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Research Article

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Synthesis and *in vitro* anti-trypanosomal activity of Vanillic Acid and *Para*-Hydroxybenzoic Acid on *T. congolense*

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Abstract The reported toxicities of antitrypanosomal drugs and the emergence of drug resistant trypanosomes underscore the need for the development of new antitrypanosomal agents. We report herein the synthesis of phenolic acid derivatives (vanillic acid and *para*-hydroxybenzoic acid) and their antitrypanosomal potential against *Trypanosoma congolense*. The compounds were synthesized by oxidizing the aldehyde functional groups (-CHO) in the starting materials to the corresponding carboxylic acids ($-CO_2H$) using Jones reagent and were then characterized by FTIR. *In vitro* testing of these compounds in five different concentrations of 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml in 10% DMSO was carried out in triplicates in 96 well micro titer plates on the blood infected with *Trypanosoma congolense*. Vanillic acid was found to be most effective showing a remarkable decrease in the number of parasite with an IC_{50} of 768 µg/ml.

Keywords Vanillic Acid, *Para*-hydroxybenzoic acid, *Trypanosoma congolense*, DMSO, Jones reagent

Introduction

African Trypanosomiasis, sleeping sickness is a parasitic disease of people and animals such as cattle, sheep, goats, pigs, horses and donkeys, caused by protozoa of the species *Trypanosoma brucei* and transmitted by the tsetse fly [1]. Trypanosomes are able to infect a wide variety of domestic animals and more than 30 species in the wild. The pattern of this disease is mainly affected by difference in the distribution of pathogenic trypanosomes [1]. *Trypanosoma congolense* (*T. congolens*), *Trypanosoma vivax* (*T. vivax*), *Trypanosome brucei* (*T. brucei*) are always found within the tsetse fly infected areas. *T. congolense* is considered the most important cause of African animal trypanosomiasis in East Asia and *T.vivax* in West Africa. *Trypanosoma congolense* is a monomorphic (12.1–17.6 µm) salivarian parasite (development take place in the mid-gut and mouthpart of tsetse flies) which lacks a free flagellum at any stage of development [2]. The parasite causes trypanosomiasis which is characterized by severe anemia, weight loss, reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease [1].

Only salts of three compounds, diminazine, homidium and isometamidium are currently in use to which resistance has been developed a decade after their introduction to the market [3]. The emergence of drug resistance trypanosome strains is considered a very serious problem in control of trypanosomiasis particularly for the resource-poor at risk populations and farmers in Africa. Recent survey in Eastern and Southern Africa and in West Africa has shown that the prevalence of trypanocidal drug resistance might even be higher than hitherto expected [4].

The limited availability and affordability of pharmaceutical medicines and parasite resistance emphasizes the need for research into a more comprehensive, formidable and cheaper sources of trypanocide [3]. Synthetic drug from phenolic acid derivatives are gaining popularity because of several advantages such as iron chelating properties, fewer side effect, better patient tolerance, relatively less expensive and its acceptance [5].

Vanillic acid and p-hydroxybenzoic acid are Phenolic acids moieties which are abundant plant secondary metabolites, and there have been reports on their iron chelating properties [5]. Trypanosomes require sufficient amount of intracellular iron for cellular activities such as DNA synthesis and energy metabolism [5]. Studies have shown the trypanocidal activity of both synthetic and siderophore derived iron chelators. The iron chelator, deferoxamine, have been shown to inhibit the growth of parasites *in vitro*, affect the activity of ribonucleotide reductase as well as the G1-S phase of the cell cycle [6]. The differential expression of the parasite's transferrin receptor and cyclin genes in response to iron deprivation has also been reported [7]. Deferoxamine has however shown some level of toxicity against mammalian cell lines [5]. Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a dihydroxybenzoic acid derivative used as flavoring agent. It is oxidized form of vanillin. It is also an intermediate in the production of vanillin from ferulic acid [8]. The highest amount of vanillic acid in plant known so far is found in the root of *angelica sinesis*, *a* herb indigenous to china which is used in traditional Chinese medicine. Vanillic acid is used in the synthesis of analeptic drug such as etamivan and can also be acetylated and converted into its acid chloride [9]. Recent studies have provided evidence of the effectiveness of vanillic acid in the management of immune or inflammatory responses [10]. Vanillic acid enhanced the activity of human lymphocyte proliferation and secretion of interferon-gamma in human peripheral blood mononuclear cells [10]. Other studies have also shown that vanillic acid has a hepatoprotective effect through its suppressive action on immune-mediated liver inflammation in concanavalin A-induced liver injury [10-11]. Accordingly, vanillic acid can significantly reduce the clinical signs of Ulcerative collitis and levels of inflammatory mediators in a mouse model of dextran sulfate sodium (DSS) induced colitis, since treatment with vanillic acid reduced the weight loss and colon shortening caused by dextran sulfate sodium (DSS) [12]. Such results suggest that vanillic acid might effectively reduce the severity of the colitis symptoms caused by dextran sulfate sodium (DSS) [12].

