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ISOLATION OF CUCURBITACIN E FROM BITTER APPLE (*Citrullus colocynthis*) FRUIT AND MEASURING THE ANTIOXIDANT ACTIVITY

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ABSTRACT

The *Citrullus colocynthis* is called bitter apple and colocynth. It is a cucurbitaceae family member, which is widely utilized in traditional medicine throughout the Middle East and Asia. *Citrullus colocynthis* is a rich source of very important compounds that can be used to fight cancer related diseases, alongside bacteria infection and help the body fight cancer. This research was concerned with the extraction, identification, and isolation of some biologically significant substance cucurbitacin E from fruits of *Citrullus colocynthis*. Extraction of these cucurbitacins were carried out using (Deionized water, n-hexane, ethyl acetate). Thin layer chromatography (T.L.C) was used for identifying cucurbitacin E using specific solvent systems tested on the extracted compounds. Iodine vapor and ultra-violet (U.V.) light were utilized for detection and then theses cucurbitacins were isolated by using column chromatography. The identification of chemical composition of *Citrullus colocynthis* (cucurbitacin E) was enhanced further by the use of high- performance liquid chromatography apparatus (H.P.L.C). In the present study, fruit extract of cucurbitacin E was screened to evaluate its free radical. The anti-radical activity of the separated compound extracted by (DPPH) (2, 2-diphenyl-1-picryl hydrazyl) assay and ascorbic acid was used for comparison. It was found that the higher the concentration of cucurbitacin E compound, the free radical scavenging will increase. The compound cucurbitacin E showed the maximum percentage of inhibition of DPPH radicals and it was about $73.67 \pm 32.68\%$, $P \leq 0.01$, and it was noted that it possesses the maximum antioxidant activity at a concentration of $1000 \mu\text{g/ml}$, standard ascorbic acid showed about $89.23 \pm 4.02\%$, $p \leq 0.01$ where it was found that DPPH radicals are inhibited at its highest concentration, which is $500 \mu\text{g/ml}$. The aim of this study is to isolate the active compound cucurbitacin E from the fruit of bitter apple and to measure the antioxidant properties of the active compound.

Key words: cucurbitacin E, *Citrullus colocynthis*, antioxidant, free radicals, active compound.

Introduction

Bitter apple (*Citrullus colocynthis*) [Cucurbitaceae family]. This perennial herb can be found growing wild in large quantities in the sandy regions of north, west and southern India. From May to October, it can also be found in arid parts of Iraq, West Asia, Arabia and the Mediterranean (Sagar *et al.*, 2020). *Citrullus colocynthis* is a small creeping perennial herbaceous plant with a climbing stem and is characterized by its very bitter taste and spherical fruits with smooth peel. The fruits are green in color with white stripes before ripening and turn yellow after ripening. (Nora *et al.*, 2016). It is an ancient herb that has been utilized in traditional medicine and by several nations to cure a variety of ailments (Nora *et al.*, 2016).

Citrullus colocynthis fruits were traditionally used to treat jaundice, constipation, fever and intestinal parasites (Ayinash & Rai, 2017).

Citrullus colocynthis has been shown to contain a number of active chemical compounds such as

colocynthin, colocynthetin, cucurbitacins, α -elaterin, cucurbitacin glycosides, tannins and flavone glycosides. cucurbitacin E (which is abundant in pulp), phenolics, alcoholic, ketonic alkyl chains, flavonoids, tannins and fatty acids. These metabolites, such as flavonoids, tannins and phenols, are critical components of the defensive mechanism against diseases caused by various bacteria and fungi. Cucurbitacins (A, B, C, D, E, J, and L) are the principal bioactive or principle compounds that confer therapeutic properties, along with caffeic acid, alkaloids, terpenoids, saponins, tannins and cardiac glycosides (Kapoor., *et al*, 2020; Joshi *et al.*, 2021). *Citrullus colocynthis* improved the glycemic profile without several other ingredients unfavorable consequences in persons with type 2 diabetes (Meybodi., *et al.*, 2020).

A variety of *Citrullus colocynthis* compounds have been studied for their cytotoxic, cardiovascular, and

cucurbitacin I and L glycosides. Cucurbitacin B has also been demonstrated to have anti-inflammatory activity (Li *et al.*, 2021).

