

Full Length Research Paper

Effect of alcoholics extract on rat livers and antibacterial screening of *Citrullus colocynthis*

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Crude ethanolic extracts of fruits, leaves, stems and roots of *Citrullus colocynthis* Schrad were examined for their antibacterial potentialities against Gram positive and Gram negative bacilli. Ethanolic extracts of fruits, leaves, stems and roots were found to be active against Gram positive bacilli, viz., *Bacillus pumilus* and *Staphylococcus aureus*, while fruit and root extracts in double strength gave positive results against Gram positive bacillus (*Bacillus subtilis*). The Gram negative bacilli viz., *Escherichia coli* and *Pseudomonas aeruginosa* showed no response, and in order for it to affect the alcoholics extract of rat liver, 50 rats were randomly divided into five groups (4 experimental and 1 controls). In the experimental groups a single daily dose of alcoholic extract of *C. colocynthis* (50, 100, 200, 400 g/kg) was administered intraperitoneally. Normal saline was administered in control group. After two weeks, the rats were killed and the livers were removed and fixed with formalin (10%). Specimens were then processed and stained with H&E and Reticuline. The results indicated that there is a morphological change in liver cells including karyrrhexis, chromatolysis, and granulation of the cytoplasm. Additionally, collagen and reticular fibers were evident in liver parenchyma in high doses. *Citrullus colocynthis* can have toxic effects on liver cells which may induce hepatocyte necrosis and liver fibrosis. These effects were dose dependent. Further studies are necessary to clarify the issue.

Key words: *Citrullus Colocynthis*, *Bacillus pumilus*, *Staphylococcus aureus*, liver, necrosis, zabol.

INTRODUCTION

Citrullus colocynthis belongs to the family *Cucurbitaceae*. Members of this family are generally dioecious herbs which may be prostrate or climbing by means of tendrils. Fruit is fleshy and many fruits are used as vegetable or as edible fruits. *C. colocynthis* is a small scarbid perennial creeping herb with prostrate or climbing stem, bearing smooth spherical fruits which are mottled green when young and some what yellow when ripe (Shah and Qadry, 1985). In moderate doses a drastic hydrogogue, cathartic and diuretic; in large doses emetic and gastro-intestinal irritant; in small doses it is expectorant and alterative. Physicians use this drug extensively as a drastic purgative in ascites and jaundice and in various uterine

conditions, especially in amenorrhoea. Colocynth in the form of the solid extract enters in to many of the purgative pills of modern pharmacy. It is useful in biliousness, fever, intestinal parasites, constipation, hepatic and abdominal, visceral and cerebral congestions, dropsy, etc. Juice of the fruit mixed with sugar is a household remedy in dropsy (Anonymous, 1970).

Root is given in abdominal enlargements and in coughs and asthmatic attacks of children. For intestinal inflammation, tumours, etc. a powder of root is given for three days in doses of 45 grains well mixed with castor oil. A poultice of root is useful in inflammation of the breast of nursing mothers (Dastur, 1962). *C. colocynthis* (CCT) is traditionally used as an antidiabetic medication in tropical and subtropical countries (Diwan et al., 2000). This plant can induce insulinotropic (Nmila et al., 2000) and mild immunostimulating effects (Beneddoug et al., 2003). There is some evidence that it may induce side effects.

The comparative toxicity of the alcoholic extract of CCT has been studied in seven insect species in which the adult honey bee was more affected (el-Naggar et al., 1989). Sheep which were fed fresh CCT fruits and leaves (0.2 to 10 g/kg) showed signs of weeks caused death in goats (Barri et al., 1983). The other side-effects of this plant are toxic acute colitis (Golfain et al., 1989), reversible infertility (Chaturvedi et al., 2003) and hepatotoxicity in rats (Barth et al., 2002). These damages were sometimes enhanced with higher doses of CCT. As the liver is a sensitive organ and many substances including toxins accumulate in this organ and induce liver toxicity, therefore the aim of this project was to study the histopathological changes in the liver after ingestion of CCT in male rats.

