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ORIGINAL RESEARCH

Inhibition of *Trypanosoma evansi* Protein-Tyrosine Phosphatase by Myristic Acid Analogues Isolated from *Khaya senegalensis* and Tamarindus *indica*

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Background: Trypanosome infections still pose severe health and economic consequences, especially in the endemic regions of Sub-Saharan Africa. Trypanosome differentiation to the procyclic forms which lack the immune evasion mechanisms for survival in the bloodstream is prevented by tyrosine dephosphorylation which is catalyzed by protein-tyrosine phosphatase; thereby promoting survival of the parasites in the host. Inhibition of Protein-tyrosine phosphatase is a strategic therapeutic target that could attenuate trypanosomiasis. This study investigated the in vitro inhibitory effect of stem bark extracts of *Khaya senegalensis* and *Tamarindus indica* on the enzymatic activity of protein-tyrosine phosphatase.

Methods: All determinations were carried out following standard procedures for analytical experiments. The analogues of myristic acid that inhibited the enzymatic activity of protein-tyrosine phosphatase were isolated by bioassay-guided fractionation of stem bark extracts of *Khaya senegalensis* and *Tamarindus indica*.

Results: Analogues of myristic acid proved to be potent inhibitors of protein-tyrosine phosphatase. Double reciprocal (Lineweaver–Burk) plots of the initial velocity data indicated non-competitive inhibition with K_i of 0.67 mg/mL for *Khaya senegalensis* and 2.17 mg/mL for *Tamarindus indica*. The kinetic parameters for the cleavage of para-nitrophenylphosphate by the enzyme showed a K_M of 3.44 mM and V_{max} of 0.19 µmol/min. Sodium orthovanadate, the enzymes' specific inhibitor, inhibited the enzyme competitively with K_i of 0.20 mg/mL. Gas chromatography-mass spectrometry analysis of the stem bark bioactive fractions of *Khaya senegalensis* and *Tamarindus indica* revealed the presence of myristic acid analogues.

Conclusion: Analogues of myristic acid are potent inhibitors of protein-tyrosine phosphatase that could be developed as trypanocide to inhibit the enzymatic activity of protein-tyrosine phosphatase in order to prevent transmission of trypanosomes.

Keywords: *Trypanosoma evansi*, protein-tyrosine phosphatase, signal transduction, regulation, inhibition, myristic acid analogues, *Khaya senegalensis; Tamarindus indica*

Introduction

African Animal Trypanosomiasis (AAT) is a parasitic disease that poses serious threat to livestock production and food security, especially in sub-Saharan Africa. Generally, the disease infects humans, livestock and wild animal reservoirs. Compared to other pathogenic *Trypanosoma* species; including its ancestral *Trypanosoma brucei, Trypanosoma evansi* (*T. evansi*) is known to have the widest range of hosts and geographical distribution worldwide,[1] implying that livestock are susceptible to infection by *Trypanosoma evansi. Trypanosoma evansi* is now

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considered as an emerging zoonotic parasite.[2] The evolution of *Trypanosoma evansi* as having the widest range of host distribution is largely attributed to the new modes of transmission acquired by the parasite, due to the loss of its genetic material (kinetoplast DNA) that aids the cyclical transmission in tsetse flies.[1] *Trypanosoma evansi* is one of the subgenus *Trypanozoon* known as the *Brucei* group. It exhibits variable clinical effects (such as anaemia and infertility) depending on the host and the geographical area.[1,2] This characteristic makes the disease not only multispecies but also a polymorphic disease.[1] *T. evansi* is transmitted mechanically from infected host to healthy animals through the *haematophagous Dipteran* flies belonging to the genera *Tabanus, Stomoxys, Haematopota, Lypersia* and *Hippobosca*.[1,3] Transmission by tabanid flies is efficient and achieved when there is a short time lapse between two interrupted blood meals that is less than 30 mins.[3,4] Transmission can be in several ways; via biting insects, sucking insects, and the vampire bats (*Desmodus rotundus*). Generally, mechanical transmission by biting insects is the most important mode of transmission in livestock and other large animals.[1,5] In Africa, the production losses in cattle due to trypanosome infections have been estimated to be up to 20% across a range of parameters, including mortality, calving rate, meat and milk production.[6] So far, there is no vaccine for the disease. The clinical effects of the disease can be reduced by the application of trypanocide and the introduction of trypanotolerant cattle breeds.[7]

As a consequence of the mechanical transmission of *Trypanosoma evansi* by blood-feeding insects, especially the tabanids, the disease has spread beyond its original sub-Saharan Africa to South and Asian countries.[8] *Trypanosoma evansi* evades the host immune system by modifying their surface coat; a homogeneous protein coat comprising a single variant surface glycoprotein (VSG). The VSG is a glycophosphatidylinositol-anchored glycosylated protein that protects the invariant antigens on the parasite surface from the immune system. As a result, the parasite undergoes rapid multiplication in the blood of the host, producing waves of parasitaemia that characterize the disease.[9,10] The impact of this surface membrane modification has resulted in the appearance of strains of the parasite that are resistant to drugs.[11] In the absence of effective vaccination, due to the surface membrane modification, control of trypanosomiasis could be achieved principally by chemoprophylactic or chemotherapeutic agents,[7] therefore, highlighting the need to explore novel plant metabolites for new and effective drug to tackle the disease.

Protein-tyrosine phosphatase is implicated in the disease of trypanosomiasis.[12,13] Trypanosome differentiation into procyclic forms which lack the immune evasion mechanisms for survival is prevented in the bloodstream by tyrosine dephosphorylation which is catalyzed by protein-tyrosine phosphatase, thereby promoting survival of the parasites in the hosts.[12,14–17] Basically, three developmental stages accompany the life cycle of trypanosomes; the stumpy form which exists in the mammalian hosts, the procyclic and the metacyclic forms which exist in the tsetse fly vector. When an infected tsetse fly bites a mammalian host, metacyclic forms are inoculated into the blood.[18] These metacyclic forms develop into slender forms that undergo rapid asexual replication, maintaining infection in the hosts.[18] As the parasite density increases, a parasite-derived factor known as stumpy induction factor (SIF) accumulate and causes the parasites to undergo cell cycle arrest and differentiate into stumpy forms.[19] Stumpy forms have a limited life expectancy in the blood because they no longer replicate or productively switch their variant surface glycoprotein (VSG) coat, but they are infective to tsetse flies and therefore provide the potential for transmission in another mammalian host,[20] then, the transmission cycle continues. Hence, stumpy forms are the forms of the parasite that tsetse fly ingests during a blood meal from an infected mammalian host. In the bloodstream of the hosts, differentiation from stumpy forms to procyclic forms is prevented by well-conserved cell signaling events of tyrosine dephosphorylation catalyzed by protein-tyrosine phosphatase. Differentiation to procyclic forms would cause the parasite to loss its glycoprotein coat (immune evasion mechanisms that allow survival in the host), thereby exposing the parasite to the eminent attack by the host immune system. This validates that protein-tyrosine phosphatase is a key regulator that propagates the survival of trypanosomes in the host, hence, a potential target for therapeutic interference against trypanosomiasis.[12] If the activity of protein-tyrosine phosphatase is inhibited, differentiation to procyclic forms would occur spontaneously,[12] this would result to the killing of the parasites in the bloodstream by the host's immune system.

