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Antibacterial activity and polyphenolic content of *Citrullus colocynthesis*

Afsaneh Moslemi Mehni[1*], Saghar Ketabchi[1], Gholam Hosein Shahidi Bonjar[2]

[1]*Msc student of Plant Pathology, University of Azad, Shiraz, Iran*

[2]*Department of Plant Pathology, Bahonar University, Kerman, Iran*

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Abstract

Plant bacterial disease is most groups of plant diseases that can be managed only with a few chemical poisons or some expensive antibiotics; to decrease chemical materials and replacing them with natural compounds, a lot of studies have been done. In this study antibacterial activity of some plant extracts of *Citrullus colocynthis* was screened in a low concentration (4µg/disc) against some plants bacterial diseases. MIC was obtained by serial dilution method. Total phenol content and polyphenol determination was revealed in range by plate reader set and High Perfect Liquid Chromatography set. At this concentration, all extracts of *Citrullus colocynthis* showed antimicrobial activity to *Erwinia amylovora* and *Bacillus subtilis*, a resistant bacterium. Other resistant bacteria like; *Xanthomonas axnopodis pv citri* and *Pseudomonas flouresence* had not showed any antibacterial effect to all extracts. Chemical analyzing detect total phenol content and about 12-15 polyphenol materials in *Citrullus colocynthis*, only one of them recognized, It was Gallic acid and was the first repot compound in this plant .

* **Corresponding Author:** Afsaneh Moslemi Mehni ✉ moslemimehni2001@yahoo.com

Introduction

Citrullus colocynthis (L.) Schrad (Cucurbitaceae) (bitter cucumber), is an annual herbaceous plant. Stem is angular and rough, leaves rough, 3 to 7 lobed, 5 to 10cm long; flowers are monoecious, solitary, peduncled, axilliary, corolla 5-lobed, and ovary villous, is a fruit commonly known for its bittern-ess, found in India, Sudan, Iraq and Iran (Trease and Evans, 1970). It is a small scarbid perennial creeping herb with prostate or climbing stem, bearing smooth spherical fruits which are mottled green when young and somewhat yellow when ripe (shah and Qadry,1985). Fruit is useful against fever, intestinal parasites, hepatic and abdominal diseases, visceral and cerebral congestions (Anonymous, 1970). Root extract is used against jaundice, urinary diseases, rheumatism etc. (Dastur, 1962). Seeds are diuretic (Vohora *and* Khan, 1981). Fruits are used against tumors of gastrointestinal tract. It is more pronouncedly used in anticancerous drug. It is effective in leukemia and joint pains. In *Citrullus colocynthis* there is a cyanogenic glycoside compound (amygdalin) is found generally in kernels and other parts of the plant (Wei-Feng *et al*, 2005). The dried pulp of *Citrullus colocynthis* L. has been used for constipation, edema, bacterial infections, cancer & diabetes (Al-Ghaithi *et al*., 2004). Fruits of *Citrullus colocynthis* L. contain seventeen compounds were broudly identified and divided in to five classes: alcohols, ketones, epoxy compounds, hydrocarbons and an acid (Gurudeeban *et al*, 2010). Some medicinal studies were done about gram positive and gram negative medicinal bacteria that were affected by different extracts (Memon *et al*., 2003; Marzoogh, 2009; Phate *et al*.,2011) and a few phyto-chemical analyzing of *Citrullus colocynthis* were screened (Ambi *et al*., 2007; Najafi *et al*., 2010; Talabani, 2012). So, a little experiment done to determination biochemical compounds of this plant and to some extent studies was performed to indicating antibacterial properties against medicinal bacteria. Because of plant bacterial diseases are still a major threat to agricultural crops and there is a little poison to managing plant bacterial diseases. To reducing chemical poison effects, replacing them with natural compounds is necessary in persistent agriculture. Therefore search for new antimicrobial substance must be continued and all possible strategies should be exploring (Clardy and Walsh, 2004).

