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ANTIBACTERIAL ACTIVITY OF CITRUS SINENSIS PEEL AGAINST ENTERIC PATHOGENS

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Abstract: The *in vitro* antibacterial activity of extract of peel from *Citrus sinensis* (Sweet orange) was investigated. Two extraction methods were used, hot and cold method. The screening of antibacterial activity was done by agar diffusion technique and Minimum Inhibitory Concentrations (MICs) by agar dilution technique. The extract were tested for antibacterial activity against various diarrhoeal pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Shigella flexneri* and *Vibrio cholerae*. The extract showed various levels of antibacterial activity on different test microorganisms. Future studies are in process to isolate the active principles responsible for the activity.

Keywords: Diarrhoea, *Citrus sinensis peel*, antibiotic resistance, gastrointestinal infection, Solvents, Extraction.



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INTRODUCTION

Diarrhoea is the passage of 3 or more loose or liquid stools per day, or more frequently than is normal for the individual. It is usually a symptom of gastrointestinal infection, which can be caused by a variety of bacterial, viral and parasitic organisms. Infection is spread through contaminated food or drinking-water, or from person to person as a result of poor hygiene. Severe diarrhoea leads to fluid loss, and may be life-threatening, particularly in young children and people who are malnourished or have impaired immunity¹. The extensive use of the antibiotics to control diseases has led to the emergence of multidrug resistance². It was warned by the World Health Organization that those multiple antibiotic-resistant pathogens would very likely bring the world back to the pre-antibiotic era. This clearly highlights the need for new antibacterial agents with fundamentally different modes of action than that of traditional antibiotics. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity³.

Citrus is one of the most important commercial fruit crops grown in all countries of the world. Citrus is a term commonly used for genus of flowering plants in the family *Rutaceae* originating in tropical and sub-tropical south-east regions

of the world⁴. Citrus fruits are commonly consumed because they contain a high amount of vitamins, minerals and antioxidant compounds, such as flavonoids. Flavonoids are a family of phenolic compounds that have many biological properties, including hepatoprotective, antithrombotic, antibacterial, antiviral and anticancer activity. These physiological benefits of flavonoids are generally thought to be due to their antioxidant and free radical scavenging properties⁵. The endocarp of orange peel is rich in soluble sugars and contains significant amounts of vitamin C, pectin, fibers, different organic acids and potassium salt which give the fruits its characteristics citrus flavor⁶. It contains volatile essential oils which are said to be effective in inhibiting microbial growth and in disinfecting wounds; among its other medicinal capabilities. There are strong evidences showing that the essential oil of *C. sinensis* have larvicidal, repellent and fumigant activities against *Aedes aegypti* L. mosquitoes⁷. It has also been used as antimicrobial⁸, antioxidant⁹, carminative, insect repellent, antibacterial, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent¹⁰.

Peel waste are highly perishable and seasonal, is a problem to the processing industries and pollution monitoring agencies. There is always an increased attention in bringing useful products from waste materials and citrus wastes are no

exceptions. Suitable methods have to be adopted to utilize them for the conversion into value-added products¹¹. In current Citrus industry, emphasis are laid only on orange fruits harnessed and marketed fresh or as processed (and canned) juice, while fruit peels produced in great quantities during the process are mainly discarded as waste. For this reason, researchers have focused on the utilization of citrus products and by-products^{12, 13}.

Materials and methods

Plant collection and Preparation of the plant extract:

Fresh sweet orange fruits were collected randomly from the shops of Chennai, India. The peels were separated and washed twice with double distilled water and the surface sterilized using 70% ethanol. The peels were shade dried for 3 to 4 weeks. The peels were then ground into coarse powder using a mixer.

Preparation of Crude Extracts:

The crude extracts were prepared by two methods. The powdered pericarp was dissolved in different solvents. The solvents used were non polar as well as polar (methanol, ethanol, acetone, chloroform, ether and water).

