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A 'Single Point' Experimental Approach of Assessing the Inactivation Kinetics of β-glucuronidase by Aqueous-based Leaf Extractives Notably Used as Enemas

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Authors' contributions

Author KJS conceived, performed the experiment and completed the data analysis, interpretations and the final completion of the manuscript. Author CMA assisted in the design of the experiment and preparation of the manuscript.

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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, jaundice and other disease conditions of the gut. Botanicals are potential sources of molecules, which can serve as dietary components for inactivating beta-glucuronidase. 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,

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k_{cat}/k_{inact} and D*i* values confirmed Ext2 and Ext3 as the more potent inactivators of β-glucuronidase than Ext1 and Ext4. Furthermore, Ext2/Ext3 inactivation mechanisms may involve the formation of a tenary enzyme-inactivator-substrate complex, which could be different from the inactivation mechanisms 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 min^{-1} compared with n = 0.09 and k_{inact} = 0.0020 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.

1. INTRODUCTION

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

2. MATERIALS AND METHODS

2.1 Samples

β-glucuronidase from bovine liver (Type B-1) and its substrate 4-methylumbeiliferyl-β-D-glucuronide hydrate (≥ 98 HPLC grade) were obtained from Sigma-Aldrich, UK. Phosphate buffer at pH 7 was prepared with double distilled water and filtered. Leaf extracts (Extn) were 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.

2.2 Spectrophotometric Determination of β-glucuronidase Activity

The reactions were initiated by addition of enzyme (0.01 μmoles) to a solution of 4-methylumbeiliferyl-β-D-glucuronide substrate in a

buffer, which has been thermally equilibrated at 27℃. β-glucuronidase was generally assayed by spectrophotometric scanning of the hydrolysis of 4-methylumbeiliferyl-β-D-glucuronide on a Jenway spectrophotometer. For the 'single point' inactivation assays, the equivalent of 5μg 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.

2.3 β-glucuronidase Time-course Assay

4-methylumbeiliferyl-β-D-glucuronide (GMU) and β-glucuronidase (GUASE) stock solutions were prepared in $Na_3PO4.12H_2O$ buffer. The total volume of the reaction mixture was 1mL containing a final concentration of 50 mM $Na_3PO4.12H_2O$ buffer (pH 7.0), 1 mM 4-methylumbeiliferyl-β-D-glucuronide and 0.01 μmoles of GUASE. 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 GUASE was taken as the micromoles of the aglycone 4-methylumbeiliferone (MU) released into the reaction mixture per minute [10].

2.4 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℃. 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.

2.5 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 experiments were repeated as duplicates.

2.6 Mode of GUASE Inactivation by Extracts

The 'single point' 5-μg 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 solubility factor (D) of 0.0, 0.1, 0.15, 0.2, 0.25, 0.35 and 0.4 in a final volume of 25 μL respectively. Solubility 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 solubility factor and plotted for each extract. Duplicate measurements were taken for each preparation.

3. RESULTS

3.1 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-methylumbeiliferyl-β-D-glucuronide GMU, which has maximum absorption at 340 nm to give 4-methyumbeiliferone product MU, which absorbs maximally at 350 nm as shown in Fig. 1.

3.2 Time-course of the Reaction of GUASE

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

3.3 Stability of GUASE 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 GUASE at GMU concentrations below 1 mM as shown in Fig. 3.

