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Research Article

Qualitative identification of the active principles in *Citrullus colocynthis* and evaluation of its teratogenic effects in albino rats

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ABSTRACT

Background: This study was designed to identify the active contents of *Citrullus colocynthis* plant and to examine their teratogenicity in rats. The fruit pulp of the poisonous plant was collected randomly from Suq-Alkhamis district, Tripoli, Libya.
Methods: The glucoside colocynthin was isolated by lead acetate method while the alkaloids and saponins were isolated by maceration method. These active principles were then identified by chemical tests, color reactions and thin layer chromatography. Possible teratogenic effects of the fruit pulp extract was investigated by its administration to twelve pregnant rats on the 7^{th} day of gestation at a dose of 40.6 mg/kg body weight that is equivalent to one fourth of the LD_{50} of the extract.
Results: Gross anatomical observation on the 20^{th} day of gestation revealed a high percentage of resorbed fetuses, smaller size and weight fetuses as well as absence of coccygeal vertebrae, metacarpal and metatarsal bones, and carpal and tarsal bones.
Conclusions: It could be concluded that the extract of fruit pulp of *Citrullus colocynthis*, obtained from Libya, contain glucosidal as well as other principles that may cause teratogenic effects if given during at the early stage of pregnancy.

Keywords: *Citrullus colocynthis*, Glucosides, Alkaloids, Saponins, Fetuses, Malformations

INTRODUCTION

Intoxication caused by poisonous plants is of an alimentary character. Animals are exposed to this danger both while grazing and in the Stable. Although there has been considerable progress in the livestock feeding, the presence of poisonous plants in the feed rations still remains an important factor limiting or reducing the overall efficiency of farm animals breeding.[1] Acute or chronic poisoning by toxic plants constitutes an important obstacle in animal production and thereby causing substantial harm to the national economy. Many species of tropical poisonous plants grow abundantly in Libya; among these is *Cucurbitaceae* family.[2] The family is composed of 90 – 100 genera and 850 species. The active substances present in the plant include a number of several toxic active components as alkaloids, glucosides, saponins and tannins depending, to a great extent, on ecological factors and genetic basis.[3]

Various studies on toxic and/or medicinal effects of *Citrullus colocynthis* have been reported, yet, a few ones have been related to its teratogenic effect and, moreover, none was in Libya. Along the toxic studies, *Citrullus colocynthis* has been found to have toxic effects in goats,[4,5] sheep,[6,7] calves,[3] chicks[8] and rats.[9]

On the other hand, medicinal effects have been recorded for *Citrullus colocynthis*, including, antioxidant,[10] antimicrobial,[11] anti-inflammatory,[11] antihistaminic,[12] antiparasitic,[13] antihyperlipidemic,[14] anti-ulcer[15] and antidiabetic[16] effects.

Phytochemical investigation of the active ingredients of *Citrullus colocynthis* fruits growing in Libya has been a subject of interest particularly because of their toxicological effect on animals and wide spread in Libyan environment. Therefore, the aim of the present study was to determine the active principles contained in the pulp of the *Citrullus colocynthis* plant growing in Libya; and to evaluate its teratogenicity.

METHODS

1. The plant: *Citrullus colocynthis* is a perennial, monoecious, spreading, roughly hispid creeping herb with simple tendrils and pinnatisect leaves. Flowers are yellow, ovary sparsely hispid and fruits are globose, yellow and smooth with a bitter taste. The plant is flowering almost throughout the year (Figure 1)[2]. The plant is distributed in different localities in Libya; but the plant used in this study was collected from Suq-Alkhamis district in Tripoli.

Figure 1: The fruit pulp of *Citrullus colocynthis* poisonous plant growing in Libya

2. Extraction of the active ingredients: Extraction of glucosides was carried out according to[17] where the minced fruit pulp was covered with an equal weight of both 96% alcohol and saturated basic lead acetate solution. Under these conditions all the hydrolytic enzymes in the mixture were destroyed. Filtration gave a clear liquid from which the excess of lead acetate was removed after precipitation with a saturated aqueous solution of potassium dihydrogen phosphate. Chloroform extraction removed the bitter principles completely from the aqueous phase. Whitish foams were obtained as residues of the evaporation of the chloroform extract and used only for detection of glucosides.

