Antibacterial Properties of *Grewia asiatica* and *Cuscuta reflexa*

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Abstract— It is estimated that more than 25% of modern medicines are directly or indirectly derived from plants**.** In this context, it is worth mentioning that Indian medicinal plants are considered a vast source of several pharmaceutically active principles and compounds that are commonly used in home remedies against multiple ailments. Among them two Indian native plants, *Grewia asiatica* commonly known as Phalsa or Falsa belonging to the family Tiliaceae and *Cuscuta reflexa* belongs to the family Convolvulaceae have enormous traditional uses against various diseases and many bioactive compounds have been isolated from these plants. The entire plant samples were collected in different months of year 2013. *Cuscuta reflexa* stem and flowers were collected in October and *Grewia asiatica* leaves, fruits and seeds were collected in June. Extracts of various plants parts were prepared using solvents like water (cold and hot) and organic solvents (methanol, ethyl acetate, acetone). Antimicrobial activities of different extracts were evaluated by the agar well-diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) of various extracts was estimated against *Escherichia coli* and *Bacillus cereus.* To check antimicrobial activity, minimum inhibitory concentrations (MIC) of different extracts were determined against *Escherichia coli* and *Bacillus cereus* using the test tube dilution method. All the plant extracts showed varying degree of antibacterial activity against the test organisms. Agar well diffusion assay was used for evaluating the zone of inhibition. GALAE produced maximum zone of inhibition against Gram positive and Gram negative bacteria under taken for the study such as *Bacillus cereus* (30±2mm), *Citrobacter* environmental isolates (19.5±2mm) and *Staphylococcus aureus* (16±2mm) followed by GALME against *Escherichia coli* (11.5±2mm), *Bacillus cereus* (11.5±2mm) *and Pseudomonas mendocena* (10±2 mm). CRSME developed zone of inhibition against *Bacillus cereus* (6±2 mm) only whereas CRSAE developed zone of inhibition against *E. coli* environmental isolates and *Bacillus cereus* of 5±2mm. MIC and MBC of GALME was 100 mg/ml and for GALAE was 250mg/ml. MIC and MBC concentrations against *E.coli* were 100mg/ml and 250 mg/ml with CRSAE. Gram positive bacteria are usually more sensitive to crude extracts and bioactive constituents because of the specific structure of their cell walls.

Key words: *Grewia asiatica* Leaf Methanol Extract; CRSME: *Cuscuta reflexa* Stem Methanol Extract; CRSAE: *Cuscuta reflexa* Stem Acetone Extract; GALAE: *Grewia asiatica* Leaf Acetone Extract

I. INTRODUCTION

World is endowed with a rich wealth of medicinal plants. Over the last few years, researchers have aimed at identifying and validating plant-derived substances for the treatment of various diseases. Interestingly, it is estimated that more than 25% of modern medicines are directly or indirectly derived from plants (Perumalsamy, 2008; Daswani, 2009; Laphookhieo, 2010). In this context, it is worth mentioning that Indian medicinal plants are considered a vast source of several pharmaceutically active principles and compounds and that are commonly used in home remedies against multiple ailments (Nagashima, 1989; Nagababu and Lakshmaiah, 1992) Among them two Indian native plants, *Grewia asiatica* commonly known as Phalsa or Falsa belonging to the family Tiliaceae and *Cuscuta reflexa* belongs to the family Convolvulaceae have enormous traditional uses against various diseases and many bioactive compounds have been isolated from these plants.

Grewia asiatica, is used as a medicinal plant in several countries. It is excellent to alleviate summer thirst and hot weather related problems. The raw fruit relieves inflammations, fevers and blood disorders. It purifies blood. A wonderful reliever of respiratory ailments like asthma, bronchitis, colds, coughs and sore throat. It relieves urinary problems like burning and the juice aids in relieving digestive problems like excess acidity and indigestion. The infusion of the bark is used to treat diarrhoea. The paste of the leaves cures skin infections like eczema and eruptions and heals wounds. The fruit relieves liver and gall bladder problems, regulates blood pressure and cholesterol. The seeds are used to treat gonorrhea and fertility problems. It protects from sunstroke, cures anaemia. The bark of the root is used to treat rheumatism (Krishi Sandesh Agriculture in India).

