ISSN: 2586-7342 © 2018 KFHCA. http://www.kjfhc.or.kr
doi: http://dx.doi.org/10.13106/kjfhc.2018.vol4.no4.10.

In-vitro Antimicrobial Activity Phytochemical and Cytotoxicity of Methanolic Fruits Extract of *Capsicum frutescent*

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Received: October 07, 2018. Revised: October 30, 2018. Accepted: November 17, 2018.

Abstract

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Capsicum frutescen is known in Sudan, is one of the most commonly used pepper species in cooking and in Sudanese folk medicine. The present study was conducted to investigate antimicrobial (bacteria and fungi) and cytotoxicity (Brine Shrimp Lethality Test) of methanolic extract of *Capsicum frutescen* (fruits). The extract have been tested in the present study to investigate the *in vitro* potential effects against Gram positive, Gram negative bacteria and fungi. The selected organisms were *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia and Candida albicans* using the cup plate agar diffusion method. The methanol extract of *Capsicum frutescen* (fruits) exhibited inhibitory effects against *Escherichia coli* with zone of inhibition (23 mm) and *Klebsiella pneumonia* with zone of inhibition (17 mm). The phytochemical screening revealed the presence of Tannins, Saponin, Alkaloids, Anthroquinoles and Terpenoids. The Cytotoxicity of methanolic extract of *Capsicum frutescens was* LD_{50} 64.68 µg/ml. The activity and presence of compounds known to be biologically active are a validation for the use of *Capsicum* as a food ingredient and as a therapeutic element of traditional medicine.

Keywords: In Vitro, Antimicrobial Activity and Cytotoxicity, Capsicum Frutescent.

1. Introduction

Traditional folk medicine is well known since thousand years ago. Commonly the ailment incidence in the rural area is treated with local plants that contain many pharmaceutical constituents (Bussmann and Sharon, 2006).

Among the 120 active compounds currently isolated from the higher plants are widely used in modern medicine, today 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (Pan et al., 2014). There is evidence that using some alternative medicines especially those evolving herbs, metals, minerals or other materials involves potentially serious risks including toxicity (Folashade et al., 2012). Drug discovery from medicinal plants are not simple but it has evolved to include numerous fields of inquiry and take advantages of different analytical procedures. The process initiated with a botanist especially with ethnobotanist, ethno pharmacologist, or plant ecologist that can easily collects and identifies their desired plant(s). Collection may involve those species with known biological activity which need to be study for their active compound(s) and new for isolation (e.g., traditionally used herbal remedies) or may also involve those taxa that have been collected randomly for a large screening purposes. It is also important to take care and respect the intellectual property rights of a given area, country where plant(s) of interest are collected (Heinrich et al., 2017).

The research of pharmacognosy or isolation of natural products facilitated by newly development of new bioassay methods. It has been found that the bioactive compounds are mostly plant secondary metabolites, which become medicine after processing to pure compounds; some are very useful dietary supplements, and many useful commercial products (Atanasov et al., 2015).

Pepper or *Capsicum frutescens* was known by humans from prehistoric times as a wild crop around 7500BC, and used for more than 6000 years ago. America is believed to be the origin of capsicum which is known as chili or pepper that was confirmed by (Orbán, 2014). Peppers found their route to the world after the discovery of the American continent by Christopher Columbus, he found there four other species of chili peppers (Stannard, 1993). Reported that, Chili or Pepper (*Capsicum frutescens*) origin in South America and spread into the new world tropics before subsequent introduction to Asia and Africa. *Capsicum frutescens* are now widely grown throughout the tropics, sub-tropics and warmer temperature regions of the world. The actual cultivation of peppers by Indians was between 5200-3400BC (Abdelmajid, 2016). Peppers contain phenolic compounds, flavonoids and carotenoids, besides being a source of vitamin C (Ghasemnezhad et al., 2011). Among these, flavonoids are ubiquitous phytochemicals found in plants with a wide group of exploitable activities, including antimicrobial activity, antibiotic synergism and bacterial virulence removal (Nascimento et al., 2014). Once absorbed, they influence several biological functions, including protein synthesis, angiogenesis, cell proliferation and differentiation, thus benefiting a variety of human diseases (Pui, 2011).The flavonoids found in most peppers are glycosides and aglycones of myricetin, quercetin, luteolin, apigenin and kaempferol (Bahorun et al., 2004).

