

Original Research Article

A 'single point' experimental approach of assessing the inactivation kinetics of β -glucuronidase by aqueous-based leaf extractives notably used as enemas.

ABSTRACT

Excess beta-glucuronidase in the caecum and colon is responsible for enterohepatic circulation of toxic aglycones, which have been implicated in development of cancer, diarrhea and jaundice and in other gut conditions. Botanicals are potential sources of molecules, which can serve as dietary components for inactivating this enzyme. Enzyme inactivation is employed in this study to categorize the potencies of the leaf extracts of *Alstonia boonie* (Ext1), *Vernonia amygdalina* (Ext2), *Heliotropium indicum* (Ext3) and *Momordica charantia* (Ext4) to inactivate beta-glucuronidase. In a 'single point' experimental approach a fixed amount of five micrograms of each samples were tested for their ability to reduce β -glucuronidase activity. Extracts reduced activity of the control (100%) to about 5-30% in 20 minutes. Ext2 and Ext3 were more potent as inactivators recording lower K_m , V_{max} and k_{cat} compared to Ext1 and Ext4. Ext2 and Ext3 treatments resulted in 619 and 843 fold decreases in β -glucuronidase activity respectively. The partitioning ratios k_{inact}/K_m and k_{cat}/k_{inact} which, do not depend on the reversibility and saturation of inactivation and dilution factor D_i values confirmed Ext2 and Ext3 as the more potent inactivators of β -glucuronidase than Ext1 and Ext4. Furthermore, Ext2/Ext3 treatments resulted in irreversible inactivation, which could involve the formation of a ternary enzyme-inactivator-substrate complex but may be different from the inactivation mechanism of Ext1/Ext4. However Ext2, Ext3 and Ext4 treatment produced same magnitude of order of the inactivation reaction, $n = 0.17$ and inactivation rate constant, $k_{inact} = 0.0024 \text{ min}^{-1}$ compared with $n = 0.09$ and $k_{inact} = 0.0020 \text{ min}^{-1}$ of Ext1 treatment which are indication of the complexity and form of the molecules in the extracts. Thus, extracts of similar chemical entities could display varying degrees of inactivation mechanism. These results show that the 'single point' experimental approach can be used to categorize the potencies of consumable botanicals for their ability to inactivate β -glucuronidase.

Keywords: β -glucuronidase, enemas, 'single point' inactivation, intestinal microflora, enterohepatic circulation

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36 INTRODUCTION

37 β -glucuronidase excreted into the intestinal lumen by the intestinal microflora play a major role
 38 in enterohepatic circulation releasing both beneficial and toxic aglycones [1,2]. Toxic
 39 endogenous and exogenous compounds are glucuronidated in the liver and transported partly
 40 through the biliary system into the intestinal lumen to be excreted with feces [3]. Naturally,
 41 glucuronidated compounds in the intestinal lumen are poorly reabsorbed into the blood stream
 42 and efficiently eliminated from the body unless they are hydrolyzed by intestinal β -
 43 glucuronidase [3]. Many of the intestinal microflora including *Ruminococcus gnavus* and
 44 *Escherichia coli* secrete β -glucuronidase into the intestinal lumen [4,5]. The greatest activity of
 45 β -glucuronidase in the intestine is in the caecum and in the large intestine [5]. At these sites, the
 46 enterohepatic circulation of toxic compounds due to the activity of intestinal β -glucuronidase
 47 activity has been suggested to promote tumor formation and diarrhea in cancer patients under
 48 chemotherapy [6,7]. The enzyme has also been implicated in neonatal jaundice, the most
 49 common cause for hospital re-admission of neonates, as a result of recirculation of bilirubin after
 50 the hydrolysis of its glucuronide in the intestinal lumen [8]. Nitro-reductase and azo reductase
 51 activities in the gut also contribute to the release of aglycones, which can also induce colon
 52 carcinogenesis [9]. To reduce the toxic effects of aglycones, identification of any beta-
 53 glucuronidase inhibitor that can be acknowledged to be a safe dietary component could be of
 54 considerable appeal. Consequently, existing foods, drinks and beverage are being investigated
 55 for molecules that can inactivate gut enzymes whose activity can lead to inflammatory and
 56 malignant diseases. Certain components of milk and milk-based products such as yoghurt have
 57 been suggested to inhibit β -glucuronidase activity in the intestine and therefore can be used to
 58 fortify milk products to reduce enterohepatic circulation of bilirubin in neonates [9].
 59 Multicomponent herbal drug, food and beverage preparations also hold great promise in this area
 60 of research as the one-shot treatment targeting multi-enzymes whose activity can cause cancer
 61 and inflammation of the intestinal mucosa cells. In this study a single inhibitor concentration
 62 'single-point' inactivation kinetic parameters has been used to evaluate extracts from the leaves
 63 of non-food plants commonly used as enemas for the presence small molecule inactivators which
 64 may have utility as novel therapeutics against the debilitating effects of β -glucuronidase activity
 65 in the gut and to increase the dose-intensification and efficacy especially in a significant fraction
 66 of cancer patients undergoing chemotherapy.