Although the scientific bases for the use of *Khaya senegalensis* and *Tamarindus indica* in the treatment of trypanosomiasis have been reported,[21–23] to gain a better insight into the trypanocidal properties of the plants, the

inhibitory effects of their stem bark extracts on the enzymatic activity of protein-tyrosine phosphatase were explored by testing the phytochemicals (inhibitors) in the stem bark extracts on the enzyme as well as the determination of the type of inhibition. In a survey that evaluated the use of plants in folklore medicine for the treatment of trypanosomiasis in domestic animals in Nigeria, it was found that *Khaya senegalensis* and *Tamarindus indica* were among the most commonly used plants.[21] In a similar study, the in vitro anti-trypanosoma activity of aqueous stem bark extract of *Khaya senegalensis* showed that the extract was trypanocidal against *Trypanosoma brucei brucei*; at a minimum concentration of 8.3 mg/mL, the parasites were immotile within 30 mins of incubation.[22] More so, it was revealed that ethanolic stem bark extract of *Khaya senegalensis* possessed both in vitro and in vivo anti *Trypanosoma evansi* activity.[23] Incubating 40 µL of *Trypanosoma evansi* infected blood (10^9 *T. evansi* per mL of blood) with 20 µL of ethanolic stem bark extract of *Khaya senegalensis* at 37°C eliminated the parasites within 5 mins of post-incubation,[23] while treatment of *Trypanosoma evansi*-infected rats with 80 mg/kg body weight of the same extract significantly lowered the parasitemia level within 13 days of post-infection.[23] Protein-tyrosine phosphatase was previously isolated from *Trypanosoma evansi* and characterized,[24] in continuation, we studied the inhibitory effect of stem bark extracts of *Khaya senegalensis* and *Tamarindus indica* on the enzyme.

Materials and Methods

Plant Material

The stem bark of *Khaya senegalensis* and *Tamarindus indica* were collected during the dry season of October from Kudigi village in Zaria, 11°5′7.9476″ N and 7° 43′11.8020″ E, Sabon-gari Local Government Area, Kaduna state, Nigeria. They were authenticated by a taxonomist at the Herbarium, Department of Botany, Ahmadu Bello University, Zaria. Their respective voucher number 900181 and 026 were obtained.

Preparation and Extraction of Samples

The stem bark of *Khaya senegalensis* and *Tamarindus indica* were thoroughly washed with clean water and air-dried at room temperature for 3 weeks. The dried stem barks were grounded into fine powder using mortar and pestle. Exactly 100g of each of the power obtained was weighed using a weighing balance (Contech® Instruments Ltd India, Model CAC-224) and initially extracted by maceration with 500 mL of methanol for 48 hrs. Few drops of chloroform were added to ensure there was no fungi growth during the 48 hrs of extraction, they were then concentrated by freeze-drying. The methanolic extract obtained was exhaustively extracted using ethyl acetate, n-butanol, and chloroform for partitioning into individual fractions. Exactly 0.5g of the methanolic extract was reconstituted in 100 mL of water and vigorously extracted in triplicates by ethyl acetate (50 mL) in a 250 mL separating funnel. After extraction, the solution was left for phase separation. The organic fraction of ethyl acetate was filtered using Whatman filter paper No.1 and concentrated by freeze-drying using bench top freeze dryer (Labconco™) at 4°C for 2 and half hours. A similar partitioning process was also carried out to obtain n-butanol and chloroform fractions. They were stored in air-tight bottles and kept in the refrigerator until used. These served as the crude extracts.

Bioassay-Guided Fractionation and Determination of the Active Plant Extract

The activity of each of the fractions (ethyl acetate, n-butanol, and chloroform) obtained was determined by bioassay-guided fractionation. An aliquot (50 mg) of each of the extracts was measured and dissolved in 10 mL of distilled water. Each extract was incubated with the enzyme under the standard assay conditions for the enzyme. The n-butanolic extracts showed activity against the enzyme and were further used. The technique of preparative thin-layer chromatography (TLC) was used to narrow down the search for the particular bioactive phytochemical in the n-butanolic extract.

Fractionation of n-Butanolic Stem Bark Extracts of *Khaya senegalensis* and *Tamarindus indica* by Preparative Thin Layer Chromatographic (TLC)

The n-butanolic stem bark extracts of *Khaya senegalensis* and *Tamarindus indica* were partially purified by fractionation on preparative thin-layer chromatography (TLC) using silica gel (60 F_{254}) coated aluminum-backed TLC plate (EMD MilliporeTH). Using sterile capillary tube, a spot of about 1mm of the n-butanolic extract was made horizontally on the TLC plate (1″ × 3″). Then, ascending thin layer chromatography was carried out by placing the plate vertically in a chromatographic tank-containing n-butanol, acetic acid, and water (6:2:1) as the solvent

system for stem bark extract of *Khaya senegalensis*. The same procedure was followed for the stem bark extract of *Tamarindus indica* using n-hexane and methanol (3:2) as solvent system. At the end of the chromatography, the TLC plates were viewed under UV light for the detection of the separated compounds which appeared as bands. These procedures were carried out in a fume-hood to prevent inhalation of chemicals. The individual bands were scraped and suspended in 2 mL of the respective solvent systems and centrifuged at 3000 ×g for 10 mins. The supernatants were concentrated to dryness by subjecting them to rotary evaporator (RE-20ID, Xingyan Kori, China) at 40°C and reconstituted with 2 mL of distilled water. Thereafter, they were freeze-dried using a bench top freeze dryer (Labconco™) at 4°C for 2 hrs. Reconstitution with 2 mL of distilled water was carried out in order to ensure that there was no trace of the extracting solvents in any of the scrapped bands. Each of the bands was verified for inhibitory effects against Protein-tyrosine phosphatase. The bands with inhibitory activity were submitted to Gas chromatography-mass spectrometry analysis (GCMS-QP2010 PLUS Shimadzu, Japan) to identify the bioactive compounds in the test sample.

Isolation of Protein-Tyrosine Phosphatase

Protein-tyrosine phosphatase was previously isolated from *trypanosoma evansi* and characterized.[24] Here, we focus on the inhibition of the enzyme.

Enzyme Assay

The assay of protein-tyrosine phosphatase (PTPase) was carried out following the standard method as described.[25] The assay was done with 10 mmol/L *para*-nitrophenylphosphate (*p*NPP) as substrate in assay buffer (10 mM sodium phosphate, pH 8.0, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, and 10% glycerol), to a final reaction volume of 1 mL (700 µL assay buffer, 250 µL *para*-nitrophenylphosphate, 50 µL PTPase) at 37°C for 10 mins. The reaction was terminated after 10 mins by the addition of 1 mL of stop reagent (0.2 M NaOH). The *para*-nitrophenol produced was determined by measuring the absorbance at 410 nm. The assay specificity for PTPase was verified by the addition of sodium orthovanadate (PTPase inhibitor) to the reaction mixture at a final volume of 5 µL, 15 µL, and 25 µL. The Extinction coefficient of para-nitrophenol was 19.03 at 410 nm.[25] One unit of activity is equivalent to 1 nmol/L of *para*-nitrophenylphosphate hydrolyzed per minute.