The present study aimed to investigate the antimicrobial activity of *Capsicum frutescens.*

2. Materials and Methods

2.1. Plant materials

The *Capsicum fruitesens* L was collected from White Nile state Kousti city in September 2017. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan. The *Capsicum fruitesens* (fruits) was air-dried under the shadow with good ventilation and then ground finely until their use for extracts preparation

2.2. Preparation of crude extracts

Extraction was carried out for the Fruits of *Capsicum frutesens* by using overnight maceration techniques according to the method described by (Altúzar-Molina et al., 2011). About 50 g were macerated in 250 ml of methanol for 3 hours at room temperature with occasional shaking for 24 h, the supernatant was decanted and clarity field by filtration through a filter paper, after filtration, the solvent was removed under reduced pressure by rotary evaporator at 55°C. Residue was weighed and the yield percentage was calculated then stored at 4°C in tightly sealed glass vial ready for use.

2.3. Test microorganisms:

The methanolic extract of *Capsicum frutescens* plant were tested against four bacterial species: one Gram-positive bacteria *Staphylococcus aureus*, three Gram-negative bacterial strains *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, one fungal strains *Candida albicans*. The bacterial and fungal strains used in the study were obtained from the Department of Microbiology, Faculty of Medical Laboratory Science, International University of Africa, Khartoum, Sudan. The bacterial cultures were maintained on nutrient agar and incubated at 37°C for 24 h and then used for the antimicrobial test.

2.4. *In vitro* testing of extracts for antimicrobial activity:

The cup-plate agar diffusion method described in (Fagere and Al Magbou, 2016) was used adopted with some minor modifications to assess the antibacterial activity of the prepared extract. One ml of the standardized bacterial stock suspension (between 10^8 and 10^9 CFU/ml) was thoroughly mixed with 100 ml of molten sterile Moller Hinton agar which was maintained at 45°C. 20 ml aliquots of the inoculated Moller Hinton were distributed into sterile Petri-dish plates. The agar was left to set and in all of these plates 5 cups (6 mm in diameter) was cut using a sterile corkborer (No. 4) and agar discs were removed. Each cups were filled with 0.1 ml sample of the methanolic extract using an automatic pipette, and thereafter the extract was allowed to diffuse at room temperature for two hours. The plates were then incubated in an upright position at 37°C for 24 hours. Two replicates were carried out for the extract against each of the test organisms. After incubation the diameters of the resultant growth inhibition zones were measured and averaged. The mean values were tabulated.

2.5. Antifungal testing:

The same method used for the antibacterial test was employed. However, the growth media used in case of fungi was Sabouraud dextrose agar instead of Moller Hinton. The inoculated medium was incubated at 25°C for two days for *Candida albicans*.

2.6. Phytochemical Screening:

The qualitative phytochemical investigations of methanol extract of fruits of *Capsicum fruitescens* were carried out using standard tests as described below.

2.7. Test for Terpenoids (Salkowski test)

To 0.25 g of extract, 2 ml of chloroform was added. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids (Odeja et al., 2017)**.**

2.8. Test for Saponins:

To 0.25 g of extract, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicated the presence of saponins(Ayaz et al., 2014).

2.9. Test for Tannins:

About 0.25 g of extract was boiled in 10 ml of water in a test tube and then filtered. The addition of a few drops of 0.1% ferric chloride to the filtrate resulting in blue, blue-black, green or blue-green coloration or precipitation was taken as evidence for the presence of tannins (Sharma et al., 2012).

2.10. Test for Flavonoids:

About 10ml of ethyl acetate was added to 0.25 g of extract and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonical layer indicated the presence of flavonoids (Khalil et al., 2013).

2.11. Test for cardiac glycosides (Keller-Killiani test):

To 0.25 gram of extract diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was under lied with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Tesfaye, 2017)**.**

2.12. Test for Alkaloids:

About 0.25 gram of extract was stirred with 5 ml of 1% Hcl on a steam bath. One milliliter of the filtrate was treated with a few drops of Mayer's reagent and another 1 ml was similarly treated with Dragendorff's reagent. Turbidity or precipitation with both reagents was taken as preliminary evidence for the presence of alkaloids (Molla, 2015).

2.13. Test for Anthraquinones (Borntrager's Test):

About 0.5 gram of extract was shaken with 5 ml of chloroform and filtered. A 10% ammonium hydroxide solution (5ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonical phase was taken as an indication of the presence of anthraquinones (Morsy, 2014).

2.14. Test for Polyphenols:

To 5 ml of the extract and 1 ml of FeCl3 (1%) and 1 ml K_3(Fe(CN)6) (1%) were added. The appearance of fresh Reddish blue color indicated the presence of polyphenols (Alam et al., 2017).

