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ENZYMATIC TRANSFORMATION OF CUCURBITACIN E GLUCOSIDE TO CUCURBITACIN E

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ABSTRACT: *Enzymes are valuable tools to modify structure and improve physicochemical and biological properties of various natural products. B-Glucosidase comes from widespread sources. It has been of a great scientific and industrial interest due to their large availability, low cost, wide substrate spectrum and no need of added cofactors in flavonoid biotransformation.*

At the optimum conditions of B-Glucosidase, the enzyme incubated with cucurbitacin E glucoside. This substrate was converted enzymatically into cucurbitacin E which represents the most useful effect.

The enzyme activity was determined from the substrate consumed such as Folin-Ciocalteu's reagent which reacts with the substrate. The enzyme activity was also detected from the substrated consumed and the product produced by the advanced instrumental techniques such as high- performance liquid chromatography (HPLC), gas chromatography / mass spectrometer (GC /MS), ultra performance liquid chromatography / mass spectrometer (UPLC–MS) and nuclear magnetic resonance (NMR) spectroscopy.

The current study showed that the B-Glucosidase has the activity to convert cucurbitacin E glucoside to cucurbitacin E and glucose by cleaving B-glucosidic linkages. Cucurbitacin E was more vitality active than cucurbitacin E glucoside so the enzyme convert the substance from less active to more active form .

Key words: *Cucurbitacin E, B-Glucosidase, HPLC, UPLC/MS, GC/MS.*

INTRODUCTION

Enzymes are valuable tools for the preparation of complex of carbohydrates and their derivatives due to the ability of biocatalysts to generate the required type and configuration of glycosidic linkage with no requirement to elaborate protection/ deprotection strategies (Davis, 2000; Murata and Usui, 2000).

The use of enzymes to modify structure and improve physicochemical and biological properties of various natural products has been of a great scientific and industrial interest due to their large availability, low cost, wide substrate spectrum and no need of added cofactors in flavonoid biotransformation (Benkovic and Hammes-Schiffer, 2003).

B-Glucosidase (E.C.3.2.1.21) comes from widespread sources including bacteria, animals, and plants. It exhibits wide substrate specificity and is capable of cleaving B-glucosidic linkages of conjugated glucosides and disaccharides (Dale *et al.,* 1986). B-Glucosidase is able to hydrolyze cellooligosaccharides and cellobiose into glucose (Ortega *et al.,* 2001), and capable of hydrolyzing anthocyanins that are the main coloring agents found in foods of vegetable origin (Martino *et al.,* 1994 ; Hang, 1995). Many previous studies suggested that B-Glucosidases are a heterogeneous group of enzymes with various functions (Esen, 1993). A B-glucosidase which purified and characterized from Penicillium decumbens showed optimal temperature. of 65–70 ^{o}C and optimal pH of 4.5–5.0. The enzyme showed the highest affinity to salicin (Chen *et al.,* 2010).

The cucurbitacins (tetracyclic triterpens contains cucurbitane skeleton) are of great interest because of the wide range of biological activities they exhibit in plants and

animals. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, cardiovascular, and antidiabetic effects (Jayaprakasam *et al.,* 2003). Additionally, cucurbitacin species inhibit the proliferation of cancer cells through different mechanisms (Sun *et al.,* 2005).

Cucurbitane-type triterpenes, cucurbitacins B and E, were reported to exhibit cytotoxic effects in several cell lines mediated by JAK/STAT3 signaling. However, neither compound inhibited phosphorylation of STAT3 in human leukemia (U937) cells at low concentrations (Nakashima *et al.,* 2010).

The recent studies showed that cucurbitacins E was reported to show cytotoxic effects in several cell lines such as A549, MDA-MB-468, HepG2, and KB, and their apoptosis-inducing activities mediated by the inhibition of Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling (Bartalis and Halaweish, 2005 ; Shi *et al.,* 2006).

MATERIALS AND METHODS

1. Preparation of B-glucosidase enzyme

The enzyme obtained by novo nordek copmany solution was prepared by dissolving 21mg in 10 ml of phosphate buffer solution at pH 6.5, to obtain a final concentration of 2.1mg/ml. five ml of enzyme solution, were then added to 45 ml of Cucurbitacin E Glycoside solution to obtain a final concentration of 1mg/ml. The reaction tank was kept at 35 °C. Complete mixing was achieved using a magnetic stirrer set at 50 rpm. Samples were taken as function of time to measure the amount of Cucurbitacin E Glycoside converted and corresponding glucose produced (Mazzei *et al*., 2009).

2. Measurement of Enzyme activity

The enzyme activity was measured using method to detect effect of reaction time and enzyme concentration.

2.1. Determination of substrate by Folin-Ciocalteu's reagent

This method used to detect the enzyme activity depending on the steroidal phenolic portion in the substrate concentration after separation of glucose molecules under the enzyme effect. The reaction time represented factor affecting the reaction result. The total phenolic portion were estimated using folin ciocalteu reagent purchased from Sigma Chemicals Co. according to method suggested by Singleton and Rossi. (1965) and modified by Roura *et al*., (2006) and Medina-Remon *et al*., (2009). This method was explained briefly by completing 0.1 ml of the aqueous extract to 0.5 ml with distilled water. After addition of 0.25 ml of folin-ciocalteu reagent and 1.25 ml of aqueous sodium carbonate solution, tube was vortexed and absorbance of blue coloured mixtures was recorded after 40 min at wave length 725 nm against blank containing 0.5 ml of distilled water without extract. The amount of total phenolic portion was calculated as a gallic acid equivalent from the calibration curve of gallic acid standard solutions obtained from Sigma Chemicals Co. covering the concentration range between 0.2 and 1.0 mg / ml.