Cucurbitacines are distinguished by the presence of (tetracyclic) triterpens containing cucurbitane (skeleton) with oxygen substitutions at various locations. Cucurbitacins are classified into seventeen compounds based on their structures Cucurbitacins B,D,E,I and their derivatives demonstrated substantial antitumor activity, while cucurbitacins F,O,P,Q and their derivatives demonstrated moderate anticancer activity (Ramezani *et al.*, 2020). Thus, the compounds of cucurbitacin are of great importance due to the many biological functions that they show, as they are largely found in the cucurbitaceae family, although they can be found in many other plants (Morales-Vela *et al.*, 2019).

Structure of cucurbitacin E

Cucurbitacin E (C₃₂H₄₄O₈) has thirty carbon atoms in its basic skeleton, and its lanostane skeleton is multisubstituted with methyl, hydroxyl, and oxosubstituents with unsaturation at positions 1,5 with 23, resulting in wide range of biological activities such as liver protection, immune enhacement, antichemical carcinogenesis, antitumor effect and antichemical carcinogenesis (Sikander *et al.*, 2016; Ramezani *et al.*, 2017). Cucurbitacin E, on the other hand, had the best anticancer outcome of any cucurbitacin in a range of cancers. (Ramezani *et al.*, 2020) Figure 1.

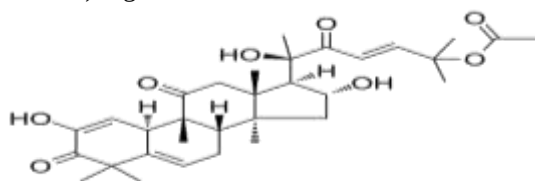


Fig. 1: chemical structure of cucurbitacin E (Ramezani *et al.*, 2020).

Furthermore, multiple investigations have shown that distinct cucurbitacine types inhibit cancer cell proliferation via diverse methods (Si *et al.*, 2019). Food antioxidant molecules serve a significant role in health protection. According to scientific research, antioxidants lessen the risk of chronic diseases such as cancer and heart disease. Whole grains, fruits, and vegetables are excellent sources of naturally occurring antioxidants. Plant-based antioxidants such as vitamin C, vitamin E, carotenes and phenolic acids. Have been identified as potentially lowering illness risk. In the diet, the majority of antioxidant compounds are from various plant sources and these compound belong to several families that possess a wide range of different chemical and physical properties. An antioxidant's fundamental trait is its capacity to trap free radicals (Gulcin, 2020).

In living systems, oxygen species such as hydroxyl radicals, superoxide anion radicals, single oxygen and other carbon/nitrogen reactive species can attack proteins, polyunsaturated fatty acids in cell membranes thus attack leads to a change in cellular

metabolism as well as gene expression of DNA and lipid peroxidation (Saba & Oridupa, 2010). Although cellular defense systems can reduce the number of free radicals, endogenous antioxidant defenses are unable to entirely scavenge them. (Halliwell, 2012). Continuous oxidative damage to DNA, proteins, lipids and other components contribute to the development of cardiovascular disease, cancer and potentially neurological illnesses (Zang *et al.*, 2003). Recently, the availability of antioxidant chemicals as phytochemical ingredients has become a prominent study focus. (Gill *et al.*, 2010). Any entity with the capacity for autonomous existence and at least one unpaired electron interacts with other molecules by either taking or giving electrons and free radicals are linked to a number of clinical diseases. because they serve an essential function in human health, helping to fight ailments such as cardiovascular disease, lung damage, and inflammation (Di - Meo & Venditti, 2020). All of these free radicals are extremely unstable, and when the body has too many of them, it can cause harm cells and tissues and may be involved in a variety of diseases; hence, natural antioxidants are required to shielding the body against illnesses generated by free - radical (Engwa *et al.*, 2018).

The aim of the current research is to study the possibility of benefiting from the active compounds present in the fruit of the *Citrullus colocynthis* by conducting isolation of the active compound cucurbitacin E from the fruit of the bitter apple, as well as conducting an in vitro test to study the effect of the compound cucurbitacin E on the displacement of the DPPH root, which is considered a free radical this is to reveal the potent antioxidant activity.