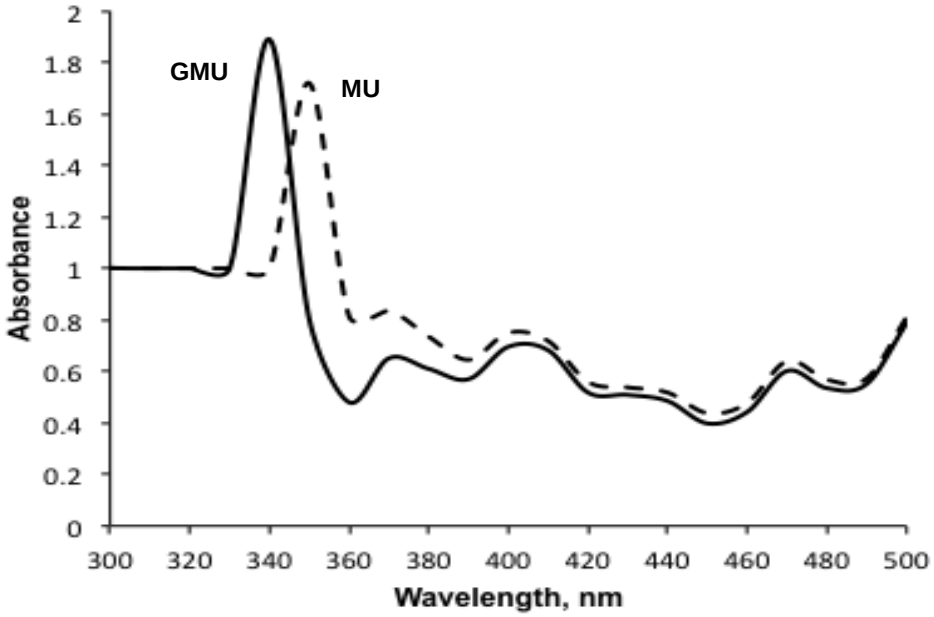

Fig. 1. Wavelength scanning of GUASE activity in a reaction mixture. After 20 minutes most of the substrate GMU has been converted to product MU

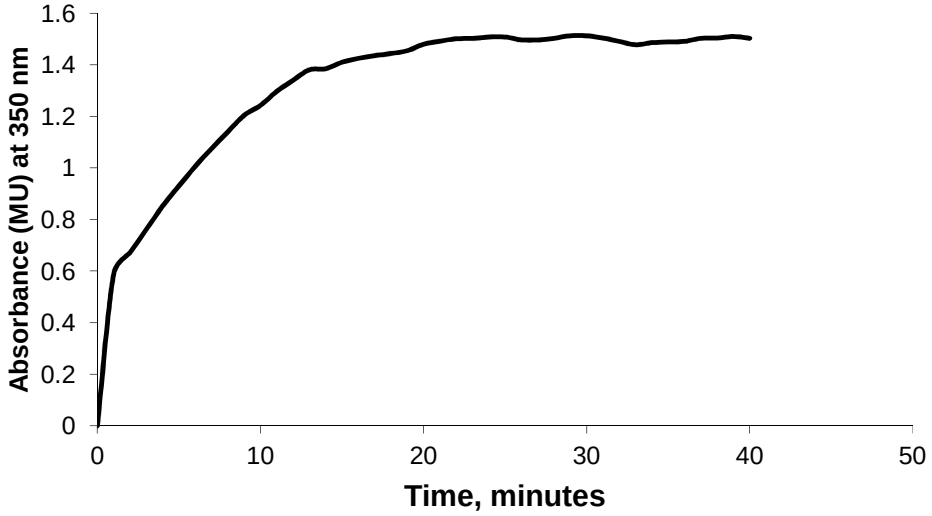

Fig. 2. Time-course for the conversion of GMU. 1 mM of GMU was treated with 0.01 µmole of GUASE. The release of MU aglycone was monitored at 350 nm for 40 minutes

3.4 Kinetic Model of GUASE Activity

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

The Lineweaver-Burk plot shown in Fig. 4b was use to estimate the parameters K_m, V_{max} and k_{cat} of the uninhibited GUASE.

3.5 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 Fig. 5, all extracts gave strong inactivation effect on the

enzyme, reducing activity of the control (100%) to about 5-30% in 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.

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 solubility 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 Fig. 6A. The differences in mechanism of inactivation were obtained by fitting data to linear and polynomial regressions are represented in Fig. 6B:

The constant k_{inact} was used to calculate the partition ratios k_{inact}/K_m and k_{cat}/k_{inact} as shown in Table 2.

4. 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.