2.2. Analysis of cucurbitacin E as reaction product

2.2.1. HPLC Analysis

The HPLC analysis was carried out by using HPLC Hewlett Packard 1100, equipped with G1322A degasser, G1311 quaternary pump, G1313A auto sampler, G1316A column thermostat and G 1314A variable wavelength detector. The analysis was performed by using ODS C18 column, 25-cm X 4.6 X 5µm, mobile phase: methanol, flow rate: 0.5 ml/min and 275nm detection wavelength. This detection wavelength was decided from spectrophotometer scanning which was carried out using UV-Visible Shimadzu spectrophotometer

The spectrophotometer scanning showed that this extract has a broad range of absorption from 300 to 220 nm. Therefore the HPLC analysis of this extract was carried out at two different wavelengths (275 and 220 nm) to choose the best detection wavelength. It was found that 275 nm was the most suitable detection wavelength in

the subsequent HPLC analyses (Torkey *et al.*, 2009).

2.2.2. UPLC–MS chromatography

The UPLC–MS chromatography used to detect the enzyme activity by qualitative detection of the substrate consumed or product produced depending on method performed by Wakuta *et al.* (2010) who prepared different concentrations of the enzyme with fixed substrate concentration dissolved in phosphate buffer (pH 4.6 – 6.5).

2.2.3. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectrometer (Varian 300MHz, DMSO-d6 at 30ºC) was used to identify the isolated expected fractions from the transformation of Cucurbitacin E 2-0-P-D-glucopyranoside E to Cucurbitacin E after splitting of the glucose unit.

2.2.4. Gas chromatography / Mass spectrometer

GC-MS analyses were carried out in a Varian CP-3800 gas chromatograph fitted with a EZ-Guard^{tm} (integrated column protection): Capillary column VF-5ms, 30 m, 0.25 mm and 0.25 µm. The gas chromatography was associated with 1200 L quadrapole MS / MS.

RESULTS AND DISCUSSION

The present study showed that B-glucosidase enzyme was able to cleave the glucosidic bond between the glucose molecules and the steroidal portion. The data in the Table (1) and graphically illustrated in the Figure (1) showed that the glucose concentration increased with increasing the enzyme concentration in dose dependent manner. This indicated that the glucose separated completely from the steroidal portion and was able to reduce benedict reagent under role of the enzyme.

The data compiled in the Table (2) and illustrated in the Figure (2). showed after 1 hr of the reaction beginning that concentration of the steroidal portion increase with the increasing the enzyme concentration till reach to 1.5 mg/ml of enzyme concentration. After that the product concentration remained unchanged even though with increasing the enzyme concentration. This indicated that the substrate concentration was insufficient to consume the enzyme completely.

Data in Table (3) and in the Figure (3). showed after 4 hr of the reaction beginning that concentration of the steroidal portion increase with the increasing the enzyme concentration till reach 0.6 mg/ml after that the product concentration remained unchanged till the point 1 mg/ml. The product concentration re-increased with increasing the enzyme concentration till the point 1.5 mg/ml after that the product concentration remained unchanged. This indicated that 4 hr of the reaction beginning sufficient to decrease the enzyme activity and the enzyme tried to retain its activity with increasing the enzyme concentration so the product re-increased slowly after 1 mg/ml of the enzyme concentration. This confirmed the concept which showed that enzyme concentration and reaction time vital factors affecting the reaction efficiency.

Table (1). The data showed increase in the enzyme concentration was associated with increase absorbance of the product with fixation of the substrate concentration.

The Substrate Conc. (mg/ml)	The Enzyme Conc. (mg/ml)	Absorbance
1	0	0.065
1	0.2	0.066
1	0.4	0.16
1	1	0.277

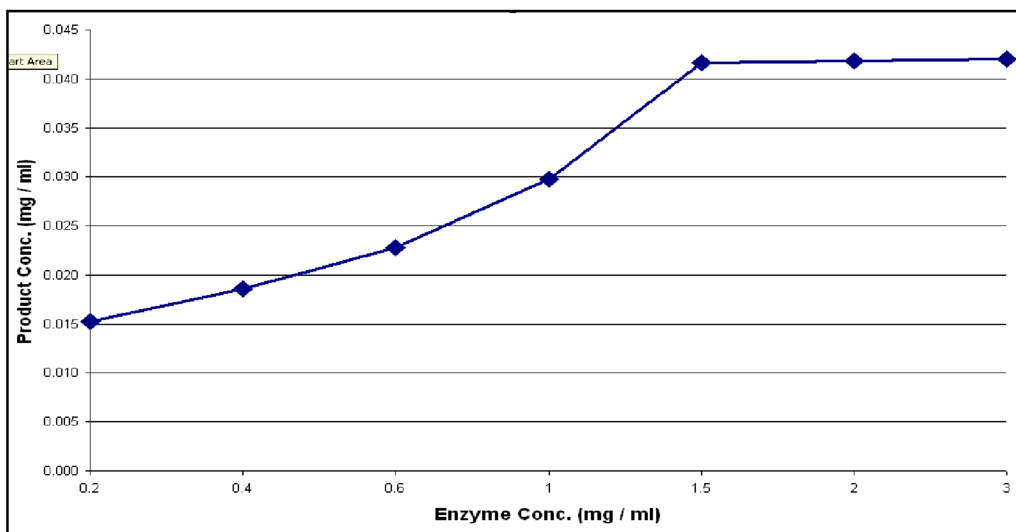

Fig. (1). Showed the relation between increase of enzyme concentration and concentration of the steroidal portion as reaction product after 1 hr of the reaction beginning.

