Comparative Study of Antibacterial, Antifungal, Antioxidant activity of bitter apple (Citrullus *colocynthis*) Plant Parts in Methanol and n_Hexane.

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Abstract

Background:

Citrullus colocynthis (Linn.) Schrad (CCT) which belongs to the family of Cucurbitaceae is used for many important medicinal purposes. In this study we have made the extracts with different parts of the plant in n-hexane, methanol extracts and investigated its in vitro activity against fungus and bacteria and also its antioxidant activity.

Methodology:

The collected sample of *C. colocynthis* and their different parts (Fruit, Leaves, Stem & Seed) were soaked into n-hexane and methanol solvent. In this experiment we used 12 bacterial and 4 fungal strains, *Citrullus colocynthis* n-hexane methanol extracts were tested against it. The minimum inhibitory concentration (MIC) of the compound was determined using broth micro dilution method. The antibacterial and anti-oxidant property and inhibition effect of fungal biomass is determined during this process. **Result:**

Extracts showed antibacterial activity against almost all tested bacteria. *C. colocynthis* was most effective against *S. aureus*. Anti-fungal activity found strong against *A. flavus* and *Rhizopus* while *A. Niger* and *S. cerevisiae* show no activity. The fruit extract showed the maximum antioxidant and free radical scavenging ability.

Conclusion:

The result of experiment shows that this extract can be used for the control of bacterial & fungal diseases as an alternative treatment to the chemical additives. Antioxidant property of the plant is the additional advantage of using the plant as a medicine.

Keywords: n_Hexane, Methanol, *Citrullus colocynthis (CCT),* Antibacterial, Antioxidant, Anti-fungal activity.

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Introduction:

Novel bio-active metabolites with many natural mechanisms had been a subject of interest for many researchers from approximately past 20 years. There are various practical applications of the numerous constituents derived from parasites and actinomycetes, microscopic organisms, and plants. The exceptionally novel particles derived from plants were exceedingly pulled in with specialists (Mohammad and Almalki A *et al.*, 2016).

Due to the inadequate availability of modern medicine, the ease and sufficiency of therapeutic plants, as well as social convictions and inclinations, therapeutic plants are used by billions of people in the majority of developing nations (Balamurugan R and Smilax CL *et al.*, 2015). Nowadays there is an increase need to explore the new and intense curative anti-bacterial and anti-fungal agents for the treatment of infectious diseases because of the increase in resistance to the conventional therapies. There are many metabolites present in the plants that can be used for the treatment of microbial diseases.

According to the World Health Organization the conventional medicines are used for 80% of the human diseases in the leading countries (Nandhini. VS and Viji S. BG., 2015). Regarding to ethnopharmacy as a means of bio-active compounds has expanded around the world, especially to explore anti-inflammatory drugs (Marazouk B *et al.*, 2011).

The best hereditarily arranged collections of therapeutic plants in the plant kingdom belongs to Cucurbitaceae family Many plants of this family are resistant to dry weather, sensitive to cold and succulents. (Riaz *et al.*, 2015). Cucurbitaceae family includes the plant called *Citrullus colocynthis (L.)* Schard which is included in the four types of the desert vine, is also a lasting herb with a tuberous taproot (Burrows B. GE and Shaik R.S *et al.*, 2015).

Fruits have a sensitive, white pulp which is stacked up with different acclaim compacted seeds. Seeds are pretty much small, ovoid, stuffed, smooth and in brown shading. Seed are smooth, pressed, ovoid-formed and around 6 mm in size. They are arranged on the parietal placenta. The seeds are light yellowish-orange to diminish dim hued in shading. The herbaceous harsh vine is non-exceptional and is extended from the base. Bitter apple plant has an immense enduring root that passes on long and thin, lean, harsh, rough vine-like stems. The parts of the plant include crude leaves which are 2-4 inches long with 3-7 folds, precise and tough stems, and solitary light-yellow flowers. Seeds of the plant are approximately 1/4 inch or less long with smooth surface and becomes brown when ripped. There are approximately 15-30 round fruits on each plant with

width of 3-4 inches, green in color with undulate yellow stripes which become all yellow when it is full ripe and dry (Borhade P *et al.*, 2013).

The fruit of Bitter apple has many therapeutic uses similar to its pharmacological and nutritional potential. (Abdullah *et al.*, 2014). WHO review showed that around 70-80% of the total populace depend on non-conventional drug, for the most of home-grown sources, in their essential medicinal services. Bitter apple plant has wide range of pharmacological actions against diseases of immunene system, cardiovascular system and respiratory system. It also has an action as antioxidant, hypolipidemic, and anxiolytic pain-reliever and antipyretic with numerous other pharmacological impacts. (Al-Snafi AE, 2016).