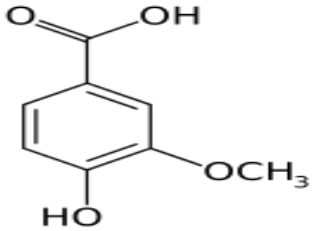

Figure 1: Structure of vanillic acid

Para-hydroxy benzoic acid (4-hydroxybenzoic acid) is a biodegradable, less bioaccumulative organic compound which can be isolated from a diverse natural sorces such as carrots (*Daucus carota*), oil palm *(Elaeis guineensis*), grapes *(Vitis vinifera*), and the rest [13]. *Para*-hydroxy benzoic acid is reported to have antibacterial, antifungal, antialgal, antimutagenic, antisickling and estrogenic activity [14]. It has been reported that *para*-hydroxybenzoic acid and trans-4-hydroxycinnamic acid isolated from rice hull demonstrated some antibacterial activity against most of Gram +ve and some of Gram -ve bacteria at 50% inhibitory concentration of 160 and 100-170µg/mL respectively. It was suggested that lipophillicity seemed to be an important factor that strongly influences the antimicrobial activity of *para*-hydroxybenzoic acid as compared to that of trans-4-hydroxycinnamic acid [15]. Induction and control of p-hydroxybenzoic acid under stress conditions are important for the antioxidative system because biosynthesis of salicylic acid is catalyzed by benzoic acid hydroxylase and connected with p-hydroxybenzoic acid [16]. Furthermore, Horvath et al., 2007 reported that *p*-hydroxy benzoic acid increases abiotic stress tolerance of winter wheat (*Triticum aestivum*) and also increases the impermeability of the cell wall, leading to increased resistance against pathogen infection [17].

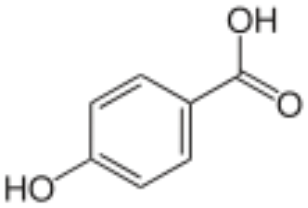

Figure 2: Structure of 4-hydroxybenzoic acid

In the light of these facts we decided to synthesize some phenolic acid derivative (vanillic acid and p-hydroxybenzoic acid) and access their antitrypanosomal potential against *trypanosoma congolense* isolated from natural infection of cattle which was infected in to albino rats.

Materials and Methods

General

All chemicals were used as received from commercial source (Sigma-Aldrich). FTIR spectroscopic data were recorded on Cary 630 (Agilent technologies). Diminazine aceturate was used as the reference drug. The test organism *T. congolense* was isolated from infected cattle at Nigerian Institute for Trypanosomiasis Research Kaduna.

Chemical Synthesis:

Preparation of Jones reagent

Jones reagent was prepared by dissolving 25g of potassium dichromate in 75mL of distilled water in 500mL beaker and 25mL of sulfuric acid was added slowly with careful stirring and was then cooled in an ice-water bath [18].

Oxidation of Vanilline to Vanillic Acid

Following reported procedure [20], 8g of vanillin was weighed and dissolve in 50mL of acetone, which was then titrated with Jones reagent until the orange-brown color of the reagent persisted for 30 second. The reaction mixture was then stirred by a magnetic stirrer for an additional of 1h at room temperature. 100mL of distilled water was added to the solution and the suspension was extracted by liquid-liquid extraction with 25mL of ethyl acetate and dried by evaporation in open air to afford vanillic acid, yield (6.314g, 69.77%), appearance white to light yellow powder, FTIR (cm^{-1}) 3200 (OH of COOH) 1700 (C=O) and 1125 (C-O).