Since no data exists on the anti phytho-pathogenic bacteria and polyphenol content of this plant, present study was conducted in order to evaluation of the potential antibacterial activity by applying ethanol50%, methanol 70%,chloroformic and aqueous extract of extracts of *Citrullus colocynthis* L. against six phyto-pathogenic bacteria at a low concentration (4µg/disc) and also estimation of the phenol content and polyphenol properties by HPLC method was done to assess the antibacterial effect and potential of organic extracts of *Citrullus colocynthis* fruits

Methods and materials

Plant materials

The fruits of *Citrullus colocynthis* L. used for the present study were collected from the Sourthern areas of Jiroft, Iran, during the months of September. The plant was acknowledged by a senior Botanist Dr. M. Vakili, in the herbarium of plant systematic laboratory of college of agricultural science, University of Azad, Jiroft. After identification, fruits were shade dried**.**

Preparing extracts

Ten grams of dried fruits was ground with a mixer and added to 100 ml of solvents. After 3 h of maceration with continuous stirring at 200 rev/ min, the mixture was then filtered using filter paper (Whatman No 1). This operation is repeated four times after each filtration with renewal of the solvent in order to exhaust the marc and increase the yield. At the end of extraction, the fractions obtained were collected in a vial and then were evaporated by rotavapor at a specific temperature to the solvent (Senhaji *et al*.,2005)

Test microorganisms

Six registered bacterial strains including: *Erwinia amylovora* , *Bacillus subtilis*, *Pectobacterium*

carotovurum, *Ralstonia solanacearum*, *Xanthomonas axnopodis pv citri*, *Pseudomonas flourescense* obtained from type culture collection of plant pathology department, Azad University of Shiraz, Iran. The bacteria rejuvenated in Mueller Hinton-Agar (MHM, E. Merk, Germany) and sub cultured as needed. For bioassay experiments, suspension of approximately 10^8 cells/ml in sterile normal saline was obtained (Forbes *et al*., 1990).

Antimicrobial bioassay

Antimicrobial activity was determined by agar disc diffusion method using Muller Hinton agar for bacteria. Plant extracts were dissolved in DMSO (Di Methyl Sulphoxside) plate was inoculated with 20mg/ml microbial suspension having a concentration of 10^8 cells/ml and 4µg/Disc concentration of extracts was added to each plate, disc paper concentration were prepared by dipping paper disc in a certain concentration of solvents for 24 hours. Then the bacterial plates were incubated at 27-32^{oC} for 48 hours. The antimicrobial activity was observed as diameter of inhibition zone which was compared with standard (A little modified) (Sandhya, 2013). To measure the Minimum Inhibitory Concentration (MIC) value of effective extracts, were determined using four- fold serial dilutions of methanol70% extracts were prepared in DMSO: methanol (1/1:v/v) solvent and assayed against the bacteria as mentioned earlier. MIC was defined as the lowest concentration able to inhibit visible bacterial growth. All data were replicated three times (Shahidi, 2004).

Estimation of total phenolics

Total phenolic content of plant extract was determined by the Folin–Ciocalteu micro-method (Slinkard and Singleton, 1977). Briefly, 20 µl of extract solution were mixed with 1.16 ml distilled water and 100 µl of Folin–Ciocalteu reagent, followed by addition of 300 µl of Na2CO3 solution (20%) after 1 min and before 8 min. Subsequently, the mixture was incubated in a shaking incubator at 40°C for 30 min and its absorbance was measured at 760 nm. Gallic acid was used as a standard for calibration curve. The phenol content was expressed as Gallic acid equivalents using the following linear equation based on the calibration curve:

$A= 0.98\ C + 9.321 \times 0.001\ \ R2= 0.9965$

Where A is the absorbance and C is concentration as Gallic acid equivalents (mg/g).

HPLC analysis

It was performed by using an Agilent 1200 series, equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a quaternary pump an online vacuum degasser, an auto sampler and a thermo stated column compartment, on an Agilent Zorbax Eclipse XDB-C18, 5 µm (ID), 4.6× 150 mm (FT) column, at a flow-rate of 1 ml min^{-1}. Solvent gradient was performed by varying the proportion of solvent A (formic acid 1% in deionized water) to solvent B (Methanol (v/v)) as follows: Methanol: formic acid 1%: (10:90), Hold Time: 0 min; Methanol: formic acid 1%: (25:75), Hold Time: 10min; Methanol: formic acid 1%: (60:40), Hold Time: 20min, Methanol: formic acid 1%: (70:30), Hold Time: 30min, the total running time was 30 min. The column temperature was 30 ^{o}C. The injected volume of samples and standards (Catechin, Quarcetin, Qumarin, Caffeic acid, Colargenic acid and Gallic acid) was 20µL and it was done automatically using auto sampler. Chromatograms were plotted at 280 and 320 nm (Justesen *et al*,. 1998)

Statistical analysis

Data obtained from antibacterial experiments were expressed as means ± SE. Results were statistically evaluated by ANOVA and using Duncan test. $P \leq 0.01$ were considered significant (Table 1).

