenase enzyme activity in *Escherishia coli* and *Staphylococcus aureus* was not sustained as *Pseudomonas auregenosa* responded more at higher concentrations of *C.odorata* extracts, making IC_{100} in *Escherishia coli* and *Staphylococcus aureus* non-determinable.

The gamma parameter model (Eqn 4) gave a strong linearization of percentage inhibition of DHA by methanol extract of *C. odorata* against

Table 3: Showing models and equations for dehydrogenase inhibition with pearson correlation coefficient in the pathogenic organisms.

Organisms	Model	Equation	R^2-value
Escherishia coli	WEIBULLCUM (abcde)	Eqn (3)	0.9969
	inverse Log(x) polynomial	Eqn (5)	0.9989
Staphylococcus aureus	logistic dose response (abcd)	Eqn (2)	0.9938
Pseudomonas aureginosa	logistic dose response (abcd)	Eqn (2)	0.9981

Pseudomonas aerugenosa. This makes this the model of choice in the determination of IC_{50} in *Pseudomonas aerugenosa*. All equations used in the modelling of the results gave high correlation coefficient ($R^2 \geq 0.99$), with very low Fit standard errors. Inhibition of dehydrogenase activity in the wound isolates showed antimicrobial activity. The extract was toxic to the organism at all concentrations and the nature of inhibition was logistic rather than hormetic (Table 3).

Extracts of the plant *C. odorata* have earlier been shown to contain phenolic compounds (Phan, *et al*., 2001). The presence of phenolic compounds in the plant *C.odorata* has been demonstrated by earlier worker (Phan *et al*., 2001). Derivatives of 4-hydroxybenzoic acid are used as anagelsics and antimicrobial substances. Also, Tannins, flavonoids, and saponins are known to have antimicrobial activities (Aziz *et al*., 1998; Evans, 2002). Hydroxybenzoic acid found in the extract is known to possess antimicrobial activity. The secondary plant metabolites identified in this extract may be acting synergistically to bring about the observed inhibition of dehydrogenase activity. These extracts may actually be exerting their antimicrobial activity via inhibition of dehydrogenase activity in the test organisms.

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