67 MATERIALS AND METHODS

68 Samples

69 β -glucuronidase from bovine liver (Type B-1) and its substrate 4-methylumbelliferyl- β -D-
 70 glucuronide hydrate (≥ 98 HPLC grade) were obtained from Sigma-Aldrich, UK. Phosphate
 71 buffer at pH 7 was prepared with double distilled water and filtered. Leaf extracts (Extn) were
 72 obtained from the medicinal plants; *Alstonia boonie* (Ext1), *Vernonia amygdalina*

(Ext2), *Heliotropium indicum* (Ext3) and *Momordica charantia* (Ext4). Extracts were prepared by mashing fresh leaves in amount of water to give the fluid consistency required for use as enema.

Spectrophotometric determination of β -glucuronidase activity

The reactions were initiated by addition of enzyme (0.01 μ moles) to a solution of 4-methylumbelliferyl- β -D-glucuronide substrate in a buffer, which has been thermally equilibrated at 27°C. β -glucuronidase was generally assayed by spectrophotometric scanning of the hydrolysis of 4-methylumbelliferyl- β -D-glucuronide on a Genway spectrophotometer. For the single point inactivation assays, the equivalent of 5 μ g dried-weight of each aqueous enema was used in each assay. Assays were always performed in duplicate determinations in total assay volume of 1 mL and the average absorbance values were recorded.

β -glucuronidase time-course assay

4-methylumbelliferyl- β -D-glucuronide (GMU) and β -glucuronidase (GUASE) stock solutions were prepared in $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ buffer. The total volume of the reaction mixture was 1 mL containing a final concentration of 50 mM $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ buffer (pH 7.0), 1 mM 4-methylumbelliferyl- β -D-glucuronide and 0.01 μ moles of β -glucuronidase. The reaction was monitored for 40 minutes. Absorbance change was recorded for every minute at a maximum absorption wavelength of 350 nm. One unit of activity of β -glucuronidase was taken as the micromoles of the aglycone 4-methylumbelliferone (MU) released into the reaction mixture per minute [10].

Michaelis-Menten kinetic assay

The conversion of GMU to MU by GUASE was assayed at final GMU concentration of 0.02, 0.06, 0.08, 0.16, 0.24, 0.32, 0.48 and 0.64 mM respectively. Absorbance change was recorded for every minute in a 20 minute reaction time at pH 7.0 and temperature of 27°C. Activity in micromoles per minute was calculated for each substrate concentration and the values were used to obtain the Michaelis-Menten plot of activity versus substrate concentration. K_m and V_{max} were determined from the Lineweaver-Burk plot. Duplicate measurements were taken for each preparation

GUASE inactivation assay

GUASE activity was repeated for each substrate concentration (0.02, 0.06, 0.08, 0.16, 0.24, 0.32, 0.48 and 0.64 mM) after the enzyme has been incubated at 0° C in 5-microgram equivalence of each of the aqueous extract for 20 minutes respectively. Duplicate determinations were performed and the average activity was recorded for each substrate. The corresponding K_m and V_{max} values for each extract were calculated with the aid of a Lineweaver-Burk plot. Similar inactivation assay was also conducted at a fixed GMU concentration of 1 mM for each extract. Same experiment was repeated as a duplicate

Mode of GUASE inactivation by extracts

The 'single point' 5- μ g weight equivalent of each extract was assessed for the mode of inactivation. This amount was diluted for each extract to give various preparations of increasing dilution factor of 0.0, 0.1, 0.15, 0.2, 0.25, 0.35 and 0.4 in a final volume of 25 μ L respectively. Dilution factor 0.4 is equivalent to the highest concentration of extract and 0.0 contains no extract. GUASE was incubated for 20 minutes at 0° C in each preparation before activity was

determined by monitoring the reaction in 1 mM GMU solution for 20 minutes at 27° C. GUASE activity was recorded for each concentration factor and plotted for each extract. Duplicate measurements were taken for each preparation