Table (2). The data showed the relation between increase of enzyme concentration and concentration of the steroidal portion as reaction product after 1 hr of the reaction beginning.

Enzyme Conc. (mg / ml)	0.2	0.4	0.6	1	1.5	2	3
Product Conc. (mg / ml)	0.015	0.019	0.023	0.030	0.042	0.042	0.042

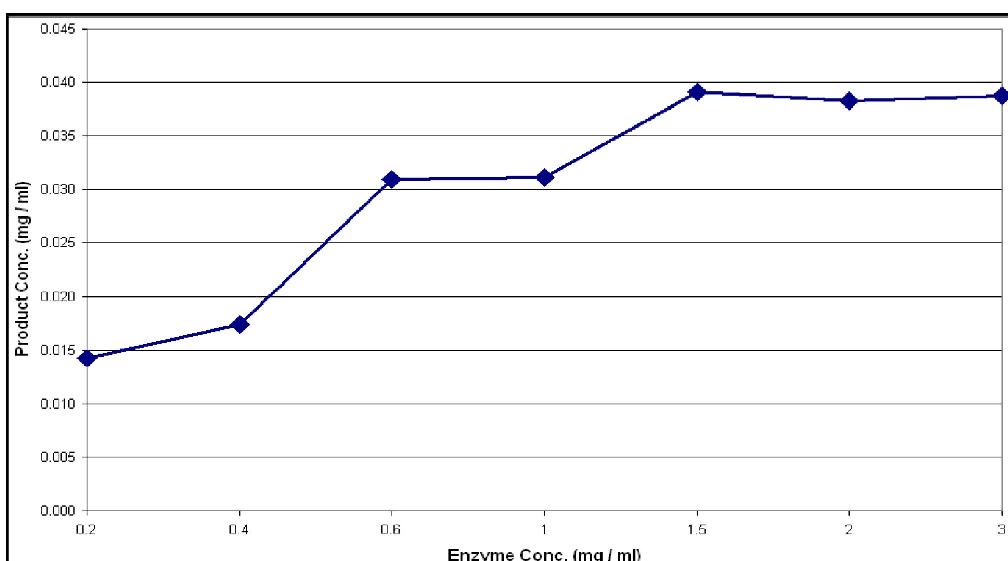

Fig. (2). The data showed the relation between increase of enzyme concentration and concentration of the steroidal portion as reaction product after 4 hr of the reaction beginning.

Table (3). The data showed the relation between increase of enzyme concentration and concentration of the steroidal portion as reaction product after 4 hr of the reaction beginning.

Enzyme Conc. (mg / ml)	0.2	0.4	0.6	1	1.5	2	3
Product Conc. (mg / ml)	0.014	0.017	0.031	0.031	0.039	0.038	0.039