In cold method of extraction (for heat-labile compounds) 1 gram of powdered peel extract is added to 10 ml of the solvent and incubated in a shaker incubator at 250 rpm at 37°C for 24 hours. Supernatant is

filtered and dried in air at room temperature. In hot method (for heat-stable compounds) 1 gram of powdered peel extract is added to 10ml of the solvent and incubated in a shaker incubator at 250 rpm at 37°C for 3-4 hours. It is placed in a water bath at 60°C for 2 hours. Supernatant is filtered and dried in air at room temperature¹⁴.

Screening of fruit peels for in vitro antibacterial activity using disc diffusion method :

Disc (5 mm) prepared from Whatmann No. 1 filter paper was sterilized and impregnated with 20 µl of various crude solvent extract (Conc: 100 mg/ml). Standard antibiotics as per the organism tested were used as positive control. Respective solvent without plant extracts are used as negative control¹⁵.

Bacterial strains used in the study

Bacterial strains used in the study were the isolates obtained from clinical samples from Institute of Communicable Disease (Cholera Hospital, Chennai, India). All the bacterial cultures, viz *Esherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Shigella flexneri* and *Vibrio cholerae* were used in the study. The reference strains of bacteria were maintained on nutrient agar slants, sub cultured regularly (every 30 days) and stored at 4°C.

Inoculum preparation:

Broth cultures of the diarrhoeal pathogens were prepared by transferring two or three isolated colonies to Nutrients broth and incubating the culture at 37°C for 4 to 6 hours in the incubator. The culture was checked for turbidity by comparing with McFarland Standard (0.5). This turbidity was equivalent to approximately $1-2 \times 10^8$ colony-forming units per millilitre (cfu/ml). This 4-h grown suspension was used for further testing.

Determination of antibacterial activity:

Antibacterial activity was tested on Muller-Hinton Agar. The test inoculums were then swabbed uniformly onto the MHA plates. A lawn culture of the organisms to be tested was made. The prepared disc were placed on the plate in a way such that each disc was at least 20 mm from one another. The plates were then incubated at 37°C for 18 to 24 hours. The zone of the incubation

around each disc both in the experiment and the control were measured.

Microbroth Dilution Assay:

The broth dilution assay was performed on a microtitre plate. Doubling dilutions of the raw juice was prepared in Mueller Hinton Broth with the first one considered as neat. Bacterial cultures of 10^6 -cfu/ml dilutions were prepared with Mc Farland Standard (0.5) and 10 microlitre were added to each well of the microtitre plate and mixed well. The microtitre plates were incubated at 37°C overnight and a loopful of the culture was streaked onto Nutrient Agar plates. Plates were incubated at 37°C overnight. The growth/no growth pattern of the organisms corresponded to the MIC of the peel extract. Final concentrations of the investigated extracts were 50 mg/ml, 37.5mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml.¹⁶

Result and discussion:

Sample Tested: Crude extract of the orange fruit peels
 Solvent Employed: Volume in each disc: 20 µl / disc
 Concentration of Disc: 100 mg / ml

Organism	Ethanol		Methanol		Acetone		Chloroform		Ether		Water	
	H	C	H	C	H	C	C	H	H	C	H	C
a	16±0.5	14±0.2	11±0.5	14±0.1	14±0.2	12±0.5	15±0.4	14±0.4	10±0.1	-	-	-
b	13±0.1	14±0.1	11±0.4	14±0.5	13±0.4	14±0.5	14±0.7	13±0.4	11±0.3	12±0.4	-	-
c	21±0.5	15±0.5	13±0.1	14±0.5	15±0.1	12±0.5	13±0.5	-	-	9±0.5	-	-
d	18±0.3	17±0.2	15±0.3	12±0.5	14±0.6	16±0.5	13±0.3	13±0.6	9±0.2	11±0.1	-	-
e	13±0.4	18±0.2	13±0.2	13±0.5	17±0.3	14±0.5	12±0.3	11±0.2	11±0.5	12±0.2	-	-
f	16±0.1	13±0.1	16±0.3	12±0.5	15±0.2	13±0.5	11±0.5	-	9±0.3	9±0.4	-	-
g	14±0.1	18±0.6	11±0.5	11	16	14	13	14±0.2	13±0.4	11±0.4	-	-
h	13±0.5	14±0.3	15±0.4	15±0.4	19±0.3	15±0.2	9±0.5	13±0.4	12±0.5	10±0.5	-	-