Cucurbitacin, flavonoids, and polyphenols are among the beneficial chemicals found in C. colocynthis. The antioxidant properties of the plants are due to flavonoids which are useful to prevent damage by reactive oxygen species, as ROS have a role in many diseases like cancer, tissue damage and inflammation. When phytochemical analysis of *C. colocynthis* was done it was found to have compounds like flavonoids, alkaloids, saponins, tannins and glycosides. When the extract of chemical components of C. colocynthis is made with chloroform and methanol the compounds like flavonoids, alkaloids and glycosides show strong anti-fungal and antibacterial properties. (Qin-Yuan Li *et al.*,2022)

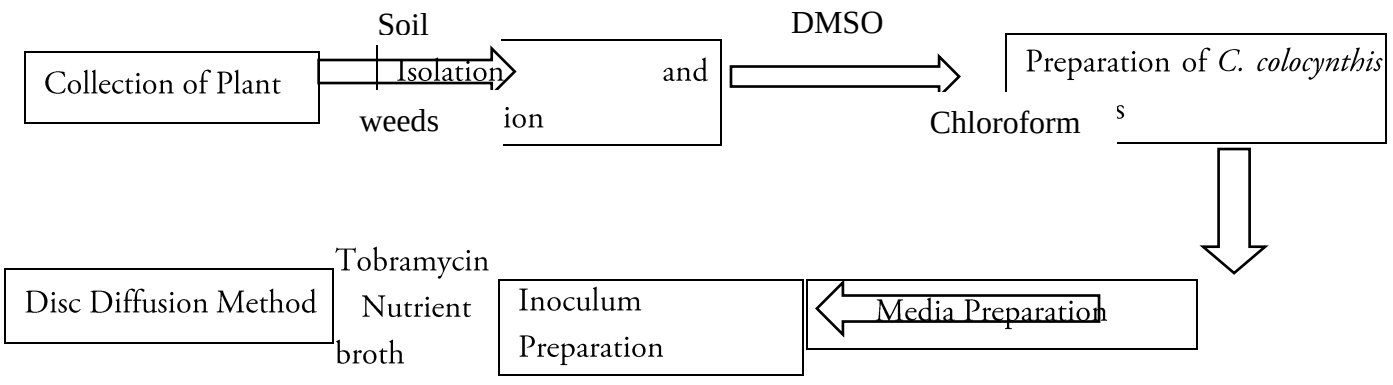
Material and Methods:

2.1. Collection of Plant:

A botanist approved the removal of various *C. colocynthis* plant parts from Rahim Yar Khan, including the root, stem, leaf, and fruit.

2.2. Isolation:

The gathered plants were meticulously separated from other unwanted materials including earth particles, weeds and other plant pieces. The fruit of *C. colocynthis* was allowed to dry for three months while the root, stem, and leaf were cleaned and dried at room temperature in dark circumstances for 3 months.



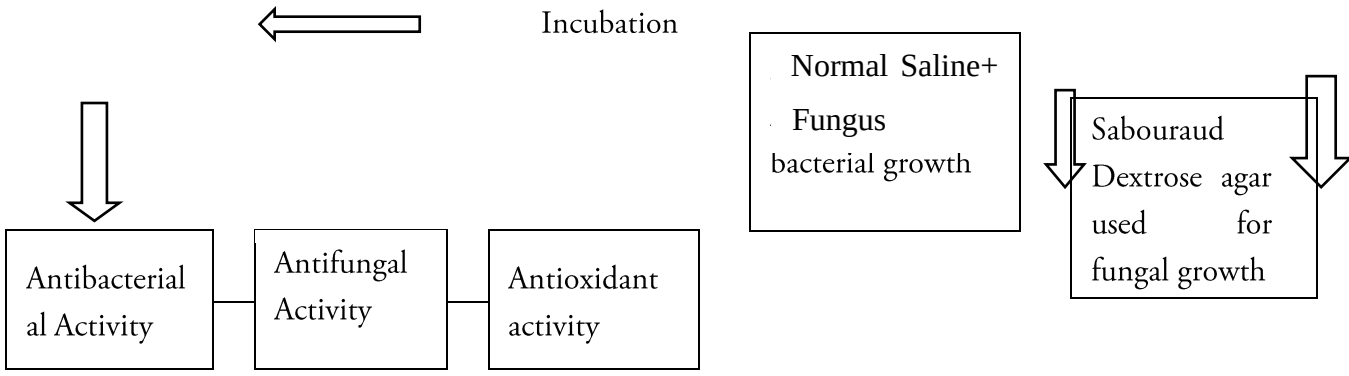


Fig:2.1 Flow Chart of *Citrullus colocynthis* against all tested Microorganisms

2.3. Preparation of *Citrullus colocynthis* extracts:

The electric blender was used to individually pulverize the root, stem, leaf, and fruit of *C. colocynthis*. Thereafter, 300 cc of n-Hexane, methanol was mixed in 30 grammes of groundup *C. colocynthis* powder per part, and the mixture was vigorously agitated by hand for five to six days, then filtered through filter papers and pour into the petri plates (Khalid AA, 2015).

