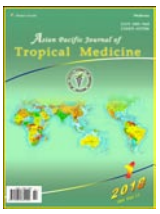

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# Cytotoxic, kinetics of inhibition of carbohydrate-hydrolysing enzymes and oxidative stress mitigation by flavonoids roots extract of *Dicoma anomala* (Sond.)

Fatai Oladunni Balogun, Anofi Omotayo Tom Ashafa✉

*Phytomedicine and Phytopharmacology Research Group, Department of Plant Sciences, University of the Free State, QwaQwa Campus, Private bag x 13, Phuthaditjhaba 9866, South Africa*

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ABSTRACT

**Objective:** To investigate the free radical scavenging, antidiabetic, kinetics and cytotoxic potentials of flavonoids extract of *Dicoma anomala* root by using standard methods. **Methods:** Antioxidant activity of the flavonoids was investigated at scavenging free radicals such as 1,1-diphenyl-2-picrylhydrazyl, nitric oxide, hydroxyl radical, reducing capabilities, 2,2-azino-bis (3-ethylbenzothiazoline-6) sulfonic acid as well as metal chelating capability at different concentrations (0.125-1.000 mg/mL) while the antidiabetic activity was evaluated via the inhibition and kinetics of carbohydrate digestive enzymes including, alpha glucosidase, sucrase and maltase. Brine shrimp lethality assay was also employed to examine the cytotoxic effects of the extract by using different range of concentrations (0.125-2.000 mg/mL). **Results:** The study revealed the best antioxidant activity of the extract in 1,1-diphenyl-2-picrylhydrazyl, 2,2-azino-bis (3-ethylbenzothiazoline-6) sulfonic acid and nitric oxide having $IC_{50}$ values of (386.90±4.91), (736.00±38.12), (629.30±9.62) $\mu$g/mL respectively compared with quercetin (standard) with $IC_{50}$ [(522.20±12.38), (1 021.00±15.61) and (1 075.00±29.35) $\mu$g/mL] respective values while it was insignificantly ($P$>0.05) at par with quercetin for reducing power. Similarly, the extract exhibited a moderate inhibition of alpha glucosidase (43.1%), sucrase (33.4%) and maltase (29.9%) activities which were significantly ($P$<0.05) better than acarbose (18.4%, 12.7% and 24.9% respectively) although acarbose (46.1%) inhibited the higher activity of alpha amylase than the extract (13.7%). The kinetics of mode of inhibition of alpha amylase, alpha glucosidase, sucrase and maltase by flavonoids extract of *Dicoma anomala* revealed an uncompetitive, non-competitive, competitive and non-competitive inhibition respectively. The result of the lethality assay showed a potent cytotoxic effect of the flavonoids with $LC_{50}$ value 0.510 mg/mL. **Conclusions:** The results obtained from this study are suggestive of the antioxidative, antidiabetic and cytotoxic potentials of flavonoids root extract of *Dicoma anomala*.

## 1. Introduction

Flavonoids, one of the secondary metabolites found in plants, are a member of polyphenolic compounds synthesized by plants (via phenyl propanoid pathway) in response to microbial invasion[1]. A number of reports revealed that most of the pharmacological

First author: Fatai Oladunni Balogun, Phytomedicine and Phytopharmacology Research Group, Department of Plant Sciences, University of the Free State, QwaQwa Campus, Private bag x 13, Phuthaditjhaba 9866, South Africa.
✉Corresponding author: Anofi Omotayo Tom Ashafa, Phytomedicine and Phytopharmacology Research Group, Department of Plant Sciences, University of the Free State, QwaQwa Campus, Private bag x 13, Phuthaditjhaba 9866, South Africa.
Fax: +2758718514444
E-mail: balogunfo@yahoo.co.uk; ashafaaot@ufs.ac.za
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activities exhibited by plants are as a result of the presence of rich amount of inherent flavonoids[2,3]. Interestingly, these activities (though structure-dependent) particularly antioxidant (produced by scavenging free radicals or chelate metal ions) are attributed to the type of functional groups, degree of hydroxylation and polymerization, substitution or conjugation reactions the concerned or class of flavonoid possessed leading to potential health-related benefits they elicit[1,4]. In terms of classification, flavonoids are sub-grouped into flavone, flavanone, flavonol, isoflavonoid, anthocyanidin, and chalcones[5].

Flavonoids as a dietary component with rich antioxidant effect are also investigated and affirmed to be effective against infectious (viral and fungal) and human degenerative diseases such as cardiovascular diseases, cancer, diabetes mellitus *etc*. probably because the etiopathology of these ailments are linked to oxidative stress caused by uncontrolled presence of reactive oxygen species/free radicals. In fact, numerous studies[3,6] had established the beneficial roles of this secondary plant's metabolite most especially in protecting enzyme systems in humans while also forming a formidable secondary antioxidant defense mechanism in plant tissues from biotic and abiotic stress.

*Dicoma anomala* (Sond.) (*D. anomala*) as described by Balogun *et al*[7,8] is a member of the Asteraceae family widely spread in most provinces in South Africa. It is medicinally used as treatment in the management of diabetes mellitus as well as against many other ailments in Sesotho tribe, Free State. The pharmacological importance of this herb as antioxidant[9], cardioprotective[10], antimalarial[11], antidiabetic[8] *etc* drug is enormous and not limited to the previously listed pharmacological activities. *D. anomala* is endowed with rich amount of polyphenolic compounds[9] including flavonoids which had been affirmed to be antioxidative, hepatoprotective, cardioprotective, antiatherosclerotic, antibacterial *etc*[12,13] in numerous investigations from different medicinal plants. In our view, there have not been any reported activities of flavonoids extract of *D. anomala* in the literature, hence, the study is aimed at investigating the antioxidant, antidiabetic and cytotoxic effects of flavonoids extract from *D. anomala* roots.

