International Journal of Advance Research in Science and Engineering
Volume No.07, Special Issue No.04, March 2018
www.ijarse.com
IJARSE
ISSN: 2319-8354

In Vitro Evaluation of Antimicrobial Activity of the Flower Juice OF *Hibiscus rosa-sinensis* for the Remedy of Gastric Complaints

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ABSTRACT

The flowers of Hibiscus rosa-sinenis are known for its medicinal properties ranging from Anidiabetic,Anticancer,Antimicrobial.In the present study various extracts of flower were made using soxlet apparatus for evaluating the antimicrobial activity by disc diffusion method against E.Coli,S.Typhi. Phytochemistry of Hibiscus rosa-sinenis after thorough literature survey reveals various important metabolites such tannins,alkaloids,flavonoids,steroids.Among all the extracts only Chloroform extract Shows maximum zone of inhibition.

***Keywords*: *Hibiscus rosa-sinenis*, Malvaceae, antimicrobial activity, zone of inhibition**

1. Introduction

The word diarrhoea is derived from the *greek* word "dia" through and "rheo" flow.Diarrhoea is commonly defined as condition having at least three bowel movement per day in some form.The long term consequences of which result in dehydration, tachycardia.[1]

1.1. The most common factors that lead to diarrhoea

- Osmotic –lactase deficiency
- Secretory –cholera, E. coli
- Deranged intestinal motility-thyrotoxicosis
- Altered mucosal morphology- viral gastroenteritis
- Allergic diarrhoea –food allergy
- Drug induced diarrhoea
- Carcinoid and medullary carcinoma of thyroid

The common approach for treating diarrhoea includes antibiotic drugs and oral/parenteral rehydration therapy[2]

Hibiscus rosa-sinenis belongs to family Malvaceae and is commonly known as china Rose. Leaves are ovate or ovate-lanceolate whereas flowers are pentamerous and complete in nature. In India, the flowers are obtained from southern part. The corolla of the flower consists of five petals which are red in colour.[3, 4]. Many chemical constituents such as cyanidin, quercetin, hentriacontane, calcium oxalate, thiamine, riboflavin, niacin and ascorbic acids have been isolated from this plant. Resistance towards antibiotics having become widespread among bacteria and fungi, new class of antimicrobial substances are urgently required. There are several studies which reveal the presence of such compounds with antimicrobial properties in various plant parts.[5]
The present study has been designed to determine the role of flower in *Hibiscus rosa-sinenis* extract in the in vitro antibacterial activity against human pathogens viz., Gram positive bacteria (*S. aureus*),*Streptococcus*, *Bacillus subtillis* (*B. subtillis*) and Gram negative bacteria (E. coli), (P. aeruginosa) and *Salmonella sp*.

2. Materials and methods

2.1. Collection of plant and extraction

The sample of Hibiscus rosa-sinenis were identified and collected. The collected sample was dried at ambient temperature and then powdered with mortar and pestle. To a certain amount of powdered sample water was added in the ratio 1:5 in a beaker and then placed in a water bath at temperature of 90°C for extraction up to the extent the filtrate becomes half the initial volume. The concentrate will be filtered through muslin cloth and the extract was collected in a beaker and the process of extraction will be repeated twice and the extract was then dried at a temperature of 90°C.

2.2. Extraction of components using different types of extraction methods

2.2.1. Cold extraction

Accurately weighed 200gm of fresh flower part of Hibiscus Rosa sinensis and then added water to in the ratio of 1:5 in a grinder.After grinding the sample is filtered and the filtered juice was placed in a cold storage for a period of 24-72 hours

2.2.2. Hot extraction

Accurately weighed 286gm of flower part of Hibiscus rosa sinenesis and then placed in an oven for drying at a temperature of 60°C and was left as such until complete drying of sample was done. After complete drying the sample weighs 32.3gms.The sample was left for grinding and after grinding it weighs 32.1gm.Now weigh 25 gm of sample and put in a 250 ml flask and added water to it in the ratiof 1:5.The flask containing the sample was kept in a water bath for 2 hrs at a temperature of 80 c.The sample was filtered via muslin cloth and the filtrate was then kept for centrifugation for 10 minutes at 7500 rpm.The supernatant so obtained was kept in a waterbath at a temperature of 80 c for drying. The extract obtained after filtration and centrifugation was left for

drying in an open air and used for soxlet extraction.The various solvents which are used for extraction are shown in the table(1) with the quantity of solvent used and time period.

