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RESEARCH ARTICLE

Inhibition of HIV-1 and M-MLV reverse transcriptases by a major polyphenol (3,4,5 tri-*O*-galloylquinic acid) present in the leaves of the South African resurrection plant, *Myrothamnus flabellifolia*

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Abstract

A polyphenol-rich extract of the medicinal resurrection plant *Myrothamnus flabellifolia* was shown to inhibit viral (M-MLV and HIV-1) reverse transcriptases. Fractionation and purification of this extract yielded the major polyphenol, 3,4,5 tri-*O*-galloylquinic acid, as the main active compound. A sensitive, ethidium bromide based fluorescent assay, was developed and used to monitor the kinetics of M-MLV and HIV-1 reverse transcriptases in the presence and absence of 3,4,5 tri-*O*-galloylquinic acid. Kinetic monitoring of these enzymes in the presence of 3,4,5 tri-*O*-galloylquinic acid revealed non-competitive inhibition with IC_{50} values of 5 μM and 34 μM for the M-MLV and HIV-1 enzymes, respectively. We propose that 3,4,5 tri-*O*-galloylquinic acid and related polymers have potential as indigenous drugs for anti-viral therapy.

Keywords: Polyphenols, anti-viral, indigenous drug

Introduction

Southern Africa possesses a tremendous diversity of plant species, many of which have medicinal value[1]. One such medicinally valued species, *Myrothamnus flabellifolia*, is present throughout Southern Africa and is used commonly to treat a number of ailments. The leaves of this species are used to make tea (for treatment of backaches, kidney disorders, coughs and colds), to produce salves (for wound healing) and can be smoked to alleviate chest complaints (for a review, see refs. [2,3]). In addition, *M. flabellifolia* is a member of a unique group of species termed resurrection plants because its vegetative organs (e.g. leaves and roots) have the ability of surviving reversible dehydration to an air dry state[3,4]. This remarkable "reviving ability" is exemplified in the action of placing dehydrated dry twigs in water and observing over the period of a few hours how the leaves are able to "miraculously" expand and turn green[3]. Hence, the plant is also used as a psychological "charm" in African culture to treat severe depression. The leaves of *M. flabellifolia* have been reported to contain essential oils such as camphor and eucalyptol[5]. These essential oils are thought to play a role in the healing properties of this plant[6]. Recent studies have also shown that *M. flabellifolia* leaves contain a substantial amount of polyphenols (40% by dry weight)[7]. We have extracted and characterized the predominant polyphenol present in the leaves of this plant to be 3,4,5 tri-*O*-galloylquinic acid[7]. The polyphenol composition of which varied among plant samples from different geographical locations[8]. Namibian plants, for instance, were shown to consist mainly of 3,4,5-tri-*O*-galloylquinic acid and certain low molecular weight galloylquinate polymers, whereas South African plants consisted of 3,4,5-tri-*O*-galloylquinic acid as well as higher molecular weight galloylquinic acids[7,8]. We have furthermore demonstrated that a crude polyphenol-rich extract of the leaves of this plant inhibited the *in vitro* production of complementary DNA (cDNA) via reverse

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transcriptase (RT)-polymerase chain reaction (PCR)[9], and a preliminary screen of related compounds have been shown to inhibit human immunodeficiency virus (HIV) RTs[10,11]. The HI-virus has infected 40.3 million people worldwide, and in South Africa almost 5.5 million people are infected (UNAIDS, 2006). Of the two distinct types of HIV, the HIV-1 is the most pathogenic and the most relevant for Southern Africa[12]. Although HIV/AIDS is a comparatively new human disease, with little in the way of an ethnobotanical history of medical research, clinical screens can be developed which evaluate different plant extracts in treating HIV/AIDS related symptoms (e.g. viral infections give rise to coughs, diarrhoea, wasting etc.) and can ultimately lead to the discovery of novel promising drugs. The HIV-1 RT is a much studied RT since it plays a crucial role in the life cycle of HIV[13]. As such, it has been a major target of many anti-viral drug therapies, one such example is nevirapine. Since nevirapine is a non-nucleoside inhibitor, nevirapine therapy for HIV infection suffers from rapid selection of resistant HIV mutant strains. This resistance has generally been associated with Tyr 181 and Tyr 188 mutations within the non-nucleoside RT inhibitor's binding pocket[14]. Thus, there is an urgent need for discovering and characterizing new inhibitors for HIV-1 RTs.

In this report we have fractionated the polyphenol-rich extract from the resurrection plant *M. flabellifolia* and isolated the most effective RT inhibiting "active" fraction. We screened the polyphenol fractions using a novel "ethidium bromide" based fluorescence assay, which we developed, for RT activity (using Moloney murine leukemia virus (M-MLV)-RT as the control enzyme). We identified this compound as 3,4,5-tri-*O*-galloylquinic acid and further determined the kinetic parameters related to the inhibition of M-MLV and HIV-1 RT enzymes in the presence of this compound. The results obtained suggest that 3,4,5-tri-*O*-galloylquinic acid and related compounds may prove useful as components of an indigenous anti-viral therapy.

Materials and methods

Chemicals and reagents

Calf thymus DNA, dAMP, ATP, and thymidine was purchased from Sigma (St. Louis, MO). The ultra-pure lithium salts of dTTP, dATP, dCTP, and dGTP were purchased from Bioline (London, UK). Thymine was purchased from Merck. Tris-HCl and ethidium bromide were from Research Organics (Cleveland, OH) and Merck (Darmstadt, Germany), respectively. Sodium pyrophosphate was purchased from Riedel-De Hahn (Seelze, Germany). Poly(rA) template (~1000 bases) was a donation from Ribotech (Cape Town, South Africa). An oligo$(dT)_{25}$ primer was synthesized (DNA synthesis service, University of Cape Town). M-MLV RT was purchased from Promega (Madison, WI). HIV-1 RT was a kind gift from Professor E. Rybicki (Department of Molecular and Cell Biology, University of Cape Town).

Extraction and fractionation of polyphenols

Naturally desiccated *M. flabellifolia* plants were collected from locations in Namibia (co-ordinates: S 22°25.497′ E 15°51.156′ at an elevation of 868 m above sea level)[8]. Leaves (~5 g) were ground under liquid nitrogen using a pestle and mortar to a fine powder. Polyphenols were extracted from the material according to the method of Makkar et al. (see ref. 15). Briefly, samples were pre-extracted (twice) using hexane with sonication treatment under a nitrogen atmosphere. The pellets obtained after centrifugation (force of 5000*g* for 10 min), were then treated with (70%) aqueous acetone twice. The acetone supernatants after centrifugation (force of 5000*g* for 10 min) were pooled, concentrated via rotary evaporation, lyophilized and then stored at –20°C. Total phenolics in the extract were determined with the Folin-Ciocalteau reagent using the method outlined in Makkar et al. (see ref. 15) with gallic acid as a standard. The crude lyophilized polyphenol-rich extract (~62 mg) was dissolved in 4 ml 10% aqueous methanol by sonication for 5 min after which any insoluble material was removed by centrifugation (force of 5000*g* for 10 min). The supernatant fraction was applied to a Sephadex LH-20 (Amersham Pharmacia Biotech AB SE-751 Uppsala Sweden) column (packed volume of 11 ml) equilibrated in water. Fractions were eluted with stepwise increasing concentrations of 0, 10, 30, 50, 70 and 100% of methanol and monitored spectrophotometrically at 280 nm. Fractions were assayed for inhibitory activity using a fluorescence-based assay (outlined later in Materials and methods).