At that time, the extract was evaporated until the sticky extract was achieved; keep the plates exposed and allow them to dry. This whole process of evacuation takes 2-3 days. After that drying process preserve the extracts in Eppendorf. At last store the extracts in freezer or at room temperature to use against anti-bacterial, anti-fungal and antioxidant activity.

2.4. Strains used

- **Bacterial strains:**

An aggregate 12 bacteria were tried to investigate antibacterial activity. The microorganisms are *Salmonella enterica* (ATCC 14028), *Bacillus subtills* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Bifidobacterium bifidum* (ATCC 29212) *Bacillus Alcalophilus* (ATCC 27647), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Saccharomyces cerevisiae* (ATCC 9763), *Listeria monocytogenes* (ATCC 13932), *E. sakazakii* (ATCC 29544), *Lactobacillus acidophilus* (ATCC 4356), *Salmonella typhi* (ATCC 14028.

- **Fungal strains:**

We used 4 following different fungi to carry out antifungal activity.

Angier (clinical), *A. flavus* (ATCC 200026), in n_ Hexane, *Angier* (clinical), *A. flavus* (ATCC 200026), in Methanol.

The University of Lahore's microbiology lab 404 had access to these microorganisms.

2.5. Preparation of media:

The media for culturing was selected for their capacity for the development of a wide assortment of bacterial and fungal strains; 12 strains of microscopic bacteria and 4 strains of fungi were utilized. Mueller Hinton agar was utilized; it is a microbiological development medium which is usually utilized for anti-microbial weakness testing. It was utilized for the bacterial and fungal development

and whatever is left of the systems were additionally carried on similar media, it was set as the essential requirement for our work. Although it was initially developed for the separation of pathogenic Neisseria spp., it is now frequently used for vulnerability testing by the Kirby-Bauer plate dissemination method.

Antibiotic drug used:

Tobramycin drug was used as positive control.

2.6. Culture media

- **Nutrient broth:**

  Peptone, sodium chloride and yeast extract are all included in this medium. 13 grammes of the medium were dissolved in 1 liter of distilled water to make it. The medium's pH was adjusted to 7.4, and it was then divided into screw-capped bottles of 10 ml each and autoclaved at 121°C for 15 minutes.

- **Mueller Hinton agar (MHA):**

  One liter of distilled water was used to dilute 38 grammes of MHA powder, which was then allowed to soak for 10 minutes. The medium was thoroughly mixed, dissolved in a water bath, autoclaved for 15 minutes at 121°C, cooled to 47°C, and then poured into sterile Petri plates.

- **Sabouraud Dextrose agar (SDA):**

  62 Sabouraud dextrose agar powder was weighed, thoroughly mixed, and autoclaved at 121°C for 15 minutes before being cooled to 47°C and placed on sterile Petri dishes. The mixture was then soaked for 10 mints (Abdalla SA *et al.*,2019)

2.6. Methods

- **Preparation of inoculum:**

  Microscopic organisms were successfully inoculated in nutritional broth and ordinary saline. In case of bacteria, 10ml of nutrient broth was placed in each of the 14 test tubes. Nevertheless, five 10 ml tubes of normal saline were made in the case of fungus. As a negative control, one additional tube that was prepared in both situations was utilized.

- **Method of Disc Diffusion:**

  Muller-Hinton agar that had been sterilized in a flask and chilled to room temperature was used to test for microorganisms on sterilized petri plates. Swabs of the arranged test inoculums were placed on the coagulated media's highest point. With the use of sterilized forceps, the discs were placed on the media's surface. The experiments were conducted using 100 l of extract concentration. With the use of a 100-l pipette, the extract was loaded. The disc had a 6mm diameter. For 24 hours, the plates were incubated. We measured the constraint zones in millimeters (mm).