Results and discussion

The antimicrobial study showed that the different fruit extracts of *Citrullus colocynthis* inhibited the growth of gram positive bacteria *Bacillus subtilis* and mentioned gram negative bacteria *Erwinia amylovora* at 4µg/disc concentration Other resistant bacteria like; *Xanthomonas axnopodis pv citri* and *Pseudomonas flouresence* had not showed any antibacterial effect to all extracts and *Pectobacterium*

carotovurum was susceptible to methanol70% and ethanol50% extracts (Table 2). There are some medicinal studies about gram positive and gram negative medicinal bacterial that were affected by different extract of this plant. Similar results about gram positive bacteria observed by Memon *et al*., (2003) that the ethanol50% extract of *Citrullus colocynthis* which is active against gram positive bacteria i.e. *Bacillus pumilus and Staphylococcus aureus* whereas it is inactive against gram negative bacteria *Eschreschia coli and Pseudomonas aeruginosa*. Another study revealed that methanol; ethanol, acetone and distilled water extract have been showed potent activity against *Escherichia coli* (Phate *et al*., 2011). Fruit and seeds were screened for activity against Gram negative and Gram-positive bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis* and various *Candida* spp. (*Candida glabrata, Candida albicans, Candida parapsilosis* and *Candida kreus*ei). The highest MICs were obtained from the fruit aqueous extracts (MIC 0.10mg/ml against Candida albicans and Candida glabrata, 0.20mg/ml against Escherichia coli and Pseudomonas aeruginosa), lowest activity from the roote xtracts (Marzouk, 2009). These different results can be of different bacteria strains. In this study, MIC values were determined for all affected strains used in this study. The highest MICs were obtained from the Ethanolic50% extracts (MIC 0.5µg/disc) against *Pectobacterium carotovorum* and after that Ethanol50% and methanol70% extracts (at MIC 1µg/disc) against to *Erwinia amylovora* and lower MIC value were equal to 4µg/disc against other strains (Table 3). Total phenol content of plant extract was revealed in table (4) and it was 3.696 mg/g in dried material. Also, by screening some poly phenols like Catechin, Quarsetin, Qumarin, Caffeic acid, Colargenic acid and Garlic acid on HPLC set, only Gallic acid was detected in this plant and it was the first report compound. Gallic acid is a tri hydroxyl benzoic acid, a type of phenolic acid, a type of organic acid, also known as 3, 4, 5-trihydroxybenzoic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants (Reynolds and Wilson, 1991).

Table 1. Analysis variants of DIZ(mm) values of test bacteria against various extracts of *Citrullus colocynthis* at 4µg/disc.

Source of various S.O.V	Free degree D.F	Means of squares
Test bacteria	5	211.6**
Different Extract	4	94.73**
Test bacteria× Different Extract	20	35.23**
Error	60	0.14
		10.75 =CV

** Very significant at p<0.01.

Table 2. Comparison means of diameter inhibition zone of bacterial growth against various plant extracts of *Citrullus colocynthis* at 4µg/disc by Duncan's test.

Bacteria	Bacteria				
	DMSO	Aqueous	Ethanol 50%	Methanol 70%	Cholorophorm
Erwinia amylovora	0.00±0.00 d	8.33±0.14c	8.00±0.14c	10.00±0.14b	17.00±0.14a
Ralstonia solanacearum	0.00±0.00d	0.00±0.00 d	0.00±0.00 d	8.00±0.14c	0.00±0.00d
Xanthomonas axnopodi Pv citris	0.00±0.00d	0.00±0.00 d	0.00±0.00d	0.00±0.00d	0.00±0.00d
Pseudomonas flourescense	0.00±0.00d	0.00±0.00 d	0.00±0.00d	0.00±0.00d	0.00±0.00d
caratovorum Pectobacterium	0.00±0.00d	0.00±0.00d	8.66±0.14c	8.66±0.14c	0.00±0.00d
Bacillus subtilis	0.00±0.00 d	8.33±0.14c	8.66±0.14c	9.66±0.14b	10.66±0.14b

Means with similar letters in each column are not significantly different. significant at p<0.01.

Table 3. Comparison means of Minimum inhibition Concentration of effective plant extracts of *Citrullus colocynthis* on mentioned bacteria in µg/disc.

Bacteria	Effective plant extracts of *Citrullus colocynthis*			
	Aqueous	Cholorophormic	Ethanolic50%	Methanolic70%
Bacillus subtilis	4c	4c	4c	4c
Ralstonia solanacearum	-	-	-	4c
Erwinia amylovora	4c	4c	1b	1b
Pectobacterium carotovorum	-	-	0.5a	4c

Table 4. Total phenol content.