Liquid chromatographic and mass spectrometric analysis

High performance liquid chromatography (HPLC) analysis of the crude and fractionated polyphenol-rich extracts was performed using a Shimadzu LC-10 system equipped with a photodiode array detector. Compounds were separated on a Jones C18 column (250 mm × 4.6 mm, 5 µm particle size), pre-equilibrated in 0.1% (v/v) aqueous trifluoroacetic acid (TFA), using a 0–100% gradient of acetonitrile at a flow rate of 0.7 ml/min[7]. Peaks present in the chromatographic profile of the crude extract, previously identified[8] were re-identified by co-injecting aliquots from selected fractions on the system. Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was performed as outlined in Moore et al.[7]. Samples were dissolved in 50% acetonitrile and applied to the sample plate. Dihydrobenzoic acid was used as the matrix. The instrument was operated in the delayed extraction mode and positive ions were analyzed.

DNA quantification by fluorimetry and poly (rA) and oligo$(dT)_{25}$ titration

Calf thymus DNA (ctDNA) was chosen as a standard for DNA quantification. ctDNA was dissolved in double distilled water and the DNA concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer). ctDNA was diluted in Tris-HCl

buffer (40 mM Tris, 100 mM NaCl, pH 7.2) to give a concentration range of 0–8 μg/ml. Ethidium bromide was added to a final concentration of 2.5 μg/ml. Tris-HCl buffer was added to bring the final volume to 1.0 ml. Tris-HCl buffer and ethidium bromide were prepared using double distilled and sterilized water. For poly (rA) and oligo$(dT)_{25}$ titration, a poly (rA) template solution was prepared by dissolving the polymer in RNase free water and the RNA concentration was determined spectrophotometrically. The poly (rA) solution was diluted to a working concentration of 1.00 μg/μl. Poly (rA) (2.5 μg) was mixed with various amounts of oligo$(dT)_{25}$ primer up to 5 μg. The final volume of the poly (rA) and oligo $(dT)_{25}$ mixture was made to 50 μl with RNase free water. Poly(rA) was omitted from the control reaction. The mixture was incubated at 65°C for 5 min, chilled on ice for at least 1 min and then incubated at room temperature for 15 min. Tris-HCl buffer (945.0 μl, pH 7.2) and ethidium bromide (2.5 μg/ml) was added to the mixture to bring the final volume to 1.0 ml. Fluorescence was measured using an Aminco SPF 500 fluorometer. The excitation wavelength was 545 nm with a 5-nm bandpass, and the emission wavelength was 605 nm with a 10-nm bandpass.

Assay for M-MLV and HIV-1 RT activity

First strand cDNA synthesis using M-MLV and HIV-1 RT was performed according to their respective manufacturer's protocol with some variation. Filter-sterilisation of reagents and mixtures was performed where necessary. Poly (rA) template (12.5 μg) and oligo $(dT)_{25}$ primer (0.25 μg) were mixed to give a total volume of 25 μl. The mixture was centrifuged (Eppendorf microcentrifuge, force of 5000*g* for 5 min) briefly and then incubated at 65°C for 5 min. The mixture was then chilled on ice for at least 1 min and allowed to stand at room temperature for 15 min. Reagents for M-MLV RT assay were added to the poly (rA).oligo$(dT)_{25}$ mixture in the order shown: M-MLV-RT buffer (5×, 25 μl), dTTP (up to 4 mM final concentration), and RNase free water. The mixture was vortexed and centrifuged briefly (force of 5000*g* for 5 min) to ensure thorough mixing. M-MLV RT enzyme (170 units, 0.071 μM) was added to the mixture to give the 100.0 μl final assay volume, mixed by gently tapping the bottom of the tube, briefly centrifuged (force of 5000*g* for 5 min) as before and then immediately incubated at 37°C. Reagents for the HIV-1 RT assay were added to the poly (rA).oligo$(dT)_{25}$ mixture as follows: HIV-1 RT buffer (10×, 16.7 μl), dTTP (0–2 mM) and RNase free water to make the final volume to 25 μl. The mixture was treated as per the M-MLV RT assay before addition of HIV-1 RT enzyme (20 units, 0.17 μM). The mixture was incubated at 42°C. Aliquots (20 μl) were taken at 5-min time intervals and immediately added to the Tris-HCl buffer (975 μl pH 7.2,) containing ethidium bromide (2.5 μg/ml) to a final volume of 1.0 ml. Ethidium bromide fluorescence was measured as described previously. The assay for M-MLV RT activity was performed in the presence of 1.0 mM dTTP and either dATP, dCTP, dGTP, dAMP, ATP, thymidine, thymine, pyrophosphate or phosphate. All additional compounds were added to a final concentration of 1.0 mM.

Inhibition of M-MLV and HIV-1 RT activity by polyphenol-rich extracts

The effect of crude and purified polyphenols (F_1, F_2–F_6) on enzyme activity was initially performed using M-MLV RT in the presence of 1.2 μg/ml final polyphenol concentration. The dTTP and Mg^{2+} concentrations were fixed at 1.0 and 3.75 mM, respectively. Polyphenol fractions which exhibited 100% M-MLV RT inhibition were diluted and re-assayed. The most effective polyphenol fraction was further analysed to establish the 50% inhibitory concentration (IC_{50}) values for both M-MLV and HIV-1 RTs. Polyphenols were omitted from control reactions. The effect of nevirapine on M-MLV and HIV-1 RT was determined using nevirapine concentrations of up to 1.0 mM and 1 μM for M-MLV and HIV-1 RT, respectively.

Inhibition kinetics of M-MLV and HIV-1 RT activity by 3,4,5 tri-*O*-galloylquinic acid

An enzyme activity assay to determine the nature of the 3,4,5 tri-*O*-galloylquinic acid inhibition of M-MLV and HIV-1 RT was carried out in the presence of 0.5 μM and 34 μM of the polyphenol, respectively. The concentration of Mg^{2+} ions was held constant at 3.75 mM and dTTP concentrations up to 1.0 mM were used for M-MLV RT, whereas 5.0 mM Mg^{2+} and dTTP concentrations up to 0.25 mM were used for HIV-1 RT.