±standard deviation (number of trials, 3)

H – Hot Method; C – Cold Method; mm – milli meter.

Table- I Table showing the zone of inhibition of different solvent extract of Citrus sinensis peel against enteric pathogens.

a-Escherichia coli, b-Klebsiellae pneumoniae, c-Pseudomonas aeruginosa, d- Salmonella typhi, e-Salmonella paratyphi A, f-

Salmonella paratyphi B, g- Shigella flexneri, h-Vibrio cholerae.

Microbroth dilution assay:

Table- II Microbroth dilution assay for the extract which showed highest activity against the pathogens.

ORGANISM USED	SAMPLE USED	CONCENTRATION OF THE EXTRACTS IN MG / ML							
		100	50	25	12.5	6.25	3.125	1.56	
<i>Pseudomonas aeruginosa</i>	HOE	+	+	+	+	-	-	-	
<i>V. cholerae</i>	HAE	+	+	+	+	-	-	-	

- → Indicates no inhibition of bacterial growth CPE-Ethanollic Cold

HOE – Ethanollic Hot Orange peel extract showed a MIC value of 12.5 mg / ml

HAE –Acetonic Hot Orange peel extract showed a MIC value of 12.5 mg / ml .

In the preliminary screening procedure using agar disc diffusion assay, *Citrus sinensis peel* showed good antibacterial activity against the wide spectrum of the pathogens tested. The concentration of the crude extract was 100mg/ml. The results were summarized in Table 1 and Fig I-IV. Extract of orange peel exhibited antibacterial against *E. coli*-10-16mm, *K. pneumoniae*-11-14mm, *P. aeruginosa*- 9-21mm, *S. typhi*- 9-18mm, *S. paratyphi A*-11-18mm, *S. paratyphi- B* 9-16mm, *S. flexneri* 11-18mm and *V. cholerae* 9-19mm. Ethanol hot extract showed highest zone of 21 mm against *P. aeruginosa* followed by Hot acetone extract which showed an activity of 19 mm against *V.cholerae*. Different solvent produced zone of inhibition ethanol-13-21mm, methanol-11-16 mm, acetone-12-19mm, chloroform 9-15mm, ether 9-13mm. No activity is observed using aqueous extract. Among the solvents used in the extraction procedures, ethanol was found

to be highly effective extracting the antibacterial components, followed by acetone, methanol, chloroform, ether, water. The water solvent was ineffective in extracting the components of the fruit peels.

Diarrhoeal diseases have been recognised since the beginning of civilization and remain one of the most prevalent public health problems of today. About two-thirds of the world population live in areas regarded as underdeveloped and it is estimated that over 1.3 billion cases of diarrhoeal illness occur each year in the underdeveloped countries. Of these over 2.7 million deaths occur in children¹⁷. The resistance of enteropathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as in developed countries. Resistance has emerged even to newer, more potent antimicrobial agents¹⁸. The emergence of

antimicrobial resistance to members of the *Enterobacteriaceae* family is posing major problem in the management of bacterial infections¹⁹.