Laboratory	Sample	Total Phenol (mg/g)
P911136	Bitter cucumber	3.696

The chemical formula is C_6 H_2 $(OH)_3$ COOH. Gallic acid is found both free and as part of hydrolysable tannins. Salts and esters of Gallic acid are termed 'gallants'. Despite its name, it does not contain gallium. Gallic acid is commonly used in the pharmaceutical industry (Fiuza *et al.*, 2004). According to Phate *et al.* (2011) tannins has been reported to prevent the development of microorganisms such as fungi, bacteria, yeast, viruses by precipitating microbial protein and converting it into unavailable form. The fruit extract of *Cirullus canadensis* contains diver's chemical compounds including alkaloids, fixed oils, cyanogenic glycosides, terpenoids, tocopherols and minerals as showing in the results; the fixed oil pattern contributes to the stability of the fruit as well as adding important nutritional value (Gurudeeban *et al*, 2010). Ambi *et al.* (2007) screened the phytochemicals of *Citrullus colocynthis* and observed that the presence of alkaloid, seteroids, glycoside and flavanoids.The earlier studies of Najafi *et al.*, (2010) also exhibited the presence of similar phytochemicals in fruit extract of *Citrullus colocynthis* the aqueous extract contained alkaloids, flavonoids and steroids. Saponins were not detected in this extract. The methanol extract reacted positively with all families of the phytochemicals tests. The chemical contents of the kernel of *Citrullus colocynthis* was up to now lesser known. The experimental results reported that this kernel could be potential sources of nutrient mainly of essential fatty acids, alkaloids, vitamin E, amygdalin, triterpenes and some important minerals. It might be important in upcoming studies to evaluate the bioactive compounds (Talabani, 2012). Different results can be of detecting different phenol compounds and different geographical conditions. HPLC Chemical analyzing, revealed Gallic acid at 0.19305 (mg/g) concentrations in methanol extract of this plant. The results of chromatograms that were plotted at 280 and 320 nm (Justesen *et al*,. 1998) showed existence of 12 phenol materials at 280nm (Figure 1) and 15 phenol materials at 320nm (Figure 2). Gallic acid in two different conditions was detected at 1.6 minutes after detecting.

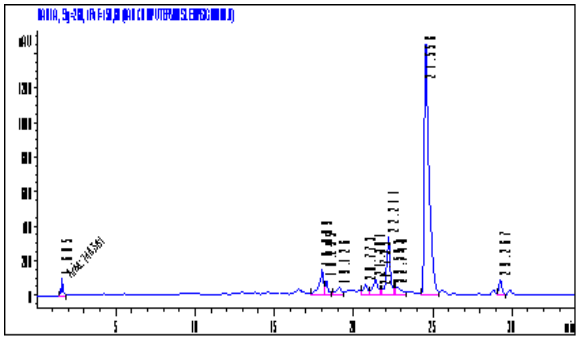

Fig. 1. Curve of poly phenols determination on HPLC technique at 280nm.

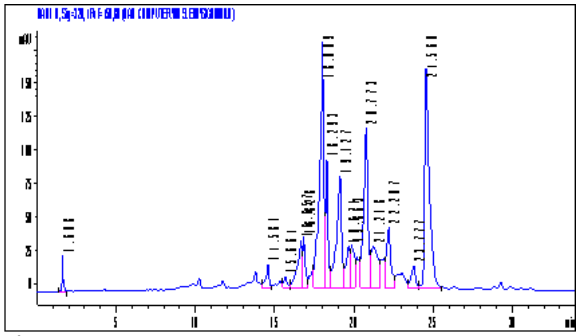

Fig. 2. Curve of poly phenols determination on HPLC technique at 320nm.

Conclusion

Present study concludes that the all applied fruit extracts of *Citrullus colocynthis* show active response against the *Erwinia amylovora* and *Bacillus subtilis* also methanol70% extracts of it showed antimicrobial activity to *Pectobacterium carotovorum* and *Ralstonia solanacearum* at 4µg/disc concentration. This antibacterial activity is may be due to total phenol content and secondary metabolites which are identified and not identified in *Citrullus colocynthis*. The study indicates that fruit extract possess considerable total phenol content and possess potent antimicrobial activity and proves that use of *Citrullus colocynthis* for the treatment of plant diseases caused by the test organisms. There is still an urgent need to identify novel substances that are active towards pathogens and phyto-pathogens with high resistance.

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