Table 1: solvents used with Total Extraction time

***Hibiscus Rosa Sinensis* flower extract**						
Solvent	**Solvent quantity**	**Extraction tim**	**No of Cylcle's/Reflex**	**Pre wt**	**Post wt**	**% age yield (gm)**
Hot water(Aq)	**150ml**	**3hrs**	**-**	**25gm**	**12gm**	**48%**
Methanol	**300ml**	**4hrs**	**3**	**12gm**	**5gm**	**41%**
Chloroform	**250ml**	**3hrs**	**3**	**6gm**	**4gm**	**66%**
Pet. Ether	**250ml**	**4.5hrs**	**4**	**2gm**	**1gm**	**50%**

3. Phytochemical screening

After complete drying, the extract was analysed for various phytoconstituents like Alkaloids,Tanins,Saponins,Flavonids and Glycosides.While analyzing the extracts the concentration of extract was kept at 1mg/ml[6]

3.1. Test for sugars (Molisch's test)

10g of α-napthol was dissolved in 100ml of 95% ethyl alcohol to get the Molisch's reagent. Now two drops of molisch's reagent was added to 1 ml of sample. 1 ml of sulphuric acid was added to this solution from the sided of the inclined tube, so that beneath the aqueous solution an acid layer is formed without mixing with it.At the common surface of liquids a red brown ring appears which reveals the presence of sugars

3.2. Test for saponins (Foam test)

To the 1ml of sample, added 2ml of water in a test tube followed by shaking for 15 minutes in a test tube. The formation of foam layer at the top of test tube indicates the presence of saponins.

3.3. Test for steroids

The sample was dissolved in 10ml of chloroform in a test tube followed by adding equal volume of sulphuric acid by sides. The upper layer turns red whereas the sulphuric acid showed yellow with green fluorescence indicates presence of steroids

3.4. Test for Flavonoids

Few drops of 10% NaOH solution was added 1ml of sample in attest tube,Yellow colour appears in the test tube.Added few drops of dilute acid it becomes colourless indicates the presence of flavonoids.

3.5. Test for Alkaloids (Mayer's reagent)

Mayers reagent which is prepared by adding 3g of potassium iodide and 1.3g of mercuric chloride in 100ml of distilled water. Now to the 1ml of extract few drops of Mayers reagent was added. The presence of alkaloid was indicated by formation of cream colour precipitate

Table 2: Phytochemical screening Results

Phytochemical extract Tests	Hibiscus rosa- sinensis flower extract					
	Hot (Aq. Extract)	Methanolic extract	Chloroform extract	Pet. Ether extract	Flower juice	Fresh flower juice
Molisch's test	++	++	++	+++	-	+
Saponin's (Foam test)	-	-	-	-	-	-
Steroids	+	+	+	+	-	+
Flavonoids	-	-	+	+++	-	-
Tannins	++	+	-	-	-	-
Alkaloids	-	-	++	+	-	
Glycosides	+	-	-	-	-	-

4. Determination of Total phenolic content

Phenolic contents of all extracts prepared in DMSO @ 10mg/ml were estimated, using method of Taga[7]. Briefly, 100 µl aliquots of sample were mixed with 2.0 ml of 2% Na_2CO_3 and allowed to stand for 2 min at room temperature (RT). After incubation, 100 µl of 50% Folin Ciocalteau's phenol reagent was added, mixed thoroughly and allowed to stand for 30 min at RT in dark conditions. Absorbance of all the solutions were measured at 720 nm using Genesys 10 UV Spectrophotometers (Spectronic Unicam). Phenolic contents were expressed as Gallic acid equivalent per gram (GE/g)

Table 3: Absorbance value at different concentrations of standard

Sr. no.	Concentration (µg/ml)	Absorbance (721nm)
1	20	0.451
2	40	0.803
3	60	1.28
4	80	1.32
5	100	1.8