Competition assay of 3,4,5 tri-*O*-galloylquinic acid binding to M-MLV and HIV-1 RT

To determine how tightly the polyphenols were bound to the M-MLV RT enzyme, soluble polyvinylpyrollidone (PVP) and bovine serum albumen (BSA) were added to the cDNA synthesis assay, either alone or in combination with 3,4,5 tri-*O*-galloylquinic acid. The final concentration of PVP and BSA in the assay mixture was 0.03 mg/ml, resulting in molar ratios of PVP to M-MLV RT and BSA to M-MLV RT of 11:1 and 63:1, respectively. The final 3,4,5 tri-*O*-galloylquinic acid concentration used was 2.4 μM. The assay was performed as follows: (i) PVP or BSA was added in the absence of 3,4,5 tri-*O*-galloylquinic acid followed by M-MLV RT and then immediately incubated at 37°C. (ii) The enzyme was initially mixed with 3,4,5 tri-*O*-galloylquinic acid, briefly vortexed and centrifuged (force of 5000*g* for 5 min) to mix the contents and then allowed to stand on ice for 5 min. PVP or BSA was then added and the mixture allowed to stand on ice for another 5 min before performing the enzyme assay. (iii) PVP or BSA was initially added to the assay mixture containing 3,4,5 tri-*O*-galloylquinic acid, mixed thoroughly and allowed to stand on ice for 5 min before adding M-MLV RT.

The mode of 3,4,5 tri-*O*-galloylquinic acid inhibition of HIV-1 RT was determined by performing the inhibition assay in the presence of 100 μM 3,4,5 tri-*O*-

galloylquinic acid and 11.0 μM BSA. The molar ratio of BSA to HIV-1 RT was 63:1. The enzyme activity assay was initially performed in the presence of 3,4,5 tri-*O*-galloylquinic acid and BSA alone. Secondly, the HIV-1 RT enzyme was mixed with 3,4,5 tri-*O*-galloylquinic acid and allowed to stand for 5 min on ice before BSA addition. Finally, BSA was mixed with 3,4,5 tri-*O*-galloylquinic acid, allowed to stand for 5 min on ice before HIV-1 RT enzyme addition. cDNA synthesis by HIV-1 RT was performed at 42°C. Ethidium bromide fluorescence was measured to follow the progress of cDNA synthesis as before.

Results

Fractionation of polyphenols

The polyphenol-rich extract from *M. flabellifolius* leaves was dissolved in aqueous methanol and fractionated using a Sephadex LH-20 column with increasing concentrations of methanol. Elution was monitored by measuring the absorbance at 280 nm and resulted in five distinct chromatographic fractions (F_1, F_2–F_6) (see Figure 1). Fraction F_2 eluted with 10% methanol did not show any significant absorbance at 280 nm. Repeated fractionations were performed to confirm the reproducibility of the elution profiles as well as to collect sufficient material for further analysis. An estimate of the quantity of polyphenols in each fraction was obtained from the absorbance at 280 nm (Table 1). This amount was expressed as a percentage of the total absorbance of the crude "unfractionated" polyphenol-rich extract. Fraction F_6 contained the most phenolic material, followed by fractions F_5 and F_4, while fractions F_1 and F_3 registered the lowest amounts. In addition, the water solubility of the lyophilized polyphenols from each fraction was tested. It was observed that lyophilized material from fractions F_1, F_3 and F_4 readily dissolved in water, whereas F_5 and F_6 were not very soluble. It has been reported[16] that non-polymeric polyphenols are eluted from the Sephadex LH-20 matrix by 60% methanol, whereas polymeric polyphenols require more hydrophobic solvents. Hence, fractions F_5 and F_6 probably contain larger phenolic polymers. This would also explain why these fractions show limited water solubility and bind with strong affinity to the Sephadex LH-20 column.

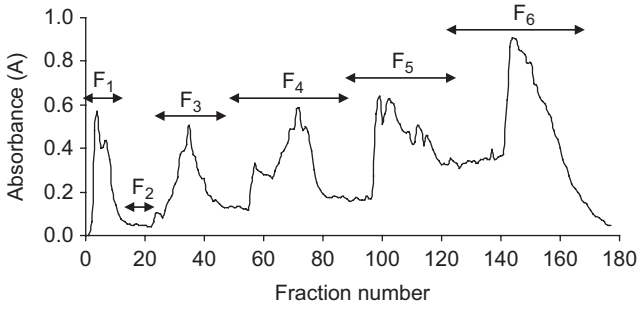


Figure 1. The elution profile of polyphenols eluted from a Sephadex LH-20 column using water (F_1), 10% MeOH (F_2), 30% MeOH (F_3), 50% MeOH (F_4), 70% MeOH (F_5) and 100% MeOH (F_6). The absorbance was measured at 280 nm.

Table 1. An estimation of the percentage of polyphenols in each fraction eluted from Sephadex LH-20.

Fraction	Amount of polyphenol (%)
F_1	11 ± 1.0
F_3	12 ± 0.5
F_4	18 ± 0.2
F_5	24 ± 6.0
F_6	38 ± 6.0

Analysis of polyphenols using liquid chromatography and mass spectrometry

Polyphenol fractions purified using Sephadex LH-20 chromatography were analysed using C18 reverse phase HPLC. The HPLC profiles for the crude and fractionated polyphenol extracts are shown in Figure 2. The HPLC profile for the crude extract showed six predominant peaks with one characteristic major peak (Figure 2). Based on previous studies, the major peak (Figure 2) was identified using a combination of nuclear magnetic resonance spectroscopy and mass spectrometry as 3,4,5 tri-*O*-galloylquinic acid[7]. This identification was re-confirmed using MALDI-TOF mass spectrometry of this peak after purification using reversed phase HPLC which revealed an m/z ratio of 648 (±1 Da), characteristic of 3,4,5 tri-*O*-galloyquinic acid[7]. Further characterisation of the fractions was performed using a combination of HPLC and MALDI-TOF mass spectrometry. HPLC analysis of the first fraction (F_1) eluted from the Sephadex LH-20 contained at least four peaks with a very small amount of 3,4,5 tri-*O*-galloylquinic acid based on retention time data. Fraction (F_2) contains very little UV absorbing material and consequently little phenolic material was present as confirmed by HPLC data. The HPLC profile for fraction F_3 showed one large peak. It was observed that the retention time for the single peak in F_3 was similar to the retention time for the major compound (3,4,5 tri-*O*-galloylquinic acid) of the crude extract profile. This was confirmed by co-injecting the two fractions onto the HPLC column (not shown). The profile showed a major peak at the identical retention time, suggesting that 3,4,5 tri-*O*-galloylquinic acid was eluted with 30% methanol. The profile for polyphenols eluted with 50% methanol (F_4) also showed a single major peak. This peak was confirmed by co-injection with F_3 to be 3,4,5 tri-*O*-galloylquinic acid, suggesting that significant amounts of 3,4,5 tri-*O*-galloylquinic acid was still bound to the column even though the column was extensively washed with 30% methanol. Confirmation that fractions F_3 and F_4 contained 3,4,5 tri-*O*-galloylquinic acid as the sole component was obtained by using mass spectrometry and nuclear magnetic resonance spectroscopy (data not shown). The spectra for fractions purified by