## 2. Materials and methods

### 2.1. Chemicals

Acarbose, *p*-nitrophenyl glucopyranoside (*p*NPG), porcine pancreatic alpha amylase ( α -AML), alpha glucosidase ( α -GCD; *Saccharomyce cerevisiae*), quercetin, sucrose, maltose, butylated hydroxyl anisole (BHA), quercetin, 2,2-azino-bis (3-ethylbenzothiazoline-6) sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside, ferrozine, *etc*. were purchased from Sigma-Aldrich (South Africa). Brine Shrimp eggs, other chemicals and reagents were obtained from local suppliers with 100% purity.

### 2.2. Plant materials and extraction of flavonoids

The collection, identity, authentication and deposition of *D. anomala* (BalMed/01/2015/QHB) at University herbarium was according to Balogun and Ashafa[8]. At preliminary preparation stage, fresh rootstock was ground into powdered plant substance.

Approximately 20 g each of the powdered samples was extracted by maceration with 220 mL aqueous ethanol 70% (v/v), filtered using Whatman No 1 filter paper and concentrated using a rotary evaporator at 45 ℃ to obtain dry brown flavonoids extract (4.005 g translating to 20.025% yield). The extract was reconstituted in distilled water for antioxidant activity and phosphate buffer (PB) (0.02 M) for antidiabetic assays. These were used to prepare a stock solution of 1.000 mg/mL from where a serial dilution was made to obtain 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL concentrations used for the assays.

### 2.3. In vitro free radical scavenging assays

#### 2.3.1. DPPH radical scavenging activity

DPPH free radical activity was according to the method described by Braca *et al*[14]. Flavonoids extract of *D. anomala* (FEDA) (100 μL) was released to 300 μL methanolic solution (0.004%) of DPPH in a 96-well microtitre plate. The absorbance of the mixture at 517 nm was checked after 1 800 seconds of incubation in the dark, and the percentage inhibition of the extract to scavenge DPPH radical was calculated using the expression $[(A_0-A_1)/A_0] \times 100$, where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of the extract/standard.

#### 2.3.2. Nitric oxide scavenging activity

The nitric oxide scavenging activity of FEDA was based on the method of Kumaran and Karunakaran[15]. Experimentally, 2 mL sodium nitroprusside (10 mM) and 0.5 mL phosphate buffered saline were reacted with 0.5 mL of varying concentrations of the extract in 10 mL test tubes. The reaction mixture was allowed to incubate at 25 ℃ for 9000 s while the same procedure was conducted for the control by replacing the aliquot with distilled water. Subsequently, the addition of 0.5 mL of 1% sulfanilamide, 2% $H_3PO_4$ and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloric acid concluded the reaction by reading the absorbance at 546 nm on a spectrophotometer (Biochrom, England).

#### 2.3.3. Reducing power

Pulido *et al*[16] method was adopted to access the reducing capacity

of FEDA / standards. Briefly, 2.5 mL of different concentrations of FEDA (0.125-1.000 mg/mL) were diluted with 0.2 mM PB (2 500 μL) and 1% potassium ferrocyanide (2.5 mL) in a 10 mL test tube. While allowing the mixture to stand for 20 min at 50 ℃, 2 500 μL of 10% trichloroacetic acid was emptied into the tube and subjected to centrifugation at 650 g for 10 min. About 2 500 μL from the upper portion of solution was diluted with distilled water in equal proportion and $FeCl_3$ (0.5 mL, 0.1%). The absorbance of the mixture was checked at 700 nm on a spectrophotometer. Increased absorbance values signified higher reducing capacity.

### 2.3.4. Metal chelating activity

The method of Dinis *et al*[17] was employed to determine the chelating capacity of FEDA. In brief, the extract (0.1 mL) was mixed with 0.5 mL of 2 mM $FeCl_2$ solution. The reaction commenced by further dilution of the mixture with 200 μL 5 mM ferrozine and subsequently agitated while afterwards allowed to maintain stability for 600 s at 25 ℃. Spectrophotometric measurement of the mixture was taken at 562 nm. The percentage inhibition of ferrozine–$Fe^{2+}$ complex formation was determined by the expression as in 2.3.1 above.

### 2.3.5. ABTS radical determination

FEDA scavenged $ABTS^+$ chromophore resulting from the reaction of ABTS solution with potassium persulfate was investigated according to the method of Re *et al*[18]. Summarily, 100 mL of 7 mM (aq) ABTS and 2.45 mM $K_2S_2O_7$ were prepared and kept in the dark for 16 h. About 20 μL aliquot was reacted with 200 μL ABTS solution and absorbance was determined following 15 min of incubation at 734 nm by using a microplate reader (BIO RAD, Japan).

### 2.3.6. Hydroxyl radical evaluation

The method of Sindhu and Abraham[19] was adopted for hydroxyl radical activity. In summary, 100 μL aliquot, 40 μL of 500 μM $FeSO_4$, 120 μL of 20 mM deoxyribose, 40 μL of 20 mM $H_2O_2$, 400 μL of 0.1 M PB were all individually pipetted into a 2 mL Eppendorf tubes. While the mixture was made to 800 μL with distilled water and incubated for 1 800 s at body temperature, the reaction was terminated with 0.5 mL and 0.4 mL of trichloroacetic acid (2.8%) and thiobarbituric acid (0.6%) solutions respectively. About 300 μL was transferred into microplate wells to read the absorption of the mixture at 532 nm following the incubation period of 1 200 s at 100 ℃.

## 2.4. In vitro antidiabetic potentials

The *in vitro* antidiabetic activity of FEDA was evaluated by the inhibition of α-AML, α-GCD, sucrase and maltase activities based on the methods described below.