Oxidation of *para*-hyroxybenzaldehyde to *para*-hydroxybenzoic Acid

Following reported procedure [20], 8g of 4-hydroxybenzoic acid was weighed and dissolve in 50mL of acetone, which was then titrated with Jones reagent until the orange-brown color of the reagent persisted for 30 second. The reaction mixture was then stirred by a magnetic stirrer for an additional of 1h at room temperature. 100mL of distilled water was added to the solution and the suspension was extracted by liquid-liquid extraction with 25mL ethyl acetate and dried by evaporation in open air to afford *para*-hydroxybenzoic acid, yield (5.729g, 64.81%), appearance white crystalline, FTIR (cm^{-1}): 3200 (OH of COOH) 1700 (C=O) and 1125 (C-O).

Sample Preparation

The two phenolic acid derivatives were weighed into eppendorf tubes and dissolved in 10% dimethylsulfoxide (DMSO) in PBS to produce effective solution of concentrations 50µg/mL, 100µg/mL, 200µg/mL 400µg/mL and 800µg/mL. The compound solutions were prepared just before use [2].

Assessment of *In Vitro* Trypanosomal Activity

Infected blood was obtained by cardiac puncturing of albino rats at peak parasitemia. Assessment of *in vitro* trypanocidal activity was done in triplicate in 96 wells microtitre plate (Flow laboratories Inc).1mL of the infected blood was obtained by cardiac puncturing of albino rat at peak parasetemia (0.0000001 tryponosome/mL) and was

put into EDTA coated tubes. Stock solutions of the compounds were first prepared in phosphate buffer saline glucose (PBSG). 20µL of the blood containing 20-25 parasites per field was added in each of the 96 well micro titre plates and was mixed with 5µL of 50µg/mL, 100µg/mL, 200µg/mL, 400µg/mL and 800µg/mL of each of vanillic acid and para-hydroxybenzoic acid respectively [19]. This produces effective concentrations of 10µg/mL, 20µg/mL, 40µg/mL 80µg/mL and 160µg/mL respectively. After incubation period of 5 minutes further analysis were carried out.

Cessation or drop in motility of the parasite in compound treated blood compared to that of parasite loaded control blood without compound was taken as a measure of anti trypanosomal activity [19]. The shorter the time of cessation of motility of the parasite, the more active the compound was considered to be. Triplicates of treated blood with diminazine aceturate was used as the positive control while untreated blood and parasite suspended in 10% DMSO alone act as negative control to ensure that the effect monitored was that of the compound alone. After 5 minutes incubation covered micro titre plate maintained at 37 degree Celsius, a drop of test mixture was placed on a separate microscopic slide covered with a cover slip and the motility of the trypanosome was observed under the microscope (400 or 40x) at 10 minutes interval for 1 hour. [20]. The IC_{50} (Half maximal inhibitory concentration) of the compound that immobilized the parasite was determined and the activity (in percentage) was plotted against log concentration by linear regression plot using Microsoft excel (2007).

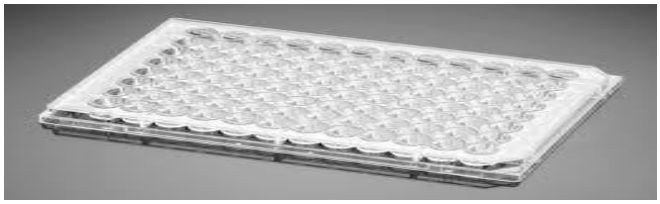

Figure 3: 96 well titer plate

Results and Discussion

To determine the *in vitro* antitrypanosomal activities, we decided to study two phenolic acid derivatives (vanillic acid and p-hydroxybenzoic acid) which were obtained by oxidizing their respective aldehydes. The FTIR spectroscopic data indicated the presence of a strong and broad carboxylic acid -OH band around 3200 cm^{-1} while the carbonyl band appeared around 1700cm^{-1}. Finally, a strong band due to the C-O of the carboxylic acid is observed at 1125cm^{-1} was indicative of successful formation of the compounds (Fig 6 and 7).