2.7. Different activities performed Antibacterial test:

Using a modified disc diffusion technique, the antibacterial activity of extracts was determined (Kil *et al.*, 2009). The extract solutions (20, 40, and 100 mg/ml) were made by diluting with 5% dimethyl sulfoxide (DMSO). The test microorganisms were planted into the appropriate medium

using the spread plate technique. After solidification, filter paper discs with a diameter of 6.0 mm were impregnated with 10 l of crude extracts and dried. As a negative control, DMSO was utilized. Antibacterial discs were placed over the surface of inoculated agar plates used in triplet for each plant component, the petri plates were incubated for 24 hours at 37°C.
The diameters of the discs' clear zones of inhibition were measured.

- **Antifungal action:**

To assess the antifungal activity, the same technique outlined for bacteria was utilized, Sabouraud Dextrose Agar. Two milliliters of the sterile, molten Sabouraud Dextrose Agar (45 to 50°C) were thoroughly combined with 20 milliliters of each fungal stock suspension before being divided among sterile Petri dishes and allowed to set. The plates were then incubated at a temperature of 37°C for 24 to 48 hours while standing upright. The diameter of the inhibitory zones was measured following the incubation times, and the mean results were recorded. (Loiy E and Ahmed H *et al.*, 2011).

- **Antioxidant Mechanism:**

The DPPH technique was used to assess the isolated molecule's antioxidant capacity. In a nutshell, different concentrations of the chemical (10–50 g/ml) were combined with newly made DPPH solution in ice-cold methanol (0.15%), and after 30 minutes in the dark, the mixture was exposed to light to measure the absorbance at 515 nm. The amount of antiradical that must be present to inhibit free radicals by 50% was used to represent the antioxidant activity as IC50 (g/ml). The standard was vitamin C. The following equation was used to determine the capacity to scavenge the DPPH radical:
Scavenging activity (%) = [1-(absorbance of sample-absorbance of blank)/absorbance of control] X 100

- **Diphenyl-1-picrylhydrazyl (DPPH) assay:**

DPPH is a cell-porous, stable, free radical that is typically used to analyses the antioxidant activity of tissue fragments and evaluate a compound's potential to serve as a free radical scavenger or hydrogen benefactor. When an antioxidant or depleting substance reacts with DPPH, it produces the related hydrazine DPPH2, which is followed by a shift in color from purple to yellow (absorbance at 515–528 nm) (Hyun Young Kil *et al.*,2009).

2.8. Statistical Investigation:

The means and S.D. for all data were shown. Microsoft Excel was used to do statistical analysis on all of the test findings.

Results:

Citrullus colocynthis is a plant of great importance. As it grows in dried places so it is uneffected to high temperature. It is therapeutic plant and contains a number of pharmacological properties. Our objective is to investigate the antifungal, antibacterial and antioxidant activities in *C. colocynthis* of methanol, n_Hexane extracts. The results of these activities are determined below.

3.1. Extracts obtained from *C. colocynthis* plant parts

Solvent Used	Plant parts Used	Wt. Of Extract (gm)
n-Hexane	Leaves	1.98
	Seed	2.02
	Fruit	2.05
	Stem	1.85

Solvent Used	Plant parts Used	Wt. Of Extract (gm)
Methanol	Leaves	1.98
	Seed	1.99
	Fruit	1.78
	Stem	1.99

Key words: *Citrullus colocynthis*, n-hexane, methanol, Weight of extract (wt), Grams (gm)

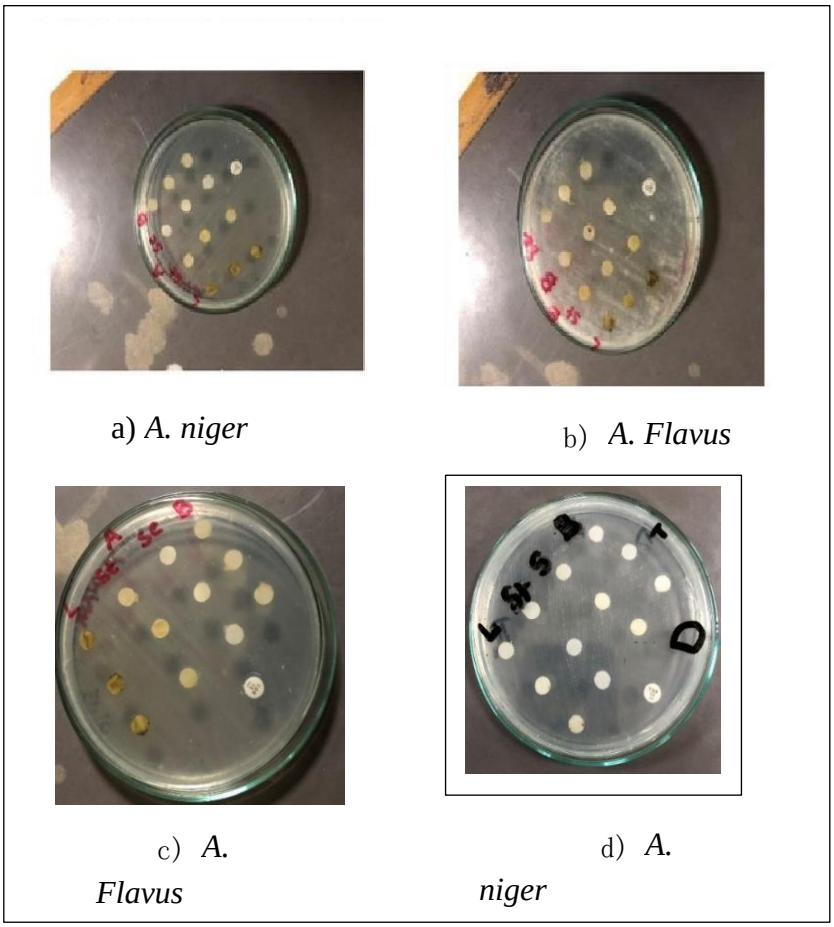
Results of antifungal activity:

Table 3.2 Antifungal activity of *Citrullus Colocynthis* (100mg/1ml DMSO)

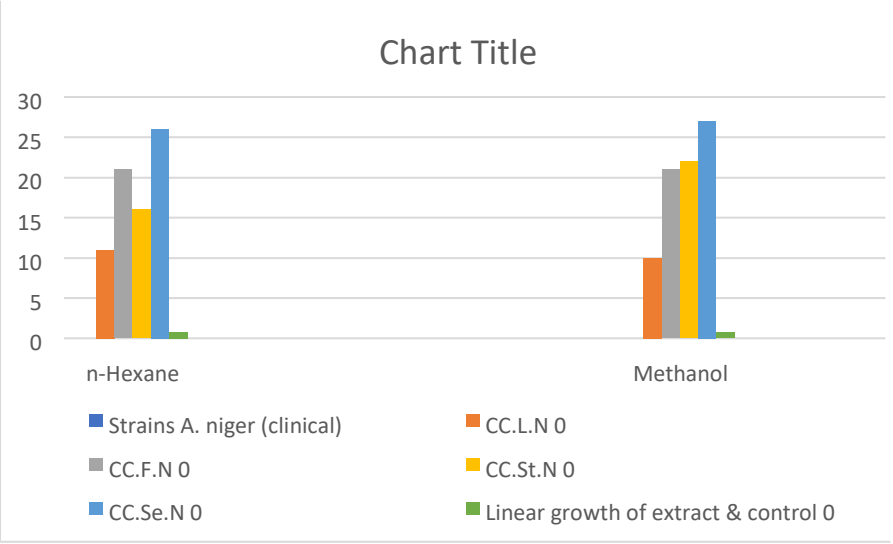
	Strains	CC.L.N	CC.F.N	CC.St.N	CC.Se.N	Linear growth of extract & control
n-Hexane	*A. niger* (clinical)	0	0	0	0	0
	A. flavus	11	21	16	26	80%
	(ATCC 200026)					
Methanol	*A. niger* (clinical)	0	0	0	0	0
	A.flavus (ATCC 200026)	10	21	22	27	70%

Key words: *Citrullus colocynthis* leaves in n-hexane, Methanol (CC.L. N), *Citrullus colocynthis* fruit in n-hexane, Methanol (CC.F.N), *Citrullus colocynthis* stem in nhexane, Methanol (CC.St. N), *Citrullus colocynthis* seed in n-hexane, Methanol
(CC.Se.N)

Fig:3.1



a) *A. niger* b) *A. Flavus*: Methanol Extract
c *A. nigar* d) *A. Flavus*: n_Hexane



Graph 3.1: Comparison between the zones of inhibition of essential parts of *Citrullus Colocynthis* against all test fungus as mean ± standard error.