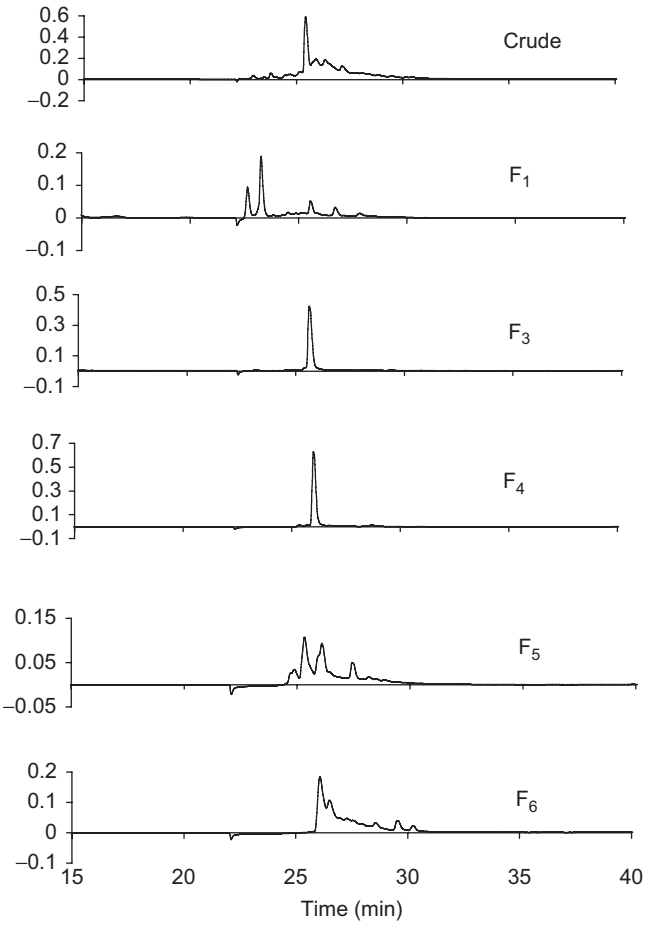


Figure 2. HPLC profile for crude and purified polyphenol fractions (F_1, F_3–F_6). The Y-axis represents the absorbance (mA) of polyphenols at 280 nm.

30% (F_3) and 50% (F_4) methanol showed two major ions at m/z 671 (±1 Da) and 687 (±1 Da). These ions were also present in the unfractionated/unpurified crude phenolic-rich extract mass spectral profiles (data not shown). It has been reported that the presence of Na^+ and K^+ ions during desorption and ionization results in the formation of $[M+Na]^+$ and $[M+K]^+$ ions, where M represents the molecular weight of the parent molecule[17]. The calculated m/z ratio of 3,4,5 tri-*O*-galloyquinic acid together with a sodium ion is m/z 671, similarly the potassium ion adduct would have an ion at m/z 687. It can therefore be concluded that the ion peaks observed were the Na^+ and K^+ adduct ions of the parent 3,4,5 tri-*O*-galloyquinic acid molecule. This is further supported by a previous analysis of phenolic fractions from *M. flabellifolia* leaves which demonstrated that sodium and potassium are common adducts of galloylquinic acid compounds[8]. The HPLC profile of the Sephadex LH-20 fractions (F_5) and (F_6) eluted with 70 and 100% methanol, respectively, showed four main peaks. Co-injecting with fraction F_3, confirmed that 3,4,5 tri-*O*-galloylquinic acid was not present in either fraction (data not shown). Based on these results, it would appear that 3,4,5 tri-*O*-galloylquinic acid was optimally eluted with a methanol concentration in the 30–50% range. The mass spectra of these latter fractions contained complex higher molecular weight compounds which have been reported to be formed by depside bonds and/or oxidative addition of gallic acid moieties to 3,4,5 tri-*O*-galloylquinic acid[8]. The compounds identified by mass spectra (data not shown) as being present in fractions (F_5) and (F_6) have been putatively identified as higher molecular weight (i.e. penta-, hexa-, hepta-, octa- galloylquinate) polymers[8].

Development of a fluorescence-based assay for RT activity

Assays for RT activity are traditionally based on the incorporation of ^{32}-P labelled deoxynucleoside triphosphates (dNTPs) into a primer-template complex. Such assays are relatively expensive and require the use of specialized facilities/equipment. We have therefore developed a convenient "inexpensive" assay for RT activity based on the fluorescence of ethidium bromide when incorporated into the double-stranded product of the reaction. The assay products could then monitored using a standard UV "lightbox" DNA gel photography and fluorescence spectroscopy system readily available in molecular biology laboratories.

Initially, we wished to confirm the linear relationship between ethidium bromide fluorescence and DNA concentration. The RT assay was designed to synthesise cDNA from a poly (rA) template of an average length of approximately 1000 nucleotides using an oligo(dT)25 primer together with dTTP. The concentration of the cDNA produced was then determined from the fluorescence at 605 nm after addition of excess ethidium bromide to the reaction mixture. Assuming that the binding of ethidium bromide to calf thymus dsDNA and its subsequent fluorescence is equivalent to binding to cDNA, a linear relationship ($R^2 = 0.997$) was obtained between the ethidium bromide fluorescence and the DNA concentration in the range of 0–8 μg/ml with a limit of sensitivity of approximately 0.1 μg/ml (see Figure 3).

Next the assay procedure was optimized using M-MLV RT and concentrations of dTTP up to 4 mM. The concentration of cDNA synthesized as a function of time was calculated from the ethidium bromide fluorescence. The linear portion of the reaction profile yielded Vo, the initial velocity of the reaction. A plot of Vo vs. the substrate concentration showed that an increased reaction rate occurred as a function of the dTTP concentration. This rate reached a maximum at 1.0 mM dTTP (Figure 4). Further addition of dTTP resulted in a marked decrease in the rate of cDNA synthesis, which was virtually zero in the presence of 4.0 mM dTTP. This behaviour, where the rate of catalysis increases with an increased substrate concentration to a maximum velocity after which the rate decreases, is known as substrate inhibition and has been reported to be a common deviation from normal Michaelis–Menten kinetics[18]. Possible causes of the observed substrate inhibition include competitive

substrate inhibition, the presence of contaminating non-competitive inhibitors in the substrate and co-factors required for enzymatic activity[19,20]. Since the effect of competitive substrate inhibition is negligible when initial rates are used as in this study[20] and the dTTP used was reported to have a purity >99%, we investigated the effect of changing the Mg^{2+} concentration present in the assay mixture. M-MLV RT requires Mg^{2+} for its catalytic activity, and this would therefore be considered to be a co-factor. Since Mg^{2+} ions bind to the triphosphate moiety of dTTP[21], it is possible that the substrate inhibition observed at high substrate concentrations was caused by insufficient free Mg^{2+} in the assay mixture. To investigate whether this was indeed the case, the assay was repeated in the presence of 7.5 mM Mg^{2+}. The results (Figure 4) showed that there was no difference in the reaction rate up to 1 mM dTTP when compared with the reaction in the presence of 3.75 mM Mg^{2+}. However, the maximum rate observed now occurred at 2-mM dTTP, after which the rate was found to decrease. This decrease was markedly less than that observed in the presence of 3.75 mM Mg^{2+}. The M-MLV RT activities in the presence of 3.75 mM Mg^{2+} and 7.5 mM Mg^{2+} were compared at Mg^{2+}:dTTP molar ratios of 1:1. These activities were approximately equivalent, suggesting that the inhibition of enzyme activity observed at high substrate concentrations was due to interaction between the Mg^{2+} ions and the dTTP.

