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The Inhibition of *Clostridium chauvoei* (Jakari strain) Neuraminidase Activity by Methanolic Extracts of the Stem Barks of *Tamarindus indicus* and *Combretum fragrans*

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The inhibition of neuraminidase from *Clostridium chauvoei* (jakari strain) with partially purified methanolic extracts of some plants used in Ethnopharmacological practice was evaluated. Extracts of two medicinal plants, *Tamarindus indicus* and *Combretum fragrans* at 100–1000 μg/ml, both significantly reduced the activity of the enzyme in a dose-dependent fashion ($P < 0.001$).

The estimated IC_{50} values for *Tamarindus indicus* and *Combretum fragrans* were 100 and 150 μg/ml respectively. Initial velocity studies conducted, using fetuin as substrate revealed a non-competitive inhibition with the V_{max} significantly altered from 500 μmole min^{-1} mg^{-1} to 240 μmole min^{-1} mg^{-1} and 340 μmole min^{-1} mg^{-1} in the presence of *Tamarindus indicus* and *Combretum fragrans* respectively. The K_M remained unchanged at 0.42 mM. The computed Index of physiological efficiency was reduced from 1.19 min^{-1} to 0.57 min^{-1} and 0.75 min^{-1} with *Tamarindus indicus* and *Combretum fragrans* as inhibitors respectively.

Keywords: *Tamarindus indicus* and *Combretum fragrans*; Neuraminidase inhibition

INTRODUCTION

Blackleg is a disease of cattle, sheep and other ruminants caused by *Clostridium chauvoei*.[1] In Nigeria, the disease was first reported in 1929 and has remained a major problem to cattle in the country.[2] Neuraminidases (sialidases, EC 3.2.1.18) are involved in the pathogenesis of some infectious diseases, whose aetiologic agents produce the enzyme.[3,4] The enzyme is of great importance in medicine and the pharmaceutical industry for the analysis of oligosaccharides and development of neuraminidase inhibitors.[5] Inhibitors of neuraminidase are central in the clinical management of some infectious diseases, such as human influenza virus infections.[6] *Clostridium chauvoei* (jakari strain) which causes blackleg infection in indigenous Nigerian cattle is known to produce neuraminidase and the enzyme is involved in the pathogenesis of blackleg infection by spreading the disease in host tissues.[7] The nomadic Fulani pastoralists of rural Nigeria, who own about 70–80% of livestock in the country, prefer the use of herbal remedies to manage livestock diseases.[8] In the present study, the effect of two medicinal plants of Nigeria (*Tamarindus indicus* and *Combretum fragrans*) used by nomads of rural Nigeria to manage blackleg[9] was tested on *Clostridium chauvoei* (jakari strain) neuraminidase *in vitro*. In this report we show for the first time, the inhibition of *Clostridium chauvoei* (jakari strain) neuraminidase by *Tamarindus indicus* and *Combretum fragrans*.

MATERIALS AND METHODS

Plant Collection, Extraction and Preparation

The stem barks of both *Tamarindus indicus* and *Combretum fragrans* used in this study were collected from Samaru village, Zaria, Nigeria in September and identified at the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. They were air dried and subsequently ground to powder. The powder materials of each of

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these plants were weighed and then individually mixed with methanol in a ratio of 1:5 w/v in an Erlenmeyer flask for 24 h. The mixture was filtered and the filtrate concentrated *in vacuo* at 50°C in a rotatory evaporator coupled to a thermocirculator. The resultant extract of each of these plants was weighed and reconstituted with distilled water to obtain a 1% stock solution.

Bacterial Strain

Clostridium chauvoei (jakari strain) isolated from clinically infected Zebu cattle was obtained in its lyophilised form from the National Veterinary Research Institute, Vom, Plateau state, Nigeria and used here.

Bacterial Cultivation

Three media were used for cultivating *Clostridium chauvoei* (jakari strain) to isolate neuraminidase: reinforced clostridial medium (RCM), blood agar (BA) and cooked meat medium (CMM). Media preparation and bacterial cultivation were carried out as previously described.[10] All the procedures for microbiological asepsis were strictly maintained.

Isolation of Neuraminidase from Culture

The growth medium CMM containing the cultivated bacteria was centrifuged at 9000 g for 40 min at 4°C. The supernatant (2 L) containing crude neuraminidase was fractionated at 55–70% $(NH_4)_2SO_4$ saturation and dissolved in 20 mL of 50 mM acetate buffer pH 5.0. The enzyme solution was dialysed overnight against three changes of the same buffer. The dialysed enzyme was used for all the experiments. Total protein was quantified using Coomasie Brilliant Blue with bovine serum albumin as standard.[11]

Enzyme Assay

Neuraminidase was assayed using fetuin as substrate by quantifying the cleaved sialic acid.[12] Fetuin was purchased from Sigma Chemical Company, St. Louis, USA. Briefly, fetuin (98 mM) was dispensed into tubes (n = 100) and 0.05 U/ml of the purified culture supernatant containing *Clostridium chauvoei* (jakari strain) neuraminidase was added to it. Aliquots (0.25 ml) of sodium periodate was added to each sample and the mixture was shaken and incubated for 20 min in a water bath at 37°C after which 0.1 ml of sodium arsenite was added. The mixture was then shaken and placed in boiling water for 10 min when a pink colour appeared after boiling for 7.5–10 min.