RESULTS

UV-spectrophotometric scanning for the conversion of GMU to MU

β -glucuronidase plays an important role in drug metabolism and has been used as target in a number of drug discovery studies. The enzyme cleaved 4-methylumbelliferyl- β -D-glucuronide GMU, which has maximum absorption at 340 nm to give 4-methylumbelliferone product MU, which absorbs maximally at 350 nm as shown in Figure 1.

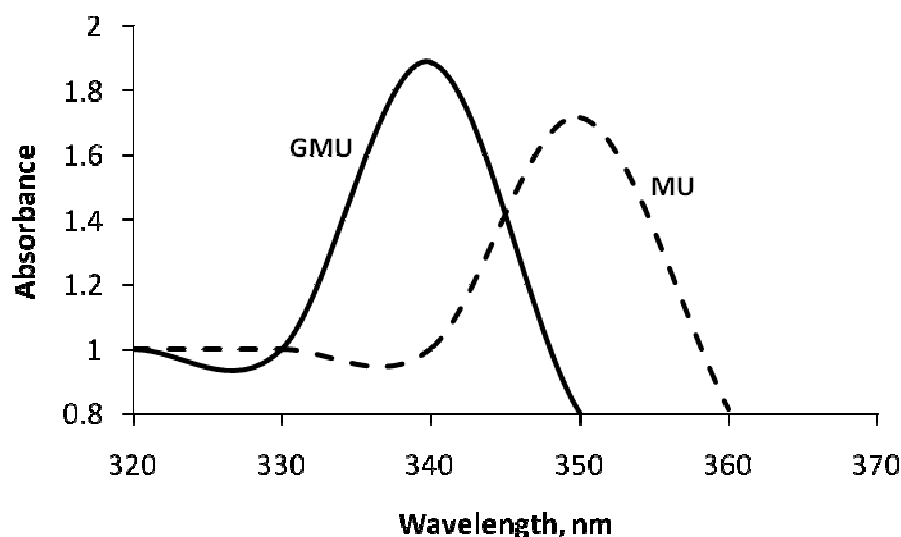


Figure 1. Wavelength scanning of GUASE activity in a reaction mixture. After 20 minutes most of the substrate GMU has been converted to product MU.

Time-course of the reaction of β -glucuronidase.

The distinct absorption peaks of the substrate GMU, and the product MU, facilitated the kinetics and inactivation studies of β -glucuronidase. In Figure 2, 1 mM concentration of GMU was used to determine the time course for the conversion of GMU to MU.

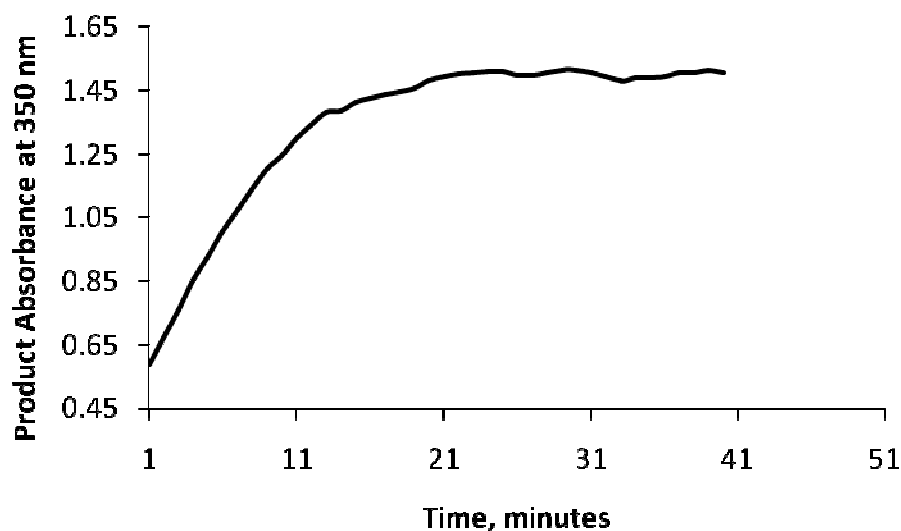


Figure 2: Time-course for the conversion of GMU. 1 mM of GMU was treated with 0.01 μ mole of GUASE. The release of MU aglycone was monitored at 350 nm for 40 minutes.