Materials and methods

Chemicals

2, 2-Diphenyl - 1 - picrylhydrazyl (DPPH) (santa croase, Germany), (L - ascorbic acid / C₆H₈O₆) was taken from Sigma / Aldrich (steinheim, Germany), methyl hydroxid (methanol / CH₃OH) was taken from (Hyman, England), hexyl hydride (n-hexane / C₆H₁₄) was taken from (BDH chemicals ltd, England), dimethyl ketone (acetone / C₃H₆O), ethanoic acid (acetic acid / C₂H₄O₂), silica gel and acetic ether (ethyl acetate / C₄H₈O₂) was taken from (Fluka, Germany), standard cucurbitacin E (≥ 98 % purity) was taken from extrasynthes/ genay/ france.

Method of extraction and isolation of the compound cucurbitacin E

Ripened fruits of *Citrullus colocynthis* were collected. The fruit pulp were cut into pieces and the seeds were removed. The fruits were reduced to powder using an electric grinder. Powder was then stored until it was used. 1 kg soaking overnight in 3 L of (Deionized water) for 2 days then placed in ultrasound vibration for 10 minutes after that filtering was done using wattman filter paper number one. (100 mL) of high purity hexyl hydride is mixed with 100 mL of the liquid in a 500 mL separating funnel. Shake the mixture for thirty minutes before letting it

separate for thirty minutes. The aqueous phase is kept in a different glass after the hexyl hydride and aqueous phases have been separated, and the remaining liquid is extracted with hexyl hydride once again. In a subsequent 500 mL separating funnel, 97 mL each of acetic ether was mixed with 97 mL of the hexyl hydride - extracted aqueous liquid. After 30 minutes of slow shaking, the mixture is allowed to separate for another 30 minutes. Following the separation of the organic and aqueous phases. The organic phase is kept and the acetic ether extraction with the remaining hexyl hydride-extracted liquid is repeated. The organic phase that results from the acetic ether extraction is then it is evaporated under reduced pressure to produce a mixture containing 0.548 g of separated cucurbitacins.

After extraction with the moderately polar solvent, the organic phase contains a mixture of partially isolated cucurbitacins. The cucurbitacins are then isolated and separated from the organic phase by column chromatography (diameter = 1cm, length = 10 cm).

After preparing the separation column (silica gel) at first, a polar solvent acetic ether is passed through to the separation column, then the organic phase is added to the separating column, then an appropriate mixture of solvents (acetic ether, dimethyl ketone and methyl hydroxid) is passed through the prepared separating column to separate cucurbitacin E compound. Cucurbitacin E is highly affinized by these solvents, ideally in a ratio of 80:5:15 by volume. 0.128 g cucurbitacin E

(Abdelwahab *et al.*, 2011; Ponsankar *et al.*, 2020).

Identification of cucurbitacin E

Thin layer chromatography (T.L.C) was used to identify cucurbitacin E using a ready-made aluminum plate coated with silica gel, (20 × 20 cm, thickness 0.1 mm). A capillary tube was used to spot sample solutions on the plate, and the reference sample was applied to one side of the plate. Methanol (50 mL) was utilized as the mobile phase. The separated compound appears as a band that is recognized by two distinct detection methods, First, 366 nm (U.V.) light was used, followed by iodine vapor in the jar., in solvent system (ethyl acetate, acetone and methanol) (80:5:15) (Halaweish & Tallamy, 2006).

HPLC analysis

The HPLC analysis was carried out by using HPLC liquid chromatography –Shimadzu 2010 LC equipped with binary delivery pump model 2010 (shimadzu, koyota, japan). Equipped with degasser, auto sampler, column thermostat and UV-VIS detector. The analysis was performed by using ODS C18 column, (250x4.6mm) x5 µm, flow rate: 1mL / min and 275 nm wavelength, this detection wavelength was decided from spectrophotometer scanning which was carried out using UV - VIS shimadzu spectrophotometer. The sample in 1 ml of the mobile phase were filtered on disposable ministers 0.2 µm (supelco company cat No 16534 K) the HPLC column

was then injected with 20µm. By contrasting the peak area of the sample with the peak area of the standard, the concentration of the compound was quantitatively calculated (Torkey *et al.*, 2009).