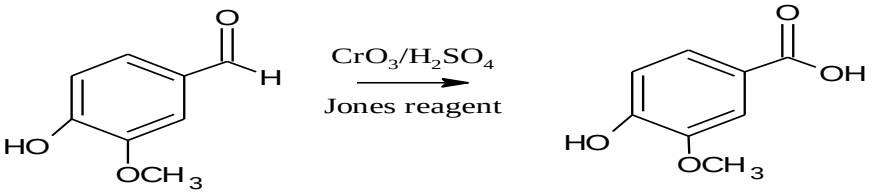

Figure 4: Oxidation of vanilline to Vanillic acid

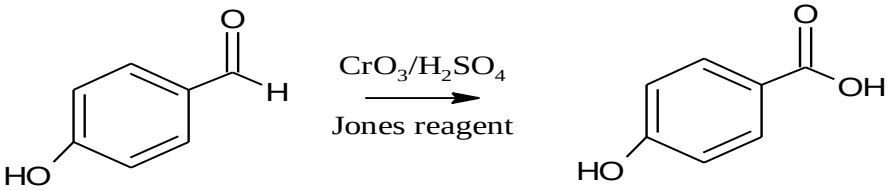

Figure 5: Oxidation of para-hydroxybenzaldehyde to para-hydroxybernzoic acid

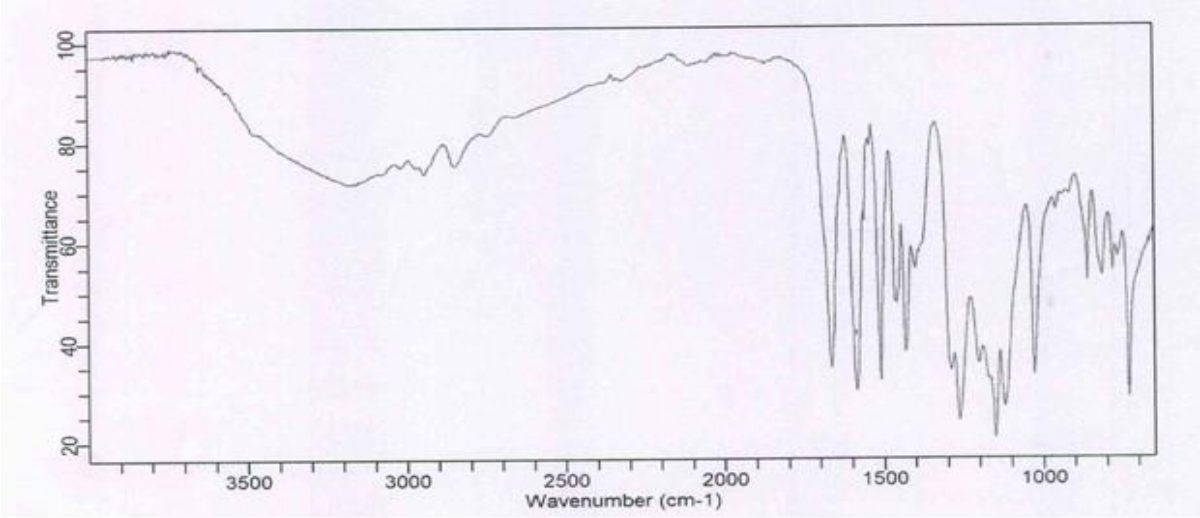

Figure 6: FTIR spectrum of Vanillic acid

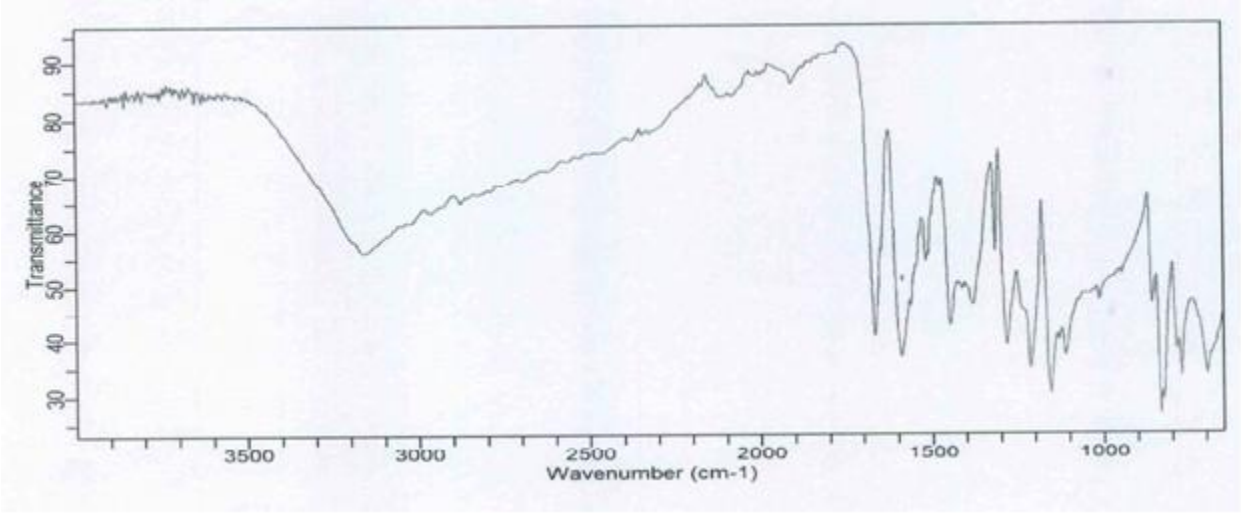

Figure 7: FTIR spectrum of p-hydroxybenzoic acid

***In vitro* antitrypanosomal activities**

The results for the *in vitro* antitrypanosomal activities (Table 1 and 2) revealed that para-hydroxybenzoic acid showed no significant decrease in the motility of parasite whereas vanillic acid showed a remarkable decrease in the number of parasite with an IC_{50} of 768g/µml (Fig 8). Diaminazene aceturate a standard drug for trypanosomes was used as a positive control which was able to immobilzed the entire parasite at all concentration after 30minute of incubation while the negative control DMSO on other hand shows no significant decrease in the motility of parasite. The activity of the vanillic acid against the parasite might be due to the presence of methoxy group at the third carbon atom of the compound which might improves the iron chelating properties of the compound, since trypanosomes require cellular iron for their DNA synthesis and energy metabolism.