Stability of β -glucuronidase activity at different substrate concentrations.

At GMU maximal concentration of 1 mM, approximately 20 minutes was required for the conversion to MU. This time period was used to assess the kinetics of enzyme at GMU concentrations below 1 mM as shown in Figure 3.

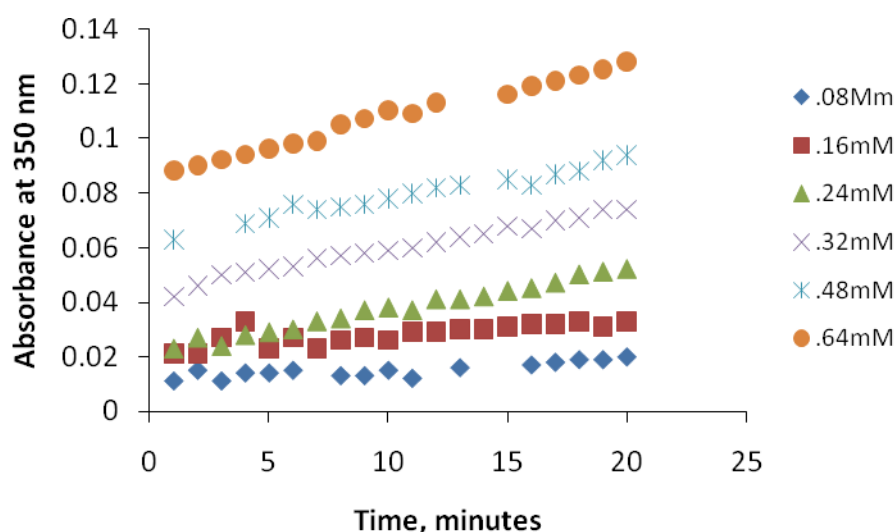


Figure 3. GUASE activity at different GMU concentrations. Activity increases with increasing GMU concentration.

Kinetic model of β -glucuronidase activity.

The Michaelis-Menten plot (Figure 4a) of the activity of the enzyme at different GMU concentrations was also conducted at the same time period of 20 minutes.

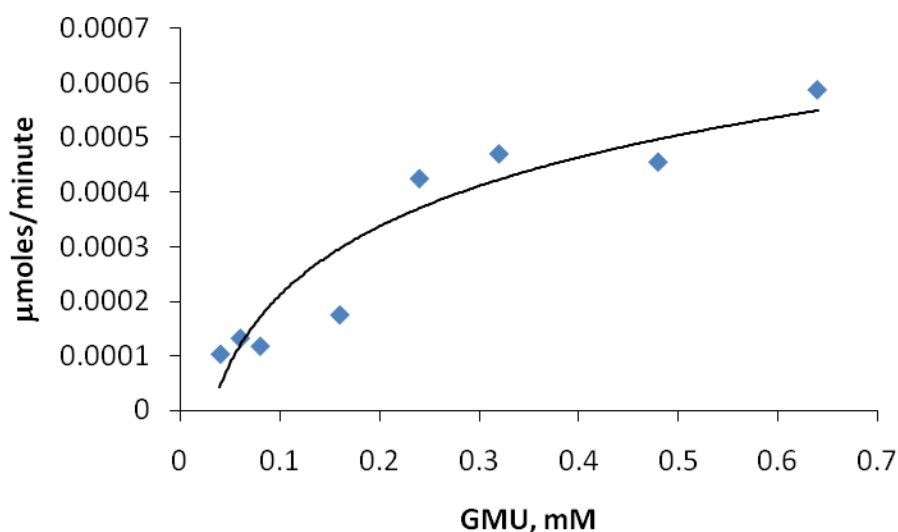


Figure 4a Michaelis-Menten analysis of β -glucuronidase. Enzyme activity increases with increasing substrate concentration till a saturation point.