Inhibition Study

The inhibition study was carried out by incubating varying concentrations; 0 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL (Table 1) of the bioactive band from n-butanolic extract. *Para*-nitrophenylphosphate was used as substrate for the measurement of enzymatic activity. The buffer solution was a mixture of 25 mM Tris–HCl (pH 7.5), 2 mM b-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). The inhibition assay was performed by adding 25 µL of each of the varying concentration to 675 µL assay buffer, 50 µL PTPase, 250 µL of 10 mM *para*-nitrophenylphosphate to a final reaction volume of 1 mL. Each of the reaction mixtures was incubated at 37°C for 10 mins. The reaction was terminated by adding 200 µL of stop reagent (0.2 M NaOH). *Para*-nitrophenol produced following the dephosphorylation of *para*-nitrophenylphosphate by protein-tyrosine phosphatase was monitored using table top Shimadzu spectrophotometer at 410 nm. Sodium orthovanadate (Na_3VO_4) was used as positive control of the inhibition. The readings obtained from spectrophotometer were used for Lineweaver–Burk plots[26] using sigma plot software (SPCC Inc., Chicago, IL) in order to determine the type of inhibition and to calculate the kinetic parameters.

Assay Specificity of PTPase by Inhibition with Sodium Orthovanadate (Na_3VO_4)

A working stock solution (0.1 M) of sodium orthovanadate was prepared by dissolving 0.92 g Sodium orthovanadate

Table 1 Incubation Pattern Using the Bioactive Stem Bark Fraction of the Extracts

Group 1: 0mg/mL [I]	Group 2: 0.2mg/mL [I]	Group 3: 0.5mg/mL [I]	Group 4: 1mg/mL [I]
0mg [I] + E + 1mM [S]	0.2mg [I] + E + 1mM [S]	0.5mg [I] + E + 1mM [S]	1mg [I] + E + 1mM [S]
0mg [I] + E + 2mM [S]	0.2mg [I] + E + 2mM [S]	0.5mg [I] + E + 2mM [S]	1mg [I] + E + 2mM [S]
0mg [I] + E + 3mM [S]	0.2mg [I] + E + 3mM [S]	0.5mg [I] + E + 3mM [S]	1mg [I] + E + 3mM [S]
0mg [I] + E + 4mM [S]	0.2mg [I] + E + 4mM [S]	0.5mg [I] + E + 4mM [S]	1mg [I] + E + 4mM [S]
0mg [I] + E + 5mM [S]	0.2mg [I] + E + 5mM [S]	0.5mg [I] + E + 5mM [S]	1mg [I] + E + 5mM [S]

Notes: [I]: inhibitor, E: enzyme, [S]: substrate.

(Sigma Aldrich, USA) in 30 mL of distilled water and placed on a bench top shaker (Thermo Scientific MaxQ™ 4000 orbital shaker) at room temperature until the yellowish solution turned colorless. Protein-tyrosine phosphatase was incubated on ice in the presences of varying concentrations (0.2 mg/mL, 0.5 mg/mL, and 1 mg/mL) of sodium orthovanadate. The assay was performed with 10 mmol/L *para*-nitrophenylphosphate as substrate in a buffer (25 mM, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), pH 7.3, and 10% glycerol), to a final reaction volume of 1 mL (695 µL assay buffer, 50 µL PTPase, 5 µL of 0.2 mg/mL sodium orthovanadate, 250 µL para-nitrophenylphosphate) at 37°C for 10 mins. This was followed for the 15 µL of 0.5 mg/mL and 25 µL of 1 mg/mL of sodium orthovanadate. The reaction was terminated after 10 mins by the addition of 10 mM dithiothreitol (10 µL). The activity was measured using table top Shimadzu spectrophotometer at 410 nm. Data obtained was used for Lineweaver–Burk plot as described above.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Aliquots of each bioactive fraction of the extracts were subjected to analysis using GCMS-QP 2010 plus Shimadzu, Japan. Standard analytical conditions for the GC-MS were followed. The chromatographic conditions were as follows: flow rate was 1.58 mL/min pressure with split ratio of 1:0. The machine was programmed with the injection syringe positioned at 250ºC temperature and 100.2 pressures and at 60% column oven. The carrier gas was helium at a flow rate of 1.58 mL/min. The transfer line and source temperature were set at 280ºC. The oven temperature was initially at 80ºC for 2 mins, then increased to 200ºC at a rate of 9ºC per min until 280ºC and held at 280ºC at a flow rate of 10ºC for 5 mins. The chromatograms were acquired in full scan mode (m/z 40–800). The NIST/EPA/NIH Mass Spectral Library (NIST/EPA/NIH 11, version 2.0) was used for identification of the compounds.

Results

Analogues of myristic acid isolated from the stem bark extracts of *Khaya senegalensis* and *Tamarindus indica* proved to be potent inhibitors of *Trypanosoma evansi* protein-tyrosine phosphatase. Interestingly, the Lineweaver–Burk plot of the inhibition indicated that the analogues of myristic acid present in the stem bark

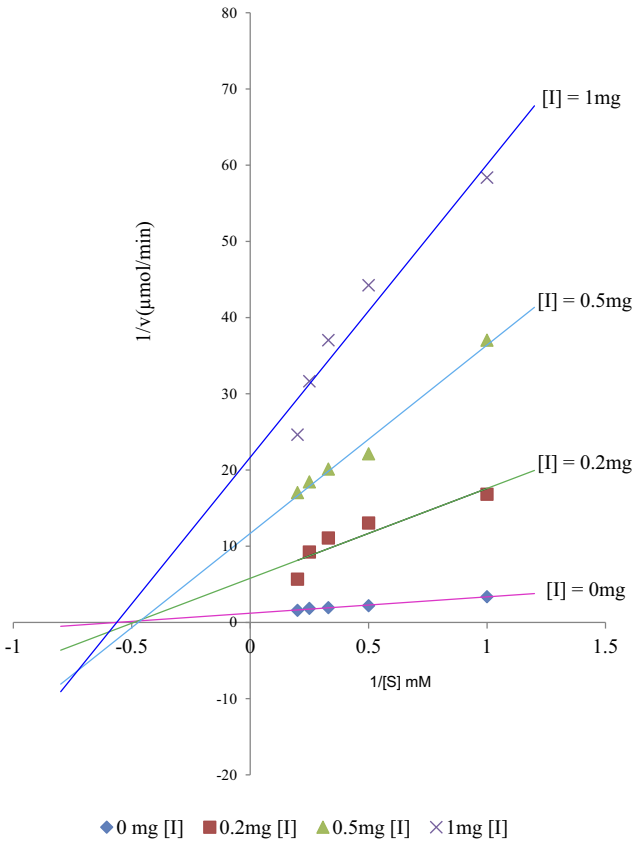


Figure 1 Lineweaver–Burk plot showing non-competitive inhibition of *Trypanosoma evansi* protein-tyrosine phosphatase by the stem bark bioactive fraction of *Khaya senegalensis*.

extracts of *Khaya senegalensis* and *Tamarindus indica* inhibited the enzyme non-competitively as shown in Figures 1 and 2, respectively. This implies that analogues of myristic acid are non-competitive inhibitors of protein-tyrosine phosphatase. The inhibitor binding constant (K_i) was 0.67 mg/mL for *Khaya senegalensis* and 2.17 mg/mL for *Tamarindus indica*. The enzyme kinetic parameters for the cleavage of para-nitrophenylphosphate substrate (Figure 3) showed a K_M of 3.44 mM and V_{max} of 0.19 µmol/min. Sodium orthovanadate (specific inhibitor of protein-tyrosine phosphatase) inhibited the enzyme competitively with a K_i of 0.20 mg/mL.