Table 4: Absorbance curve of standard

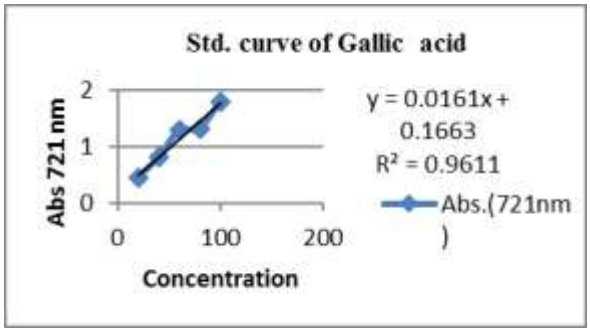

Table 5: Absorbance curve of sample

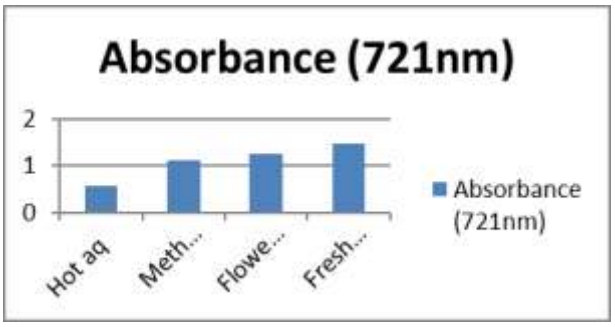

Table 6: Absorbance value at Different concentrations of sample

Extract	Absorbance (721nm)
Hot aq	0.561
Methanol	1.123
Flower Juice	1.239
Fresh Flower Juice	1.456

5. Evaluation of Antioxidant activities

Total antioxidant activities of extracts (10 mg/ml of DMSO) were determined using method described by [8] Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Reaction mixture was incubated at 950C for 90 min in a water bath. Absorbance of samples mixture were measured at 695 nm using Genesys 10 UV Spectrophotometers (Spectronic Unicam). Total antioxidant activity was expressed as number of equivalents of ascorbic acid.

Table 7: Absorbance curve of sample

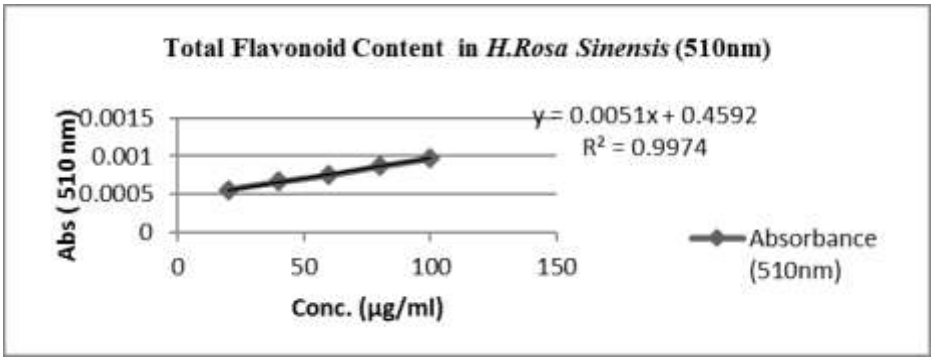

Table 8: Absorbance value at Different concentrations of sample

Sr. No.	Conc. (µg/ml)	Abs. (510nm)
1	20	0.56
2	40	0.67
3	60	0.75
4	80	0.87
5	100	0.967

6. Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

Radical scavenging ability of extracts was measured according to Mensor[9] with little modifications. Briefly, one ml from 0.3mM methanolic solution of DPPH was added to 2.5 ml of samples containing different concentrations of herbal formulation extract. Samples were kept at room temperature in dark conditions and after 30 min optical density (OD) was measured at 518 nm. Antiradical activity (AA) was determined using following formula:

AA% = 100 – {[(Abs. Sample – Abs. Empty Sample) × 100] / Abs. control}

Where empty samples – 1 ml ethanol + 2.5 ml from various concentrations of all C. iberica extract; control sample – 1 ml 0.3mM DPPH + 2.5 ml methanol. OD of samples, control and empty samples were measured in comparison with methanol.