### 2.4.1. α-AML inhibitory assay

The modified procedure of McCue and Shetty[20] was employed for α-AML assay. About 50 μL of the extract / acarbose (0.125-1.000 mg/mL) was reacted with 50 μL α-AML (0.5 mg/mL) in 0.02 M sodium phosphate buffer (pH 6.9), pre-incubated at 25 ℃ for 600 s, followed by addition of 50 μL of 1% starch solution (prepared in sodium phosphate buffer). The second incubation was repeated as above and the reaction mixture stopped with the introduction of 100 μL of dinitro salicylic acid reagent. At last, the mixture was incubated in boiling water for 300 s, cooled and diluted with 1 mL distilled water for absorbance reading at 540 nm. Distilled water was replaced with the extract to represent the control following similar procedure. The α-AML inhibitory activity was calculated as percentage inhibition following the above expression in 2.3.1.

### 2.4.2. Mode of α-AML inhibition

The method of Ali *et al*[21] was used to determine the mode of inhibition of FEDA. In brief, 0.25 mL of the extract (5 mg/mL) was pre-incubated with 0.25 mL α- AML solution for 600 s at 25 ℃ in one set of 5 test tubes with concurrent pre-incubation of 0.25 mL PB with 250 μL of α-AML in another set of 5 test tubes. Introduction of 0.25 mL starch solution in increasing concentrations (0.30-5.00 mg/mL) to all test tubes signified the beginning of the reaction which was then brought to a halt with dinitro salicylic acid following the procedure in 2.4.1. A maltose standard curve was used to determine the amount of reducing sugars released and converted to reaction velocities. The kinetics of inhibition of FEDA on α-AML activity was thereafter evaluated using Lineweaver and Burk[22].

### 2.4.3. α-GCD inhibitory assay

The effect of the flavonoids extracts on α-GCD activity was determined according to the method described by Kim *et al*[23] using α-GCD from *Saccharomyce cerevisiae*. The substrate solution *p*NPG (5 mM) was prepared in 0.02 M PB (pH 6.9). Briefly, 50 μL of the different concentrations of the extract (0.125-1.000 mg/mL) was pre-incubated with 100 μL of α-GCD (0.5 mg/mL) in a test tube and 50 μL of *p*NPG was added to initiate the reaction while 2 mL of 0.1 M $Na_2CO_3$ terminates the process following incubation at 37 ℃ for 1 800 s. The α-GCD activity was determined by measuring the yellow coloured *para*-nitrophenol (*p*NP) released from *p*NPG at 405 nm. Percentage inhibition was determined by using the expression in 2.3.1.

### 2.4.4. Mode of α-GCD inhibition

The modified method of Ali *et al*[21] was used to assay the mode of inhibition of α-GCD by flavonoids extract. Summarily, 50 μL of the extract (5 mg/mL) reacted with 100 μL of α-GCD solution

for 600 s at 25 ℃ in one set of 5 test tubes while at the same time α - GCD was mixed with 50 μL of PB (pH 6.9) in another set of 5 tubes. Fifty μL of *p*NPG at increasing concentrations (0.25-2.00 mg/mL) was subsequently added to the two sets of test tubes to initiate the process. The resulting mixtures were allowed to stand at 25 ℃ following stopping the process with 500 μL of $Na_2CO_3$. A *p*NP standard was used to determine spectrophotometrically the amount of reducing sugars released and kinetics of FEDA on α -GCD activity determined by using Michaelis-Menten kinetics.

### 2.4.5. Sucrase and maltase inhibition assays

The FEDA actions on sucrase and maltase activities were determined according to the methods described by Kim *et al*[23] and Adisakwattana *et al*[24] by using α -GCD from *Saccharomyce cerevisiae*. The substrate solutions, sucrose (50 mM) and maltose (25 mM) were prepared in 0.02 M PB (pH 6.9). Briefly following the procedure described in 2.4.3, the substrate (*p*NPG) was replaced by sucrose and maltose while the absorbance was taken at 540 nm instead of 405 nm. The sucrase and maltase activities were determined as percentage inhibition according to the expression in 2.3.1.

### 2.4.6. Kinetics of sucrase and maltase inhibition

The modified method of Ali *et al*[21] was used to determine the mode of inhibition of sucrase and maltase by flavonoids extract of *D. anomala* root. Based on the 2.4.4 procedure above, the kinetics of sucrase and maltase was determined by replacing *p*NPG with sucrose and maltose.

## 2.5. Brine shrimp lethality assay (BSLA)

The method of Meyer *et al*[25] was used to obtain the larvae from the brine shrimp eggs. This was achieved by dissolving 17 g of brine shrimp eggs into 500 mL of filtered, artificial seawater in a 1 000 mL plastic beaker (hatching chamber) with a partition for dark (covered) and light areas. The shrimp eggs were introduced into the dark side of the chamber while the lamp above the other side (light) was positioned to attract the hatched shrimps. The whole set-up lasted for 2 880 min for the eggs to hatch and develop into mature nauplii (larva). Following 48 h of matured shrimp larvae, lethality assay was carried using the method of Olajuyigbe and Afolayan[26]. Briefly, five concentrations (0.125, 0.250, 0.500, 1.000, 2.000 mg/mL) of FEDA prepared in filtered seawater were considered for the assay. About 5 mL of each concentration of FEDA was emptied into a 10 mL vial and tested in triplicates after the introduction of 10 shrimps each into the tubes. Three tubes containing 10 shrimps each inside the 5 mL seawater served as negative control and all the test tubes were left open under the lamp. To determine the mortality of the larvae, brief observation of the vials was made to see if there is no movement of the larvae. Similarly, in order to ensure that the mortality witnessed was not due to starvation but as a result of the extract or bioactive compounds in FEDA, comparison on the dead larvae between the treatments and control was made. The number of surviving shrimps in all the vials were counted and recorded after 24 h. Using probit analysis, $LC_{50}$ (lethality concentration resulting in 50% mortality of the larvae) was assessed at 95% confidence intervals. The percentage mortality (%M) was also calculated by using the expression below:

% M = Number of dead nauplii / Total of nauplii × 100

It is worthy of mention that in many experiments where limited numbers of treatments are used, it is preferable or advisable to adjust or correct the death witnessed in the treatments to the negative control. Traditionally, correction of control mortality was via the use of mathematical expression by Abbot[27] and the acceptable adjusted value should be less or equal to 20%. Hence, the corrected mortality in this assay was obtained following the expression:

$$\text{Corrected \% M} = [(M_{observed} - M_{control}) / (100 - M_{control})] \times 100$$

## 2.6 Statistical analysis

Data analysis was done by one-way analysis of variance, followed by Bonferroni's multiple comparison test and results were expressed as mean ± SEM using Graph Pad Prism version 3.0 for windows, Graph Pad software, San Diego, California, USA.

# 3. Results

## 3.1. Antioxidant (in vitro) assays

The ability of FEDA to scavenge tested free radicals was shown in Table 1. It was observed that the inhibition of the radicals was dose dependent *i.e.* there was an increase in the inhibition by the extract with increasing concentrations. From the result, FEDA revealed the best activity in DPPH, ABTS and nitric oxide scavenging capabilities with $IC_{50}$ values of (386.90±4.91), (736.00±8.12), (629.30±9.62)

**Table 1**

Metal chelating and free radical scavenging activities of flavonoids extracts of *D. anomala* root (mean ± SEM) (*n*=3) ( μ g/mL).

| Extracts | DPPH | ABTS | Hydroxyl radical | Nitric oxide | Metal chelating |
|---|---|---|---|---|---|
| *D. anomala* | 386.90±4.91[a] | 736.00±8.12[a] | 933.00±9.76[a] | 629.30±9.62[a] | 1 454.00±23.97[a] |
| Quercetin | 522.20±12.38[b] | 1 021.00±15.61[b] | 342.50±21.50[b] | 1 075.00±29.35[b] | 252.30±26.23[b] |
| BHA | 592.00±19.90[c] | 1 166.00±27.80[c] | 747.90±41.80[c] | 639.56±11.26[c] | 1 363.00±8.96[c] |

Values with different superscripts in the column are significantly different ($P < 0.05$) to each other.

μg/mL respectively which were significant ($P$<0.05) when compared with the standards such as quercetin [(522.20±12.38), (1 021.00±15.61) and (1 075.00±29.35) μg/mL respectively] and BHA [(592.00±19.90), (1 166.00±27.80), (639.56±11.26) μg/mL respectively]. The standards, however, performed better than the extract for hydroxyl radicals and metal chelating. Moreover, FEDA competed favourably with quercetin and BHA for reducing capabilities though the standards showed significant ($P$<0.05) difference than FEDA at the lowest concentration (Table 2).

**Table 2**
Reducing capabilities of standards and flavonoids root extract of *D. anomala* (mean ± SEM) ($n$=3) .

| Extracts (Absorbance at 700 nm) | Concentrations (mg/mL) | | | | |
|---|---|---|---|---|---|
| | 0.125 | 0.250 | 0.500 | 0.750 | 1.000 |
| FEDA | 1.04±0.27$^{a}$ | 1.96±0.01$^{a}$ | 2.23±0.01$^{a}$ | 2.32±0.01$^{a}$ | 2.40±0.04$^{a}$ |
| Quercetin | 2.19±0.01$^{b}$ | 2.23±0.03$^{a}$ | 2.30±0.02$^{a}$ | 2.38±0.01$^{a}$ | 2.41±0.02$^{a}$ |
| BHA | 2.29±0.01$^{b}$ | 2.34±0.01$^{a}$ | 2.35±0.01$^{a}$ | 2.49±0.01$^{a}$ | 2.56±0.00$^{a}$ |

Values with different superscripts in the column are significantly different ($P$ < 0.05) to each other.

### 3.2. In vitro antidiabetic assays

The inhibitions of the four evaluated hydrolysing enzymes were shown in Table 3. As observed, the inhibition of α-GCD by FEDA was dose-dependent and there was a significant increase ($P$<0.05) on the inhibition of the enzyme activity from 0.125 mg/mL to 0.750 mg/mL when compared with acarbose though, they both competed favourably at 1.000 mg/mL. For α-AML, there was a moderate or mild inhibition of the enzyme by both the extract and acarbose though the inhibition significantly ($P$<0.05) rose with increasing concentrations. In fact, the effect of acarbose on inhibiting the activity of the enzyme was better than FEDA. The inhibition of the sucrase by acarbose was poor (below 50%) even at the highest concentration of 1.000 mg/mL. FEDA significantly ($P$<0.05) and dose-dependently inhibited the enzyme at all concentrations when compared with acarbose except at 0.750 mg/mL where they competed favourably with each other. Moreover, it was observed that at concentrations from 0.125 mg/mL to 0.500 mg/mL, there was insignificant difference ($P$>0.05) on the inhibition of maltase activity by FEDA and acarbose. However, FEDA revealed an astonishing maltase activity (almost 100%) at 1.000 mg/mL significantly better than acarbose ($P$<0.05).