The extraction of the alkaloids, saponins and tannins from the fruit pulp was carried out by maceration. The minced fruit was covered with double volume of distilled water for 24 hrs. and then filtrated, purified and dried on drying cabinet at 36 ^{o}C for 7 days.[18]

3. Qualitative identification of the active ingredients

3.1.1. Chemical tests: Molisch's, Baljet's, Fehling's and Benedict's tests were used for detection of glucosides while Mayer's, Wagner's, Dragendorff's, Hager's and tannic acid tests were used for detection of alkaloids; foam, mercurous chloride and silver nitrate tests were used for identification of saponins; and ferric chloride and tannic acid tests were used for detection of tannins.

Molisch's reagent test: The reagent composed of 1% alcoholic alpha-napthol. The test was carried out by adding a few drops of 1% alcoholic alpha-naphthol to5 ml of the plant extract and then 1 to 3 drops of concentrated sulphuric acid onto the wall of the tube.

Baljet's reagent test:The reagent composed of 95ml of 1% of picric acid solution in ethanol and 5 ml of 10% alcoholic potassium hydroxide solution. The solution was filtrated and the filtrate was used as the reagent. To a small portion of the ethanolic extract an equal volume of theBaljet's reagent was added.

Fehling's test: Equal amounts of Fehling's solution and aqueous extract of the minced fruit pulp were mixed and heated in a boiling water bath for 10 minutes.

Benedict's test: Ten drops of the aqueous extract of the minced fruit pulp was added to 5 ml of Benedict's reagent in a test tube and heated in a boiling water bath for 10 minutes.

Mayer's test: Ten grams of the minced extract were boiled with 50 ml of 1% acidulated water and filtrated after cooling. Thirteen and half grams of mercuric chloride were dissolved in one liter of 5% aqueous solution of potassium iodide to prepare Mayer's reagent. The test was performed by adding a few drops of the freshly prepared Mayer's reagent to 5ml of acidulated plant filtrate.

Wagner's test: Fifty five grams of iodine dissolved in 10% solution of potassium iodide in water. A few drops of the freshly prepared Wagner's reagent were added to 5 ml of the acidulated filtrate of fruit plant extract.

Dragendorff's test: Two grams of bismuth sub-nitrate in 25ml acetic acid along with 40grams of potassium iodide diluted to 100ml of water.

Hager's test: A cold saturated aqueous solution of picric acid was added to equal amount of the acidulated filtrate that was prepared as mentioned above.

Tannic acid test: Five grams of tannic acid were diluted with 95ml distilled water, the test was carried out by

mixing 3ml of the filtrated extract with 2ml of the diluted tannic acid.

Foam test: The extract (50 mg) was diluted with distilled water and made up to20 ml. The suspension was shaken in a graduated cylinder for 15 min. A long lasting two cm layer of foam indicated the presence of saponins.

Mirror test: Equal amounts of ammoniated silver nitrate (1%) and the aqueous filtrate are mixed and boiled for 10 min in water bath.

Mercurous chloride test: Two ml of mercurous chloride (1%) solution was added to 5 ml of aqueous filtrate.

Ferric chloride test: The extract (50 mg) was dissolved in 5 ml of distilled water. To this, a few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of tannin or phenolic compounds.

3.1.2. Color reaction tests: The color reactions were carried out according to the method described by.[19] The reagents used were: Marquis (sulphuric formaldehyde reagent; two drops of formaldehyde solution were mixed with 1ml of sulphuric acid),alizarin red (1% Alizarin in 50% ethanol), bromo-cresol green (1% bromocresol in ethanol), Frohde (a micro-drop of 0.5% aqueous ammonium molybdate solution is added to a micro-drop of the test solution, evaporated, and then moistened with sulphuric acid and the color is noticed), Vitali (a micro-drop of the test solution is allowed to dry and a micro-drop of a fuming nitric acid is added; the residue is then moistened with freshly prepared ethanolic potassium hydroxide and the color is noticed), Libermann (one g of potassium nitrate in 10 ml of sulphuric acid), mercurous nitrate (a saturated solution of mercurous nitrate in water), Beam's (5 g of potassium hydroxide is dissolved in sufficient ethanol to 100 ml), ferric chloride (five percent solution of ferric chloride; 5 g of ferric chloride dissolved in 95 ml distilled water) and finally, diluted acetic acid (five percent aqueous acetic acid; 5 volumes of acetic acid diluted to 100 volumes with distilled water).