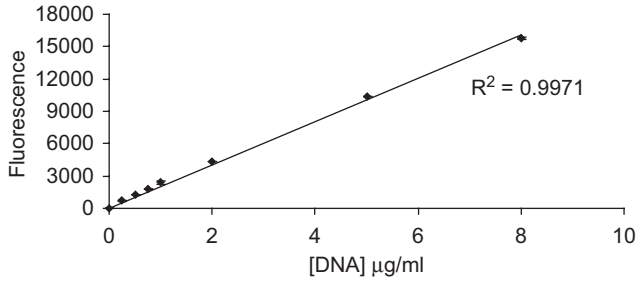


Figure 3. Calibration curve for the determination of DNA in solution using fluorescence spectroscopy. Ethidium bromide in water was added to a final concentration of 0.025 mg/ml to various concentrations of calf thymus DNA in Tris-HCl pH 7.2. The fluorescence was determined at 605 nm (excitation 545 nm). The data represent the mean (± SD) of three replicate samples. Error bars not visible are within the symbols.

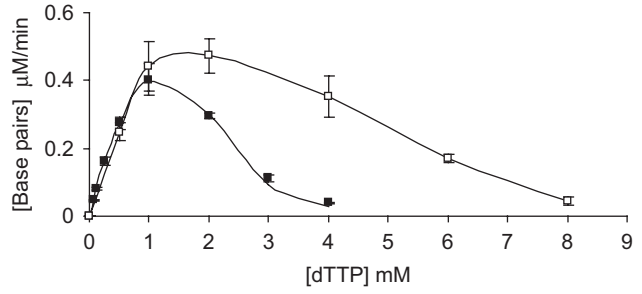


Figure 4. Initial rate (Vo) for the formation of cDNA as a function of the dTTP concentration using M-MLVV RT. The assay was carried out in the presence of either 3.75 mM $MgCl_2$ (▪) or 7.5 mM $MgCl_2$ (□). The data represent the mean (± SD) of two replicate samples.

A double reciprocal Lineweaver–Burk plot ignoring the contribution of higher substrate concentrations allowed us to calculate the V_{max} and K_m for the reaction. V_{max} was found to be 0.8 ± 0.2 µM (bp)/min and K_m was found to be 1.06 ± 0.22 mM irrespective of whether the Mg^{2+} concentration was 3.75 or 7.5 mM. K_{cat} and K_{cat}/K_m were determined to be 11 ± 3 min^{-1} and 1.1 ± 0.3 × 10^4 $min^{-1}M^{-1}$, respectively.

A number of nucleotide triphosphates and similar molecules were investigated as to whether these inhibited the M-MLV RT reaction. The Mg^{2+} and dTTP concentrations were fixed at 3.75 and 1.0 mM, respectively, and the compounds tested were present at a final concentration of 1.0 mM. The inhibition of M-MLV RT activity of these compounds was compared to the inhibition observed with 2-mM dTTP. The results (Table 2) showed that all the nucleotide triphosphates used (dGTP, dCTP, dATP and ATP) inhibited M-MLV RT activity significantly, and were comparable to the inhibition observed in the presence of 2.0 mM dTTP. Pyrophosphate also showed a significant inhibition but all the other compounds tested did not show any significant inhibition.

Preliminary data showed that crude polyphenol extracts of *M. flabellifolius* leaves inhibited M-MLV RT activity *in vitro*. The crude extract was separated into various fractions using Sephadex LH-20 chromatography, which were analysed by HPLC and MALDI-TOF mass spectrometry (previously discussed). To determine which fractions contained active compound, each of the six fractions was tested for inhibitory action on M-MLV RT using the fluorescence assay. The final concentration of each polyphenol fraction used to test for the inhibitory activity of M-MLV RT was prepared at 1.2 µg/ml. At this concentration, the crude extract and fractions F1, F5 and F6 inhibited M-MLV RT by up to 84% (Table 3). In contrast, complete inhibition was found for fractions F_3 and F_4. These fractions were diluted 4-fold and re-assayed. At a concentration of 0.3 µg/ml, these fractions exhibited identical inhibitory activity of approximately 35%. All further work on polyphenol-mediated RT inhibition was performed using fraction F_3, pure 3,4,5 tri-*O*-galloylquinic acid (previously determined using HPLC and mass spectrometry), which possessed the greatest inhibitory activity towards M-MLV RT.

Inhibition kinetics of 3,4,5 tri-*O*-galloylquinic acid and nevirapine on M-MLV RT activity

We investigated the effect of 3,4,5 tri-*O*-galloylquinic acid on M-MLV RT activity by adding various concentrations of this compound up to a final concentration of 0.9 µM in the enzyme assay. We found an exponential response of M-MLV RT inhibition in this concentration range, with an approximate 0.5 ± 0.004 µM concentration of 3,4,5 tri-*O*-galloylquinic acid required for 50% inhibition (Figure 5). Although polyphenols are known to chelate divalent metal ions, it is unlikely that the observed inhibition was due to the polyphenol binding to Mg^{2+} ions since these were present in a 1000-fold

Table 2. M-MLV RT activity inhibition by potential competitive inhibitors.

Compound	Inhibition (%)
dTTP	35 ± 3
dGTP	39 ± 3
dCTP	41 ± 6
dATP	43 ± 4
dAMP	7 ± 5
Ribonucleotides	
ATP	38 ± 0.3
ADP	7 ± 8
Bases	
Thymine	9 ± 7
Deoxyribonucleoside	
Thymidine	10 ± 4
Other compounds	
Sodium pyrophosphate	23 ± 4
Phosphate	0

1 mM of each test compound was mixed with 1 mM dTTP and then assayed for enzyme activity. The data were compared with the activity observed in the presence of 2.0 mM dTTP which resulted in 35 ± 3% inhibition. The data represent the mean (± SD) of three replicate samples.

excess. Using 0.5 μM 3,4,5 tri-*O*-galloylquinic acid and 3.75 mM Mg^{2+}, a Lineweaver-Burk plot of Vo vs. the dTTP concentration showed that 3,4,5 tri-*O*-galloylquinic acid was a non-competitive inhibitor (Figure 6) with $V'_{max} = 0.36 \pm 0.03$ (μM (bp)/min), $K'_m = 0.90 \pm 0.15$ mM and $K_i = 0.31 \pm 0.05$ μM (Table 4 and 5). The effect of a non-nucleoside RT inhibitor on M-MLV RT activity was also investigated. Nevirapine, a derivative of dipyrido-diazepinone, is a non-nucleoside RT inhibitor widely used in the prevention of mother to child HIV transmission[22]. Although nevirapine has been shown to bind to HIV-1 RT non-competitively, resulting in disruption of the enzyme catalytic site[23], it has been reported that it is ineffective against HIV-2 RT and RTs from simian immunodeficiency and feline leukemia viruses[24,25]. The effect of nevirapine on M-MLV RT activity was investigated using concentrations up to 1.0 mM. No effect was observed (data not shown).