The kinetics of inhibition of α-AML by FEDA as revealed from Lineweaver Burk plot signified a reduction in both Vmax and Km values from 1.47 to 1.27 mM/min and from 0.03 to 0.02 mM respectively for FEDA and control suggesting an uncompetitive inhibition (Figure 1A). A non-competitive inhibition was observed for α-GCD based on the calculated values of Km and Vmax obtained from double reciprocal plot. A constant Km value (0.14 mM) between FEDA and control with a reduction in Vmax value from 0.017 (mM/min) for the extract to 0.005 (mM/min) for the control were obtained (Figure 1B). The probable mechanism of inhibition of sucrase by FEDA as revealed by Lineweaver Burk plot showed a constant Vmax (0.027 mM/min) for FEDA and control as well as an increase in Km value for the extract from 0.203 mM to 0.617 mM (control). This was suggestive of a competitive inhibition (Figure 1C). Moreover, constant Km values (0.25 mM) for both FEDA and control were obtained for the kinetics of maltase as revealed by the double reciprocal plot. However, a slight reduction in Vmax values from 0.023 to 0.020 mM/min (control) was determined and suggestive of a non-competitive inhibition (Figure 1D).

**Table 3**
Inhibition of carbohydrate-hydrolysing enzymes by acarbose and flavonoids extract of *D. anomala* root (mean ± SEM) (% inhibition).

| Parameters | Concentrations (mg/mL) | | | | |
|---|---|---|---|---|---|
| | 0.125 | 0.250 | 0.500 | 0.750 | 1.000 |
| α-AML | 3.32±0.35$^{a}$ | 4.13±0.11$^{a}$ | 9.85±0.09$^{a}$ | 21.86±0.13$^{a}$ | 29.40±0.09$^{a}$ |
| Acarbose$^{\alpha}$ | 40.66±0.70$^{b}$ | 43.86±0.93$^{b}$ | 45.23±2.97$^{b}$ | 48.17±0.35$^{b}$ | 55.61±0.29$^{b}$ |
| α-GCD | 18.66±0.26$^{a}$ | 27.08±0.53$^{a}$ | 47.58±1.31$^{a}$ | 48.90±0.79$^{a}$ | 73.34±4.73$^{a}$ |
| Acarbose$^{\beta}$ | 2.58±0.21$^{b}$ | 3.94±0.31$^{b}$ | 4.84±0.37$^{b}$ | 6.47±0.16$^{b}$ | 74.19±1.05$^{a}$ |
| Sucrase | 7.79±0.50$^{a}$ | 8.80±0.25$^{a}$ | 22.37±1.01$^{a}$ | 29.91±1.76$^{a}$ | 98.05±2.25$^{a}$ |
| Acarbose$^{\mu}$ | 0.84±0.08$^{b}$ | 1.51±0.00$^{b}$ | 2.34±0.72$^{b}$ | 23.27±0.25$^{a}$ | 35.52±1.64$^{b}$ |
| Maltase | 8.17±3.04$^{a}$ | 13.31±0.00$^{a}$ | 14.01±2.80$^{a}$ | 16.11±0.00$^{a}$ | 98.04±1.40$^{a}$ |
| Acarbose$^{\Delta}$ | 10.01±0.69$^{a}$ | 13.53±1.24$^{a}$ | 21.40±0.00$^{a}$ | 26.37±3.22$^{b}$ | 53.27±1.53$^{b}$ |

Values with different superscripts in the column are significantly different ($P$<0.05) to each other between similar parameters (such as AML/acarbose$^{\alpha}$; GCD/acarbose$^{\beta}$; sucrose/acarbose$^{\mu}$; maltase/acarbose ).

### 3.3. BSLA

The effect of the extract (FEDA) on BSLA was presented in Table 4. While the maximum mortality was found at 83%, the degree of lethality was dose-dependent and found at 0.510 mg/mL as revealed by the regression equation ($R^2$ = 0.889 3). $R^2$ is measure of how closely related a parameter fell within a particular set of experimental data and it ranged from 0 (indicating no correlation between the data and the line) to 1 (perfect statistical correlation between the data and the line drawn).

**Table 4**
Cytotoxic effect of FEDA on BSLA.

| Conc. (mg/mL) | Conc. log | Number of larvae | Number of survivors | | | % Mortality | C% Mortality |
|---|---|---|---|---|---|---|---|
| | | | V1 | V2 | V3 | | |
| 0.125 | 2.10 | 10 | 8 | 7 | 6 | 30 | 12.5 |
| 0.250 | 2.40 | 10 | 4 | 5 | 6 | 50 | 37.5 |
| 0.500 | 2.70 | 10 | 5 | 6 | 4 | 50 | 37.5 |
| 1.000 | 3.00 | 10 | 4 | 4 | 4 | 60 | 50.0 |
| 2.000 | 3.30 | 10 | 2 | 2 | 1 | 83 | 78.8 |
| Control | | 10 | 8 | 9 | 7 | 20 | |

Conc.: concentration; C: corrected; V: vial.