Gas Chromatography–Mass Spectroscopy (GC-MS) Analysis

Results of the gas chromatography-mass spectrometry analysis of the bioactive stem bark fractions of *Khaya senegalensis* and *Tamarindus indica* showed that seven compounds were present in the bioactive fraction of *Khaya senegalensis*, namely, 4-hydroxy-methylolacetone, methyltrimethylene glycol, 2-methylpropyl ester,

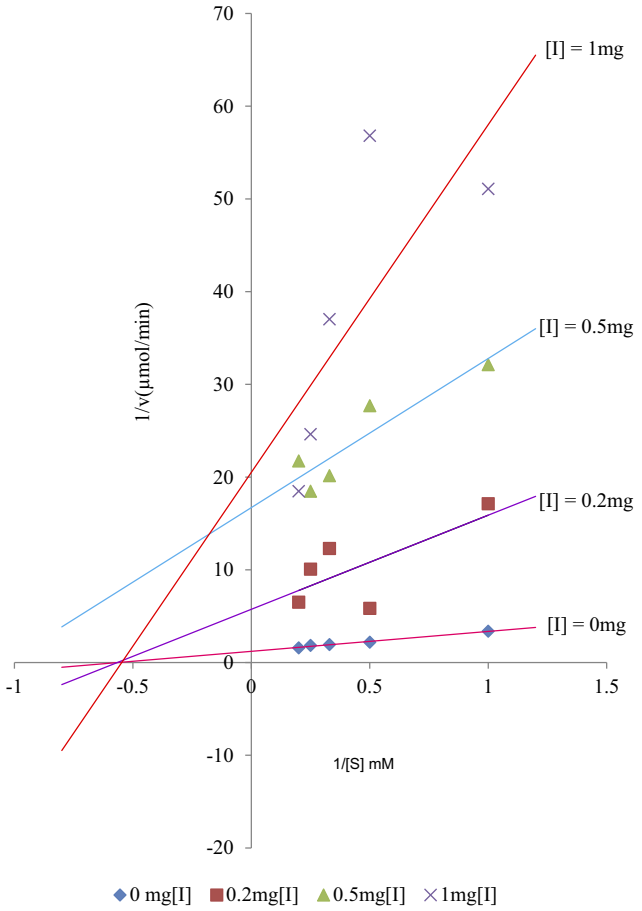


Figure 2 Lineweaver–Burk plot showing non-competitive inhibition of *Trypanosoma evansi* protein-tyrosine phosphatase by the stem bark bioactive fraction of *Tamarindus indica*.

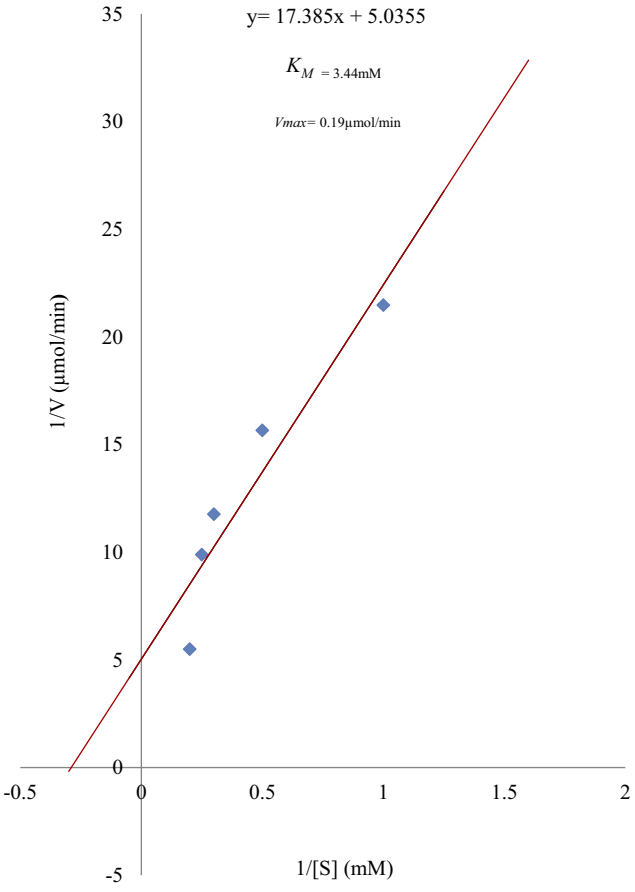


Figure 3 Lineweaver–Burk plot for the cleavage of para-nitrophenylphosphate by *Trypanosoma evansi* protein-tyrosine phosphatase.

n-tridecanoic acid, n-tetratriconate, n-hexadecanioc acid, and 14-pentadecanoic acid, with their corresponding molecular weight and structures as shown in Figure 4. Compounds present in the bioactive fraction of *Tamarindus indica* were five, namely, n-decanoic acid, n-tridecanoic acid, 1-(+)-ascorbic acid 2,6-dihexadecanoate, methyl trans-9-octadecanoate, and octa-9-enoic acid, with their corresponding molecular weight and structures as presented in Figure 5. The GC-MS revealed that these compounds are analogues of myristic acid.

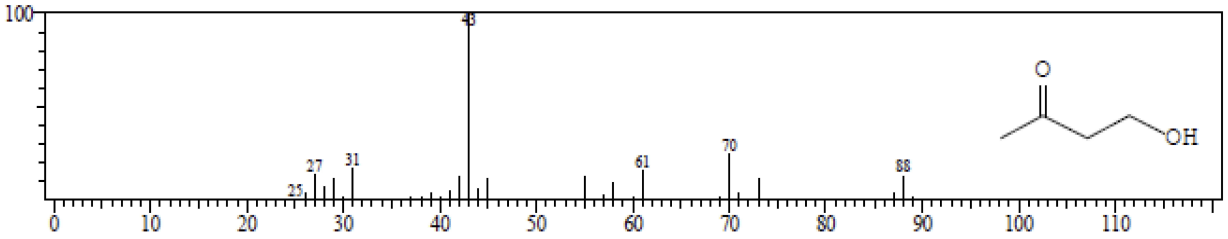
Discussion

Protein-tyrosine phosphatases are enzymes that dephosphorylate proteins on tyrosine residues and together with protein-tyrosine kinases that phosphorylate proteins on tyrosine residues are responsible for the regulation of signaling events that control fundamental biological processes in living cells. As a key mechanism for trypanosome survival in which differentiation to procyclic forms is prevented in the bloodstream by tyrosine dephosphorylation,[12] protein-tyrosine phosphatase plays major role in the disease of trypanosomiasis, hence, an important therapeutic target. This study investigated the inhibitory effect of stem bark extracts of *Khaya senegalensis* and *Tamarindus indica* on the enzymatic activity of protein-tyrosine phosphatase, with the view to establishing a novel and potent trypanocide that targets the activity of protein-tyrosine phosphatase in trypanosomes. This was inspired by the use of the plants in folklore medicine for the treatment of African Animal Trypanosomiasis.[21–23] During this study; it was found that the bioactive fractions of the extracts inhibited *trypanosoma evansi* protein-tyrosine phosphatase dose-dependently. This may probably be the scientific rationale for the use of stem bark extracts of the two plants for the treatment of animal trypanosomiasis in folklore medicine. Considering its dephosphorylation mechanism which aids the survival of the stumpy forms of the parasite in the host's bloodstream and for uptake