3.1.3. Thin layer Chromatography: Chromatographic technique is one of the most outstanding methods for the purification, separation, detection and identification in toxicological studies. The separation and identification of the active ingredients of the poisonous plant *Citrullus colocynthis* was carried out chromatographically according to[20&21]. Chromatography (color writing) is used for the separation of small amounts of closely related substances by continuous redistribution between two phases, stationary and mobile phases.[22] The idea is based on the selective absorption of the components from a solution on the active surface of a finely divided solid. Closely related substances exhibit different abilities to adsorption, so that separations which are impractical by ordinary chemical methods may be easily done by this means. A solution, of the substances to be separated in a suitable solvent is moved post the stationary phase which has a tendency to adsorb different substances in varying degrees and rates.[20] When the plates are being developed, the solvent moving through the thin layer tends to draw substances being analyzed along with it and spots the stationary phase, the rate of flow (R_f value) of the more strongly adsorbed substances will be the more retarded. The result is that the substances spread out along the plate depending on the relative affinities for the solvent and the layer. Substances that are not adsorbed at all will move directly with the solvent flow. Filield and Kearly[23] described the rate of flow as:

$$\text{Rf value} = \frac{\text{distance travelled by the center of solvent spot}}{\text{distance travelled by the front of mobile phase}}$$

Under suitable conditions, the resulting different rates of flow can bring about complete separation of substances. The rate of flow (R_f value) is dependent on three factors: the activity of the layer, the saturation of the chamber, the uniform thickness of the layer.[19]

***4. Experimental animals*:** Twenty two pregnant female albino rats, selected as explained below, were obtained from the experimental animal unit, University of Tripoli, Libya. Animals were clinically healthy, weighing 180-250 g. All animals were housed in stainless steel cages with hard wood chips as bedding. Animals were maintained on a balanced diet composed of barley, milk and green fodder, with water *ad libitum* throughout the experimental period.

5. Teratological study

5.1. Determination of zero day of pregnancy: Female albino rats were monitored for estrus by taking daily vaginal smears for detecting cornified cells microscopically. The female that was proved to be in estrus was paired with a mature male in a separate cage. Sperm positive smear was taken as an indication of the zero day of pregnancy.[24]

5.2. Dosing and time of administration: The twenty two proved pregnant female albino rats were randomly divided into two groups. The first group (12 animals) was treated with 40.6 mg/kg.b.wt of the plant extract on the 7thday of gestation[25,26] while the second group (10 animals) was kept as a control without treatment. The above mentioned dose is equivalent to ¼ of the LD_{50} of the extract which was calculated as 162.4 mg/kg.b.wt according to the method described by Behrens & Karber.[27]

5.3. Implantation sites: On the 20th day of pregnancy, the treated as well as control dams were sacrificed. The uterus was exposed by necropsy and photographed externally to observe the resorption sites. The uterine horns were examined with a magnifying lens for tracing

the implantation sites that appeared as black spot after being wet with 10% ammonium sulphide for 20 minutes.[28] The total number of the foeti in each animal was counted and alive and dead foeti were recognized and recorded (the survivability of the foeti was indicated by the rosy color, heart beating and active circulation). Foeti were also examined for sex determination, their weight and length of crown-rump were also recorded.