3. Cytotoxicity

3.1. Toxicity testing against the brine shrimp (*Artemia Salina*):

Hatching shrimp:

Brine shrimp eggs, *Artemia Salina* were hatched in artificial seawater prepared by dissolving 38g of sea salt in one liter of distilled water. After 24-72h incubation at room temperature (37°C), the larvae were attracted to one side of the vessel with a light source and then were collected with pipette. Larvae were separated from eggs by liquating them three times in small beakers containing artificial seawater (Abdalaziz et al., 2017).

Brine shrimp Toxicity assay:

The bioactivity of the extracts was evaluated by the brine shrimp lethality test (Ramachandran et al., 2011).Brine shrimp lethality bioassay was carried out to investigate the Cytotoxicity of plant extracts. 50 mg of *Artemia Salina* (Leach) eggs were added to a hatching chamber containing artificial sea water (75ml). The hatching chamber was kept under an inflorescent bulb for 48h for the eggs to hatch into shrimp larvae. 20 mg of the tested extracts of the various plant species were separately dissolved in 2 ml of methanol, then 500, 50, and 5µl of each solution was transferred into vials corresponding to 1000, 100, and 10 µg/ml, respectively. Each dosage was tested in triplicate. 10 larvae of *A. Salina* Leach taken 48 – 72hours after the initiation of hatching were added to each vial. The final volume of solution in each vial was adjusted to 5ml with Sea water immediately after adding the shrimps. One drop of dimethylsulphoxide (DMSO) was added to the test and control vials before adding the shrimps to enhance the solubility of test materials. LD_{50} values were determined at 95% confidence intervals by analyzing the data on a computer loaded with a "Finney Programme." The concentration at which it could kill 50% larvae (LD_{50}) was determined. LD_{50} values below 200ppm are generally considered as significant according to (Ghareeb et al., 2014).

Statistical analysis:

The obtained data were presented as means ± S.D. Statistical analysis for all the assays obtained data carried out using Microsoft Excel Program. Student Test was used to determine the significant difference between the control and the plant extract at level of $P < 0.05$.

4. Results and Discussion:

The fruits of *Capsicum fruitescens* (family: Solanaceae) were screened for antimicrobial activity against 3 Gram negative bacteria *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeroginosa* and one Gram positive bacteria *Staphylococcus aureus* and one fungi *Candida albicans* using the cup plate agar diffusion method. The extract obtained from the fruits of *Capsicum frutescens* exerted pronounced activity against two tested bacteria strains (*Klebsiella pneumoniae* and *Escherichia coli*) as indicated by diameter of growth inhibition zones that varied from 17 and 23 mm respectively. As can be seen from Table 1, Figure 1 the extract of *Capsicum fruitescens* dissolved in methanol (1:10) showed high activity against *Escherichia coli* 23mm at the highest concentration checked (200 mg) and moderate activity 17mm against *Klebsiella pneumoniae*. Therefore this result showed that the tested extract inhibited the growth of some microorganisms though the sensitivities of microorganisms varied. This result is in agreement with that reported previously (Nascimento et al., 2014). The methanol extracts of *C. frutescens* showed the highest activity against the tested microorganism. This result is in agreement with that reported by (Vargas-Hernández et al., 2017) all the pepper varieties' extracts tested showed antibacterial properties against Gram- positive, Gram-negative bacteria and fungi used in this investigation.

Table 1: Antimicrobial activity of *Capsicum frutescens* against tested microorganisms

N	Tested microorganisms	Concentrations (mg/ml)

o.		100	50	25	12.5	6.25
		Zone of Inhibition in mm ± (SD)				
1	*Escherichia coli*	20±0.5	18±0.5	14±1	11.33±0.5	00
2	*Staphylococcus aureus*	00	00	00	00	00
3	*Klebsiella pneumonia*	13.6±0.5	11.6±0.5	00	00	00
4	*Pseudomonas aeruginos*	00	00	00	00	00
5	*Candida albicans*	00	00	00	00	00

The results showed that *C. fruitescens* extracts inhibited *E. coli* within the tested range of concentration (200-12.5mg/ml) (MIC =12.5mg/ml) whereas they were inhibited *K. pneumoniae* at 100 and 5o mg/ml only (MIC =5o mg/ml). On the other hand No inhibitory effects were shown against *S. aureus*, *P. aeroginosa* and *C. albicans*.

Comparison of the observations given in Tables 1 showed that, the methanolic fruits extract of *Capsicum fruitescens* inhibited *Klebsiella pneumoniae* higher than 5µg/ml exhibited by Ampicillin and less than 5 µg/ml of Gentamicin. It also inhibited *E.coli* higher than that of 20µg/ml Ampicillin. It inhibited *E. coli* less than that of 10µg/ml Gentamicin. While, extracts didnot inhibits of *Candida albicans*.