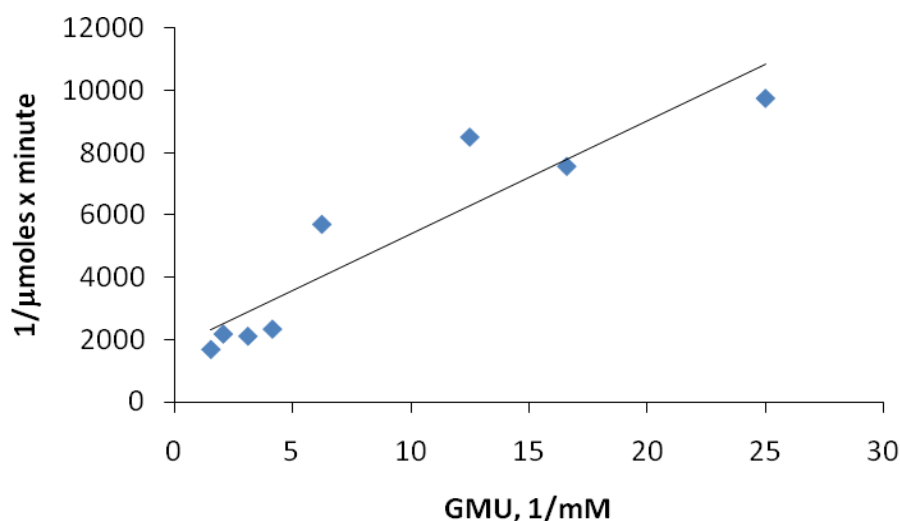


Figure 4b. Lineweaver-Burk analysis of Figure 4a. The plot was used to estimate the kinetic parameters K_m and V_{max} .

The 'single point' inactivation experiments

A time-course assay was performed after 20 minutes of incubation with extracts. This was aimed at confirming the marked reduction in activity after incubation with five micrograms equivalent weight of extract. As shown in Figure 5, all extracts gave strong inactivation effect on the enzyme, reducing activity of the control (100%) to about 5-30% in 20 minutes.

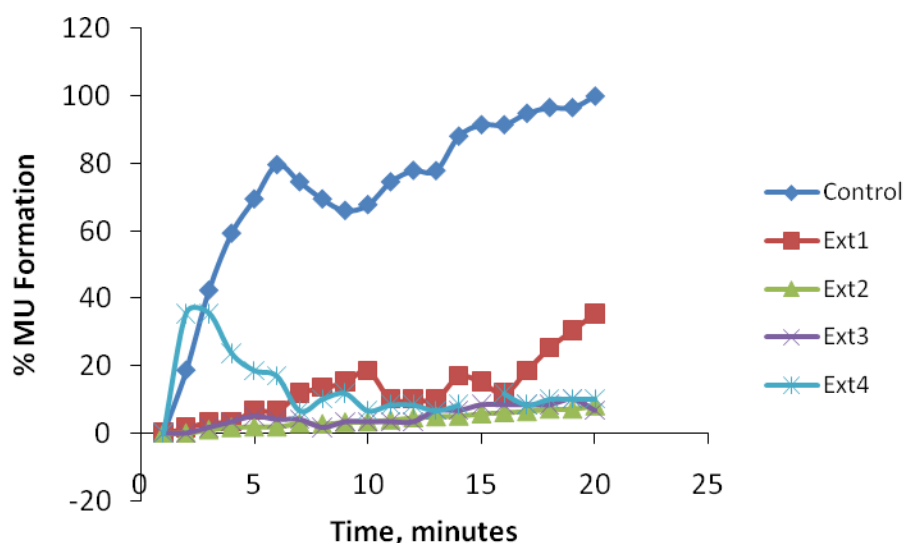


Figure 5: Time-course inactivation of GUASE by extracts. Each reaction was monitored at 350 nm at one-minute interval for 20 minutes.

The kinetic assay was repeated for each GMU concentration in the presence of 5 μg of each of the enemas and the reduction in activity was used to determine the change in K_m , V_{max} and k_{cat} for each extract Extn, as shown in Table 1 below:

Table 1. The changes in K_m , V_{max} and k_{cat} corresponding to the treatment of β -glucuronidase with extracts (Ext1, Ext2, Ext3 and Ext4). k_{cat} and k_{cat}/K_m were calculated for the monomer (M) and tetrameric (T) forms of GUASE.