The effect of substrate concentration on HIV-1 RT activity using the fluorescence assay

The effect of substrate concentration on HIV-1 RT activity was next determined using the fluorescence assay described previously. A plot of the initial velocity (Vo) against the substrate concentration for HIV-1 RT showed that an increased reaction rate occurred as a function of the dTTP concentration (data not shown). This rate reached a maximum at 0.25 mM dTTP. Further addition of dTTP resulted in a gradual decrease in the rate of cDNA synthesis. Inhibition of HIV-1 RT was unlikely to be due to Mg^{2+} depletion, since the Mg^{2+} concentration used was 5 mM. Failure by HIV-1 RT to obey Michaelis–Menten kinetics has been reported previously[26]. These authors reported that substrate inhibition occurred at dTTP concentrations above 50 μM.

Table 3. Effect of crude and pure polyphenol fractions on M-MLV RT activity.

	Inhibition (%)	
Polyphenol fraction	1.2 μg/ml	0.3 μg/ml
Crude extract	54 ± 10	
F_1	34 ± 8	
F_3	98 ± 0.2	31 ± 5
F_4	98 ± 0.3	41 ± 8
F_5	33 ± 2	
F_6	84 ± 2	

The data represent the mean (± SD) of three replicate samples. Fractions F_3 and F_4 were diluted and then re-assayed at a final concentration of 0.3 μg/ml.

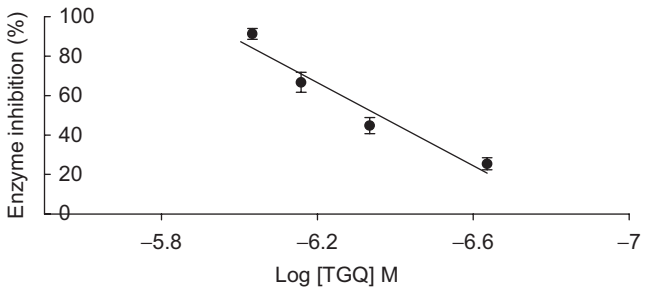


Figure 5. Inhibition of M-MLV RT activity as a function of the log (3,4,5 tri-*O*-galloylquinic acid) concentration. The data represent the mean (± SD) of three replicate samples.

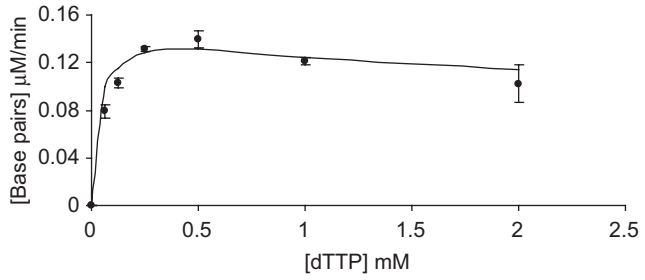
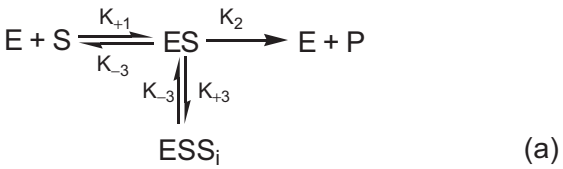


Figure 6. Initial rate (Vo) for the formation of cDNA as a function of the dTTP concentration using M-MLV RT. The assay was carried out in the presence of 5.0 mM $MgCl_2$. The data represent the mean (± SD) of two replicate samples.

The kinetic parameters of the reaction were therefore estimated using a model that accounts for data that exhibit substrate inhibition[27]. It is based on the assumption[20] that an ineffective (dead end) enzyme substrate complex with two substrate molecules (ESSi) is formed as shown (A):



The rate Equation (1) is derived from the reaction scheme (A):

$$V = \frac{V_{max}[S]}{K_m + [S] + ([S]^n / K_i)} \quad (1)$$

The data. for the initial rate of cDNA formation as a function of the dTTP concentration fitted well when n, the total number of substrate molecules bound to an ineffective complex, was assigned a value of 2. K_i was therefore given by the Equation (2):

$$K_i \frac{[ES][S]}{[ESS_i]} \quad (2)$$

K_m, K_i. and V_{max} were estimated to be 0.097 ± 0.004 mM, 10.82 ± 1.20 mM and 0.223 ± 0.004 μM/min, respectively. K_{cat} and K_{cat}/K_m were determined to be 1.31 ± 0.02 min^{-1} and 1.2 ± 0.2 × 10^4 $min^{-1}M^{-1}$, respectively (Table 4). The previous value reported for K_i was 195 ± 37 μM[26]. The data were further analysed using a Hill plot to assess if the binding of the first dTTP molecule to HIV-RT had an effect on the binding of the second molecule. A Hill coefficient of one was obtained at low dTTP concentration and less than one at high dTTP concentration, suggesting zero and negative co-operativity, respectively[28].

Inhibition kinetics of 3,4,5 tri-*O*-galloylquinic acid and nevirapine on HIV-1 RT activity

We next investigated whether 3,4,5 tri-*O*-galloylquinic acid also inhibited HIV-1 RT activity. The results showed an exponential response of HIV-1 RT inhibition in the concentration range up to 82 μM (Figure 7) with an estimated IC_{50} of 34 μM. The nature of the inhibition observed was investigated by assaying HIV-1 RT activity in the presence and absence of 34 μM 3,4,5 tri-*O*-galloylquinic acid. The Mg^{2+} concentration used was 5.0 mM. A Lineweaver–Burk plot showed an apparent change in the values of both the K_m and V_{max} in the presence of 3,4,5 tri-*O*-galloylquinic acid (Table 6). This suggested mixed non-competitive inhibition. This type of inhibition demonstrated that 3,4,5 tri-*O*-galloylquinic acid bound HIV-1 RT at a site other than the active site, and also that its binding influenced the binding of dTTP. The inhibition constants for mixed non-competitive inhibition are not the same. The value of K'_i of 26 ± 2 μM (bp)/min was less than that of K_i, which was 135 ± 89 μM (bp)/min, suggesting that binding of 3,4,5 tri-*O*-galloylquinic acid to the enzyme substrate complex (ES) was stronger than to the free enzyme (E). The effect of nevirapine on HIV-1 RT activity was next investigated. The results showed that addition of nevirapine to the RT assay at 1 μM caused double the inhibition observed at 100 nM, but that the higher concentration was insufficient to reduce the activity by 50% (not shown). No effect was observed for DMSO alone used as a control. Although the median 50% inhibitory concentration for nevirapine has been reported to be 100 nM[25], this study was performed using an RT from a different HIV strain to that used in the reported study.