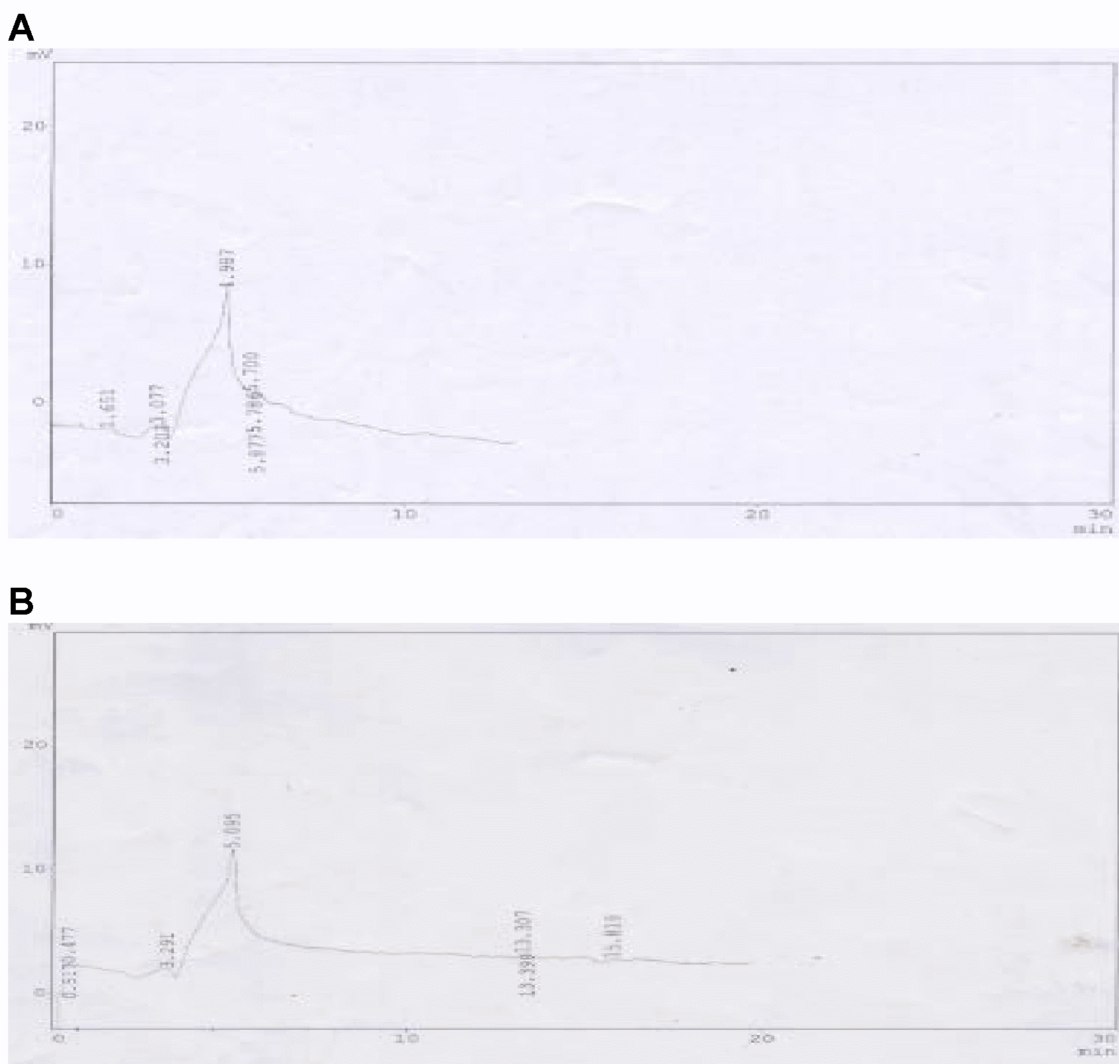

Fig.(3). The chromatograms showed the substrate without the enzyme activity (A) and the substrate after incubation with the enzyme (B) showing effect of the enzyme on concentration of the substrate which represented by area under peak.

The Folin-Ciocalteu reagent is sensitive to reducing compounds, including phenols, thiols, vitamins, amino acids, proteins, nucleotide bases, unsaturated fatty acids, carbohydrates, organic acids, inorganic ions, metal complexes, aldehydes and ketones (Everette *et al.*, 2010) thereby producing a blue colour upon reaction. This blue colour is measured spectrophotometrically. Thus total phenolic content can be determined (Swain and Hillis, 1959 ; Savitree *et al.*, 2004 ; Pourmorad *et al.*, 2006).

Phenolic compounds are a class of antioxidant agents which acts as free radical terminators (Shahidi and Wanasundara, 1992). The high scavenging property may be due to hydroxyl groups existing in the phenolic compounds (Gyamfi *et al.*, 1999).

The steroids have reducing properties and react with folin by the same reaction mechanism as phenolics (Reichestein and Shoppee, 1943).

It is believed that the Folin-Ciocalteu assay functions as a nonspecific antioxidant assay. It may prove useful for measuring the antioxidant capacities of compounds of biomedical interest.

Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reagent (Amin and Yazdanparast, 2007) However, it should be noted that some chemical group of amino acids, proteins, organic acids, sugars and aromatic amines could react with the reagent. Hydrogen donation is the main mechanism of phenolics as antioxidants. The lower strength of the O–H bond present in phenolics corresponds to a higher scavenging activity (Prasad *et al.*, 2009). The overall antioxidant activity might be attributed to the polyphenolic, flavonoid and phytosterol constituents (Dashora *et al.*, 2011).

-O-B-D-glucopyranosylcucurbitacin E can be hydrolyzed enzymatically to D-glucose and cucurbitacin E. Both of the two products contain free functional group and can reduce the folin components. So, folin reagent used determine the B-glucosidase activity through the two separated products.

D-Glucose can be determined by Folin-Ciocalteu due to presence of free anomeric carbon (the carbon of Carbonyl group present in Carbohydrates). Due to which the carbon of Carbonyl attains a partial positive charge and hence able to gain a electron from a electron donating specie.

Folin & Ciocalteu's phenol reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically.

The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence (Bray and Thorpe, 1954).

The Folin–Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically. The electron transfer reaction is not specific for phenolic compounds (Ainsworth and Gillespie, 2007).

The data in the Table (4) and graphically illustrated in the Figure (3) showed that the enzyme consumed to hydrolyze substrate. The HPLC analysis of the butanol fraction at 275 nm showed presence of one compound at retention time (Rt): 5.095 min. Its area 556631 and after the enzyme activity, 448902. The decrease in area of substrate after the enzyme activity showed that the enzyme caused hydrolysis part of the substrate giving indication to the enzyme activity. This was in agreement with Chang, and Juang, (2007) who suggested that One activity unit (U) of beta-glucosidase is defined to be the amount of this enzyme required hydrolyzing 1μmol of substrate per minute.

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The Figure (4) showed structure of 2-O-â-D-glucopyranosylcucurbitacin E which represents substrate for beta-glucosidase enzyme. The enzyme catalyzed breakage of the beta glucosidic bond causing splitting of the glucose unite at the anomeric carbon atom. The complete structure appeared at Rt 5.095 min but the hydrolysed fractions did not appear because the wave length and the flow rate were not suitable for the detection.

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Gas chromatography / Mass spectrometer

As shown in Figure (5), it was found that the GC/MS confirmed that data obtained analytically and by the different instrumental techniques. The chromatogram showed that substrate appeared with the molecular weight 718. This represented the substrate with the sugar unit before the enzyme action. After the incubation with the enzyme, the Fig. 6 showed that the cucurbitacin E appeared in the chromatogram with molecular weight 480. This indicated that the sugar unit splitted from the substrate.

Table (4). The data showed the relation between decrease in area of the substrate after reaction with enzyme giving indication to activity of enzyme to hydrolyze part of substrate.