Sample	K_m mM	V_{max} $\mu\text{mole}/\text{min}$	k_{cat} min^{-1}		k_{cat}/K_m $\mu\text{mole}^{-1}\text{min}^{-1}$		Fold Decrease
			T	M	T $\times 10^3$	M $\times 10^6$	
Control	0.22 ± 0.02	0.134 ± 0.007	13.5	335	61.363	1.522	NIL
Ext1	0.33 ± 0.15	0.00056 ± 0.0001	0.056	1.4	0.169	0.0042	241
Ext2	0.16 ± 0.08	0.000218 ± 0.00005	0.022	0.545	0.138	0.0034	619
Ext3	0.03 ± 0.01	0.000162 ± 0.00001	0.016	0.405	0.533	0.0135	843
Ext4	0.64 ± 0.20	0.00033 ± 0.00011	0.033	0.825	0.051	0.0013	409

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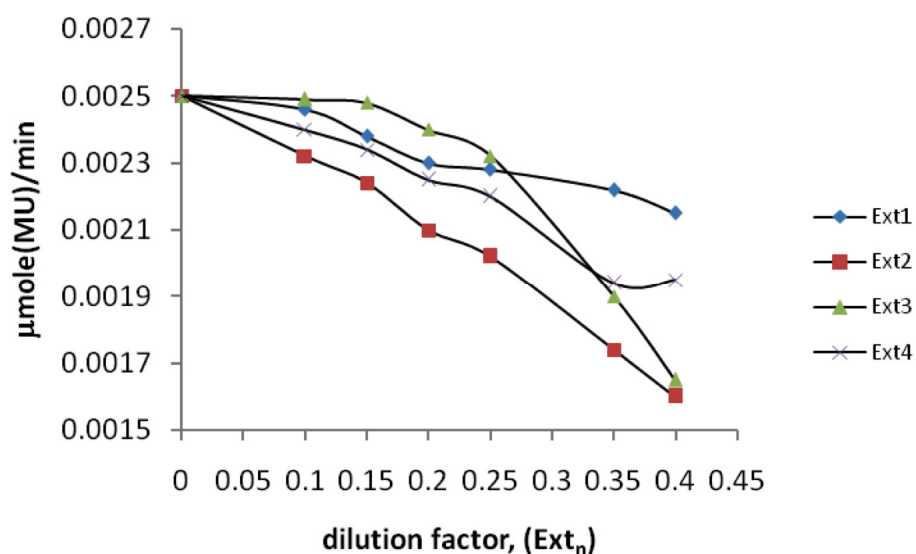
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The mode of inactivation of GUASE of the ‘single point’, 5-microgram weight of each extract was determined by incubating GUASE in different dilutions of the 5-microgram weight of each extract representing dilution factors of 0.0, 0.1, 0.15, 0.20, 0.25, 0.35 and 0.40 such that factor 0.0 contains no extract and 0.40 has the highest amount of extract. No saturation was observed in all four cases shown in Figure 6A. The differences in mechanism of inactivation were obtained by fitting data to linear and polynomial regressions are represented in Figure 6B below:

A.



B.

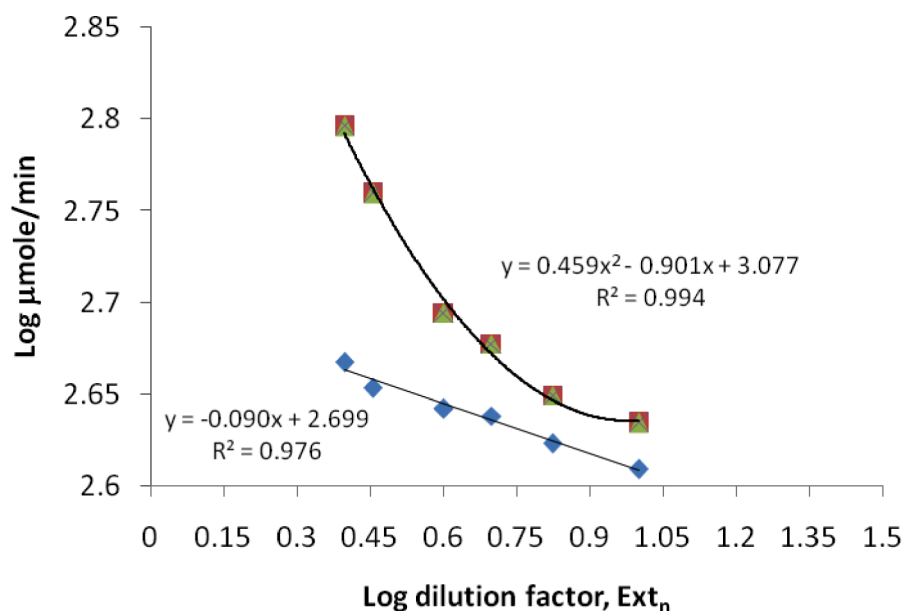


Figure 6: Patterns of GUASE inactivation by extracts. A. Different diluted solutions were prepared from the 5-microgram weight of each extract representing the dilution factors: 0.0, 0.1, 0.15, 0.2, 0.25, 0.35, and 0.4. Activity of GUASE was determined for each solution after 20