Inhibition reversal of 3,4,5 tri-*O*-galloylquinic acid on RT activity by BSA and PVP

To determine whether 3,4,5 tri-*O*-galloylquinic acid bound strongly to M-MLV RT, an enzyme activity

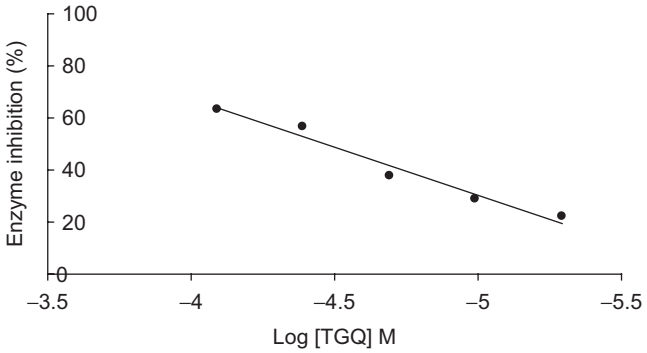


Figure 7. Inhibition of HIV-1 RT activity as a function of 3,4,5 tri-*O*-galloylquinic acid concentration. The data shown represent a single value.

Table 4. Kinetic parameters for cDNA synthesis by M-MLV and HIV-1 RTs.

	Enzyme	
Parameter	M-MLV RT	HIV-1 RT
V_{max} (M/min)	0.8 ± 0.2 × 10^{-6}	0.22 ± 0.004 × 10^{-6}
K_{cat} (min^{-1})	11 ± 3	1.31 ± 0.02
K_m (M)	0.98 ± 0.14 × 10^{-3}	0.11 ± 0.015 × 10^{-3}
K_{cat}/K_m (min^{-1} M^{-1})	1.1 ± 0.3 × 10^4	1.2 ± 0.2 × 10^4

The K_m value is an average of K_m values from two independent experiments.

Table 5. Kinetic parameters for dTTP incorporation into poly (rA):oligo$(dT)_{25}$ complex using M-MLV RT, in the presence and absence of 3,4,5 tri-*O*-galloylquinic acid.

Parameter	No TGQ added	TGQ added
V_{max} (μM/min)	0.95 ± 0.03	
V'_{max} (μM/min)		0.36 ± 0.03
K_m (mM)	0.90 ± 0.15	
K'_m (mM)		0.90 ± 0.15
K_i (μM)		0.31 ± 0.05

Reaction velocity units represent the concentration of base pairs incorporated per minute.

Table 6. Kinetic parameters for dTTP incorporation into poly (rA):oligo$(dT)_{25}$ complex using HIV-1 RT in the presence and absence of 3,4,5 tri-*O*-galloylquinic acid.

Parameter	No TGQ added	TGQ added
V_{max} (μM/min)	0.169 ± 0.002	
$V/_{max}$ (μM/min)		0.072 ± 0.004
K_m (mM)	0.13 ± 0.03	
K'_m (mM)		0.07 ± 0.01
K_i (μM)		135 ± 89
K'_i (μM)		26 ± 2

Reaction velocity units represent the concentration of base pairs incorporated per minute.

assay was performed in the presence of 3,4,5 tri-*O*-galloylquinic acid together with either PVP or BSA, which were used as a polyphenol binding agent and a competing protein, respectively. The Mg^{2+} concentration was fixed at 3.75 mM. The presence of PVP and BSA alone in the assay mixture showed no effect on M-MLV RT activity (Figure 8), whereas addition of 0.9 μM 3,4,5 tri-*O*-galloylquinic acid resulted in 93% inhibition. If PVP at a PVP:M-MLV RT molar ratio of 106:1 or BSA at a molar ratio of 63:1 were subsequently added, no increased M-MLV RT activity was observed. The molar ratios of PVP and BSA to 3,4,5 tri-*O*-galloylquinic acid used were 8:1 and 5:1, respectively, sufficient to bind all the 3,4,5 tri-*O*-galloylquinic acid present[15,29], demonstrating strong binding of the polyphenol to the enzyme. If the same amounts of PVP and BSA were present when the 3,4,5 tri-*O*-galloylquinic acid was added to the assay mixture, 78 and 63% inhibition were observed, respectively. The binding of 3,4,5 tri-*O*-galloylquinic acid to M-MLV RT therefore appeared significantly stronger than that to either BSA or PVP. 3,4,5 tri-*O*-galloylquinic acid through its planar aromatic groups has the potential to either enhance or quench the fluorescence of the ethidium bromide-DNA complex in solution. The effect of 3,4,5 tri-*O*-galloylquinic acid on the fluorescence of calf thymus DNA-ethidium bromide complex was investigated. The results (data not shown) showed no significant change in the fluorescence of the ctDNA-ethidium bromide complex in the presence of 34 μM final 3,4,5 tri-*O*-galloylquinic acid concentration. Furthermore, fluorescence measurements for enzyme assays were performed after a 50-fold dilution of the assay mixture (including the polyphenol concentration) while ethidium bromide concentration remained constant at 2.5 μg/ml negating a quenching contribution.

The competitiveness of 3,4,5 tri-*O*-galloylquinic acid binding to HIV-1 RT was determined by performing an enzyme activity assay in the presence of 100 μM 3,4,5 tri-*O*-galloylquinic acid and 11.0 μM BSA and 5.0 mM Mg^{2+} (Figure 9). This BSA molar concentration has been reported to be sufficient to bind all the 3,4,5 tri-*O*-galloylquinic acid present[29]. The molar ratio of BSA to HIV-1 RT was 63:1. Although the presence of BSA alone had no effect on HIV-1 RT activity, the presence of 100 μM 3,4,5 tri-*O*-galloylquinic acid resulted in approximately 95% inhibition. BSA addition subsequent to 3,4,5 tri-*O*-galloylquinic acid addition restored the activity of HIV-1 RT to 53% of its original value, demonstrating that the binding of 3,4,5 tri-*O*-galloylquinic acid to HIV-1 RT was partially competed by the presence of BSA. When the polyphenol addition to HIV-1 RT was performed after the addition of BSA, only 37% inhibition was observed. The effect of nevirapine on HIV-1 RT activity was next investigated. We found that the presence of 100 nM nevirapine resulted in 14 ± 2% inhibition and that the presence of 1 μM resulted in double the inhibition, 31 ± 6%.