	Area	Conc.
Substrate Without Enzyme (1 mg/ml)	556631	1 mg / ml
Substrate (1 mg/ml) With Enzyme (2 mg/ml)	350677	0.629 mg / ml

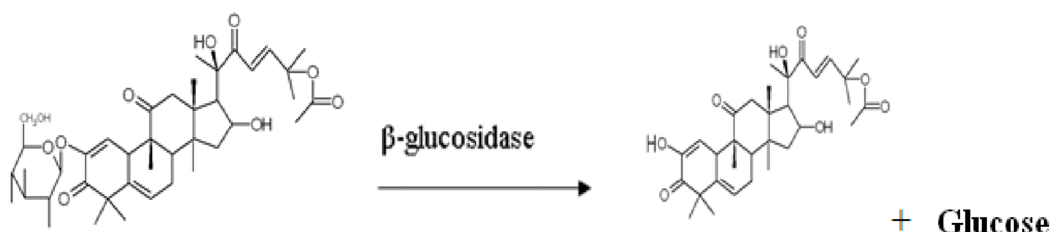


Fig. (4). Scheme of the first step of the reaction catalyzed from Beta-glucosidase from almond having 2-O-â-D-glucopyranosylcucurbitacin E as substrate.

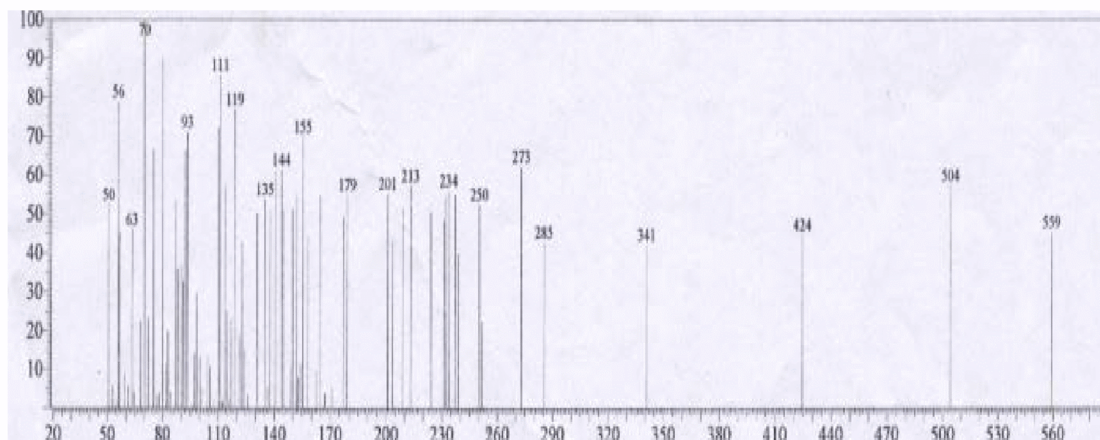


Fig. 5 : Chromatogram of GC / MS for the substrate before the enzyme activity and splitting of sugar unit.

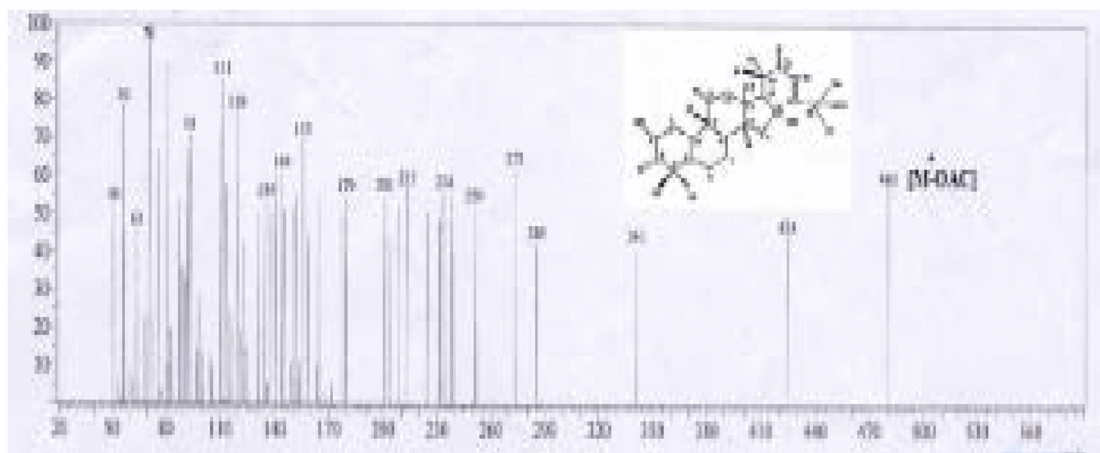


Fig. 6 : Chromatogram of GC / MS for the product after the enzyme activity and splitting of sugar unit.

NMR Spectroscopy

Analytical samples of Cucurbitacin -E- glucoside and isolated metabolites (24R-hydroxy-23, 24-dihydrocucurbitacin-E and 24S- hydroxyl-23, 24-dihydrocucurbitacin E) were obtained as pale-yellow amorphous powders. Compund-1, cucurbitacin-E was isolated from *Citrullus Colocynthis shard*. The analytical specimen gave: mp: 157-158 ^{0}C. ^{1}H-NMR and ^{13}C-NMR spectral properties of isolated Cucurbitacin -E- glucoside is recorded in Tables 1 and 2, respectively. These data recorded in Table 5 and illustrated in Fig. 7.