minutes of pre-incubation. B. Data were fitted using the equation $V_{\text{inact}} = k_{\text{inact}}[I]^n$ [11] to determine the order n , the dilution constant D_i (dilution that give 50% inactivation) and the rate of enzyme inactivation V_{inact} .

The constant k_{inact} was used to calculate the partition ratios k_{inact}/K_m and $k_{\text{cat}}/k_{\text{inact}}$ as shown in Table 2 below:

Table 2. Kinetic parameters derived from a single-point dilutions of extracts Ext1, Ext2, Ext3, Ext4

	n	k_{inact} min^{-1}	k_{inact}/K_m mM^{-1} min^{-1}	$k_{\text{cat}}/k_{\text{inact}}$	D_i mL
Ext1	0.09	0.0020	0.006	28.0	3.3
Ext2	0.17	0.0024	0.015	9.0	1.13
Ext3	0.17	0.0024	0.08	6.7	0.3
Ext4	0.17	0.0024	0.004	13.8	16.2

DISCUSSION

Multi-component herbal preparations are gaining increasing prominence in medicine equally as the pure isolated mono-compounds or their synthetic drug analogs. However, the one major problem of their use has been with the difficulty of understanding the mode of action due to the multiplicity of molecules any single botanical preparation being it enzyme inhibitor/activator or receptor agonist/antagonist can present. Consequently the single substrate Michael-Menten reversible model of evaluating the potencies of mono-compound inhibitors based on K_i and IC_{50} values; strictly do not apply to the use of herbal medicines, which could involve reversible and also irreversible processes, which are time-dependent. Similar approach has been used to categorize the potency of synthetic mono-compound drugs based on k_{inact} and K_i [12]. In this article, the single substrate Michaelis-Menten steady-state enzyme kinetic model as shown in the equation below;



where **E** and **ES** represent the enzyme and the enzyme substrate complex and **P**, the product of the reaction has been employed to evaluate and categorized the potency of four crude aqueous plant extracts notably used as enemas by their ability to inactivate β -glucuronidase in *in vitro* assay. The batch of enzyme obtained for this study showed a typical β -glucuronidase (GUASE) activity as shown in Figure 1, giving distinct GMU and MU peaks at 10 nm apart. A time-dependent experiment of this activity using 1mM GMU and 0.01 μ M of GUASE produced a hyperbolic curve of Figure 2 asymptomatic after 20 minutes of reaction. At this concentration of enzyme and fixed reaction time of 20 minutes the stability of GUASE in increasing substrate concentration was established in Figure 3. Within this concentration range GUASE exhibit a typical Michaelis-Menten stead-state kinetic shown in Figure 4a. The data from this kinetic were fitted to the Lineweaver-Burk equation to produce Figure 4b, which was used to extract the Michaelis constant K_m of 0.22 ± 0.02 mM, and maximum rate of reaction V_{max} of 0.134 ± 0.007 μ mole/min. The ‘single point’ involved pre-incubation (20 minutes) of GUASE with a single dried mass of 5 μ g obtained after evaporation of 1 mL of each of the four aqueous extracts; at fluid consistency required for use as enemas, as the inhibitory substance in each inactivation assay. In a reaction mixture of 1 mL the 5 μ g of each enema material in Figure 5 gave over 60 % reduction of GUASE activity after 20 minutes of reaction. In addition, the ‘single point’ produced different modes of inactivation by the marked variations in the K_m , V_{max} , k_{cat} and k_{cat}/K_m values as shown in Table 1. Ext3 (*Heliotropium indicum*) and Ext2 (*Vernonia amygdalina*) gave the lowest K_m , V_{max} and k_{cat} . The two extracts also gave the highest fold decrease of GUASE activity compared to the control. This result is supported by the curves of Figure 1 where the inactivation curves of Ext1 (*Alstonia boonie*) and Ext4 (*Momordica charantia*) lie above that of Ext2 and Ext3. Ext2 and Ext3 appeared to form a tenary enzyme-inactivator-substrate complex during the inactivation process [13, 14]. Furthermore, the catalytic efficiency of 0.533 μ mole/min under Ext3 treatment was an improvement over the other extracts, however Etx3 in the same reaction gave the highest value of fold decrease of 843 compared to 409 of Ext4 treatment, which corresponds to the lowest catalytic efficiency of 0.015 μ mole/min. These events are indicative of the different modes of inactivation by the extracts. The extents of these mechanisms by the ‘single point’ approach are depicted by the marked variations in the partition ratios k_{inact}/K_m and k_{cat}/k_{inact} [14], in Table 2. Ext2 and Ext3 gave the lowest k_{cat}/k_{inact}