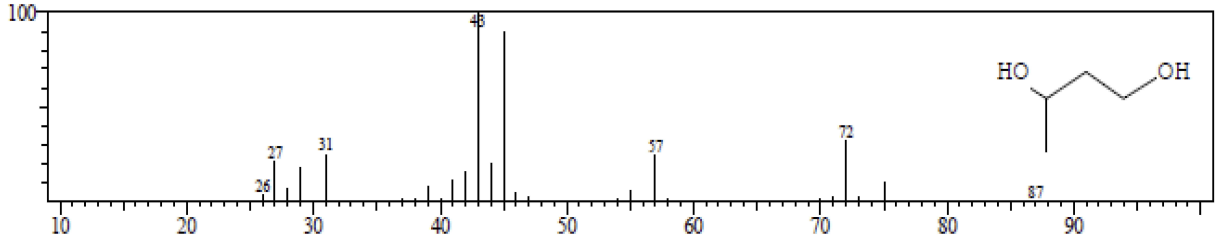
Peak Report TIC								
Peak	**R. Time**	**I. Time**	**F. Time**	**Area**	**Area%**	**Height**	**Height %**	**A/H**
1	3.463	3.425	3.600	2397037	3.63	1024462	10.21	2.34
2	4.170	4.142	4.308	2816558	3.31	672784	6.71	3.25
3	5.065	5.025	5.133	1065446	1.61	333383	3.32	3.20
4	16.751	16.683	16.842	2646123	4.01	724494	7.40	3.56
5	18.185	18.008	18.467	16568934	25.11	1384602	13.80	11.97
6	20.502	20.350	20.650	16539132	25.06	2849401	28.41	5.80
7	23.362	23.200	23.525	24590488	37.26	3022975	30.14	8.13
				65993718	100.00	10033101	100.00	

TIC= Total Iron Current, R. Time= Retention Time, I. Time= Initial Time, F. Time= Final Time, A/H= Area/ Height

The Spectra



SI:95 Formula: C4H8O2 CAS: 590-90-9 MolWeight:88 RetIndex:798
CompName:2-Butanone, 4-hydroxy-2-Butanone, 4-hydroxy-Methylolacetone, Monomethylolacetone, 3-Ketobutan-1-ol, 3-Oxo-1-butanol.



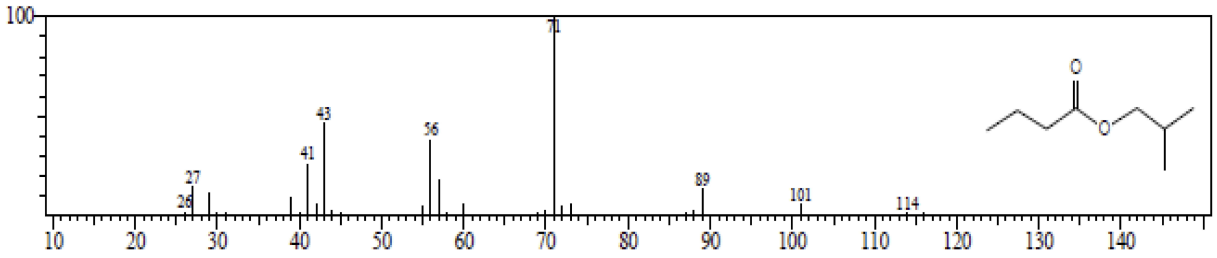
SI:97 Formula: C4H10O2 CAS:107-88-0 MolWeight:90 RetIndex:824
CompName:1,3-Butanediol, beta-Butylene glycol, Methyltrimethlene glycol, 1-Methyl-1,3-propanediol, 1,3-Butylene glycol, 1,3-Dihydroxybutane

Figure 4 Gas chromatography–mass spectrometry spectra of the stem bark bioactive fraction of *Khaya senegalensis*.

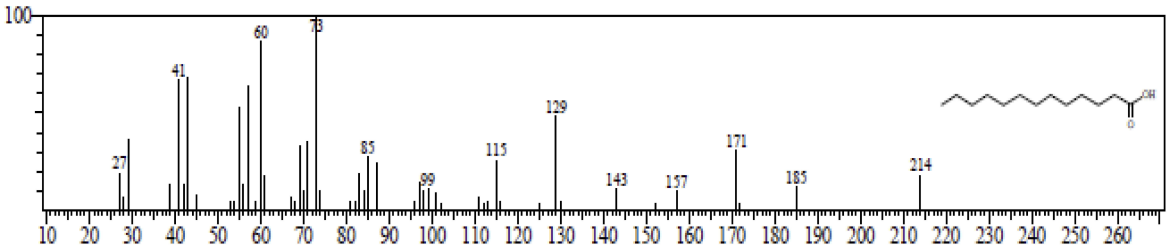
into tsetse flies,[19,27] the inhibition of *Trypanosoma evansi* protein-tyrosine phosphatase by the bioactive fractions suggests that the compounds in the fractions could serve as potential candidates for effective trypanocide.

The non-competitive inhibition of *Trypanosoma evansi* protein-tyrosine phosphatase indicated that the inhibitors were allosterically bound to a site on the enzyme-substrate complex other than the enzyme active site. The observed reductions in the enzyme activity could be attributed to the effectiveness of the phytochemicals in the bioactive fractions, which were identified as analogues of myristic acid. This shows that the phytochemicals (analogues of myristic acid) are capable of interacting efficiently with the enzyme; suggesting that the agents are potential trypanocides.

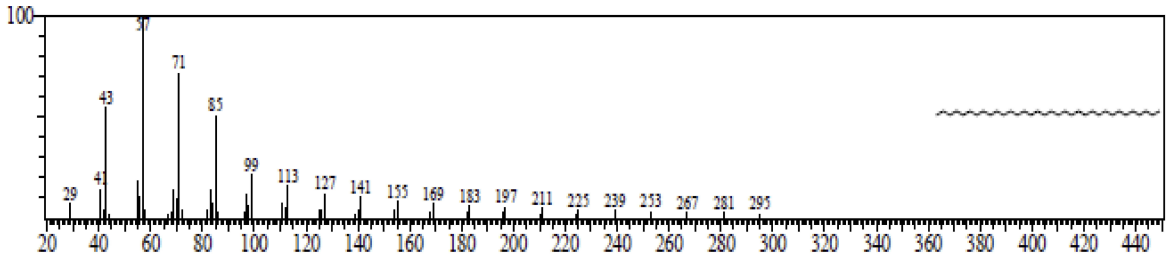
A hallmark of the classical protein-tyrosine phosphatase is the strictly conserved primary structure, including cysteine and arginine residues in the catalytic domain which constitutes a phosphate-binding pocket.[28–31] The