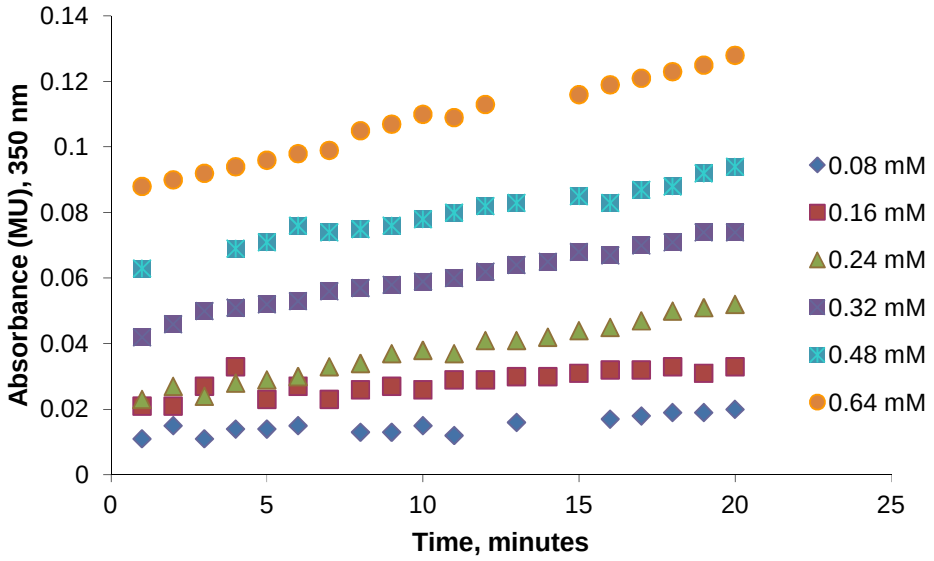

Fig. 3. GUASE activity at different GMU concentrations. Activity increases with increasing GMU concentration

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

Sample	K_m mM	V_{max} µmole/min	k_{cat} min^{-1}		k_{cat}/K_m µmole^{-1}min^{-1}		Fold decrease
			T	M	T x10^3	M x10^6	
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

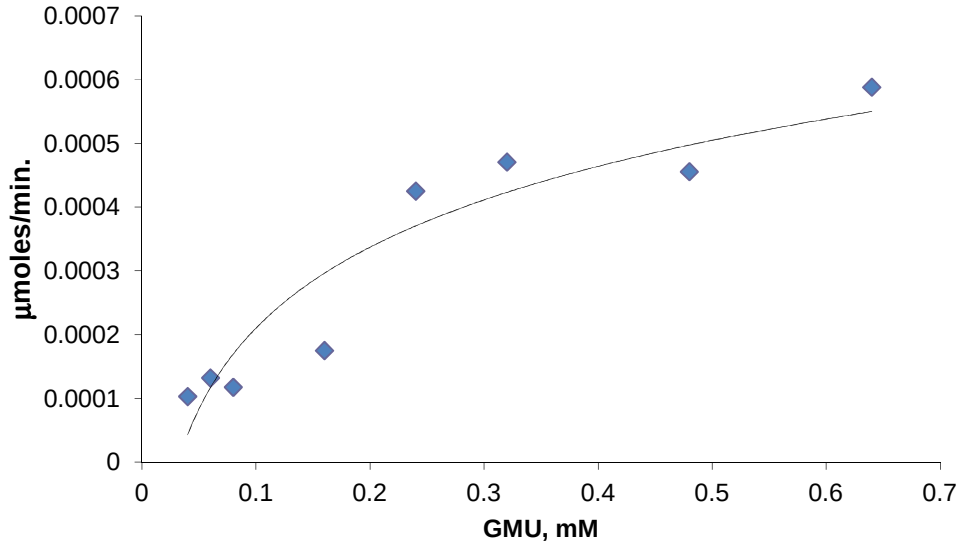

Fig. 4a. Michaelis-Menten analysis of GUASE activity. Enzyme activity was determined at different GMU concentration in the absence of the inactivator