Table 9: Absorbance curve of sample

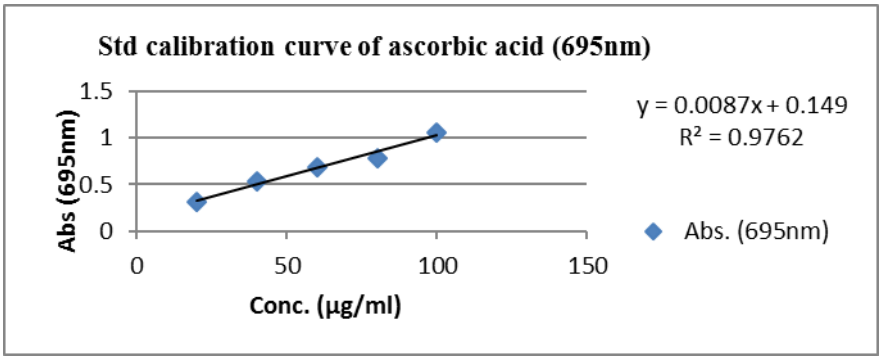

Table10: Absorbance value at Different concentrations of sample

Sr. No.	Conc. (µg/ml)	Abs. (695nm)	Mean ±Error
1	20	0.31	0.674 ±1.988
2	40	0.53	
3	60	0.69	
4	80	0.78	

Table 11: Absorbance curve of sample

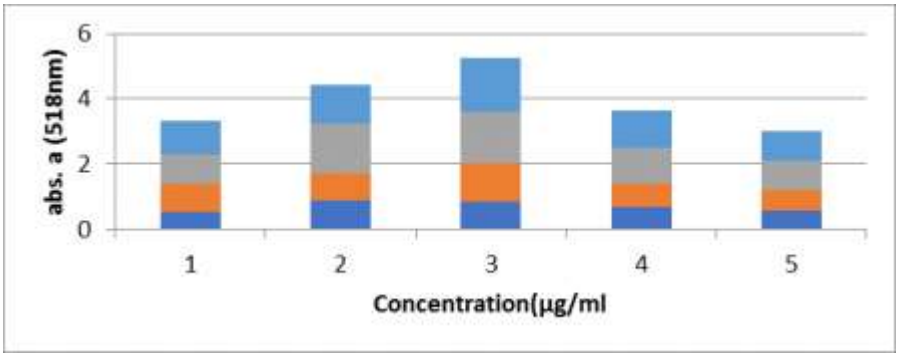

Table 12: Absorbance value at Different concentrations of sample

Experimental plant name	Solvent of Extraction	Absorbance (518 nm) at different Conc. of Extracts				
		2.5	5	7.5	15	30
H.Rosa sinensis	**Hot Aq.**	0.415	0.516	0.619	0.703	0.899
	Methanol	0.288	0.238	1.165	0.713	0.689
	Chloroform	0.926	1.424	1.688	1.202	0.493
	Flower juice	0.346	1.341	1.548	1.146	0.799
	Fresh Flower juice	0.488	1.512	1.466	1.413	0.512

7. Detection of hydroxyl radicals by Deoxyribose assay

The assay was performed as described by Halliwell[10] with minor modifications. Required solutions were freshly prepared for this study. One ml of reaction mixture contained 100 µl of 28mM 2-deoxy-D- ribose (dissolved in KH2PO4–K2HPO4 buffer, pH 7.4), 500 µl solution of various concentrations of the extract, 200 µl of 200 µM FeCl3 and 1.04mM EDTA (1:1 v/v), 100 µl H2O2 (1.0mM) and 100 µl ascorbic acid (1.0mM). After 1 hour of incubation at 370C, extent of deoxyribose degradation was measured by thiobarbituric acid (TBA) reaction. One ml of TBA (1% in 50mM NaOH) and 1.0 ml of TCA were added to reaction mixture and tubes were heated at 1000C for 20 min. After cooling, absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). All the tests were performed three times and mean was calculated. Ascorbic acid was used as a positive control. Percent inhibition in hydroxyl radical was calculated by the following expression:

$$I (\%) = [(A0 - A1) / A0] \times 100,$$

Where A0 is absorbance of control and A1 is absorbance of sample. Data obtained at each point was the average of three measurements.