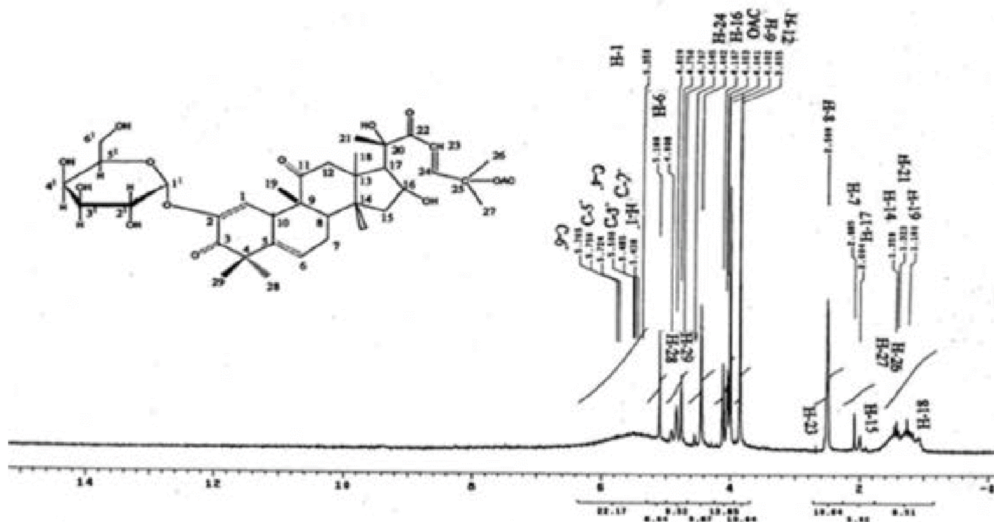
The Cucurbitacin -E- aglycone, 24R-hydroxy-23, 24-dihydrocucurbitacin-E (Compund-2) was obtained in 60% yield. The compound was crystallized as colorless prisms from CH_3OH-$CHCl_3$, and it exhibited the following physical properties: mp 232 ^{0}C and ^{1}H-NMR and ^{13}C-NMR spectral data are recorded in Table 1 and 2, respectively. These data recorded in Table 6 and illustrated in Fig. 8.

Table (5) : ^{1}H-NMR spectral properties of cucurbitacin metabolites.

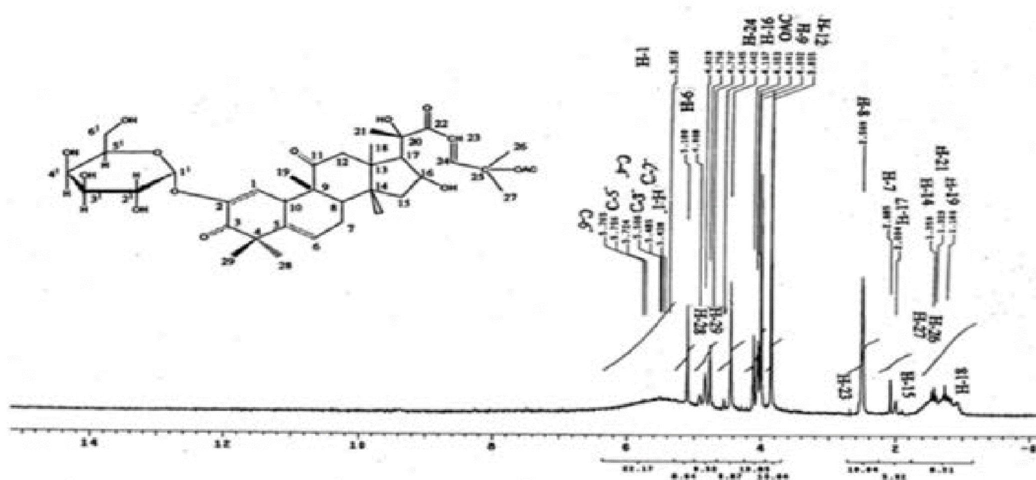
Proton	Compound-1	Compound-2
1	5.3	2.5
6	5.1	5.2
7	1.97	1.99
8	2.509	2.493
9	4.002	2.499
12	3.855	3.3
14	1.359	1.454
15	1.95	1.955
16	4.107	3.9
17	2.004	2.127
18	0.99	0.926
19	1.16	1.11
21	1.323	2.176
23	2.66	2.505
24	4.44	3.78
26,27	1.22	1.202
28,29	4.829	4.759
OAC	4.014	3.4
1^{-}	5.438	Absent
2^{-}	5.485	Absent
3^{-}	5.506	Absent
4^{-}	5.724	Absent
5^{-}	5.756	Absent
6^{-}	5.765	Absent

Table (6) : 13C-NMR spectral properties of cucurbitacin metabolites.

Carbon	Compound-1	Compound-2
1	127.875	129.380
2	122.45	127.857
3	120.914	120.914
4	22.084	22.215
5	28.647	28.815
6	28.813	29.315
7	25.374	29.740
8	39.504	39.504
9	48.47	41.78
10	38.668	38.947
11	174.615	174.915
12	38.223	42.34
13	40.061	39.782
14	40.339	40.016
15	14.003	14.033
16	69.77	69.45
17	24.615	62.347
20	22.215	21.76
22	186.34	183.45
23	38.947	38.221
24	29.135	31.027
25	31.413	31.413
OAC	106.38	105.33
1ˉ	102.33	Absent
2ˉ	72.57	Absent
3ˉ	64.75	Absent
4ˉ	63.197	Absent
5ˉ	66.48	Absent
6ˉ	60.28	Absent



¹H-NMR spectral data of compound (1)



¹H-NMR spectral data of compound (1)

Fig. 7: This chart showed the 1H-NMR and 13C-NMR spectral data of the substrate without action of the enzyme.