Determine the total activity of antioxidants

In laboratory experiments, the (DPPH) assay is used to measure the activity of free radicals and the antioxidant potential of any compound can be estimated on the basis of its scavenging activity for the stable (2, 2 – Diphenyl -1- picrylhydrazyl) (C₁₈ H₁₂ N₅ O₆) (Aadesariya *et al.*, 2017).

Principle

Using the stable free radical DPPH, the extract's capacity to scavenge free radicals was evaluated from hydrogen formation ability or radical-scavenging potential. The DPPH free- radical's odd electron produces a significant absorption maximum with a purple color at 517 nm. The hue changes from purple to yellow when the reduced DPPH-H is formed when the odd electron of the DPPH radical pairs with an antioxidant to scavenge free radicals at 517 nm. The methanolic solution of the DPPH radical exhibits peak absorbance at 517 nm and is a stable radical. Because of the conversion of 2, 2 - diphenyl-1-(2,4,6 - trinitro phenyl) - hydrazyl radical (purple solution) to 2, 2- diphenyl-1- (2,4,6- trinitro phenyl) - hydrazine, absorbance vanishes (yellow solution). The resulting color change is stoichiometric with respect to the electrons gained. (Baliyan *et al.*, 2022).

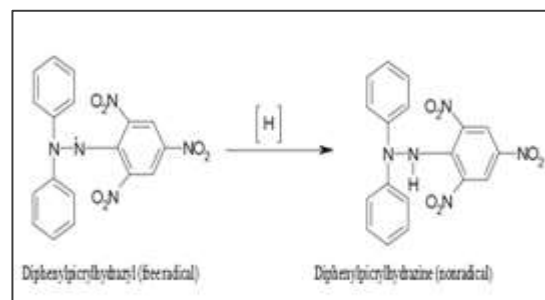


Fig. 2: Reduction of free radical by DPPH (Ionita, 2012)

DPPH radical scavenging activity

Cucurbitacin E has free radical scavenging activity that was determined by calculating the descent in the absorption of a methanolic solution of DPPH using a spectrophotometric technique.

(1.5 mL) cucurbitacin E methanolic solution at various concentrations (25, 50, 300, 500, 700, 900, 1000 µg/ml) were combined with (1.5 ml) of a methanolic DPPH solution (100 µm). After shaking the mixture, it was left in a dark place to complete the reaction at temperature of the room. similarly (1.5 ml) methanolic solutions of ascorbic acid (25,50,100,300,500 µg/ml) were combined with (1.5 ml) of DPPH. As a control, a merge of (1.5 ml) DPPH methanolic solution (100 µm) and methanol was used. After stirring all solutions were kept in dark place for 20 minutes and samples absorbance was measured at 517 nm. Scavenging was measured by inhibition percentage and samples were measured in triplicate (Jayaraman *et al.*, 2013).

$$\text{DPPH scavenge (\%)} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100$$

Where:-

A control- is the control reaction's absorbance

A test- is the absorbance when a sample of the extracts is present (Jayaraman *et al.*, 2013).

Statistical analysis

The statistical analysis system – SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference – LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Estimate of correlation coefficient between variables in this study (SAS , 2018).

Results and discussion

Nature has been a rich storage site for medicinal plants for thousands of years, and a great number of modern medications have been isolated from natural sources, particularly plant sources. (Khan *et al.*, 2021).

Citrullus colocynthis fruits contain cucurbitacin E, which is one of the cucurbitacins. (T.L.C) of cucurbitacin E extracts derived from Citrullus colocyn dried fruit pulp In comparison to the standard, this indicates the existence of cucurbitacin E in the extraction portion . As illustrated in Table 1.

(ROS) from both endogenous and exogenous sources have a role in the pathogenesis of a variety of human diseases, including ischemic heart disease, injury arteriosclerosis, neurodegenerative diseases and tumor, in addition to inflammatory and getting old (Liguori *et al.*, 2018). There is evidence that indigenous antioxidants can help avoid the negative effects of oxidative stress, where it was found that there is an increasing importance of the protective biochemical function of the antioxidants present in medicinal herbs and plants.