Table 13: Absorbance curve of sample

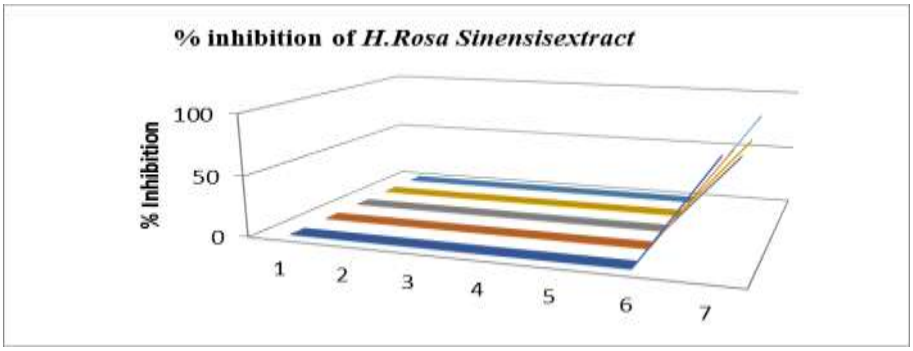

Table 14: Absorbance value at Different concentrations of sample

Experimental Plant Name	Solvent of Extaction	Absorbance (532nm) at different Conc. of Extracts				
		2.5	5	7.5	15	30
H.Rosa sinensis	Hot Aq.	0.214	0.101	0.217	0.218	0.089
	Methanol	0.316	0.254	0.189	0.061	0.233
	Chloroform	0.233	0.144	0.147	0.244	0.127
	Flower juice	0.135	0.341	0.169	0.290	0.167
	Fresh Flower juice	0.389	0.080	0.190	0.190	0.180

8. In vitro antimicrobial profile extract

8.1. Bacterial strain

Staphylococcus aureus and E. coli were used for the activity

8.2. Preparation of the extract

The stock solution of extract was prepared in 10% DMSO in water with final concentration 10 mg/ml.[11] Each fraction along with stock solution of extract was sterilized by filtration through 0.22um sterilizing Millipore express filter.Discs impregnated with 10% DMSO in water were as negative controls. Diffusion Discs ciprofloxacin which are commercially available were used as positive controls among all bacterial strains.

8.3. Well Diffusion Assay

Petri plates were prepared by adding Mueller Molten agar in molten in a total volume of 20 ml at the base layer followed by formation of wells.Then each petri plate was inoculated with bacterial suspension at the concentration of 15ul.After complete drying in a sterile hood the wells were filled with test samples and ciprofloxacin were used as positive control and 10% DMSO as negative control.Finally all the plates were kept for incubation at a temperature of 37 C for 24 h.The zone of inhibition was evaluated in millimeters.Extracts which show zone of inhibition around 3mm were considered as antimicrobial. In Each extract the tests were performed in triplicate and the antimicrobial activity was expressed as mean of inhibition diameters.[11]

9. RESULT

While analysing all the extracts it was found that chloroform extract shows more zone of inhibition as compared to other extracts.Thus can be used for further analysis such as isolation and characterisation purpose.Futher we

after isolating the active moiety we make various analogs of the isolate moiety. All the extracts along with their concentration and the zone of inhibition shown by each extract is shown in the table(3)

Table 15 : Zone of inhibition of various extracts

	Zone of inhibition(mm)							
Strain	***S. aureus***				***E. coli***			
Concentration(µg/ml	**10**	**20**	**40**	**50**	**10**	**20**	**40**	**50**
Hot AQ								
Methanol								
Chloroform		**12**	**14**	**15**		**12**	**14**	**15**
Pet.ether								
Flower juice								**14**
Fresh Flower Juice								**14**

10. CONCLUSION

From the above data we come to the conclusion that the flower juice of Hibiscus rosa- sinensis do possess the antimicrobial activity and the active moiety responsible for it can be isolated, characterised by NMR,Mass spectrometry,IR,XRay crystallography for further studies

11. Discussion

While evaluating the Hibiscus rosa-sinensis for its antimicrobial activity almost all the eextract s show antibacterial activity against the disease causing organisms which include E.coli, B.Subtilus, S aureus.The in-vitro inhibition of bacterial growth could be due to presence of some active constituents present the extract.These constituents in combination or alone show antibacterial activity.As the extract contains multiple organic compounds which include Tannins,Flavonoids,Alkaloids,Triterenoids, All are having antimicrobial activity.However the flower extract mainly contains phenolic compounds like Tannins which are having efficient antimicrobial acrivity[12]. Besides Tannins, Flavonoids are also known for their antimicrobial activity against wide array of microrganisms due to their ability to form complexes with extra cellular proteins and bacterial cell wall[1].Thus the extracts of Hibiscus rosa-sinensis acts as good source of remedy for gastric complaints as the results are encouraging however for there is a need to isolate the active moiety which is actually responsible for the acivity

12. AKNOWLEDGEMENT

We would to acknowledge BIRAC, SRISTI Ahmedabad for the for providing financial assistance and providing laboratory facilities for this work as well as the University of Kashmir.

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