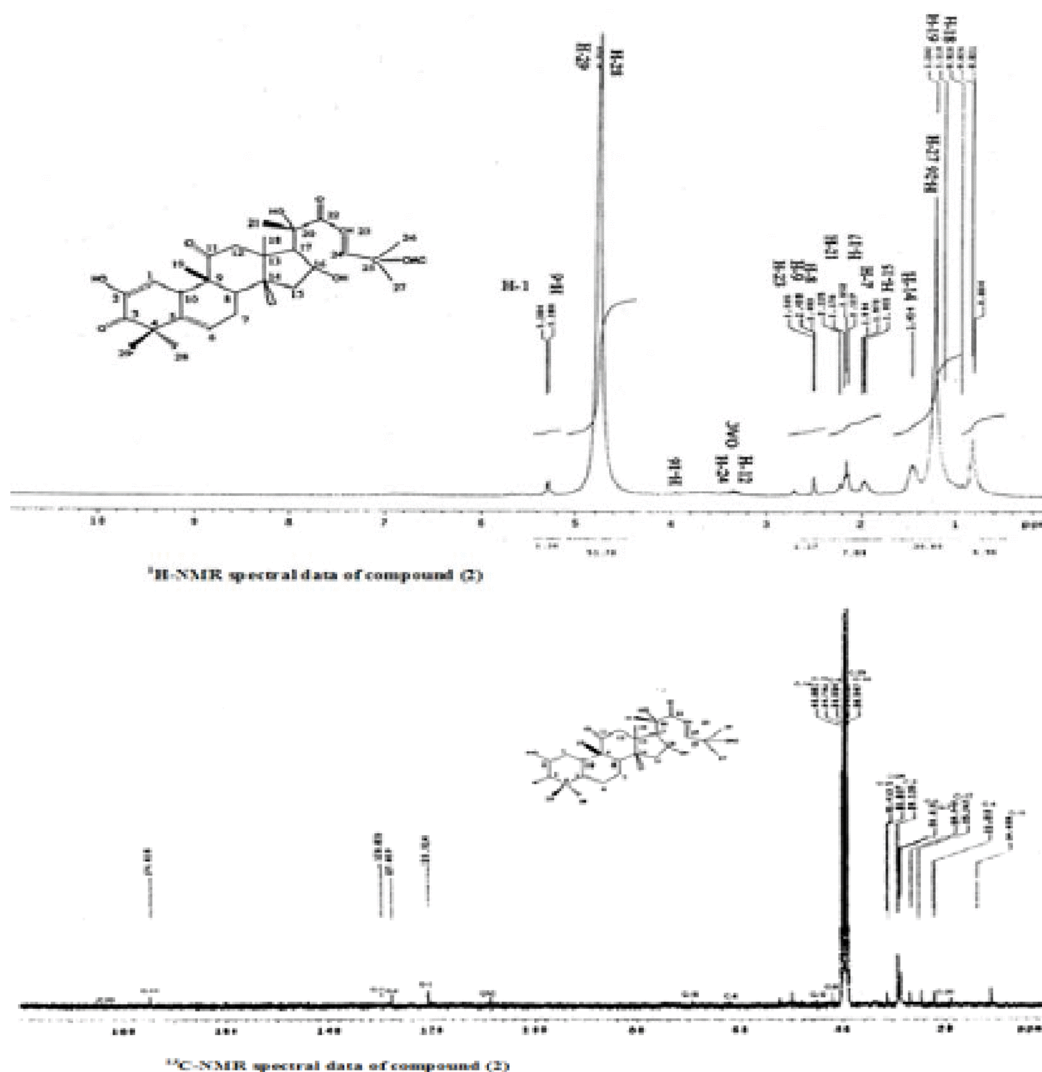


Fig. 8 : This chart showed the 1H-NMR and 13C-NMR spectral data of the product and splitting of the sugar unit (after action of the enzyme).

UPLC–MS chromatography

As shown in graphically illustrated in Figure 9. , and , it was showed that the substrate appeared at the molecular weight 740 before hydrolysis and splitting of the glucose sugar. After the enzymatic hydrolysis, the substrate degraded into two products detected instrumentally. The two products appeared with the molecular weights 179 for glucose sugar and 559 for cucurbitacin E.

All the experimental data showed that the substrate was converted into cucurbitacin E and glucose. This indicated that the enzyme catalyzed the hydrolysis process by splitting of glucose unit from the anomeric carbon atom. The experiment carried out under the conditions which represent the optimum conditions for the enzyme activity.

The instrumental analysis used to detect activity of the enzyme from the substrate and / or the product qualitatively from response of each instrument due to absence of pure substance used as standard for the substrate and product.