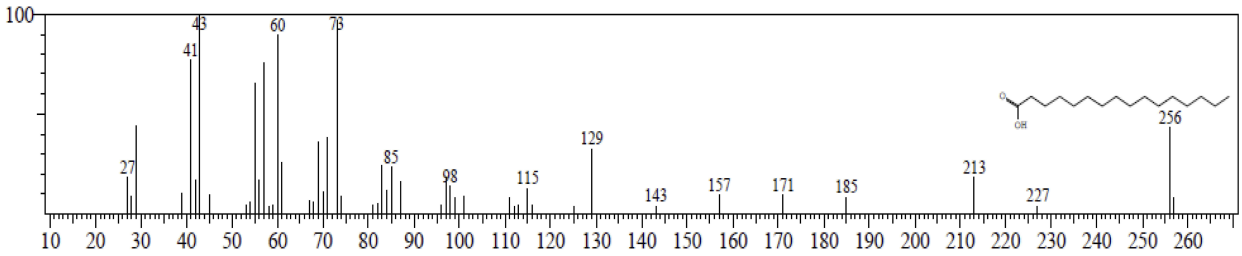
SI:91 Formula:C8H16O2 CAS:539-90-2 MolWeight:144 RetIndex:920
CompName: Butanoic acid, 2-methlypropyl ester, Butyric acid, Isobutyl ester, Isobutyl butyrate, Isobutyl n-butyrate, 2.Methylpropyl butyrate



SI:91 Formula:C13H26O2 CAS:638-53-9 MolWeight:214 RetIndex:1670
CompName: Tridecanoic acid, n-Tridecanoic acid, n-Tridecoic acid, Tridecylic acid



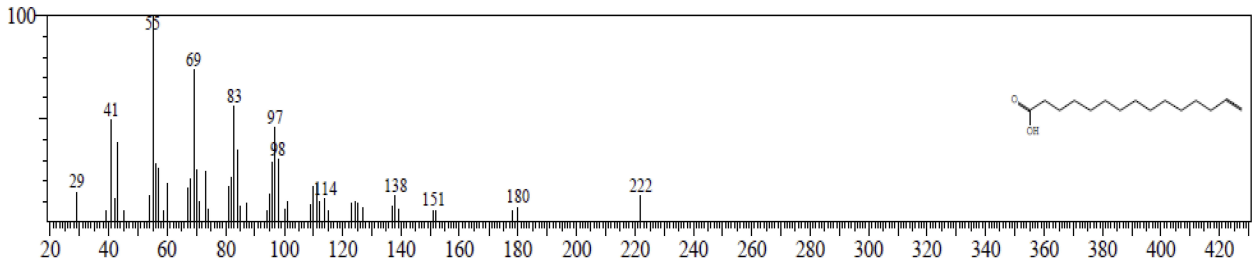
SI:92 Formula: C34H70 CAS:14167-59-0 MolWeight:478 RetIndex:3401
CompName: Tetratriaconate, n-Tetratriacontane



SI:90 Formula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968
CompName:n-Hexadecanoic acid, Hexadecanoic acid, n-Hexadecoic acid, palmitic acid, Pentadecanecarboxylic acid, 1- Pentadecanecarboxylic

Figure 4 Continued.

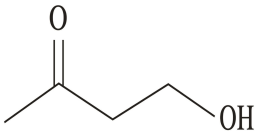
inhibition of the enzymatic activity by the analogues of myristic acid could probably be through the oxidation mechanism; particularly through oxidation of the cysteine residue, leading to the inactivation of the enzyme. The cysteine residue localized in the catalytic center exists in a thiolate anion form and is susceptible to oxidation.[28] The



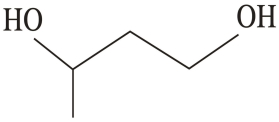
SI:86 Formula:C15H28O2 CAS:17351-34-7 MolWeight:240 RetIndex:1859
CompName:14-Pentadecenoic acid.

Structure of the compounds in the spectra

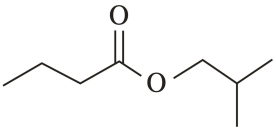
Peak 1: 4-hydroxy-methylolacetone



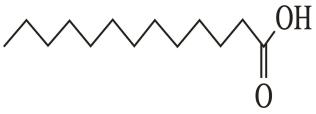
Peak 2: methyltrimethylene glycol



Peak 3: 2-methylpropyl ester

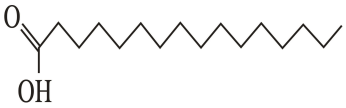


Peak 4: n-tridecanoic acid



Peak 5: n-tetratriaconate

Peak 6: n-hexadecanioc acid



Peak 7: 14-pentadecenoic acid

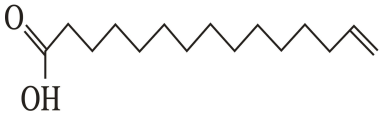


Figure 4 Continued.

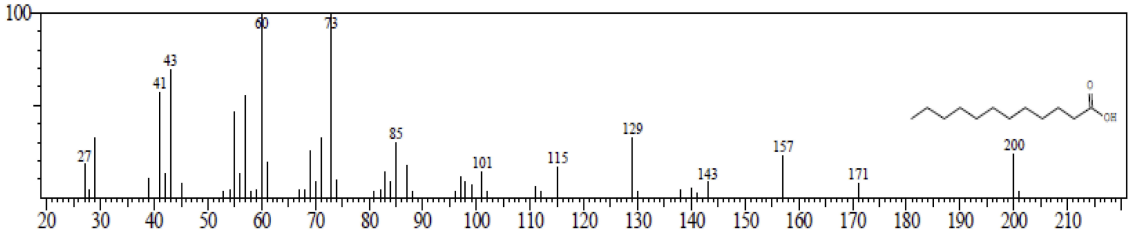
myristic acid analogues were bound irreversibly to the enzyme as evident by the non-competitive inhibition, suggesting that they may be highly oxidizing. Highly oxidizing conditions can induce oxidation to the sulfinic and sulfonic acid residues of protein-tyrosine phosphatase, which is considered irreversible under physiological conditions.[32]

In the band regions of the GC-MS spectra of bioactive stem bark fraction of *Khaya senegalensis*, the percentage abundance of 4-hydroxy-methylolacetone was 3.63%, methyltrimethylene glycol was 3.31%, 2-methylpropyl ester was 1.61%, n-tridecanoic acid was 4.01%, n-tetratriconate was 25.11%, n-hexadecanioc acid was 25.06%, and 14-pentadecanoic acid was 37.26% as shown in Figure 4. Also, for the compounds present in the bioactive fraction of *Tamarindus indica*, the percentage abundance of n-decanoic acid was 2.47%, n-tridecanoic acid was 6.17%, 1-(+)-ascorbic acid 2.6-dihexadecanoate was 35.78%, methyl trans-9-octadecanoate was 4.30%, and octadec-9-enoic acid was 51.27 as shown in Figure 5. From the percentage abundance of the various compounds, for *Khaya senegalensis*, 14-pentadecanoic acid was the highest in abundance while 2-methylpropyl ester was the least in abundance. For *Tamarindus indica*,