Medicinal plants (Tripathy & Mohanty, 2017; Liguori *et al.*, 2018).

Citrullus colocynthis plant's fruit and fruit pulp have been studied for antioxidant activity. (Gill *et al.* 2011) have found that at a concentration of 500 g/ml, the arerial portion of Citrullus colocynthis demonstrated the highest antioxidant activity. Citrullus colocynthis is an excellent source of antioxidants; leaf and root preparations of Citrullus colocynthis have also been shown to exhibit antioxidant properties: Hexane, aqueous, and ethanol leaf extracts inhibited DPPH by 45.9%, 39.81%, and 36.65%, respectively, whereas root extracts inhibited it by 29.12%, 35.51%, and 33.83% (Ahmed *et al.*, 2019). The presence of tannins, terpenoids, flavonoids, and coumarins in Citrullus colocynthis extracts has been discovered, which may be responsible for the plant's prominent antioxidant properties and other biological activities (Benariba *et al.*, 2013). (Ahmed *et al.*, 2022) have also reported that Citrullus colocynthis initial phytochemical screening revealed the presence of several flavonoids and phenols, as well as high antioxidant activity: At a concentration of 2500 g

mL¹, it contains 88.8% fruit extract and may have free radical scavenging properties.

DPPH free radical scavenging activity

In laboratory research, the free radical molecule 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which is stable at room temperature and appears as a black, crystalline powder, is frequently utilized for antioxidant assays. To transform into a stable diamagnetic molecule, it either takes an electron or receives a hydrogen-radical. Through the low absorption at 517 nm resulting from the presence of a large number of antioxidants, the ability to decrease the DPPH radical was evaluated. Because of the occurrence of a chemical reaction between the advancement of radicals and antioxidant molecules, the decrease in absorbance of the DPPH radical produced by antioxidants results in radical scavenging via hydrogen donation. At the various concentrations studied, cucurbitacin E displayed antioxidant activity comparable to that of normal ascorbic acid. Data on percent DPPH inhibition were acquired using various doses of cucurbitacin E and ascorbic acid as standard and were shown in (Table 2 and 3). The results demonstrated that the highest percent DPPH inhibition 73.67 ± 32.68 % highly significant (p ≤ 0.01) a concentration of was obtained (1000 µg/ml) of the cucurbitacin E followed by (65.78 ± 2.97 %, 61.33 ± 3.08 % and 57.24 ± 2.41%) inhibition with concentrations of (900 µg / ml, 700 µg / ml and 500 µg / mL) sequentially as show in the Table 2. It was also found that the highest inhibition percentage of standard ascorbic acid showed about 89.23 ± 4.02 % highly significant (p ≤ 0.01) where it was found that DPPH radicals are inhibited at its highest concentration, which is (500 µg/ml). As show in schedule 3.

Table 1: Displayed the cucurbitacin E RF values and their standard for creating solvent components in (T.L.C).

Solvent components of spots	Ethyl acetat, Acetone and methanol	Color
Rf value of standard cucurbitacin E	0.76	brown
Rf of value of cucurbitacin E in Citrullus colocynthis	0.73	brown

Table 2: DPPH inhibition percentage of cucurbitacin E

Cucurbitacin E concentration (µg/ml)	DPPH % inhibition
25	15.84 ± 0.71 f
50	21.42 ± 1.05 e
100	42.92 ± 2.66 d
300	51.53 ± 3.27 c
500	57.24 ± 2.41 bc
700	61.33 ± 3.08 b
900	65.78 ± 2.97 b
1000	73.67 ± 32.68 a
LSD	7.028**
P-value	0.0001

In the same column there is a significantly different with different letters. $^{**}(P \leq 0.01)$. a, b, c, d: small letters refer to the significant differences. The same letters mean there is no significant difference.

Table 3: DPPH inhibition percentage of standard ascorbic acid

Ascorbic acid concentration ($\mu\text{g/ml}$)	DPPH % inhibition
25	1.84 e 31.56 $\pm$
50	2.29 d $\pm$ 44.82
100	60.72 $\pm$ 3.04 c
300	$\pm$ 3.51 b 75.92
500	$\pm$ 4.02a 89.23
LSD	9.573 **
P-value	0.0001

In the same column there is a significantly different with different letters. $^{**}(P \leq 0.01)$. a, b, c, d: small letters refer to the significant differences. The same letters mean there is no significant difference.