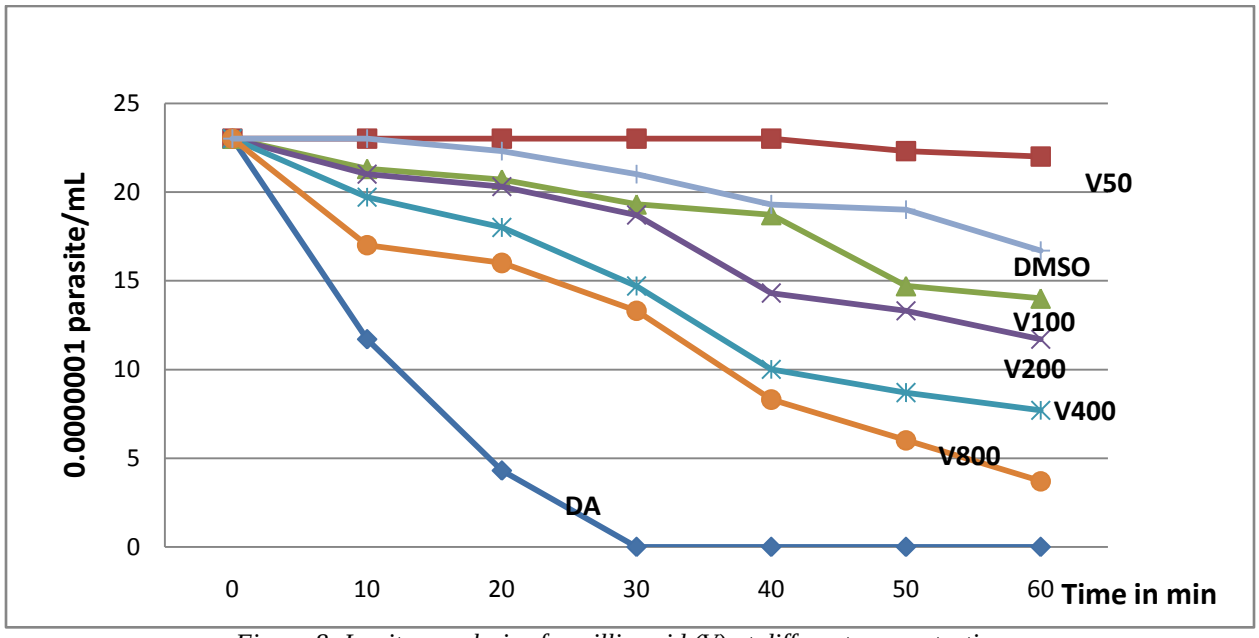

Figure 8: In-vitro analysis of vanillic acid (V) at different concentrations

V50, V100, V200, V400 and V800 depict *in-vitro* analysis of vaniline (4-hydroxy-3-methoxybenzoic acid) with concentrations 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml respectively. The vanillic acid showed a remarkable decrease in the number of the parasite. The positive