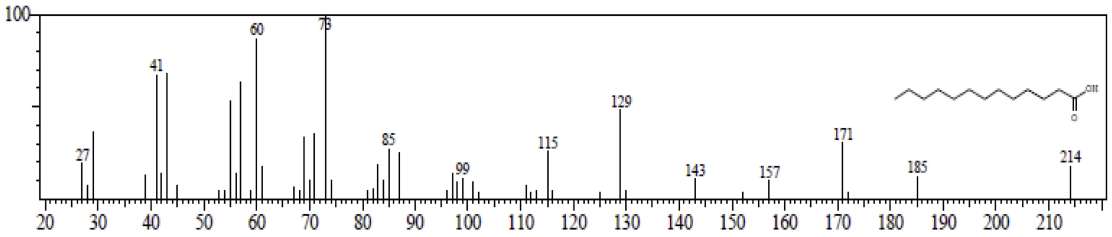
Peak Report TIC								
Peak	**R. Time**	**I. Time**	**F. Time**	**Area**	**Area%**	**Height**	**Height %**	**A/H**
1	14.172	14.117	14.267	1973141	2.47	536705	4.67	3.68
2	16.750	16.683	16.858	4927667	6.17	1363574	11.87	3.61
3	20.523	20.342	20.700	28591193	35.78	4146192	36.09	6.90
4	22.378	22.342	22.442	3438767	4.30	1191744	10.37	2.89
5	23.380	23.183	23.550	79897991	51.27	4250108	37.00	9.64
					100.00	11488323	100.00	

TIC= Total Iron Current, R. Time= Retention Time, I. Time= Initial Time, F. Time= Final Time, A/H= Area/ Height.

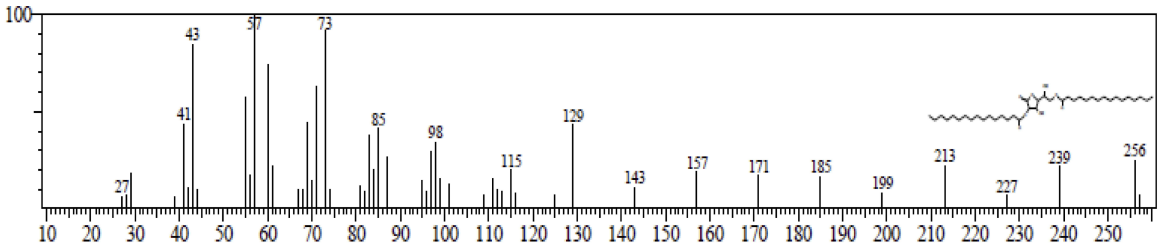
Spectra



SI:92 Formula:C12H24O2 CAS:143:07-7 MolWeight:200 RetIndex:1570
CompName: Dodecanoic acid, n-Dodecanoic acid, Dodecylic acid, Lauric acid, Laurostearic acid



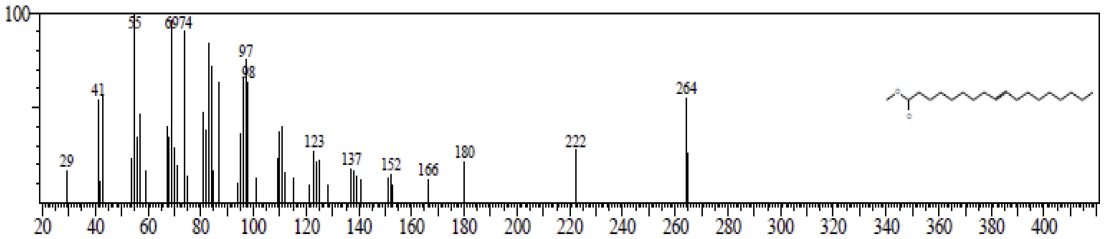
SI:90 Formula: C13H26O2 CAS:638-53-9 MolWeight:214 RetIndex:1670
CompName: Tridecanoic acid, n-Tridecanoic acid, n-Tridecoic acid, Tridecylic acid



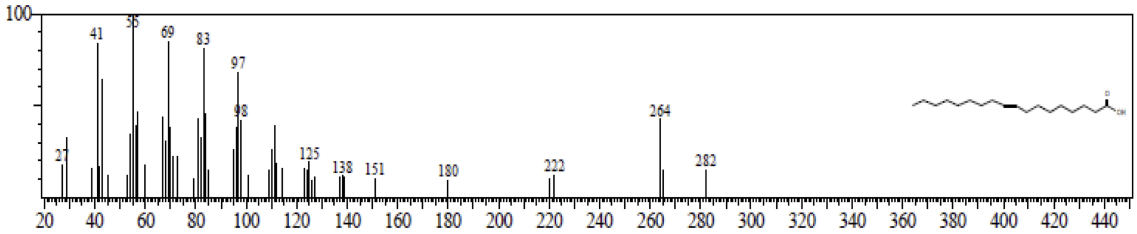
SI:88 Formula:C38H68O8 CAS:28474-90-0 MolWeight:652 RetIndex:47
CompName: 1-(+)-Ascorbic acid 2,6-dihexadecanoate

Figure 5 Gas chromatography–mass spectrometry spectra of the stem bark bioactive fraction of *Tamarindus indica.*

octa-9-enoic acid had highest percentage of 51.27% while n-decanoic acid had the lowest abundance of 2.47%. Probably, the proportions of the percentage abundance of the compounds may not be a significant factor that



SI:84 Formula:C19H36O2 CAS:2462-84-2 MolWeight:296 RetIndex:2085
CompName: 9-Octadecanoic acid, methyl ester, Methyl 9-octadecanoate, Methyl (9E)-9-octadecanoate



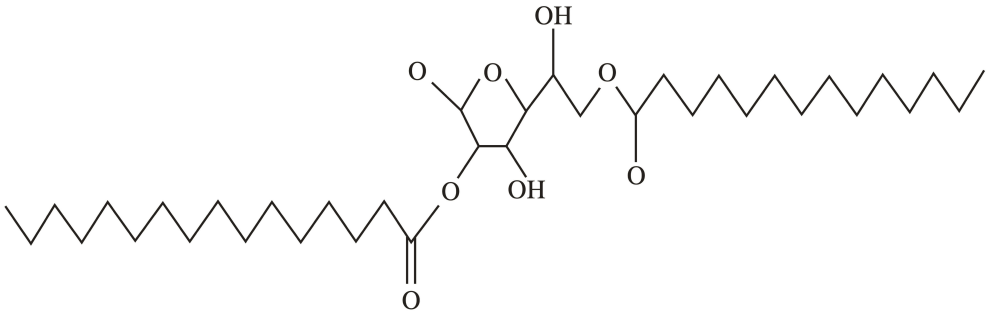
SI:87 Formula:C18H34O2 CAS:0-00-0 MolWeight:282 RetIndex:2175
CompName: Octadec-9-enoic acid

Structure of the compounds in the spectra

Peak 1: n-decanoic acid

Peak 2: n-tridecanoic acid

Peak 3: 1-(+)-Ascorbic acid 2,6-dihexadecanoate



Peak 4: Methyl trans-9-octadecanoate

Peak 5: Octadec-9-enoic acid

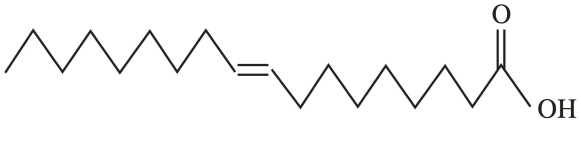


Figure 5 Continued.

determines the compound that exhibited the highest degree of inhibition; the compounds may have acted synergistically towards inhibition of the enzyme.