Cucurbitacin E was tested for potential antioxidant activity in the current study utilizing the DPPH assay, as shown in Figure 3 and 4. The present study indicates that there is a positive correlation between the % inhibition of DPPH and concentration $\mu\text{g/ml}$. A linear correlation between cucurbitacin E contents and percent antioxidant activity were established, as shown in Figure 3 the regression values of concentration ($\mu\text{g/ml}$) versus percent antioxidant activity for cucurbitacin E compound was recorded (DPPH, $R^2 = 0.8567$) whereas for standard ascorbic acid the value recorded was (DPPH, $R^2 = 0.8835$), as shown in Figure 5. The obtained data in this study reveals antioxidant activity increases as the concentration of cucurbitacin E increases.

The DPPH scavenging capabilities of active components including phenolic acids, phenolic diterpenes, and flavonoids and cucurbitacins increased. These active ingredients include a number of hydroxyl groups, particularly ortho-dihydroxy groups, which exhibit strong antioxidant and radical scavenging action.

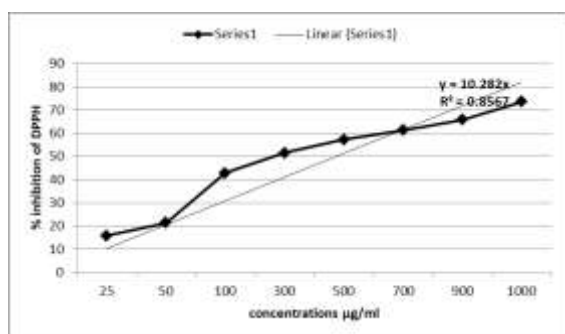


Fig. 3: correlation between percent antioxidant activity versus ncentrations of cucurbitacin E .

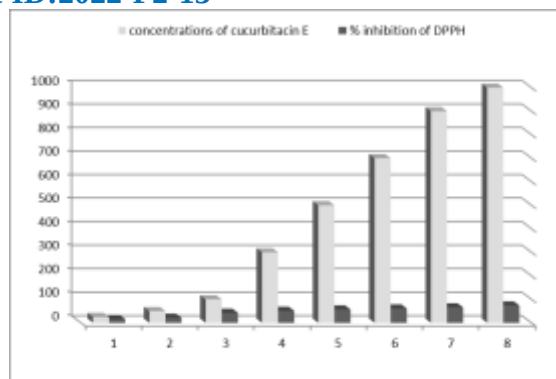


Fig. 4: Scavenging activity of cucurbitacin E using DPPH assay.

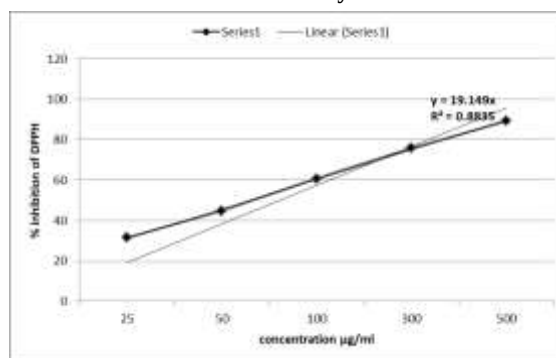


Fig. 5: correlation between percent antioxidant activity versus concentrations of standard ascorbic acid.

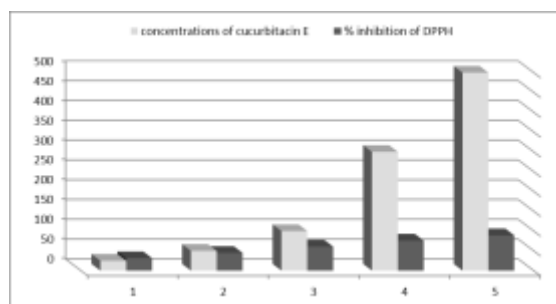


Fig. 6: Scavenging activity of standard ascorbic acid using DPPH assay.