5.4. Examination of the foetal skeleton for teratogenicity: The picked out alive and dead foeti were eviscerated and kept in 95% ethyl alcohol for 7 days, stained by Alizarin red as described by.[29] The muscles of the foeti were digested in 2% potassium hydroxide for 6 - 8 hours according to the size of foeti. The transparent foeti were immersed in Mallasch's solution with Alizarin red up to 24 hours for staining the skeleton. After that the foeti were passed into Mallasch's solution for 1 - 2 days for decoloration of stained muscles and then passed in graded concentration of glycerine-water solution (50%, 70%, 90% & 100%). All foeti were thoroughly examined according to the scheme advised by Cook and Fairweather[30] as follows:

Dam No.:

Position of the fetus in the uterus:

Sex:

Skull:

Vertebral column:

- Cervical vertebrae
- Thoracic vertebrae
- Lumber vertebrae
- Sacral vertebrae
- Coccygeal vertebrae

Fore Limb (left and right):

- Humerus
- Radius
- Ulna
- Meta carpals
- Phalanges

Thorax and Pelvis:

- Ribs
- Sternaebrae
- Pelvic girdle

Hind Limbs (left and right):

- Femur
- Tibia
- Fibula
- Patella
- Meta tarsal
- Phalanges

RESULTS

1. Qualitative identification of the active ingredients:

1.1. Phytochemical screening tests: Table 1 and Figure 2 show the reaction of the toxic active ingredients of the *Citrullus colocynthis* with different chemical reactions.

Table 1: The chemical tests for detection of the toxic active ingredients of the *Citrullus colocynthis* poisonous plant.

	Test	Result
Detection of Glucosides (carried out on Lead acetate extract)	Molische's test	Violet ring between the two reagents
	Baljet's test	Dark orange precipitate
	Fehling's test	Reddish brown precipitate
	Benedict's test	Reddish brown precipitate
Detection of Alkaloids (carried out on macerated extract)	Mayer's test	Turbidity
	Wagner's test	Brown precipitate
	Dragendorff's test	Orange precipitate
	Hager's test	Yellow precipitate
	Tannic acid test	White precipitate
Detection of saponins (carried out on macerated extract)	Mercurous chloride test	White precipitate
	Ammoniated silver nitrate test	Black precipitate and mirror onto the inner wall of the test tube.
Detection of tannins (carried out on macerated extract)	Ferric chloride test	Dark bluish green color
	Tannic acid test	Dark color

Figure 2: The chemical tests used for detection of the toxic active ingredients of the *Citrullus colocynthis* poisonous plant.

1.2. Color reactions: the plant extract gives different color reactions with the used different chemical reagents as shown in Table 2 and Figure 3.

Table 2: The color reactions of *Citrullus colocynthis* with the different color reagents.

Reagent	Color developed	Reagent	Color developed
Marquis	Brown precipitate	**Libermann**	Yellow precipitate
Alizarin red	Bloody precipitate	**Mercurous nitrate**	No reaction
Bromocresol green	Deep blue precipitate	**Beam's**	No reaction
Frohde	No reaction	**Ferric chloride**	Oily precipitate
Vitali	Yellow precipitate	**Diluted acetic acid**	Transparent

Figure 3: The different color reactions of *Citrullus colocynthis* extract with different reagents.

1.3. Thin layer chromatography: The visualization of colocynthis spots on silica gel was accomplished by spraying the compound with a spraying solution to help the location and characterization of the compound spots on chromatogram. The different adsorption rates of the reagent on the background substratum and that of the substance cited on spots offer a helpful mean of location purposes. The R_f value of colocynthis using different eluents are shown in Table 3 and Figure 4.

Table 3: The R_F value of *Citrullus colocynthis* on silica gel chromatoplates using mixture of some aromatic hydrocarbons.

Eluents			RF value
Ratio (A:B)	Solvent B	Solvent A	
Chloroform	Methanol	9 : 1	0.86
Benzen	Ethyl acetate	7 : 1	-ve
Toluene	Ethyl acetate	8 : 2	-ve
Sulphuric acid	Methanol	3 : 7	-ve
Chloroform	Benzene	1 : 1	-ve
Ethyl acetate	Benzene	1 : 1	-ve

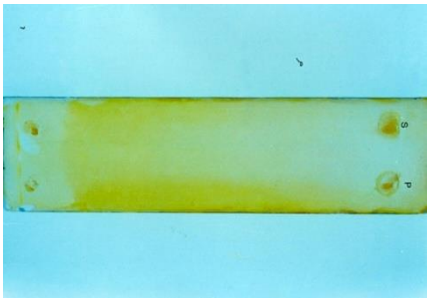

Figure 4: Flow rate (R_F) value of the glucoside colocynthin on chromatogram plate.