MATERIALS AND METHODS

Different parts of the plant *C. colocynthis*, that is, roots stems, leaves and fruits were collected in summer 2009 and January, 2010 from the desert area near zabol, Iran. The fruits, stems, leaves and roots of *C. colocynthis* were carefully separated from weeds, soil particles, added adulterants and other extraneous matter, and were dried at room temperature for one month except fruit which took three months to dry. The fruits, stems, leave and roots were crushed to coarse power separately. 50 g of the powder of fruits, leaves, stems and roots were homogenized in 100 ml of ethyl alcohol (90%) separately in homogeniser and operated for 5 min. The process was repeated for three to four times with the same quantity of alcohol. The extract was filtered through filter paper under vacuum. The alcohol extract was evaporated through rotary evaporator under reduced pressure at 40°C.

The weights of residue obtained from 50 g powders of fruits (2.37 g), leaves (8.27 g), stems (2.24 g) and roots (5.00 g) were obtained too. The alcoholic extract of this plant in order to effect on rat livers was obtained using the percolation method.

Growth media

All the bacteria *Bacillus pumilis*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were cultivated on nutrient agar medium.

Nutrient agar medium

Beaf extract 3.0 g
Peptone 5.0 g
Sodium chloride 5.0 g
Ag 15 to 20 g
Distilled water 1000 ml
pH 7.4

All the ingredients were mixed and dissolved in distilled water then sterilized by autoclaving at 121°C (151 lb/sq. inch), for fifteen minutes (Cruickshank et al., 1975).

Preparation of Inoculum

The test organism was maintained by bi-weekly transfer on agar slants of nutrient agar medium. Growth was washed from slants with sterilized 3 ml of normal saline. This suspension was used to inoculate in wide based flask containing 200 ml of the same

medium supplemented with 10 g of agar per litre (Ayoub and Yankov, 1981).

Antibacterial screening

The antimicrobial activity of extract from fruits, leaves, stems and roots against various Gram-positive and Gram-negative bacteria were observed. The organisms given below were collected from Central Drugs Testing Laboratory Zabol in south East of Iran Pharmaceuticals.

Gram-positive

1. *B. pumilis*
2. *B. subtilis*
3. *S. aureus*

Gram-negative

1. *E. coli*
2. *P. aeruginosa*

Preparation of dilution

All the glass apparatus used were sterilized in an oven at 200°C, for thirty minutes. A series of four dilutions were prepared of each extract and marked n1, n2, n3 and n4. The dilutions were prepared as follows: i) First dilution (n1) was prepared by dissolving one gram of dry extract in 5 ml of ethyl alcohol (200 mg/ml). ii) Second dilution (n2) was prepared by taking 1 ml of dilution n1 and 1 ml of ethyl alcohol (100 mg/ml). iii) Third dilution (n3) was prepared by taking 1 ml of dilution n2 and one ml of ethyl alcohol (50 mg/ml). iv) Fourth dilution (n4) was prepared by taking one ml dilution n3 and one ml of ethyl alcohol (25 mg/ml).

Preparation of assay plates

Petri dishes were sterilized in an Autoclave at 121°C (15 lb pressure/sq in) for 15 to 20 min then labeled with the name of bacterias whose inoculum were prepared. Each Petri dish was divided into four equal parts and each part was marked accordingly. One part was marked as control, while other three parts used for each sterilized in conical flask (500 ml) by autoclaving at 121°C (15 lb pressure/sq. in.) for 15 to 20 min. Some quantity of media was prepared for each part of the plant and for different inoculums. 0.1 ml of bacterial inoculum was transferred in (100 ml) sterilized melted nutrient agar medium for bacterial inoculation at temperature not more than 45°C and was gently shaken to mix the inoculum. In each sterilized Petri dish 20 ml of inoculums media was poured carefully at sterilized environment to avoid contamination and then allowed to solidify at room temperature. Now small uniform and superficial holes were made by the help of sterilized borer in the center of each part of Petri dish then each hole was sealed with one drop of same melted media. One drop of extract dilution of n1, n2, n3 and n4 were dropped with the help of pipette very carefully in three holes of individual Petri dishes, respectively with the help of pipette. The fourth one hole in each Petri dish was left as control and one drop of ethyl alcohol was dropped in it.