Cuscuta reflexa, is indigenous medicinal plant and is a well-known folklore remedy for the treatment of liver disorder. The seeds contain amarbelin and kaempferol, stem gives cuscutin, cuscutatin, beta-sitosterol, luteolin, bergenin and kaempferol. The parasitic plant accumulates alkaloids from the host plant. The climber growing on *Mangiera indica* has been found to contain mangiferin. It is found to be hepatic, laxative and carminative. It contains flavonoids, including kaempferol and quercetin, hydroxycinnamic acid derivatives. Cuscutalin (1%) and cuscutin (0.02%) are main active principles of the plant. Seeds contain amarvelin, resins, oil (3%) and reducing sugars. It is used in treatment of urinary, spleen and liver disorders (Khare, 2007).

II. METHODOLOGY

A. SAMPLE COLLECTION

The entire plant samples were collected in different months of year 2013. *Cuscuta reflexa* stem and flowers were collected in October and *Grewia asiatica* leaves, flowers and seeds were collected in June.

B. STANDARD BACTERIAL CULTURES

The standard bacterial cultures used for this study were listed as under:

Micro organism	MTCC code
Escherichia coli	
Salmonella typhimurium	KMC1
Staphylococcus aureus	5022

Pseudomonas mendocina	
Bacillus cereus	2458
Citrobacter envt.	
Klebsella	

C. PREPARATION OF PLANT EXTRACTS USING AQUEOUS AND ORGANIC SOLVENTS

Extracts of various plants parts (leaves, fruit, flowers etc.) of *Cuscuta reflexa and Grewia asiatica* were prepared using solvents like water (cold and hot) and organic solvents (methanol, ethyl acetate, acetone). Fresh plant parts collected were surface sterilized with 0.1% $HgCl_2$ and washed repeatedly with sterile phosphate buffer saline (pH 7.2) followed by distilled water. Plant parts were than dried at 50ºC using electric drier and crushed with the aid of a mechanical grinder to powdered form. These powdered plant parts were used to prepare different extracts as described below.

1) Aqueous extract

Fifty grams of dried coarse powdered plant parts were soaked in autoclaved triple distilled water under constant stirring. The filtrate was collected three times at 24 h intervals during a total extraction period of 72 h. The aqueous dry extracts were obtained by concentrating the extract liquid under reduced pressure at 40°C using a vacuum rotary evaporator. The dry extracts were stored at –20 °C until use.

2) Organic solvent extracts

The dried samples were ground to coarse powder form and phyto-constituents were extracted by Soxhlet extractor at 60˚C using various solvents like methanol, ethyl acetate and acetone. The extracts were evaporated to dryness on the rotary evaporator and stored in a refrigerator at 4˚C until required for use. Dry weight of powder before and after extraction was taken to calculate expected total amount of phyto-constituents extracted with given solvent.

D. QUALITATIVE ESTIMATION OF PHYTOCONSTITUENTS

These extract were subjected to chemical test for different phytoconstituents viz. alkaloids, carbohydrates, phenolics, flavonoids, proteins, amino acids, saponins using the standard procedures described (Gupta and Sharma, 2011; Tease and Evans, 1989).

1) Alkaloids

Alkaloids were detected by using Wagner's test. To 1 ml of extract, 2-3 drops of wagner's reagent was added. Appearance of reddish brown precipitate indicated the presence of alkaloids.

Saponins by Froth test

Extracts were diluted to 20 ml with distilled water and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

2) Steroids by Salkowski test

To the few mg of extract, 2 ml of chloroform and 2 ml of conc. Sulphuric acid was added. Tubes were shaken and allowed to stand. Appearance of golden yellow red colour indicated the presence of phytosterols.