2. Teratological study

2.1. Morphological examination: The total number of implantation sites in control and treated groups were 106 and 157, respectively; and the number and percentage of resorptions were highly significantly increased in the treated group compared to the control one. Fetal weight and the number of fetuses/dam were decreased compared to those in the control group as shown in Table 4 and Figures 5 & 6.

Table 4: The effect of *Citrullus colocynthis* extract on female rat reproduction.

	Item	Non-treated	Treated
1	Dam bred	20	20
2	Dam pregnant	10	12
3	Litters examined	90	91
4	Total implantation sites	106	157
5	Litters with resorption	16	66
6	Resorption %	15.09%	42.04%
7	Total fetal weight	434.3 g.	353.9 g.
8	Average fetal weight	4.82 g.	3.8 g.
9	No. of fetuses / dam	9	7

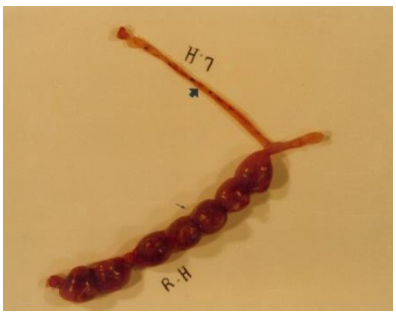

Figure 5: Uterus of a female rat treated with *Citrullus colocynthis* extract on the 7thdayof gestation showing complete resorption of fetuses in the left horn (thick arrow) and dead fetuses in the right horn (thin arrow).

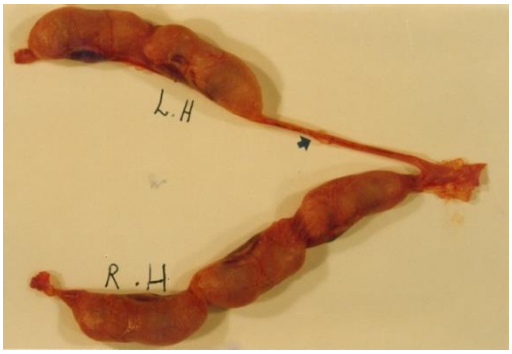

Figure 6: Uterus of a female rat treated with *Citrullus colocynthis* extract on the 7th day of gestation showing resorption of some fetuses in the left horn (arrow).

2.2 Skeletal examination: Skeletal abnormalities in the fetuses of the treated dams were recorded in Table 5 and Figures 7 & 8. They were in the form of incomplete bipartite sternbrae, missing of sternbrae, metacarpal and carpal bones, metatarsal and tarsal bones, coccygeal vertebrae, rudimentary rib, incomplete ossification of parietal and interparietal bones of skull, incomplete ossification of occipital bone as well as incomplete union of vertebral column.

Table 5: The effect of *Citrullus colocynthis* on skeletal system of rat fetuses.

Effect	Non-Treated			
	No.	%	No.	%
Fetuses examined	90	100	91	100
Fetuses with anomalies	3	2.7	91	100
Incomplete bipartite sternbrae	-	-	3	3.3
Missing sternbrae	-	-	18	20
Missing metacarpal bones	1	0.9	18	20
Missing carpal bones	1	0.9	18	20
Missing metatarsal bones	1	0.9	68	75.5
Missing tarsal bones	1	0.9	61	67.7
Missing coccygeal bones	2	1.8	75	83.3
Extra – rib	-	-	-	-
Rudimentary rib	-	-	14	15.5
Incomplete ossification of :				
Parietal bone	-	-	72	80
Interparietal bone	-	-	72	80
Occipital bone	1	0.9	79	87.7
Incomplete union of vertebral column	1	0.9	82	91.1

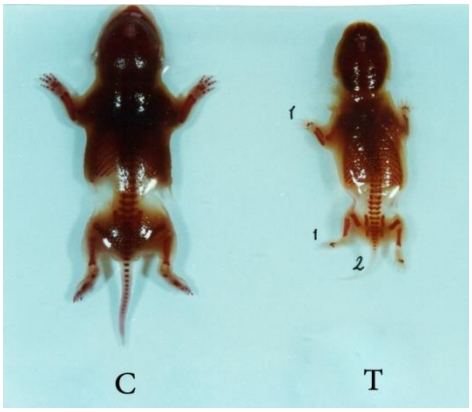

Figure 7: Skeletal abnormalities: (1) absence of digits in fore and hind limbs. (2) absence of coccygeal vertebrae in fetuses of rats treated with *Citrullus colocynthis* extract on the 7th day of gestation; (C: Control; T: Treated).