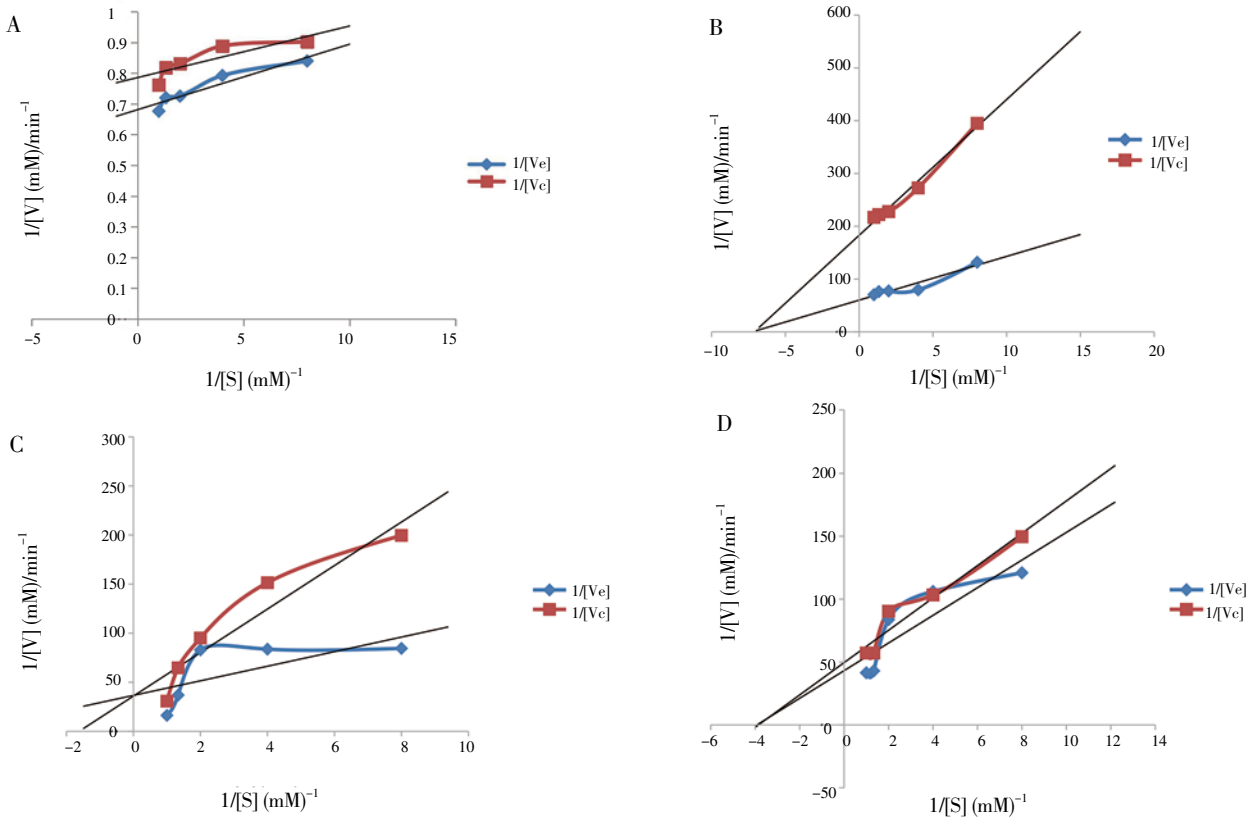


**Figure 1.** Kinetic results.

A: Kinetics of alpha amylase by flavonoids roots extract of *D. anomala* (Sond.); B: Kinetics of alpha glucosidase by flavonoids roots extract of *D. anomala* (Sond.); C: Kinetics of sucrase by flavonoids roots extract of *D. anomala* (Sond.); D: Kinetics of maltase by flavonoids roots extract of *D. anomala* (Sond.).Ve: reaction velocity of FEDA; Vc: reaction velocity of the control.

## 4. Discussion

Antioxidants are substances that hinder reactions or oxidation influenced by peroxides, oxygen or free radicals. They are involved in the management of human degenerative diseases such as cancer, diabetes, cardiovascular diseases, inflammatory diseases *etc*. which are probably due to the ill effects of oxidative stress caused by overproduction of free radicals[28]. Plant-based antioxidant or secondary metabolite such as flavonoids is an excellent antioxidant which can be effective against free radicals or associated diseases. They achieved or curbed these excesses via various mechanisms such as scavenging and suppression of radical species (reactive oxygen or reactive nitrogen) formation and by way of protecting antioxidant defense system[29]. ABTS and DPPH are the most common, reliable and extensively used methods for the evaluation of antioxidant capacity of plant substances. The latter decolourizes DPPH solution by the inclusion of a radical species or an antioxidant that scavenges DPPH while the former generates a blue $ABTS^{+}$ chromophore upon the interaction of ABTS with potassium per sulphate. In the present investigation, the ability of FEDA to scavenge four (ABTS, DPPH, nitric oxide and reducing power) of the six tested free radicals suggests and buttresses the antioxidant potentials of FEDA whose activities are better than quercetin (standard flavonoids) and BHA (known synthetic antioxidant). This also corroborates the submission of Dai and Mumper[30] who maintained that flavonoids are more effective than some synthetic antioxidants including ascorbic acid, tocopherol *etc*.

Free radicals as reactive oxygen species are linked to the development of numerous diseases including hyperglycaemia in diabetic state. They result in auto-oxidation of membrane-bound lipids or proteins leading to cellular damage[31]. However, the use of antioxidants could be employed as suitable antidote at salvaging the ravishing effects of free radicals which cause the destruction of the pancreatic beta cells thereby paving way for onset of diabetes[32]. Hence, plant-derived antioxidants and or its secondary metabolite such as flavonoids may come in handy for preserving beta cell function, inhibiting reactive oxygen species-induced diabetes and ultimately assisting in managing type II diabetes.

Pancreatic α-AML and intestinal α-GCD are prominent enzymes responsible for the breaking-down of starch to disaccharides and from disaccharides (maltose and sucrose) to smaller absorbable glucose units respectively[33-35]. Moreover, maltase is a class of α-GCD that catalyses the conversion of maltose to two glucose residues while sucrase on the other hand, catalyzes the breakdown of sucrose to glucose and fructose[36]. In postprandial hyperglycaemia, the regulation of these enzymes particularly α-AML and α-GCD

is an effective measure of controlling blood glucose (sugar level) in diabetic state. The percentage inhibition results obtained from the study revealed FEDA moderately inhibiting the activity of α-AML, strongly inhibiting α-GCD and other glucosidases effectively than acarbose (a good synthetic inhibitor of these enzymes). The moderate and strong inhibition of α-AML and α-GCD respectively by FEDA is indicative of its potent hypoglycaemic effect since a good antidiabetic agent will produce little or no side effect (flatulence, constipation) unlike the synthetic drugs (acarbose, miglitol *etc*) if it exhibits mild and strong inhibition against α-AML and α-GCD respectively[37]. This is in consonance with what we obtained with the crude water extract of the plant against α-AML and α-GCD[8]. Interestingly, Lu *et al*[38] and Vinayagam and Xu[39] revealed in different scientific reports the antidiabetic activity of flavonoids from numerous medicinal plants.