Incubation of the analogues of myristic acids with *trypanosoma evansi* protein-tyrosine phosphatase showed that the enzymatic activity was irreversibly inhibited. It has been established

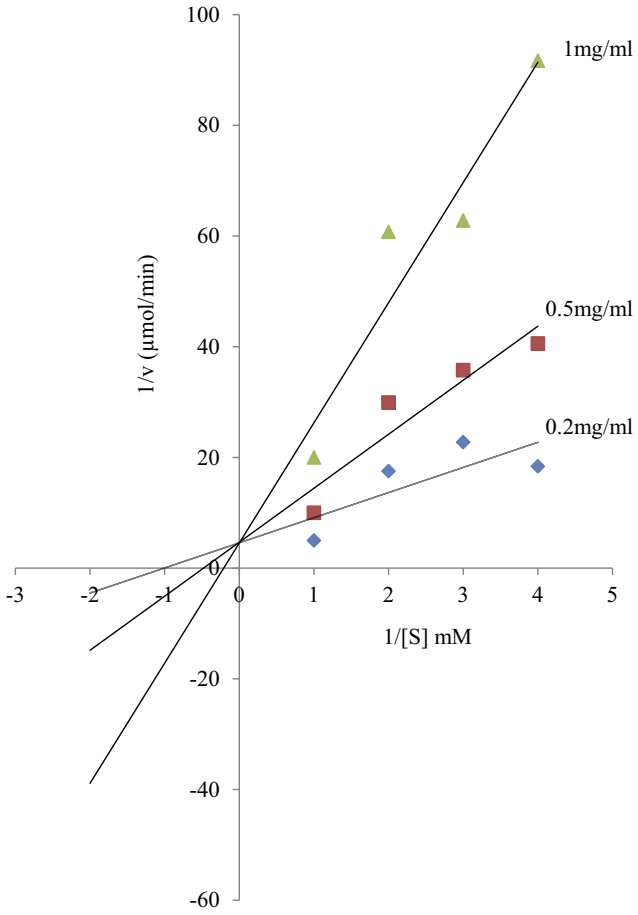


Figure 6 Lineweaver–Burk plot for the inhibition of protein-tyrosine phosphatase by sodium orthovanadate. Orthovanadate inhibited the enzyme competitively, while the bioactive fraction of the plant extracts inhibited the enzyme non-competitively in a dose-dependent fashion as shown in Figures 1 and 2.

that myristoylation is an irreversible and co-translational protein modification essential for membrane targeting and signal transduction.[33] In a previous study that investigated the effects of peroxytetradecanoic acid, an analogue of myristic acid on the enzymatic activity of Protein-tyrosine phosphatase CD45, it was found that peroxytetradecanoic acid is a potent inhibitor of protein-tyrosine phosphatase CD45. Peroxytetradecanoic acid was found to be effective in nanomolar concentrations of 50 nM which caused 50% decrease in the enzymatic activity.[33] The observation supports the findings of this study; incubation of the enzyme with a low concentration of 0.2 mg/mL of the bioactive fraction of the extracts caused potent inhibition with an inhibitor binding constant (K_i) of 0.67 mg/mL for *Khaya senegalensis* and 2.17 mg/mL for *Tamarindus indica*. The low K_i which indicates that the inhibitors in the extracts bound the enzyme with high affinity, suggests that they would be effective inhibitors. In a related study, it was found that the growth of bloodstream forms of *Trypanosoma evansi* in axenic culture was inhibited by incubation with 11-oxatetradecanoic acid, an analogue of myristic acid.[34,35] It was shown that the concentration of 11-oxatetradecanoic acid that inhibited trypanosome growth by 50% (LD_{50}) was 3.7±0.2 µM as measured by direct counting of survivors using a haemocytometer, 5.1±2.0 µM in a colorimetric test based on the formation of a formazan product, and 8.8±3.7 µM by estimation of pyruvate.[34] This also supports the observed inhibition of *Trypanosoma evansi* protein-tyrosine phosphatase by low concentrations of the analogues of myristic acids isolated from stem bark of the plants. In a study of the utilization of heteroatom-containing analogues of myristate both in a cell-free system and in vivo,[35] the result indicated that 10-(propoxy)decanoic acid, myristic acid analogue was highly toxic to *Trypanosoma brucei* in culture, although it was nontoxic to mammalian cells.[35] The study reported that at 10 µM, 11-oxatetradecanoic acid killed >99% of cultured trypanosomes within 24 hrs, 13-oxatetradecanoic acid inhibited the growth rate by 40%, and 6-oxatetradecanoic acid inhibited the growth rate by 10%.[35] In another study that assayed 244 different fatty acid analogues, with chain lengths comparable to that of myristate for trypanocidal effects, in addition to their trypanocidal effects upon incorporation in the parasite's variant surface glycoprotein (VSG), it was found that myristate analogues are useful for studying the mechanism of glycosyl phosphatidylinositol (GPI) myristoylation, and they are candidates for antitrypanosomal chemotherapy.[36] The usefulness of these agents reported in the previously mentioned studies as candidates for antitrypanosomal chemotherapy correlates with the findings of this study which indicated that the myristic acid analogues isolated from the bioactive fractions could be the antidote to trypanosomiasis and could as well constitute a novel and effective trypanocide without side effects. Although the aforementioned studies have demonstrated that myristic acid analogues are effective inhibitor of protein-tyrosine phosphatase, but being synthetic products would most likely pose the challenge of toxicity and adverse effects known to be associated with synthetic agents,[37,38] unlike inhibitor from natural sources that have little or no side effects and adverse reactions.[39–41]

In a side-by-side comparison of inhibition of protein-tyrosine phosphatase by the bioactive fraction of the stem bark extracts and sodium orthovanadate (specific inhibitor of protein-tyrosine phosphatase), results showed that while the inhibitors in the extracts inhibited the enzyme non-competitively, sodium orthovanadate inhibited the enzyme competitively as shown in Figures 1, 2 and 6, respectively. Compared to the competitive inhibition by sodium orthovanadate, it implies that the non-competitive inhibitors in the bioactive fraction of the stem bark extracts of *Khaya senegalensis* and *Tamarindus*

indica are better inhibitors of the enzyme. This is because increase in substrate concentration would not reverse the inhibition unlike in competitive inhibition where increase in substrate concentration reverses inhibition. 4-2-Hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) was used in the inhibition assay of protein-tyrosine phosphatase because it does not complex with sodium orthovanadate, unlike other buffers, hence, an ideal buffer. Although ethylenediaminetetraacetic acid (EDTA) is commonly included in protein-tyrosine phosphatase assays, it was excluded here because it is known to chelate and form a complex with sodium orthovanadate.[42] Protein-tyrosine phosphatase is specific for dephosphorylating phosphotyrosyl residues of proteins and peptides,[43] making it a key regulator of a variety of fundamental cellular processes. This work has introduced naturally occurring myristic analogues that are potent inhibitors of protein-tyrosine phosphatase which can be possibly used to attenuate the disease of trypanosomiasis.

Conclusion

The experiment described in this paper has shown that analogues of myristic acid isolated from stem bark of *Khaya senegalensis* and *Tamarindus indica* are non-competitive inhibitors of *Trypanosoma evansi* protein-tyrosine phosphatase. If harnessed, the myristic acid analogues could be developed as effective and potent trypanocides that would inhibit the enzymatic activity of protein-tyrosine phosphatase in trypanosomes.

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Disclosure

The authors report no conflicts of interest in this work.

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