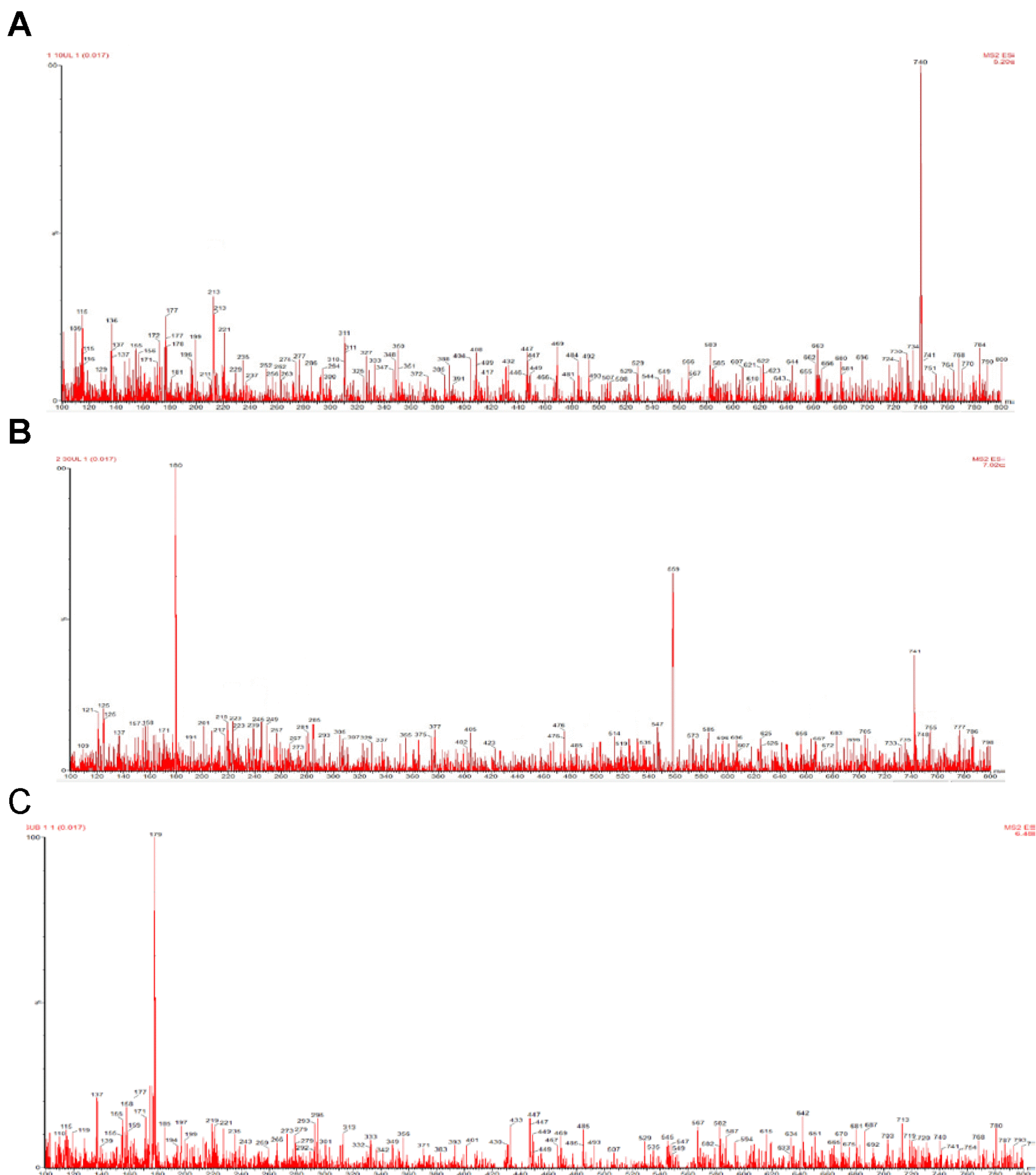


Fig. 9: Chromatogram A; showed the substrate only, B; showed substrate with some of the products formed after the enzyme activity and C; showed the sugar unit splitted after the enzyme action.

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التحول البيولوجى الانزيمى من كركيوبيتاسين إى – جلوكوسيد إلى مادة كركيوبيتاسين إى

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الملخص العربى

الانزيمات هي أدوات قيمة لتعديل هيكل وتحسين الخواص الفيزيائية والكيميائية والبيولوجية للمنتجات الطبيعية المختلفة و بيتاجلوكوسيداز الذى يأتي من مصادر واسعة الانتشار. كما ان له اهميه علمية وصناعية كبيرة جداً كما يتوافر بكميات كبيرة وتكلفتة منخفضة، ومتعدد التاثير على مواد تفاعل عديدة و لا حاجة لاضافة عوامل مساعدة عند استخدامه فى التحول الأحيائي للفلافونويد.

في الظروف المثلى للبيتاجلوكوسيداز، تم تحضين الانزيم مع مادة كيركيوبيتاسين أى جلوكوسيد. تم تحويل هذا المتفاعل عن طريق الانزيم الى كركيوبيتاسين أى الذي يمثل الأثر الأكثر فائدة. تم تحديد نشاط انزيم عن طريق الكشف عن استهلاك المتفاعلات بطريقة كاشف فولين-سيكالتو والذي يتفاعل مع المتفاعلات بشكل مباشر. تم الكشف عن نشاط انزيم أيضا من المتفاعل المستهلك والمنتج واستخدمت تقنيات متقدمة لتحليل النواتج مثل عالية الأداء اللوني السائل (HPLC)، اللوني للغاز / مطياف الكتلة (MS / GC)، الترا الأداء اللوني السائل / مطياف الكتلة (MS-UPLC) والرنين المغناطيسي النووى (NMR) الطيفي

وأظهرت الدراسة الحالية أن بيتاجلوكوسيداز لديه نشاط لتحويل كركيوبيتاسين أ جلوكوسيد إلى كركيوبيتاسين أ والجلوكوز عن طريق كسر الروابط بانزيم بينهم بالانزيم . اصبح الكركيوبيتاسين أ أكثر حيوية نشاطا من كركيوبيتاسين أجلوكوسيد وبالتالي فإن الإنزيم يحويل المادة من أقل نشاطا الى شكل أكثر نشاطا.