The kinetics of these enzymes which are a measure of determining or understanding the mode of inhibition of these enzymes by FEDA as evaluated using Lineweaver Burk plot revealed an uncompetitive inhibition (decreased Km and Vmax) against α-AML. This is indicative of FEDA at binding to enzyme-substrate (ES) complex to form ES-inhibitor (I) complex[40,41] thereby resulting in a reduced affinity of S for the active site of E and consequently lowering the reaction rate or prolonging the breakdown of starch to disaccharide[42,43]. Moreover, α-GCD and maltase were inhibited by FEDA non-competitively (decrease in Vmax but constant Km), this suggests that FEDA binds to other site aside the active sites of the ES resulting in its affinity for both E as well as ES complex[44]. However, FEDA revealed a competitive inhibition (constant Vmax, increased Km) against sucrase. This signifies the tendency of FEDA to compete with S at binding at the active site of E, implying that FEDA has a structural resemblance with the S thereby slowing down the digestion of disaccharide to glucose.

The development of chemotherapeutic drugs for cancer treatments in recent times involves the use of alternative therapeutic approach from plant sources screened for the presence of cytotoxic effects. BSLA is one of the screening assays adopted in determining the anticancer potential of medicinal plants[45] probably due to the ease of use (requires no sterile procedure), rapid (result available within 24 h), efficiency, affordability and availability. In this experiment, lethality was observed following exposure to varying concentrations of FEDA and the $LC_{50}$ was found at 0.510 mg/mL suggesting a good cytotoxic effect. According to Meyer *et al*[25], extract with $LC_{50}$ below 1 000 μg/mL (1.000 mg/mL) is toxic (active) while those above 1 000 μg/mL are non-toxic (inactive). Interestingly, Murakami *et al*[46] among other revelations submitted the antitumor and cytotoxic properties of flavonoids from plants.

Above all, the present investigation revealed the cytotoxic effect of flavonoids roots extract of *D. anomala*. Additionally, it also revealed the underlying mode of action of sucrase, maltase, α-GCD and α-AML inhibitory activities of FEDA and the plausibility to its use as an antihyperglycaemic and antioxidative agent.

## Conflict of interest statement

The two authors declare that there is no conflict of interest.

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## References

[1] Kumar S, Pandey AK. Phenolic content, reducing power and membrane protective activities of *Solanum xanthocarpum* root extracts. *Vegetos* 2013; **26**(1): 301-307.

[2] Mahomoodally MF, Gurib-Fakim A, Subratty AH. Antimicrobial activities and phytochemical profiles of endemic medicinal plants of Mauritius. *Pharm Biol* 2005; **43**(3): 237-242.

[3] Pandey AK. Anti-staphylococcal activity of a pan-tropical aggressive and obnoxious weed *Parihenium histerophorus*: an *in vitro* study. *Nat Acad Sci Lett* 2007; **30**(11-12): 383-386.

[4] Kumar S, Mishra A, Pandey AK. Antioxidant mediated protective effect of *Parthenium hysterophorus* against oxidative damage using *in vitro* models. *BMC Complement Altern Med* 2013; **13**: 120.

[5] Harborne JB. *Phytochemical methods, a guide to modern techniques of plant analysis*. 3rd edition. New Delhi: Springer Pvt. Ltd; 1998.

[6] Trifunsch IT, Ardelean DG. Flavonoid extraction from *Ficus carica* leaves using different techniques and solvents. *J Nat Sci* 2013; **125**: 79-84. Doi: 10.2298/ZMSPN1325081T.

[7] Balogun FO, Tshabalala NT, Ashafa AOT. Antidiabetic medicinal plants used by the Basotho tribe of Eastern Free State: a review. *J Diabetes Res* 2016; **2016**(3): 1-13.

[8] Balogun FO, Ashafa AOT. Aqueous roots extract of *Dicoma anomala* (Sond.) extenuates postprandial hyperglycaemia *in vitro* and its modulation against on the activities of carbohydrate-metabolizing enzymes in streptozotocin-induced diabetic Wistar Rats. *S Afr J Bot* 2017; **112**: 102-111

[9] Balogun FO, Ashafa AOT. Antioxidant and hepatoprotective activities of aqueous root extracts of *Dicoma anomala* (Sond.) against carbon tetrachloride-induced liver damage in rats. *J Trad Chin Med* 2016a; **36**(4): 505-513.

[10] Balogun FO, Ashafa AOT. Aqueous root extracts of *Dicoma anomala* (Sond.) ameliorates against isoproterenol-induced myocardial infarction in Wistar rats. *Trop J Pharm Res* 2016b; **15**(8): 1651-1657.

[11] Becker JVW, van der Merwe MM, Brummelen AC, Pillay P, Crampton BG, Mmutlane EM, et al. *In vitro* antiplasmodial activity of *Dicoma anomala* subsp. *gerradii* (Asteraceae): identification of its main active constituent, structure-activity relationship studies and gene expression

profiling. *Malaria J* 2011; **10**: 295-304.

[12]Salvamani S, Gunasekaran B, Shaharuddin NA, Ahmad SA, Shukor Y. Antiartherosclerotic effects of plant flavonoids. *Biomed Res Int* 2014; **2014**: 1-11.

[13]Gupta A, Sheth NR, Pandey S, Yadav JS, Joshi SV. Screening of flavonoids rich fractions of three Indian medicinal plants used for the management of liver diseases. *Rev Bras Farmacogn* 2015; **25**(5): 485-490.

[14]Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, Morelli I. Antioxidant principles from *Bauhinia terapotensis*. *J Nat Prod* 2001; **64**(7): 892-895.