In this study, in agar well diffusion the of orange peel extract showed activity against all the pathogens tested. Nannapaneni et al (2008)²⁰ showed that natural compounds of *citrus sinensis* peel have inhibitory effects in different strains of *Escherichia coli*, *Salmonella* and some food pathogenic bacteria which is in accordance with our study. *Citrus sinensis* peel extracts has appropriate antimicrobial effects on gram positive and gram negative bacteria²¹. The essential oils from *C. sinensis* peels shows antibacterial activity against *S. aureus* (16mm), *S. typhimurium* (15mm), *Enterobacter aerogenes* (12mm), *B. subtilis* (11mm), *E. coli* (10mm) and the tested oil has shown nearly equal antibacterial effect on both Gram negative and positive bacteria *B. subtilis*, *S. aureus* and *E. coli*, *S. typhimurium*, *E. aerogenes*²². The antibacterial activity of essential oil from *C. sinensis* against *E. coli*, *S. aureus*, *K. pneumoniae*, *B. cereus*, *Micrococcus luteus*, *Proteus vulgaris*, *Mycobacterium smegmatis*, *Listeria monocytogenes*, *P. aeruginosa* has been reported²³. The ethyl acetate extract of *Citrus* peel showed antibacterial activity against *S. aureus* and *E. coli*²⁴. The four citrus fruit peel extract used *Citrus sinensis* (Orange), *Citrus limon* (Lemon), *Citrus aurantifolia* (Lime) and *Citrus limetta* (Sweet lime) against the common gastrointestinal pathogens

showed high antimicrobial activity and lesser side effects than the synthetic ones used to combat gastrointestinal pathogens²⁵. Based on the results of Pourhossein et al., (2012),²⁶ using dried *citrus sinensis* peel reduced *Escherichia coli* and coliforms count.

According to the present study, preparing an extract with organic solvent was shown to provide a better antibacterial activity in accordance with the results obtained by Nair et al., 2005²⁷. In this study, it is observed that the potency of Citrus fruit peel is enhanced by the type of solvent used indicating that there are some active ingredients in orange peel which have high antimicrobial effect but which would not be released except when orange fruit peel is used in conjunction with a particular solvent. In the microbroth dilution assay to determine the minimum inhibitory concentration of the extract *Citrus sinensis* peels showed an MIC value of 12.5 mg/ml against most of the bacterial pathogens tested.²⁸

This study revealed that the extract of *Citrus sinensis* contains active ingredients with various medicinal properties which qualify them for medicinal use against diarrhoeal infections. Further considering the cost and availability, it can be considered and used as a cheap alternative to substitute antibiotics. However, taking into consideration, safety aspects, toxicity and isolation of active compounds and phytochemicals, further studies need to be

carried out to unravel the search for bioactivity contributing to the antimicrobial

activity of the orange peel to assist in the treatment of diarrhoea.