The tubes containing all the mixtures above were cooled by placing them under a running tap. Thereafter, 2.5 ml of acid butanol was added to each tube and the mixture vigorously shaken. The tubes were then centrifuged at 1000 g for 5 min. The supernatant for each mixture was carefully aspirated into cuvettes using Pasteur pipettes and the absorbance was read against a blank using a Sp6-400 spectrophotometer at 549 nm. The amount of sialic acid cleaved from the substrate, was calculated using a sialic acid standard curve.

Inhibition Studies

Various concentrations of the methanolic extracts 100–1000 μg/ml were added to the reaction mixture containing 98 mM of fetuin, 0.05 U/ml of the *Clostridium chauvoei* neuraminidase and 50 mM acetate buffer pH 5.5 adjusted to 3 mL. The reaction was conducted at 37°C for 4 h. After stopping the reaction, the released sialic acid was quantified as described previously and the results statistically analysed.[13] For each concentration of methanolic extract used, the test was repeated (n = 100) to check for reproducibility.

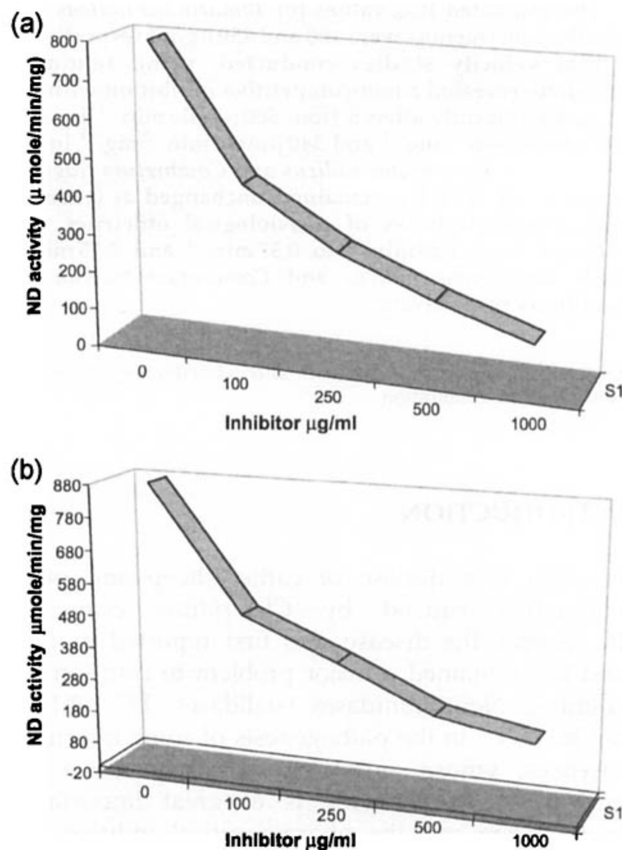

FIGURE 1 (a) *Clostridium chauvoei* Neuraminidase activity in the presence of different levels of the methanolic extract of *Tamarindus indicus* (b) *Clostridium chauvoei* Neuraminidase activity in the presence of different levels of the methanolic extract of *Combretum fragrans*.

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The kinetic analysis of the enzyme was performed in the absence and presence of the extracts at pH 5.5 and 37°C. Fetuin at 9.8–49 mM was added to individual tubes and adjusted to 3 mL with acetate buffer, pH 5.5. Aliquots of the enzyme (0.05 U/mL) was added to the reaction mixture and the reaction was allowed to proceed for 2 h. On termination of the reaction, the released sialic acid was quantified as previously described.

RESULTS AND DISCUSSION

The plants *Tamarindus indicus* and *Combretum fragrans* are used recurrently in the treatment of Blackleg.[9] In the present work, a high level of inhibition of neuraminidase was observed by the methanolic extracts of *Tamarindus indicus* and *Combretum fragrans*. Mean neuraminidase activity was computed as enzyme activity ± Standard Deviation (SD) and there was inhibition of mean neuraminidase activity from $6.1 \pm 4.2 \times 10^2$ to $1.3 \times 10^2 \pm 1.6 \times 10^1$ and $1.0 \times 10^2 \pm 4.9 \times 10^1$ μmole min^{-1} in the presence of *Tamarindus indicus* and *Combretum fragrans* extracts respectively. Our results have shown at least a target of action by the plants which corroborates their use in traditional practice. Neuraminidases have been implicated in the pathologies of several diseases which include; cell invasion in Chagas disease,[14] anaemia in trypanosomiasis[15] and viral invasion in Newcastle disease.[16] The foregoing makes the sourcing of neuraminidase inhibitors mandatory for treatment and amelioration of clinical symptoms related to the physiological activity of the enzyme. In most cases the inhibitors are synthetic and indeed costly, e.g. Zanamivir used in the treatment of influenza virus infection.[6] Moreover some of the synthetic inhibitors become less effective on account of mutations which are rampant in sialidases. This makes recourse to plants as source of neuraminidase inhibitors an appealing alternative, because their active compounds are synthesised in direct response to bacterial and viral invasion. In the present study, the two medicinal plants were tested because of their reported role in ameliorating blackleg infection in traditional veterinary practice. Both *Tamarindus indicus* and *Combretum fragrans*