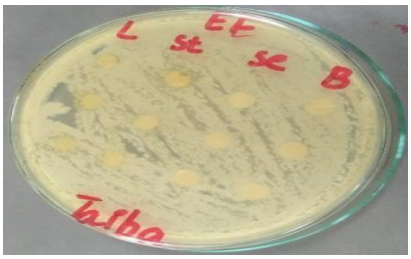
Results of antibacterial activity:

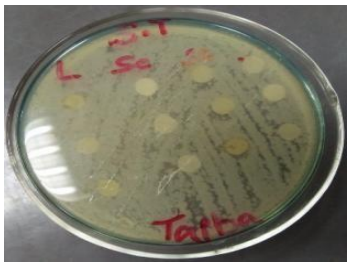
Table 3.3. Anti-bacterial activity of *Citrullus coloscynthis* (100mg/1ml DMSO)

Strains	CC.L.C	CC.F.C	CC.St.C	CC.Se.C	McFarland standard
Escherichia coli (ATCC 25922)	9	8	10	8	100%
Salmonella enterica (ATCC 14028)	9	9	10	10	100%
Staphylococcus aureus (ATCC 25923)	14	9	9	7	100%
S. typhimurium (ATCC 14028)	8	9	9	7	100%
Bacillus subtilis (ATCC 6633)	9	7	7	10	100%

Bifidobacterium bifidum (ATCC 29212)	10	9	9	9	100%

Key words: *Citrullus colocynthis* stem in n-hexane (CC.L. N), *Citrullus colocynthis* seed in nhexane (CC.F. N), *Citrullus colocynthis* leaves in n-hexane (CC.St. N), *Citrullus colocynthis* fruit in n-hexane (CC.Se. N)





b) *S. typhimurium* c) *S. aureus enterica* d) *S.* e) *B. subtilis* f) *B. bifidium*

Fig 3.2. Effects of n_Hexane extract on isolates by disc diffusion method

Table 3.4. Anti-bacterial activity of *Citrullus coloscynthis* (100mg/1ml DMSO)

Strains	**CC.L.C**	**CC.F.C**	**CC.St.C**	**CC.Se.C**	**McFarland standard**
Escherichia coli (ATCC 25922)	8	8	7	9	100%
Salmonella enterica (ATCC 14028)	9	9	9	9	100%
Staphylococcus aureus (ATCC 25923)	14	8	8	8	100%
S. typhimurium (ATCC 14028)	7	9	8	10	100%
Bacillus subtilis (ATCC 6633)	9	11	8	37	100%
Bifidobacterium bifidum (ATCC 29212)	8	9	17	8	100%

Key words: *Citrullus colocynthis* seed in methanol (CC.L. N), *Citrullus colocynthis* leaves in methanol (CC.F. N), *Citrullus colocynthis* fruit in methanol (CC.St. N), *Citrullus colocynthis* stem in methanol (CC.Se. N).