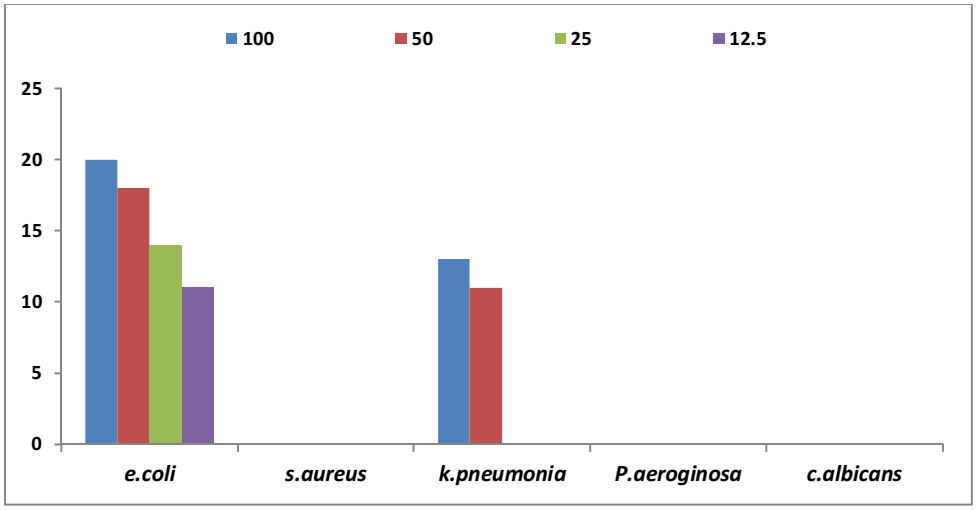

Figure 1: Minimum inhibitory concentrations of *Capsicum fruitescens* extract against tested microorganisms.

Table 2: Antibacterial and antifungal activities of reference drugs against the tested microorganisms:

Drugs	Concentrations (mg/ml)	Tested bacteria used (M. D. I. Z mm)			
		S. a	*E. c*	*K. p*	*Pa. a*
Ampicillin	40	25	-	35	16
	20	20	-	26	13
	10	18	-	25	12
	5	15	-	21	-
Gentamicin	40	35	32	26	23
	20	33	30	24	22

	10	30	17	21	21
	5	28	-	20	19
Tested antifungal used (M. D. I. Zmm)					
Clotrimazole	Concentrations (mg/ml)	***Candida albicans***			
	40	42			
	20	40			
	10	33			
	5	30			

The methanolic extracts screening for phytochemical compounds are seen in Table 3. Alkaloids, flavonoids, phenols, tannins, Anthroquinoles, terpenoids and saponins were found in the methanolic extract with the exception of flavonoids and phenols which for absent in *Capsicum fruitescens* extracts.

Table 3: phytochemical screening of *Capsicum fruitescens* methanolic fruits extract.

Test	**Tannins**	**Saponin**	**Flavonoids**	**Alkaloids**	**Phenols**	**Anthroquinoles**	**Terpenoids**
Capsicum fruitescens	+	+	-	+	-	+	+

The Cytotoxicity of methanolic extracts of *Capsicum frutescens* was performmed by using Brine Shrimp (*Artemia saline* L) leathality bioassay at different concentrations (1000, 100, 10 µg/ml). The results are shown in Table 3.

LD_{50} of *Capsicum frutescens* extracts was found to be 64.68µg/ml and this is considered as highly toxic according to Bussmann *et al* (2011). They stated that LD_{50} values below 249µg/ml are considered as highly toxic, 250– 499 µg/ml as medium toxicity and 500–1000 µg/ml as light toxicity. While values above 1000 µg/ml are regarded as a non-toxic. Which were considered to be highly toxic as the number of dead Brine shrimps was higher than the number of alive Brine shrimps.

Table 4: Cytotoxicity screening of *Capsicum frutescens.*

Name of Extracts	**Total Number of Shrimps**	**Concentrations (µg/ml)**						**LD_{50} (µg/ml)**	**The degree of toxicity**
		1000	**100**	**10**	**1000**	**100**	**10**		
		Number of dead			**Number of survive**				
Capsicum frutescens	10	09	06	02	01	04	08	64.7	High toxic

5. Conclusion

The extract of *Capsicum fruitescens* showed various degree of inhibitory activity against the tested Gram positive, and Gram negative organisms tested. These encouraging results indicated that, the plant might be exploited as natural sources of antibiotics for the treatment of several infectious diseases. This studied plant used has proven their toxicity by using Brine Shrimp lethality test. The antimicrobial activity of the extract from of *Capsicum fruitescens* may be directly associated with their major constituents or the presence of synergy between the major and minor constituents within these extracts.

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