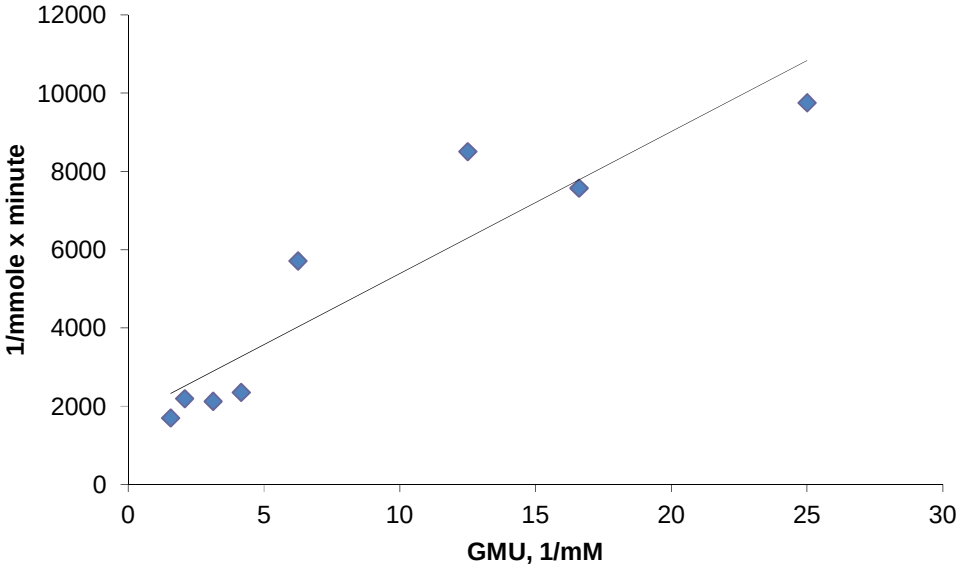

Fig. 4b. Lineweaver-Burk representation of Fig. 4a. The linear relation allowed for the estimation of the kinetic parameters K_m and V_{max}

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 time-dependent reversible and irreversible processes. 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;

$$E + S \leftrightharpoons ES \longrightarrow E + P$$

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 activity as shown in Fig. 1, giving distinct GMU and MU peaks at 10 nm apart. A time-dependent experiment of this activity using 1 mM GMU and 0.01 μM of β-glucuronidase produced a hyperbolic curve of Fig. 2 asymptomatic after 20 minutes of reaction. At this concentration of enzyme and fixed reaction time of 20 minutes the stability of β-glucuronidase in increasing substrate concentration was established in Fig. 3. Within this concentration range the enzyme exhibited a typical Michaelis-Menten steady state kinetic shown in Fig. 4a. The data from this kinetic were fitted to the Lineweaver-Burk equation to produce Fig. 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 μmole/min. The 'single point' involved pre-incubation (20 minutes) of β-glucuronidase with a single dried mass of 5 μg 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 μg of each enema material in Fig. 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 enzyme activity compared to the control. This result is supported by the curves of Fig. 1 where the inactivation curves by 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 μmole/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 μmole/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 β-glucuronidase 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 enzyme inactivation. This study introduced a solubility parameter, which reflects the necessity to obtain

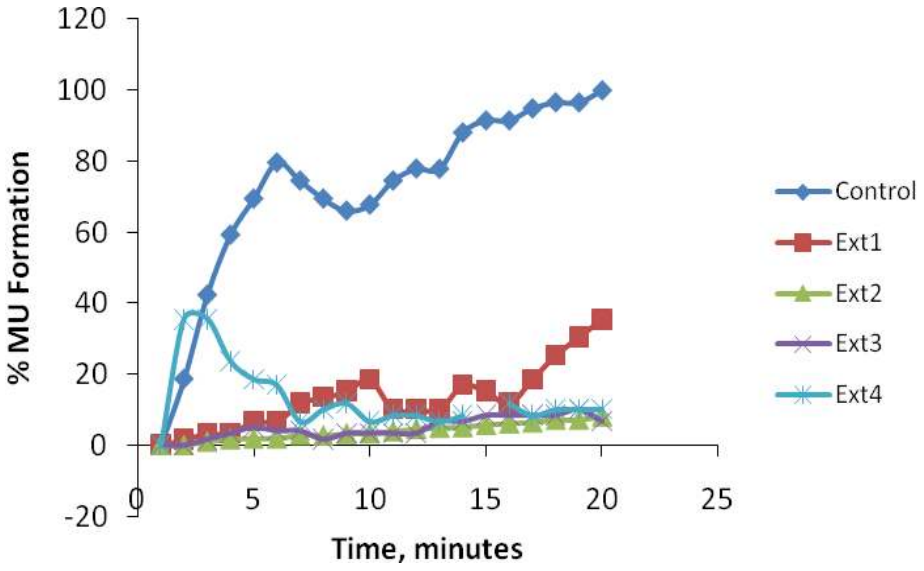

Fig. 5. Time-course inactivation of GUASE by extracts. Each reaction was monitored at 350 nm at one-minute interval for 20 minutes

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_{cat}/k_{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