This process was repeated for each part of the plant and also for individual microorganism. All the Petri dishes with bacterial inoculation were incubated at 37°C for 24 to 48 h. After incubation period the zones of inhibitions were measured results were noted.

Table 1. Average zone of inhibition for *Staphylococcus aureus*.

Dilution	Zone of inhibition (cm)			
	Fruit extract	Leaves extract	Stem extract	Root extract
First	1.30	1.31	1.27	1.32
Second	1.22	1.12	1.24	1.14
Third	1.19	1.10	1.21	1.12
Fourth	1.18	1.10	1.10	1.09

Table 2. Average zone of inhibition for *Bacillus pumilus*.

Dilution	Zone of inhibition (cm)			
	Fruit extract	Leaves extract	Stem extract	Root extract
First	1.27	1.23	1.23	1.21
Second	1.26	1.16	1.20	1.17
Third	1.203	1.163	1.19	1.11
Fourth	1.173	1.083	1.1	1.06

Table 3. Average zone of inhibition for *Bacillus subtilis*.

Dilution	Zone of inhibition (cm)			
	Fruit extract	Leaves extract	Stem extract	Root extract
First	1.02 (1.20 with	-ve	-ve	1.05 double conc.)
Second	-ve	-ve	-ve	1.02
Third	-ve	-ve	-ve	-ve
Fourth	-ve	-ve	-ve	-ve

Effect of alcoholics extract on rat livers

Fifty male rats, weighing 200 to 250 g were obtained from the Animal House of Shiraz Medical School and were maintained under standard conditions (light, temperature, humidity and free access to water and food). The rats were then selected and randomly divided into 5 groups (4 experimental and 1 control). In the experimental groups a single daily dose of the alcoholic extracts of *C. colocynthis* (50, 100, 200, 400 mg/kg) was injected intraperitoneally. Each of the experimental groups received only one particular dose for the duration of 14 days. Normal saline was administered in the control group. After 14 days, the rats were sacrificed under deep anesthesia and their livers were removed and fixed with 10% formalin. Specimens were processed routinely and sections with 5 micron thickness were prepared and stained with Hematoxiline-Eosine (H&E), Reticuline methods. The slides were studied by light microscopy by Knodell scoring system for assessing histological activity in asymptomatic chronic active hepatitis (Knodell et al., 2003) and the results were recorded. The results were analyzed using Chi-square and ANOVA tests. P-value less than 0.05 was considered statistically significant

RESULTS AND DISCUSSION

Antibacterial screening

The results obtained from the parts of *C. colocynthis* Schrad, that is, fruits, leaves, stems and roots are shown

in the Tables 1, 2 and 3. A sufficient time period (24 h) was given for thorough and complete extraction but same result and quantity was obtained when extraction was made in homogenizer with ethyl alcohol (400 ml as menstrum) for 15 min and evaporated the ethyl alcohol under reduced pressure using rotary evaporator and drying the extract at 40°C in oven. Another change was made in the extraction process by using normal saline as menstrum, and soaked over night instead of ethyl alcohol. Then same series of dilutions were prepared but results were negative. Changes were also made in dilution series after first dilution of ethyl alcohol, then further dilution was prepared with sterilized water and results were negative. For experimental work the bacterial inoculum was prepared in normal saline as mentioned in method and the results were compared by changed method, that is, inoculum was prepared in sterilized water and phosphate buffer, positive results were obtained in the changed method. The same results were obtained by prolonging the incubation period from 24 to 48 to 72 h. The same zone of inhibition was obtained by changing the size of borer one number less.