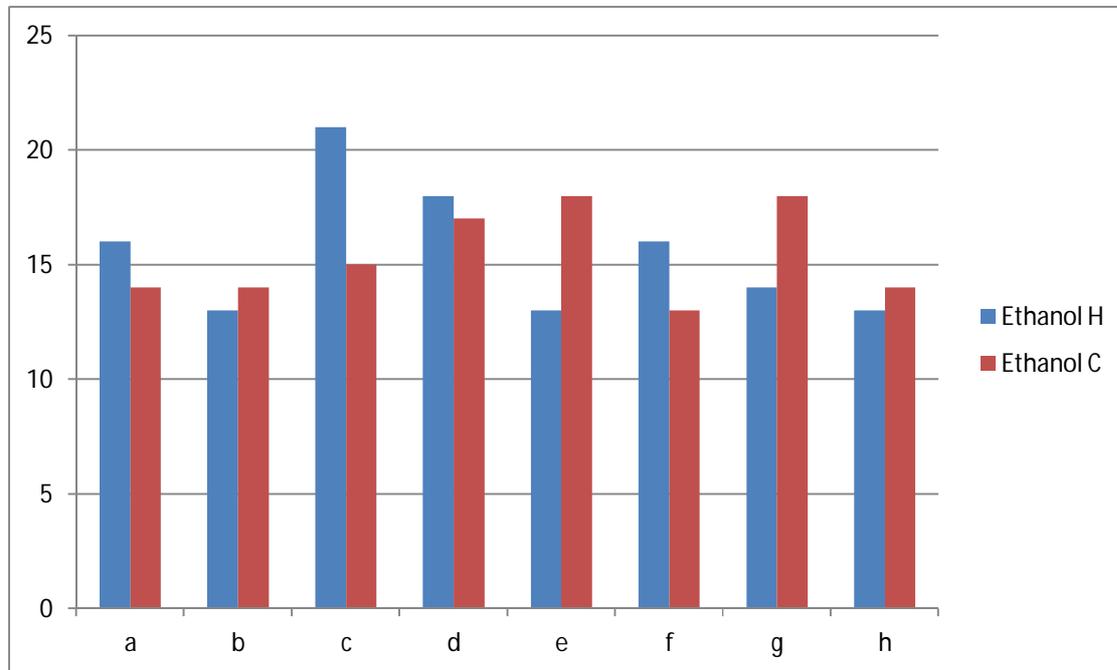


Fig.1. Inhibition zones produced by the hot and cold ethanolic extract of orange peel against all pathogens

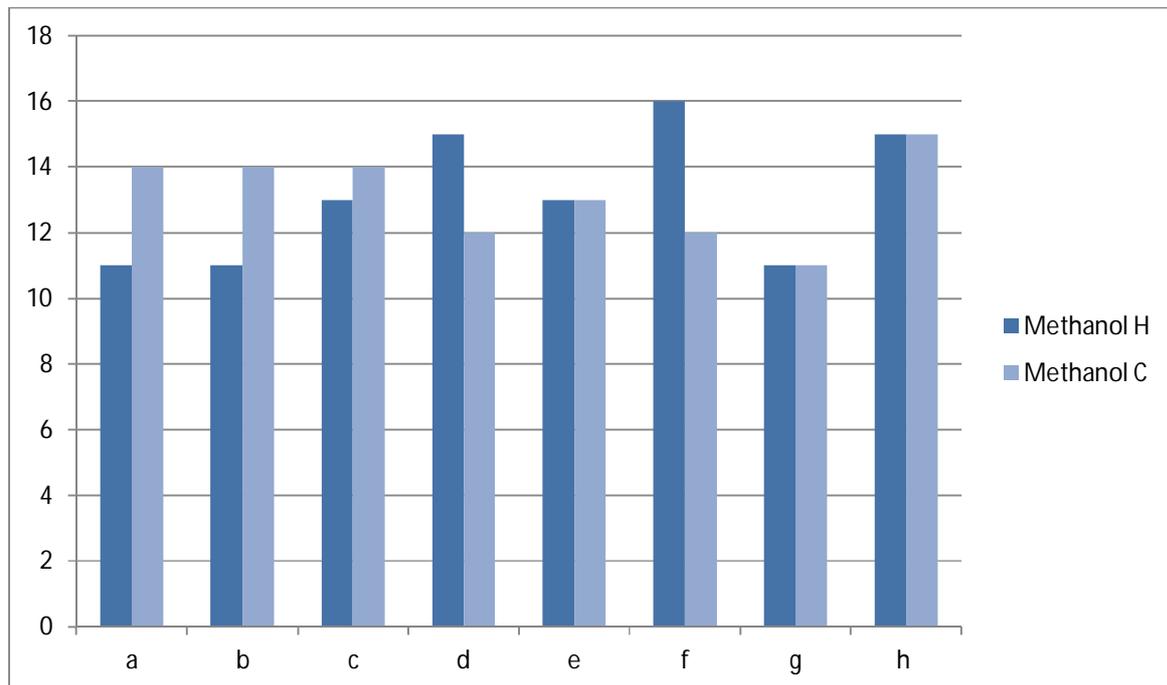


Fig.2. Inhibition zones produced by the hot and cold methanolic extract of orange peel against all pathogens