Discussion

In this report, we have fractionated the major polyphenols present in the leaf extract of *M. flabellifolia*. We screened the fractions for inhibitory activity towards M-MLV RT using a simple cost-effective fluorescence-based assay to determine RT activity. We were able to show that the main active compound was 3,4,5 tri-*O*-galloylquinic acid and used the assay to determine kinetic parameters related to inhibition. The assay was shown to be semi-quantitative by comparing the ethidium bromide fluorescence of the reaction product with the fluorescence of known concentrations of DNA. Our results suggested that the reasons for the substrate inhibition observed were very different for the two RT enzymes used. Thus HIV-1 exhibited genuine substrate inhibition, the data for which could be mathematically modelled suggesting that an ineffective enzyme substrate complex with two substrate molecules was formed. In contrast, the substrate inhibition observed with M-MLV RT appeared to be due to binding of Mg^{2+} ions by the substrate since this inhibition was shifted to higher substrate concentrations

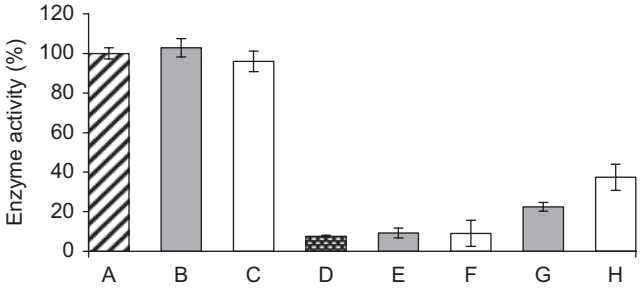


Figure 8. Effect of PVP and BSA on M-MLV RT activity in the absence and presence of 0.9 μM 3,4,5 tri-*O*-galloylquinic acid. The enzyme activity assay was performed as follows: (A) no addition,; (B) PVP; (C) BSA; (D) 0.9 μM 3,4,5 tri-*O*-galloylquinic acid; (E) enzyme + 3,4,5 tri-*O*-galloylquinic acid, then PVP; (F) enzyme + 3,4,5 tri-*O*-galloylquinic acid, then BSA; (G) PVP + 3,4,5 tri-*O*-galloylquinic acid, then enzyme; (H) BSA + 3,4,5 tri-*O*-galloylquinic acid, then enzyme. The data represent the mean (± SD) of three replicate samples.

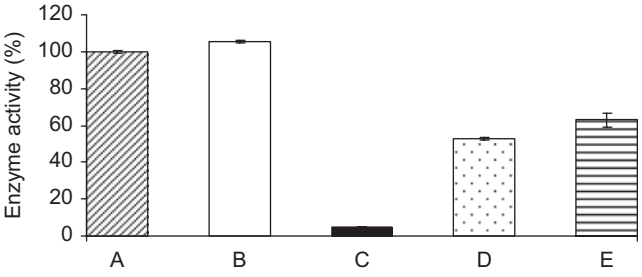


Figure 9. Effect of BSA on HIV-1 RT activity in the absence and presence of 100 μM 3,4,5 tri-*O*-galloylquinic acid. The enzyme activity was performed as follows: (A) no addition; (B) BSA; (C) 100 μM 3,4,5 tri-*O*-galloylquinic acid; (D) HIV-1 RT + 3,4,5 tri-*O*-galloylquinic acid, then BSA; (E) BSA + 3,4,5 tri-*O*-galloylquinic acid, then HIV-1 RT. The data represent the mean (±SD) of duplicate samples.

when the Mg^{2+} concentration was increased. Moreover, a variety of nucleotide triphosphates showed identical inhibition to dTTP. No difference was observed if the sugar moiety of the nucleotide was ribose or deoxyribose but the presence of a triphosphate was crucial, presumably as the dissociation constant for $Mg.ATP^{2-}$ shows approximately one order of magnitude tighter binding of Mg^{2+} for ATP (0.087 mM) compared with that of Mg^{2+} for ADP (0.676 mM). The pyrophosphate inhibition observed might result from two distinct causes. Firstly, pyrophosphate might bind to the positions where the X and Y phosphates bind thereby competing with nucleotide triphosphate binding. Secondly, pyrophosphate is a product of the RT reaction and so increased pyrophosphate concentrations would favour the reverse reaction and reduce the forward enzyme activity.

Although 3,4,5 tri-*O*-galloylquinic acid inhibited both the M-MLV and the HIV-1 RTs, K'_i, the IC_{50} for the former occurred at a concentration almost two orders of magnitude lower, 0.5 μM vs. 34 μM. It has been reported that the type and strength of the interaction between polyphenols and proteins is influenced by their corresponding chemical structures[29]. The hydroxyl groups on the galloyl moiety are thought to play a crucial role in the interaction of galloyl-containing polyphenols with proteins[29] with phenylalanine and proline residues considered to be the polyphenol binding sites[30,31]. Although the total phenylalanine and proline contents of BSA and M-MLV RT are similar at 10.2 and 10.6 mol%, respectively, BSA has significantly higher phenylalanine content (5.4 mol%), whereas M-MLV RT has significantly higher proline content (8.0 mol%). We would propose that, since phenylalanine residues would tend to be buried on account of their hydrophobic nature, hydrophilic superficial proline residues, more plentiful on M-MLV RT, would be the only effective polyphenol binding site. The inhibition observed for both RT enzymes was brought about by binding of the polyphenol at an allosteric site. Although inhibition was purely non-competitive for M-MLV RT, mixed non-competitive inhibition was observed for HIV-1 RT. Moreover, 3,4,5 tri-*O*-galloylquinic acid binding to M-MLV RT, suggested strong interactions between the polyphenol and the enzyme. Weaker interactions, however, existed between 3,4,5 tri-*O*-galloylquinic acid and HIV-1 RT as the activity of the enzyme was partially restored on subsequent addition of BSA to a 3,4,5 tri-*O*-galloylquinic acid inhibited enzyme. Clearly limitations currently exist for the use of 3,4,5 tri-*O*-galloylquinic acid as an anti-viral (specifically anti-HIV) therapy, these include; (i) the relatively high concentration (IC_{50} = 34 μM) of 3,4,5 tri-*O*-galloylquinic acid required to inhibit the HIV-RT means substantial quantities need to be used, (ii) competitive and nonspecific binding of 3,4,5 tri-*O*-galloylquinic acid to other proteins such as serum albumin would reduce the effective concentration of the compound, and (iii) transport constraints within the human body would limit their efficacy. However, preliminary results have shown that 3,4,5 tri-*O*-galloylquinic acid reduces the viral count in tissue culture experiments (W.F. Brandt, unpublished observations). It would appear this polyphenol reduces the entry of HIV-1 into CD-4 cells (unpublished data). This could be due to the binding of 3,4,5 tri-*O*-galloylquinic acid to the viral coat proteins or to interacting with the cell membrane and/or membrane proteins. Hence, 3,4,5 tri-*O*-galloylquinic acid and related molecules may have potential as antiviral therapies and the lack of specificity of action may be an advantage. The HI-virus mutates incredibly rapidly, meaning that effective control with tight (i.e. highly specific) binding molecules can be countered by the virus evolving structurally altered binding sites thus abolishing the action of the molecule on new "mutated" HIV variants. This is less likely with 3,4,5 tri-*O*-galloylquinic acid which binds with lower affinity but has more viral target "sites". We are currently investigating the "mode of action" of 3,4,5 tri-*O*-galloylquinic acid on inhibiting HIV components (e.g. RTs) *in vitro* and also *in vivo* by blocking HIV entry into cultured cell lines.

Declaration of interest

The authors report no declarations of interest.

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