values and highest k_{inact}/K_m values, which further support the two extracts as stronger inactivators of GUASE than Ext1 and Ext4. The partitioning ratios also show some levels of relatedness between Ext1 and Ext4 and between Ext2 and Ext3 on mechanism of GUASE inactivation. This study introduced a dilution parameter, which reflects the necessity to obtain the right fluid consistency when botanicals are prepared for use as enemas. Increasing dilution can help increase the levels of vital soluble metabolites from botanicals. D_i in this study is the aqueous dilution factor that gives 50% of GUASE inactivation by enemas. The use of D_i is analogous to K_i ; lower D_i value corresponds to an effective enema that can inactivate GUASE in a very diluted state and the vice versa. D_i of Ext2 and Ext3 were lower than D_i values of Ext1 and Ext4; an additional proof of the effectiveness of the two extracts to inactivate GUASE. In spite of the above relationships, the order of reaction n and rate of inactivation k_{inact} parameters present Ext1 in different category of GUASE inhibitors compared to Ext2, Ext3 and Ext4 as shown in Table 2. Values of k_{inact} were similar for all extracts which confirm the rapidity of the inactivation process observed in Figure 5. This justified the use of the partitioning ratios k_{inact}/K_m and $k_{\text{cat}}/k_{\text{inact}}$ as the true reliable parameters to compare the inactivation rates of the extracts [15]. The order of reaction n , however placed Ext1 in a different category compare to Ext2, Ext3 and Ext4; a strong indication that Ext2, Ext3 and Ext4 might present complexity of metabolites, which is different from Ext1 but the pairs Ext1/Ext4 and Ext2/Ext3 share the same mode of GUASE inactivation respectively.

CONCLUSION

A herbal medicine with its complex set of compounds presents a unique difficulty in understanding its mechanism of action. This understanding may be even more daunting when such mechanism is studied for a multifactorial disease; disease condition where a multi-compound-medicine may have advantage over a single-compound medicine in a multi-target therapy approach. Complex diseases may sometimes be triggered by one-step pathway. In such situation inactivating the enzyme/receptor involved in this pathway will prevent the occurrence of a disease and the multiplicity of bioactive and non-active molecules in a medicine may not be an issue in the understanding of the mechanism of such inactivation process. Inactivation of GUASE in the gut to avoid enterohepatic circulation of toxic aglycones may represent the latter scenario. In this study partitioning ratios k_{inact}/K_m , $k_{\text{cat}}/k_{\text{inact}}$, fold decrease of enzyme activity, a dilution factor, D_i , and the order of the inactivation reaction, n have been used to categorize four different enemas of their potential to inactivate beta-glucuronidase activity. The 'single point' approach did not require the prior knowledge of the form or concentration of the active and non-active components. It was possible by this approach to evaluate the inactivation without having to depend on any active molecule and therefore avoid the usual ranking parameters K_i and IC_{50} , which depend on the reversibility of the inhibition/inactivation and a saturation inhibition/inactivation kinetic. Ext3 is potent inactivator, which is possible to involve an irreversible formation of a ternary enzyme-inhibitor-substrate complex. Ext2 may have a lesser degree of this mechanism. Ext1 and Ext4 have inactivation mechanism different from Ext2 and Ext3, even though Ext2, Ext3 and Ext4 appeared to have the same complexity of metabolites, which may be different from Ext1. Consumable botanicals; foods and medicines can be screened with this approach to categorize them in the search for food and micronutrient supplements to serve as blocking agents in the special conditions of jaundice in neonates, patients with chemotherapy induced-diahrroea, cancer development in the colon and other similar conditions, which are triggered by enterohepatic circulation.

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