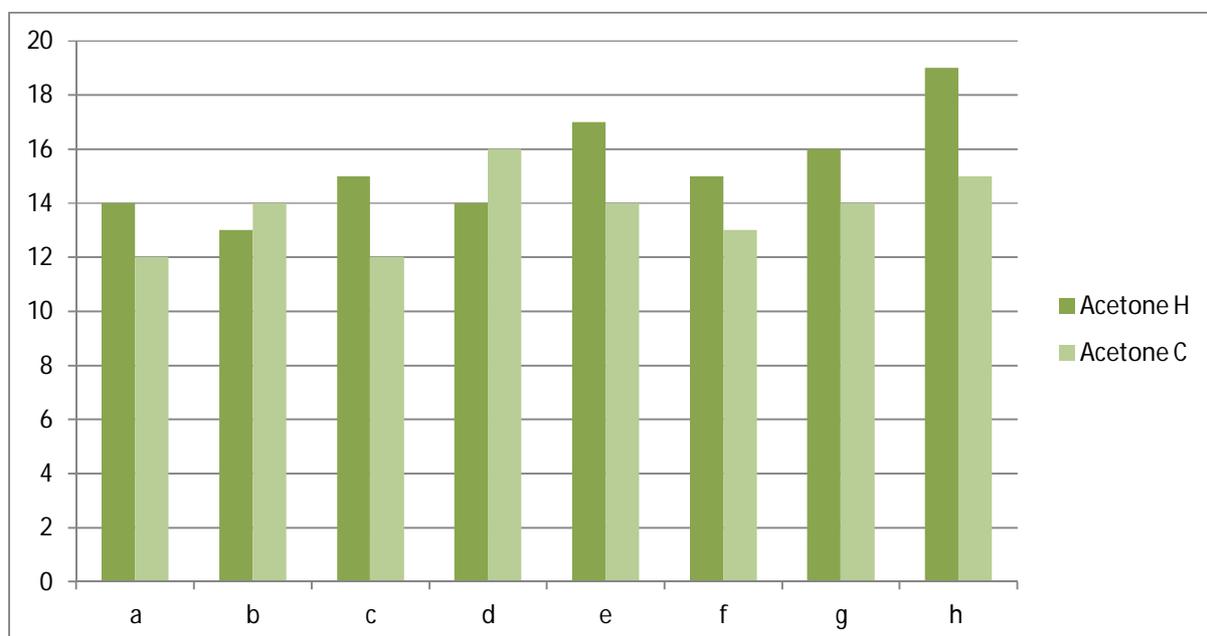


Fig.3. Inhibition zones produced by the hot and cold acetone extract of orange peel against all pathogens