During performing the experiment for checking the

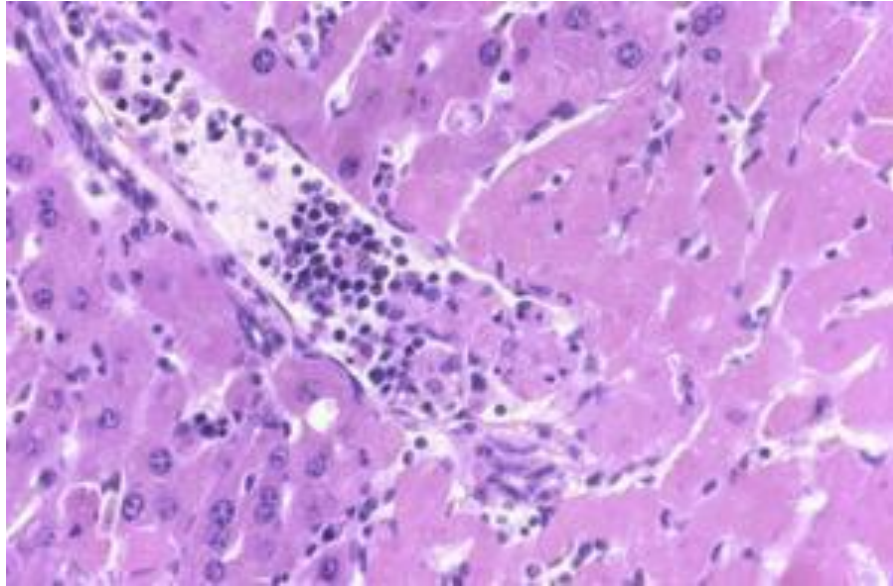


Figure 1. Liver cell necrosis in dose 400 mg/kg with H&E staining (400×).

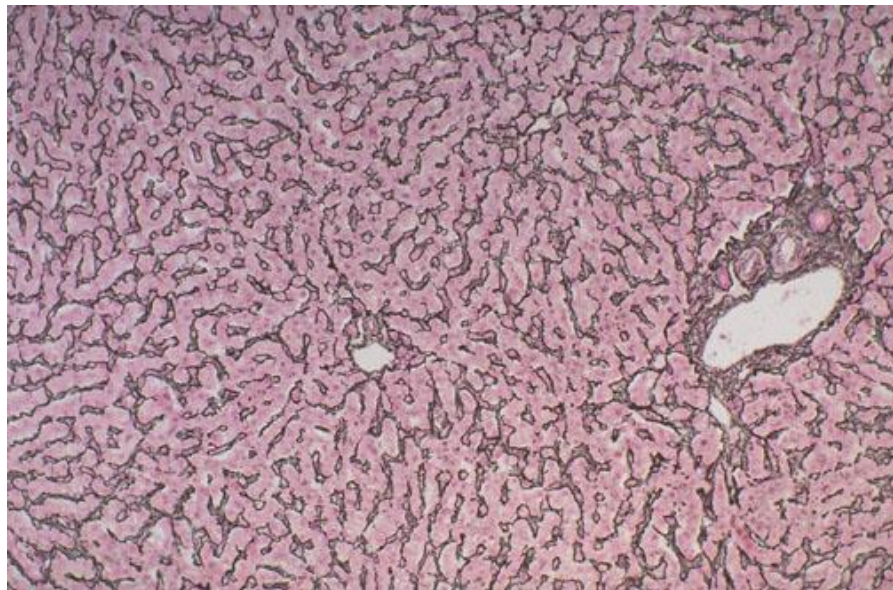


Figure 2. Reticular fiber in liver parenchyma in dose 400 mg/kg with Reticulin staining (100×).

antibacterial activity of crude extracts extreme care was taken to prevent the contamination. All the apparatus and growth media were sterilized for this purpose using oven and autoclave. A sterilized environment was made for inoculum. However, the crude extract was significantly active which shows the prominent antibacterial activity. The crude extract also shows appreciable inhibition of the growth of *Staphylococcus aureus*, *B. pumilus* and *B. subtilis*. The antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa* were negative.