control (diminazine aceturate) immobilized the entire parasite within 30 minutes whereas the negative control showed no significant decrease in the number of parasite.

Key: V- Vanillic acid; Negative control- 10% Dimethysulfoxide; Positive control (DA)- Diminazine aceturate

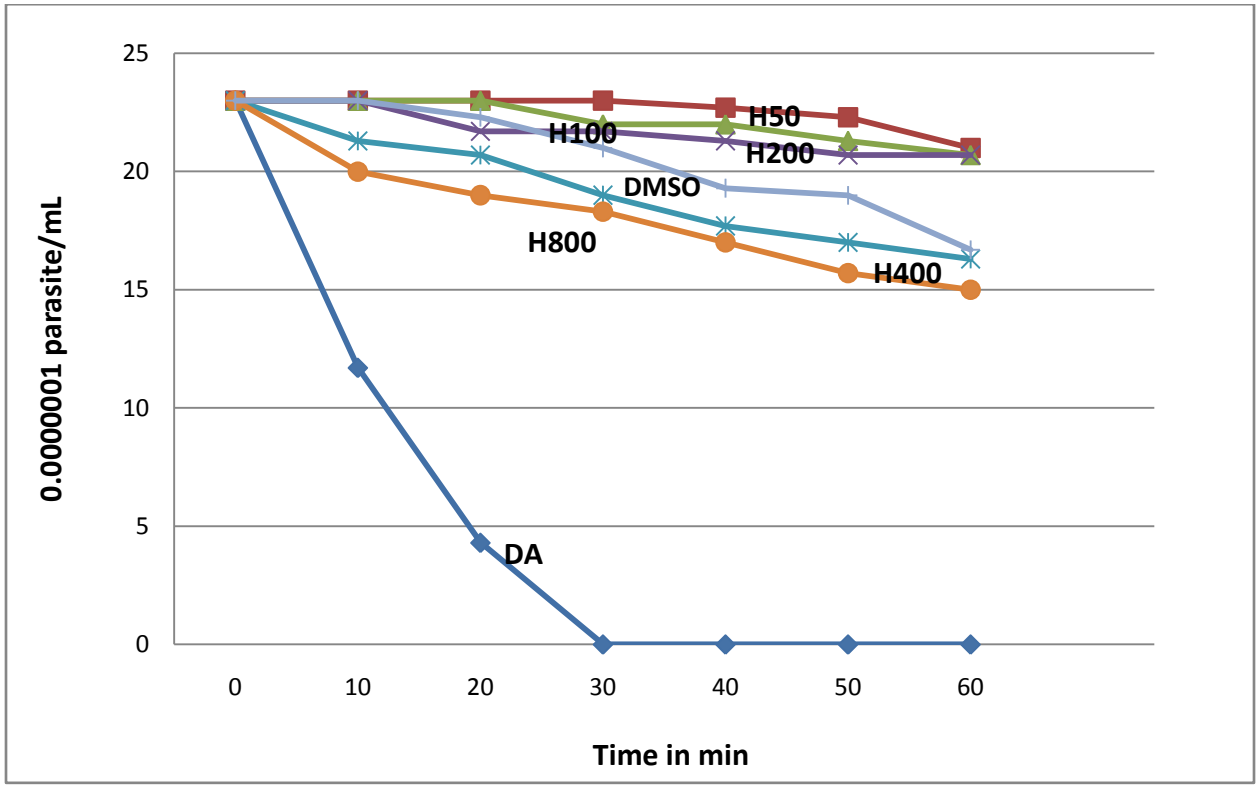

Figure 9: In vitro analysis of p-hydroxybenzoic acid (H) at different concentrations