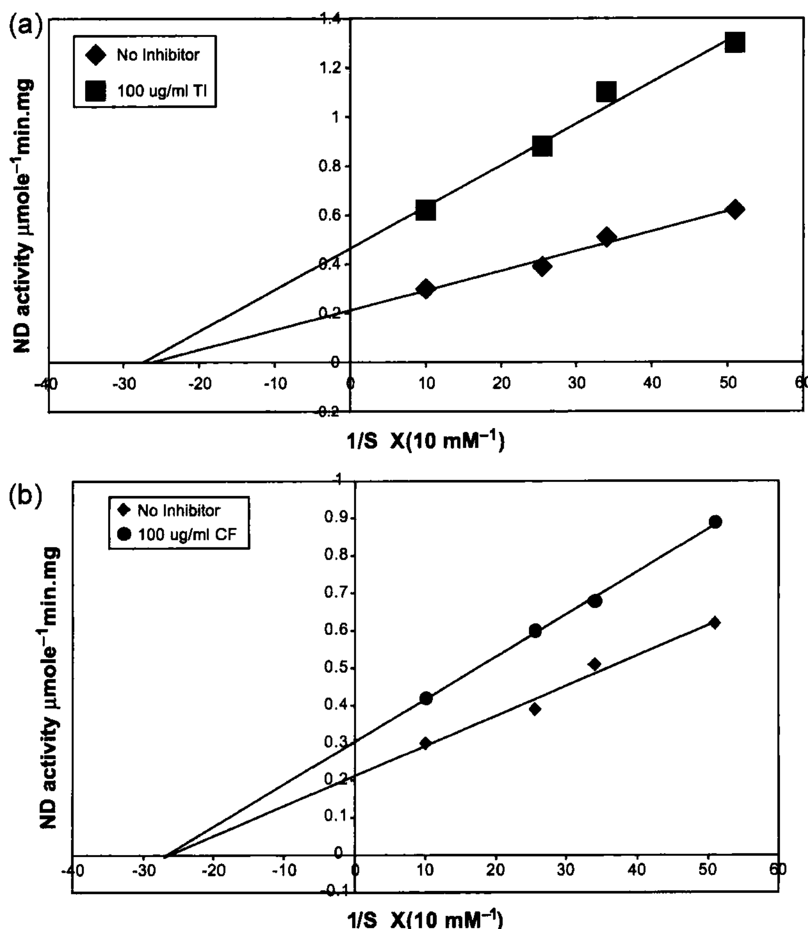

FIGURE 2 (a) Line weaver–Burk plots of *Clostridium chauvoei* neuraminidase catalysed hydrolysis of Fetuin in the absence and presence of 100 µg/ml of methanolic extract of *Tamarindus indicus*. (b) Line weaver–Burk plots of *Clostridium chauvoei* neuraminidase catalysed hydrolysis of Fetuin in the absence and presence of 100 µg/ml of methanolic extract of *Tamarindus indicus*.

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clearly showed a significant level of inhibition which was dose-dependent. As shown in Figure 1, the estimated IC_{50} for *Tamarindus indicus* and *Combretum fragrans* extracts were 100 and 150 μg/ml respectively.

In order to determine neuraminidase inhibition by both plant extracts, a steady state analysis of neuraminidase activities was conducted at varied concentrations of fetuin, which assays the sialic acid cleaved by the enzyme. From the array of experiments, both *Tamarindus indicus* and *Combretum fragrans* exhibited non-competitive inhibition patterns against fetuin. As shown in the Figure 2a the extract from *Tamarindus indicus* reduced the V_{max} from 500 μmole/min/mg to 240 μmole/min/mg. The V_{max} was reduced to 340 μmole/min/mg when *Combretum fragrans* was used as inhibitor (Figure 2b). The K_M value remained unchanged at 0.42 mM. Further analysis of the inhibition data showed a significant change in the index of physiological efficiency (V_{max}/K_M). This was reduced from 1.19 min^{-1} to 0.57 min^{-1} and 0.75 min^{-1} i.e. 50% and 40% inhibitions with *Tamarindus indicus* and *Combretum fragrans* as inhibitors respectively. It will be expedient to further characterize the active constituents of both plants which could be exploited in drug development.

Acknowledgements

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