The conclusion which drawn from the above discussion

is that crude extracts showed active response against the some strains of bacteria may be due to carbohydrates, flavonoids, glycosides and tannin which are reported in the literature.

Effect of alcoholics extract on rat livers

A morphological change in hepatocyte including karyorrhexis, chromatolysis and granulation of the cytoplasm was seen using H&E staining (Figure 1) especially

Table 4. The reaction of liver induced–CCT using H&E, PAS, staining between control and experimental groups.

Groups	Lesion	Hepatocyt necrosis H&E staining	Fibrosis inflammatory cell	Reticuline H&E staining
Control		0	0	0
50 mg/kg		0	0	0
100 mg/kg		5	0	2
200 mg/kg		9	0	6
400 mg/kg		12	3	9

With doses of 200, 400 mg/kg. Collagen and reticular fiber were observed around more than the control group in parenchyma at a dose of 400 mg/kg using reticuline staining (Figure 2). All of these effects were dose dependent. These changes are shown in (Table 4). There is growing concern about the hepatotoxicity of herbal remedies (Larry, 1997). Herbal hepatotoxicity has been recognized for many years, but new agents are constantly being identified (Cruickshank et al., 1975). *Citrullus colocynthis* extract was found free of hepatotoxic effect at concentrations up to 100 µg/ml (Chaturvedi et al., 2003). But, higher concentrations seem to have some degree of hepatotoxicity. Male wistar rats that were fed diets containing 10% CCT ripe fruits showed body weight loss, inefficiency of feed utilization, diarrhea, ruffled hair and enterohepatonephrotoxicity (Adam et al., 2001). In this study, the effect of different concentrations of CCT on the liver was investigated. The results showed some histological changes in the nucleus and cytoplasm of hepatocytes. The changes observed in the nuclei included karyorrhexis and chromatolysis. The mechanism for these changes is not clear but other reports have shown that CCT has a damaging effect on different cells. The ethanol extract of CCT decreases the concentration of sialic acid in serum of mice. This decrease is concomitant with an increase in the unmasking of galactose residues that is recognizable by macrophages in apoptotic cell (Itzhaki et al., 2003). Therefore; it seems that CCT by decreasing sialic acid induces cell degeneration. In addition, CCT causes an increase in neutrophils (Elawad et al., 1984) which confirms the above finding. Elevation of alkaline and acid phosphates is a useful marker for diseases of the liver such as liver cirrhosis (Israeli and Bogin, 1986; Rynniak, 1986). However, in some studies alkaline and acid phosphatase concentration was decreased by CCT (Chaturvedi et al., 2003). So, it seems that CCT has probably no effect in causing liver cirrhosis. On the other hand, it is a well-known fact that inflammation can be precursor of liver fibrosis. In our study, we observed scattered neutrophil and lymphocytes in liver parenchyma. This phenomenon could potentially lead to liver cirrhosis. CCT has been shown to increased oxidative damage (Shivakumar and Geoffrey, 2000). On the other hand CCT extract has stimulated lipid peroxidation, H₂O₂ formation and has amplified chemiluminescence in rat liver microsomes (Barth et al.,

2002). Therefore, it seems that a decrease in iron and an increase in lipid peroxidation induce the generation of free radicals which damages hepatocytes. In conclusion, CCT can have toxic effects on liver cells which may induce hepatocyte necrosis and liver fibrosis. However; more research is needed to clarify the issue.

Conclusion

In conclusion, the studied plants could provide some activity against antibacterial and anti fungal; however, it is not known that which component of the extract is responsible for this effect. Further studies effect using isolated constituents instead of the whole extract should be carried out.

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