H50, H100, H200, H400 and H800 depict *in-vitro* analysis of p-hydroxy-benzoic acid with concentrations 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml respectively. The 4-hydroxy benzoic acid showed no significant decrease in the number of the parasite. The positive control (diminazine aceturate) immobilized the entire parasite within 30 minutes whereas the negative control showed no significant decrease in the number of parasite

Key: H ------- p-Hydroxybenzoic acid

Negative control---------- 10% Dimethysulfoxide

Positive control (DA) ----------Diminazine aceturate

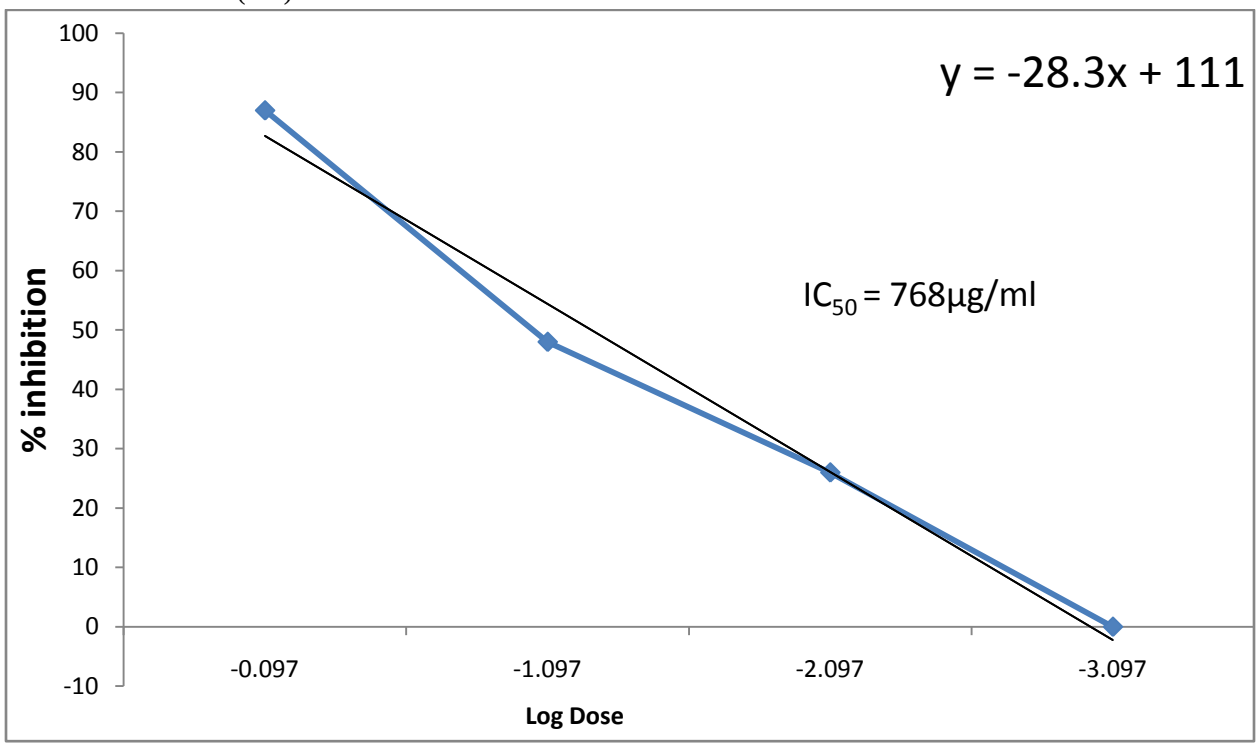

Figure 10: The half maximal inhibitory concentration (IC50) value determination

The IC_{50} (Figure 8) below gives the half maximal inhibitory concentration of vanillic acid at 768µg/ml.

Conclusions

In this study we presented the synthesis of phenolic acid derivatives (vanillic acid and para-hydroxybenzoic acid), which were obtained in good yield and their antitrypanosomal activities were evaluated against *trypanosoma congolense*. *In vitro* activity testing of these compounds were evaluated at five different concentrations and it was observed that vanillic acid exhibited the most significant decrease in the motility of the parasite with an IC_{50} of 768µg/ml. Conclusively, phenolic acid derivatives, especially vanillic acid are a good candidates for the discovery of new potent anti trypanosomal drugs.

Conflict of Interests

The authors declare no conflict of interests.

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