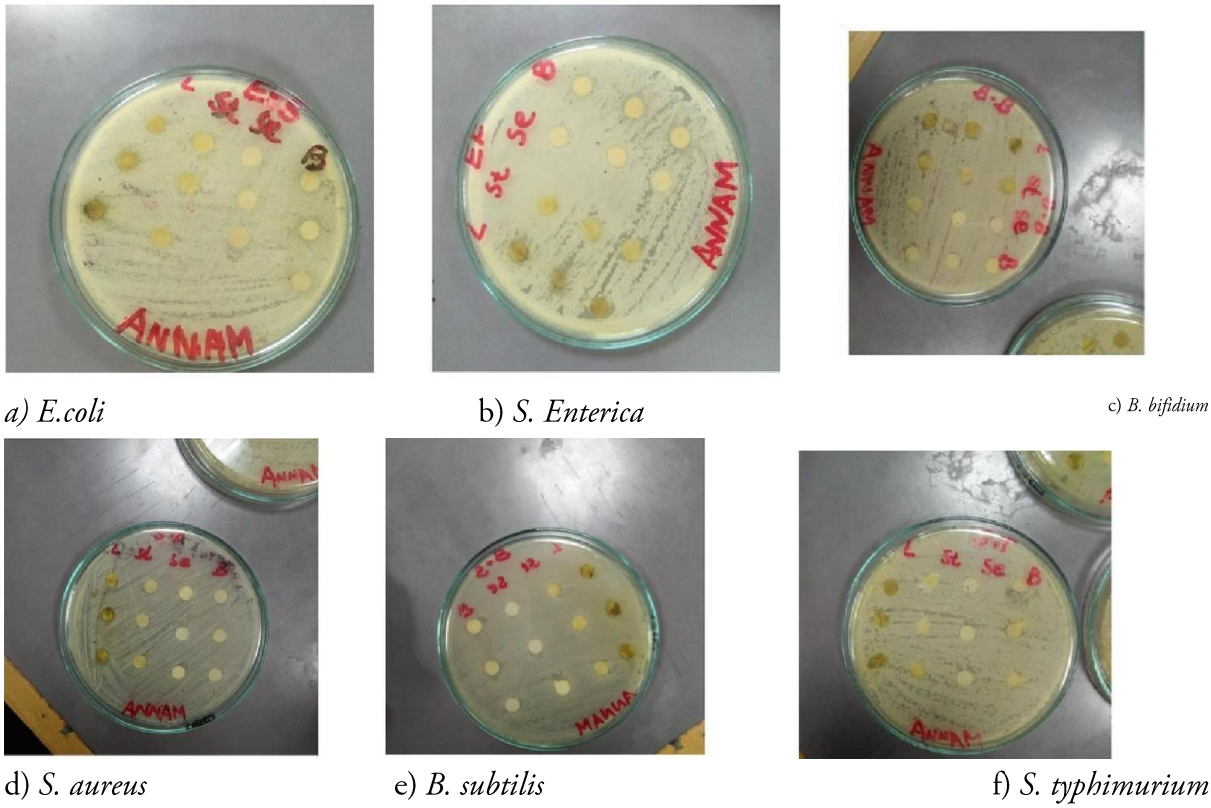


Fig 3.3. Effects of methanol extract on isolates by disc diffusion method

3.4. Results of antioxidant activity:

The results mentioned below demonstrates the free radical scavenging activity of nhexane extracts of *C. colocynthis.* Among all the parts of *C. colocynthis* the fruits part is more antioxidant and having more free radical scavenging activity.

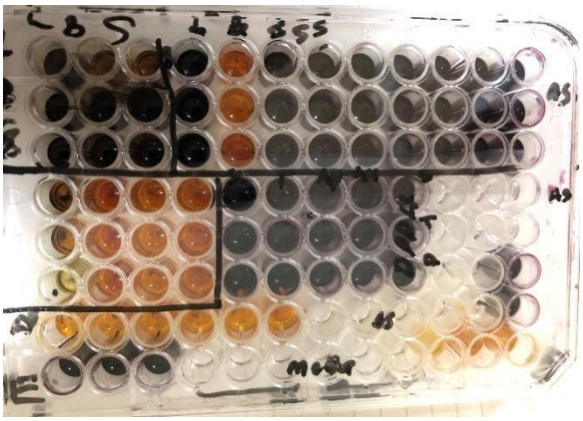


Fig 3.4. 96 well plate used for antioxidant activity

Table 3.5. Antioxidant activity of *Citrullus colocynthis* (100mg/1ml DMSO)

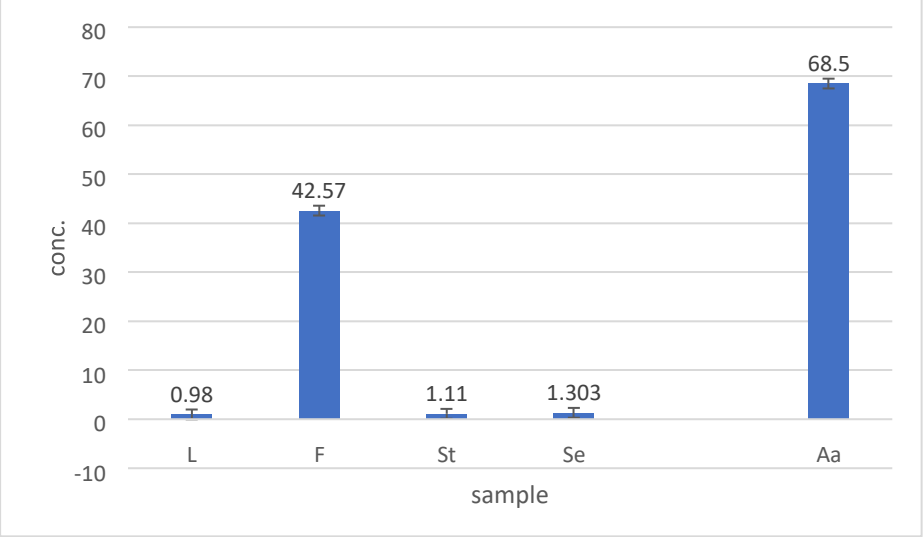
Plant part *(C. colocynthis)*	**% of scavenger±SD 100mg/ml**
Leaves	0.98±0.120
Fruit	42.57±0.418
Stem	1.11±0.095
Seed	1.303±0.121

Key word: *Citrullus colocynthis,* Standard deviation (SD)

3.2. Chart for results of antioxidant activity

This chart represents the values of the parts (leaf, fruit, stem & seed) of bitter apple which is also mentioned above in table. These values indicate their antioxidant potential. Ascorbic acid used as a standard exhibit 68.5% of antioxidant activity. The all other parts of plant are compared to this standard value. Leaves display 0.98, fruit 42.57%, stem 1.11% & seed shows 1.303% antioxidant activity. Fruit exhibits highest antioxidant potential.