[15]Kumaran A, Karunakaran RJ. *In vitro* antioxidant activities of methanol extracts of five *Phyllanthus* species from India. *LWT* 2007; **40**(2): 344-352.

[16]Pulido R, Bravo L, Saura-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J Agric Food Chem* 2001; **48**(8): 3396-3402.

[17]Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* 1994; **315**(1): 161-169.

[18]Re R, Pellegrini N, Proteggente A. Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Rad Bio Med* 1999; **26**(9-10): 1231-1237.

[19]Sindhu M, Emilia Abraham T. *In vitro* antioxidant activity and scavenging effects of Cinnamonium verum leaf extract assayed by different methodologies. *J Food Chem Toxicol* 2005; **44**(2): 198-206.

[20]Mccue P, Shetty K. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase *in-vitro*. *Asia Pac J Clin Nutr* 2004; **13**(1): 101-106.

[21]Ali H, Houghton PJ, Soumyanath A. Alpha-amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *J Ethnopharmacol* 2006; **107**(3): 449-455.

[22]Kim YM, Jeong YK, Wang MH, Lee WY, Rhee HI. Inhibitory effects of pine bark extract on α-glucosidase activity and postprandial hyperglycemia. *Nutrition* 2005; **21**(6): 756-761.

[23]Lineweaver H, Burk D. The determination of enzyme dissociation constants. *J Am Chem Soc* 1934; **56**(3): 658-666.

[24]Adisakwattana S, Charoenlertkul P, Yibchok-Anun S. α-glucosidase inhibitory activity of cyanidin-3-galactoside and synergistic effect with acarbose. *J Enzyme Inhib Med Chem* 2009; **24**(1): 65-69.

[25]Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. *Plant Med* 1982; **45**(5): 31-34.

[26]Olajuyigbe OO, Afolayan AJ. Pharmacological assessment of the medicinal potential of *Acacia mearnsii* De Wild: antimicrobial and toxicity activities. *Int J Mol Sci* 2012; **13**(4): 4255-4267.

[27]Abbot WS. A method of computing the effectiveness of an insecticide. *J Econ Entomol* 1925; **18**(2): 265-267.

[28]Krishnaiah D, Rosalam S, Rajesh N. A review of the antioxidant potential of medicinal plant species. *Food Bioprod Process* 2011; **89**(3): 217-233.

[29]Cotelle N. Role of flavonoids in oxidative stress. *Curr Topics Med Chem* 2001; **1**(6): 569-590.

[30]Dai J, Mumper R. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* 2010; **15**(10): 7313-7352.

[31]Kajaria D, Tripathi JS, Tiwari SK. Phytochemical composition and *in vitro* antimicrobial and antioxidant activities of anti-asthmatic polyherbal compounds. *Elixir Hum Physiol* 2013; **61**: 16666-16671.

[32]Liu CT, Sheen LY, Lii CK. Does garlic have a role as an antidiabetic agent? *Mol Nutr Food Res* 2007; **51**(11): 1353-1364.

[33]Manohar V, Talpur NA, Echard BW, Lieberman S, Preuss HG. Effects of a water-soluble extract of maitake mushroom on circulating glucose/insulin concentrations in KK mice. Diabetes *Obes Metab* 2002; **4**(1): 43-48.

[34]Rolfes SR, Pinna K, Whitney E. *Understanding normal and clinical nutrition*. Belmont, California: Cengage Learning Inc; 2008.

[35]Gropper SS, Smith JL. *Advanced nutrition and human metabolism*. Hampshire: Cengage Learning; 2012.

[36]Toda M, Kawabata J, Kasai T. α-glucosidase inhibitors from Clove (*Syzgium aromaticum*). *Biosci Biotechnol Biochem* 2000; **64**(2): 294-298.

[37]Kwon YI, Apostolidis E, Kim YC, Shetty K. Health benefits of traditional corn, beans and pumpkin: *in vitro* studies for hyperglycemia and hypertension management. *J Med Food* 2007; **10**(2): 266-275.

[38]Lu X, Ross CF, Powers JR, Aston DE, Rasco BA. Determination of total phenolic content and antioxidant activity of garlic (*Allium sativum*) and elephant Garlic (*Allium ampeloprasum*) by attenuated total reflectance–Fourier Transformed Infrared Spectroscopy. *J Agric Food Chem* 2011; **59**(10): 5215-5221.

[39]Vinayagam R, Xu B. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. *Nutr Metab* 2015; **12**: 60. Doi: 10.1186/s12986-015-0057-7.

[40]Bisswanger H. *Enzyme kinetics: principles and methods*. 2nd edition. London, UK: John Wiley & Sons; 2008.

[41]Cornish-Bowden A. *Fundamentals of enzyme kinetics*. 4th edition. London, UK: John Wiley & Sons; 2013, p. 510.

[42]Cornish-Bowden A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem J* 1974; **137**(1): 143-144.

[43]Bachhawat JA, Shihabudeen MS, Thirumurugan K. Screening of fifteen Indian ayurvedic plants for α-glucosidase inhibitory activity and enzyme kinetics. *Int J Pharm Pharma Sci* 2011; **3**(4): 267-274.

[44]Berg JM, Tymoczko JL, Stryer L. *Glycolysis and gluconeogenesis.* New York: Freeman WH and Company; 2002, p. 425-464.

[45]McLaughlin JL, Chang CJ, Smith DL. "Bench-Top" bioassays for the discovery of bioactive natural products: an update. *Stud Nat Prod Chem* 1991; **9**: 101.

[46]Murakami A, Tanaka T, Lee JY, Surh YJ, Kim HW, Kawabata K, et al. Zerumbone, a sesquiterpenes in subtropical ginger, suppresses skin tumor initiation and promotion stages in ICR mice. *Int J Can* 2004; **110**(4): 481-490.