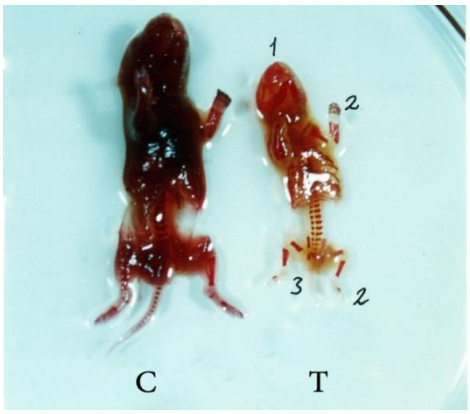

Figure 8: Skeletal abnormalities: (1) Incomplete ossification of cranial bone (2) Absence of digits in fore and hind limbs (3) Absence of coccygeal vertebrae (4) Incomplete union of vertebral column in fetuses of rats treated with *Citrullus colocynthis* extract on the 7th day of gestation; (C: Control; T: Treated).

DISCUSSION

The extraction of putative active principles from plant tissue was carried out by lead acetate for glucosides and by ordinary maceration method for alkaloid and saponins. The color tests for identification of the active ingredients of *Citrullus colocynthis* plant depend on variation of colors with the different compound and reagents used.[31] Alizarin red gave bloody precipitate, Marquis gave brown precipitate, Liebermann's gave yellow precipitate and bromocresol green gave deep blue precipitate. As the color tests are not enough as a method of identification, a combination of thin layer chromatography and color reaction have been used to identify various compounds.[32]

Spraying of a chromatoplate using certain reagents produce different characteristic spots. These are used to

determine the presence of a number of compounds. Isolation of the studied compounds could be done by thin layer chromatography which has the advantage of being able to indicate the presence of all the compounds.[20,22] The solvent system chloroform: methanol (9:1) gave complete separation for all active ingredients.

With regard to the chemical tests, the glucoside colocynthin, alkaloids and saponins gave different identified colors and precipitates with the different reagents that were in agreement with those obtained by Rehm et al.,[17] and those of Clark[31].

Appearance of structural and functional congenital defects have been observed in animals for centuries;[33] later on, specific plants have been incriminated as etiologic agents.[34,35] At this time, it is known that wide variety of plants, many of them common range plants, are capable of inducing congenital defects in offspring from pregnant individuals consuming the plant during some stages of gestation. In addition, the lack of any other available literature mentioning the congenital deformities in the offspring induced by *Citrullus colocynthis* led us to study these effects. The lack of information may be due to the fact that the appearance of these congenital deformities is delayed from the time of plant ingestion by the dam until the time of parturition. Our experimental work proved that *Citrullus colocynthis* can pass transplacentally, crossing barriers and inducing deformities in concept.

As it is known that the conceptus genotype determines susceptibility.[25] Cows seem to be affected mainly by the lupines teratogen while sheep seem to be mainly affected by the veratum teratogen.[36] Thus we can expect variation in susceptibility to *Citrullus colocynthis* among species, breed and strains of livestock. Variation in effect and incidence was found to be related to gestation period at which administration was given.

The previous fact is also of economic value in veterinary gynaecology and animal resources. Both malformation and growth-retarded fetuses were recorded in the same individuals at birth. No clinical signs were observed in dams giving malformed or retarded growth fetuses at the dose level given. This indicates that the embryo is more susceptible than the mother. Teratogenesis of *Citullus colocynthis* may be attributed to its effect on the replicating cells *via* alteration in replication, transcription, translation or cell division which accompanied by excessive necrosis[37] as the embryo can tolerate substantial depression in DNA synthesis.[38,39]

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