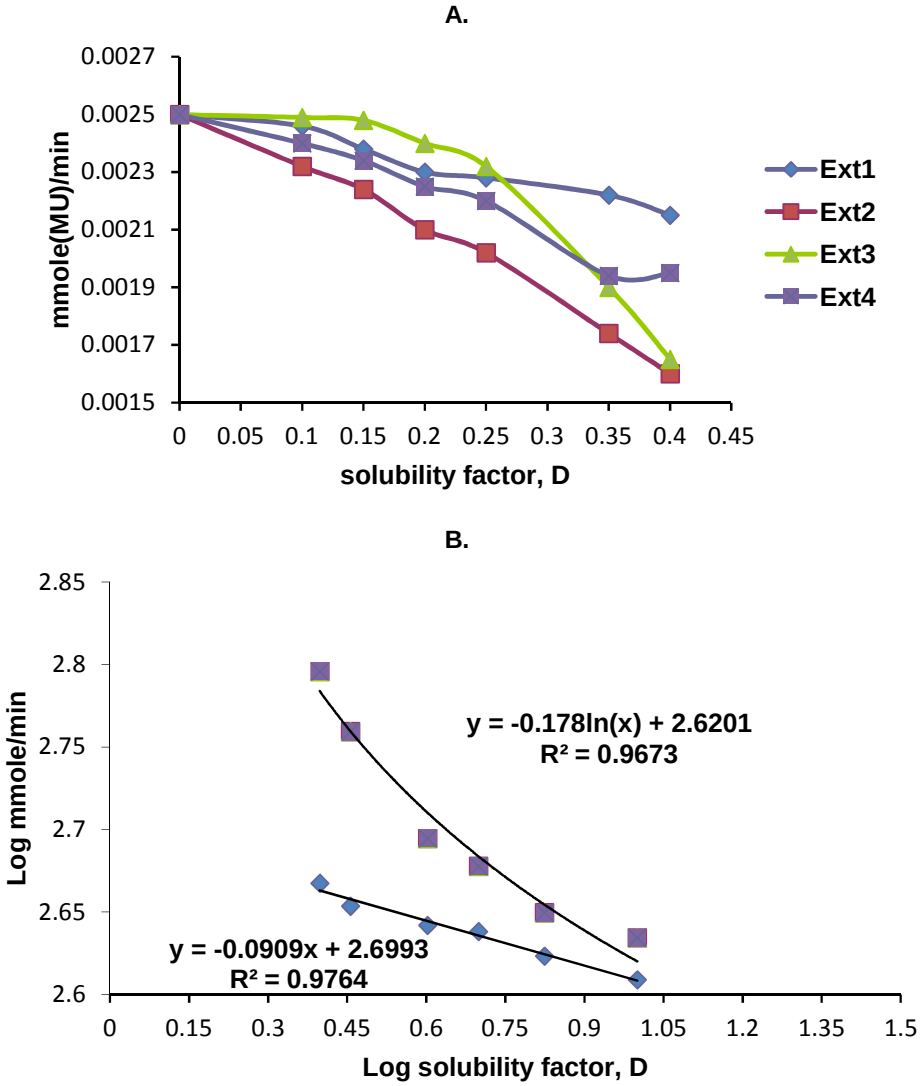

Fig. 6. Patterns of GUASE inactivation by extracts. A. Different diluted solutions were prepared from the 5-microgram weight of each extract representing the solubility factors: 0.0, 0.1, 0.15, 0.2, 0.25, 0.35, and 0.4. Activity of GUASE was determined for each solution after 20 minutes of pre-incubation. B. Data were fitted using the equation $V_{inact} = k_{inact}[D]^n$ [11] to determine the order n, the solubility constant D_i (solubility factor that give 50% inactivation) and the rate of enzyme inactivation V_{inact}

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 solubility factor that gives 50% of enzyme inactivation (solubility constant, D*i*) 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 β-glucuronidase. In spite of the above relationships, the order of reaction n and rate of inactivation k_{inact} parameters present Ext1 in different category of inactivators 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 Fig. 5. This justified the use of the partioning ratios k_{inact}/K_m, k_{cat}/k_{inact} and the solubility constant D*i* 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 β-glucuronidase inactivation respectively.

5. CONCLUSION

A herbal medicine with a 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 β-glucuronidase 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_{cat}/k_{inact}, fold decrease of enzyme activity, solubility constant, 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 tenary 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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