High- Performance Liquid Chromatography (H.P.L.C) approach for qualitative and quantitative measurement of cucurbitacin E

The HPLC analytical technique is used to isolate a variety natural substance. The various components of a combination can be identified, measured, purified, and qualified using the chromatographic method known as (H.P.L.C) in analytical and phytochemical chemistry (Yasodha & Suresh, 2017) (Ahuja , 2006). This method is currently dominating other analytical methods and is the top option for fingerprinting research for herbal plant quality control (Fan *et al.*, 2006). The cucurbitacin E was found to (32.881 $\mu\text{g/ml}$) as show in Table 4. (H.P.L.C) analysis for the separated compound from the *Citrullus colocynthis* revealed the presence of the cucurbitacin E with retention times (6.524) min, as show in Figure 7 and retention time of standard cucurbitacin E was found (6.139) min, as show in Figure 8.

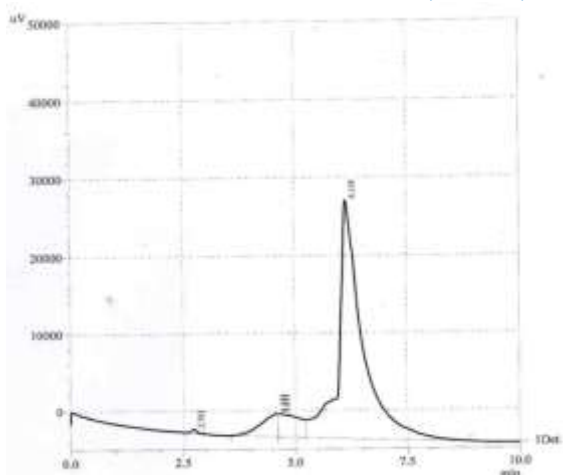


Fig. 7: HPLC chromatogram of the cucurbitacin E standard. The analysis conditions were ODS C18 column, mobile phase were (0.05% acetic acid) in deionized water solvent A: methanol solvent B, using linear gradient from (0% B to 100% B) in 7 min. flow rate: (1.0 mL) min and detection wavelength 275 nm.

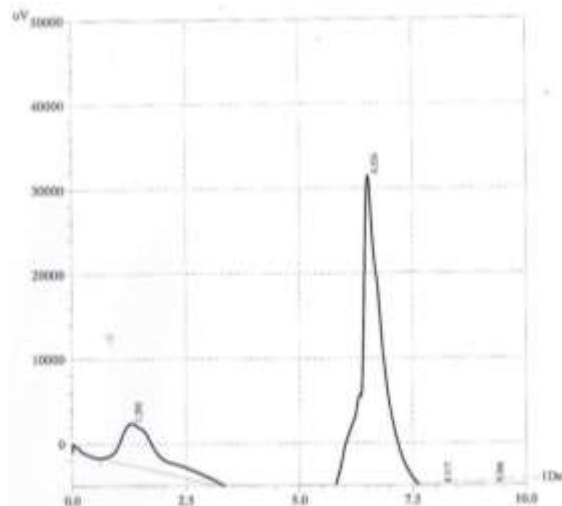


Fig. 8: HPLC chromatogram of the cucurbitacin E. The analysis conditions were ODS C18 column, mobile phase were (0.05% acetic acid) in deionized water solvent A : methanol solvent B, using linear gradient from (0% B to 100% B) in 7 min. flow rate: (1.0 mL) min and detection wavelength 275 nm.

Table 4: Area of HPLC, retention time (Rt) and the cucurbitacin E content in Citrullus colocynthis.

Compound	Retention time	Area/standard	Area/sample	Concen of standard	Concen of sample
Cucurbitacin E	6.524 min	1149335	511692	25 µg/ml	32.88 µg/ml

Conclusion

The rough this study, it can be said that bitter apple (*Citrullus colocynthis*) has a good return in terms of the natural active compounds it contains, which is credited with showing antioxidant activities. It was also concluded from this study that the active compound cucurbitacin E isolated from the fruit of the bitter apple, showed an antioxidant activity, thus

reducing the effectiveness of free radicals on causing functional and organic imbalances, as well as reducing the possibility of oxidation of some vital molecules such as the oxidation of lipids, proteins and DNA molecules, which leads to reduction in the emergence of diseases that are closely related to the formation of free radicals.

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