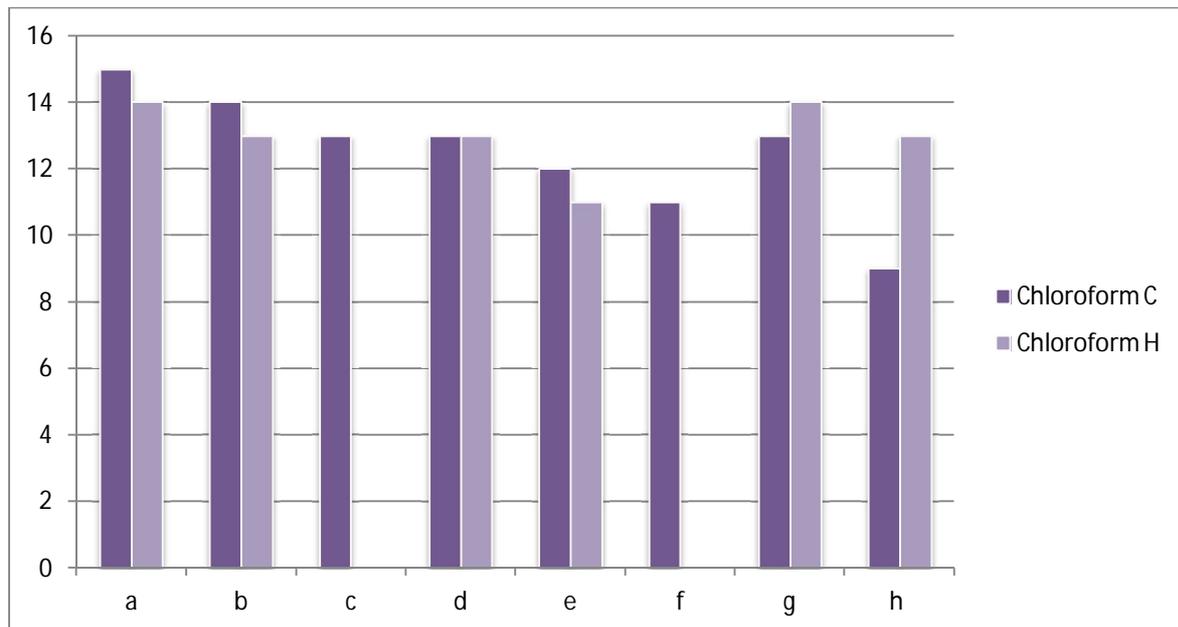


Fig.4. Inhibition zones produced by the hot and cold chloroform extract of orange peel against all pathogens

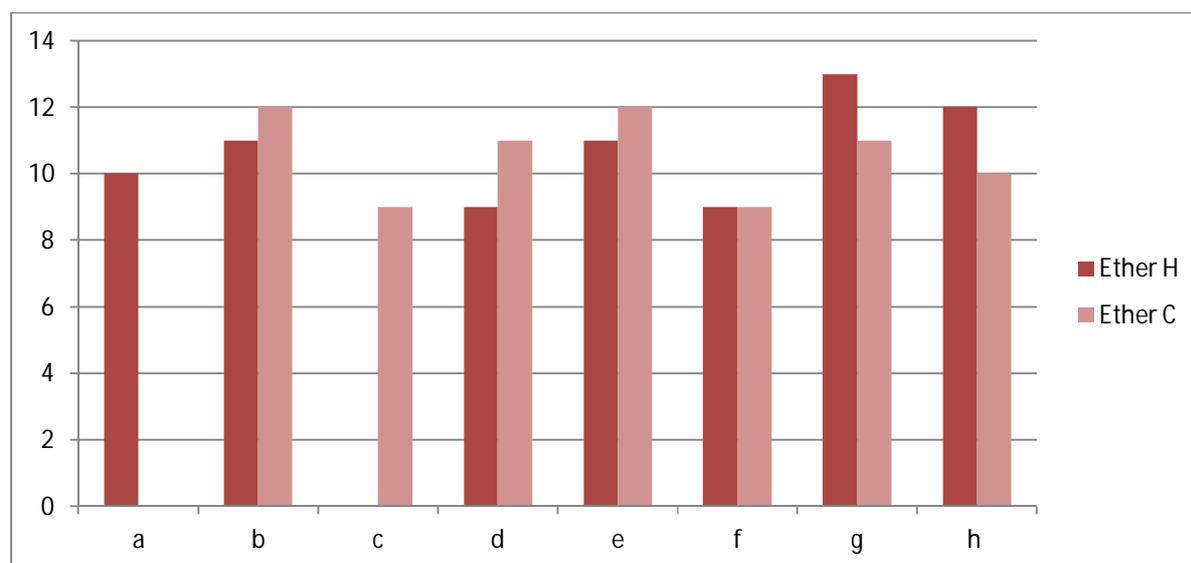


Fig.5. Inhibition zones produced by the hot and cold ether extract of orange peel against all pathogens

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