% inhibition of n-hexane *Citrullus colocynthis* extract



Discussion:

In a prior work, both fruit and seed were employed as substrate, and various solvents (ethanol and hexane) were used with both at varying concentrations. The study's findings show that *Citrullus colocynthis* has both antifungal and antibacterial action against the tested microorganisms. The two fruit and seed extracts have this action. These findings are consistent with Ali Al-thoughtful Snafi's

analysis, which identified *Citrullus colocynthis* as a potential medicinal plant with a wide range of pharmacological actions that might be applied in a few therapeutic applications due to its amazing effect and secure nature (Belsem M et al., 2011). In another examination it was presumed that antibacterial affectability of leaf extract was evaluated utilizing the plate spread technique by estimate the restraint zone measurement. The *Citrullus colocynthis* extract of acetone and chloroform demonstrated high anti-bacterial activities against every one of the microscopic organisms tried. Antibacterial and antifungal possibilities of the recognized mixtures were controlled by assessing its minim-um inhibitory concentration. The outcomes demonstrated that the recognized components indicated active in Gram-negative than the Gram-positive microbes. For all fungus isolates, the ethanolic extract's inhibitory concentration was the lowest at 0.625 mg/ml fixation. At 0.625 mg/ml convergence of the methanolic extract with a zone of 6 mm and 7 mm, respectively, minimum inhibitory fixation was seen in A. niger and Rhizopus spp. For Rhizopus, A. niger, and Gliocladium, the MIC values for hexane extract were 0.625 mg/ml, 2.5 mg/ml, and 5 mg/ml, respectively (Gurudeeban S *et al.*, 2011). This same effectiveness of extracts against all living things is dependent on the plant extract, which is often a crude balance of non-active and active mixtures. Plants are known to produce dangerous synthetic chemicals that are toxic to microorganisms, and a large number of journals have validated the antimicrobial effect of plant extracts, revealing amazing potential, particularly against multidrug resistant strains (Ilham B *et al.*, 2011). In current study, n-hexane, methanol extracts of *C. colocynthis* used against two different fungal strains. The results mentioned in the table 3.2 exhibits that *A. niger* do not show any inhibitory action both methanol and n_Hexane extracts so they are resistant to *C. colocynthis.* While *in A. Flavus* (ATCC 200026) all parts show zones of inhibition so it is more sensitive than others in methanol and n_Hexane extracts. In recent investigation, n-hexane, extracts of *C. colocynthis* used against 6 bacterial strains. The results mentioned in table 3.3 determines that all bacteria exhibit inhibitory action and are sensitive to *C. colocynthis. S. aureus* (ATCC 25923) shows high effect of zones of inhibition. In which Leave shows most noteworthy impact of zone of hindrance which is 14 mm with anti-microbial. While all remaining bacteria demonstrates moderate antibacterial activity. But in case of methanol extract B. subtilis (ATCC 6633) show great zone of inhabitation and this microorganism are more sensitive to C. *colocynthis* methanol extract, while other bacteria show moderate inhibitory action in C. *colocynthis* methanol extract. Tobramycin which is used as a negative control, when understanding with Leaves (11mm), Seed (21mm) and Stem (16mm). All parts of plant with the exception of Fruit indicates zone of inhibition. Free radical scavenging activity was assessing by the use of several in vitro experiments, the free radical scavenging activity of CCF extract was evaluated using the DPPH radical as a substrate. Moreover, the discovered chemical demonstrated considerable antioxidant activity. The molecule showed comparable activity to the reference chemical at higher concentrations, despite the fact that the antioxidant activities were dose-dependent. Conventional plant compounds with phenolic functional groups have been shown to exhibit antioxidant action. The identified novel

compound, like the other plant molecule, demonstrated significant antioxidant action. In this research we observe that the fruit part of *Citrullus colocynthis* exhibits highest antioxidant activity. CCF's free radical scavenging action rises with concentration, and maximal antioxidant activity was reported.

Conclusion:

We tested the antibacterial, antifungal, and antioxidant mechanism of *Citrullus colocynthis* (leaves, stem, seed and root) in n-hexane, Methanol solvent. It was concluded from the results obtained that the leaf and stem part amongst all plant part exhibits strong antibacterial activity. The seed part comparatively exhibits maximum antifungal activity. The antimicrobial impacts of bitter apple plant parts extract against the microscopic organisms recommend that, they have amazing activity this can support the plant's traditional usage in the treatment of bacterial and fungal diseases. Free radical scavenging impact of fruit extracts with maximum antioxidant activity was observed. Antioxidant activity might be because of phenolic mixes in fruit.

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