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3) Tannins

Few drops of 1% lead acetate were added to 2 ml of extract. The formation of yellowish precipitate indicated the presence of tannins.

4) Tannins (Ferric Chloride Test)

Extract solutions were treated with 5% ferric chloride solution. As per Culet et al., (2010) formation of blue colour indicated the presence of hydrolysable tannins and formation of green colour indicated the presence of condensed tannins.

5) Flavonoids

As described by Edeoga *et al*. (2005), a portion of extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed, indicating a positive test for flavonoids.

6) Carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used for Molisch's test for the presence of carbohydrates and Fehling's test for the presence of reducing sugars.

7) Proteins (Biuret's Test and Ninhydrin Test)

To 1 ml of test extract, 4% of sodium hydroxide solution and few drops of 1% copper sulphate solution were added. Formation of a violet red colour indicated the presence of proteins. To 2 ml of test extract, few drops of 0.25% of ninhydrin in acetone were added and heated in boiling water bath for 10 min. The formation of bluish purple colour indicated the presence of amino acid or proteins.

F. QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

1) Estimation of total phenolic content

The total phenolic content in extracts was determined with slight modifications of Folin Ciocalteau method (Singleton and Rossi, 1965). An aliquot of the extract in ethanol: triple distilled water (1:1) was treated with 5 ml Folin-Ciocalteu reagent (diluted with 1:10 v/v) and 4 ml of 1 M sodium carbonate. The tubes were incubated at room temperature for 15 min and centrifuged. The absorbance of supernatant was measured against blank at 765 nm. An equivalent amount of distilled water instead of extract was added in blank. Results were expressed as mg/g Gallic acid equivalent.

2) Estimation of total Flavonoids

The total Flavonoids content was estimated by aluminum chloride colorimetric method given by Woisky and Salatino (1998) with slight modifications. The standard calibration curve was prepared from (100µg/ml-1000µg/ml) Quercetin. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Then the reaction mixture was incubated at room temperature for 30 min. After that the absorbance was measured at 415 nm. In blank, 0.1 ml of distilled water was added instead of 10% aluminum chloride. The amount of flavanoids was expressed as mg quercetin equivalent per gm dry powder weight.

3) DPPH radical scavenging assay

The *in vitro* antioxidant activity was carried out using Dpph radical scavenging assay of these plants extract.The hydrogen donating ability of the extracts was determined in the presence of DPPH (1,1 diphenyl-2-picrylhydrazyl) stable radical . One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentration (2µg-80µg) and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus plant extract solution (2.5ml) was used as a blank, DPPH solution (1.0ml, 0.3mM) plus ethanol (2.5ml) served as negative control. The positive controls were those using the standard ascorbic acid solutions (Brand-Williams, 1995).

G. Microbiological Screening

Antimicrobial activities of different extracts were evaluated by the agar well-diffusion method (Olurinola, 1996; Murray, 1995). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) (Akinyemi, 2005) of various extracts was estimated against E. coli and Bacillus cereus.

1) Media preparation and its sterilization

For the agar well-diffusion method antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial culture, nutrient agar was used for developing surface colony growth. The MIC and MBC values were determined by the test tube dilution method. All the media prepared were then sterilized by autoclaving the media at (121°C) for 20 minutes.

2) Determination of minimum inhibitory concentrations (MIC)

To check antimicrobial activity minimum inhibitory concentrations of (MIC) of different extracts were determined against *Escherichia coli* and *Bacillus cereous* using the test tube dilution method (Cruickshank *et al*. 1975; Jain *et al*, 2012). Each of the extracts was constituted by dissolving 1 mg of the concentrates in 1 ml of nutrient broth, making the concentration to be 1mg/ml. Nineteen tubes of 1 ml of nutrient broth were set up, and appropriate amount of the 100 mg/ml of the extracts were added to different eighteen tubes of the nutrient broth to give the concentrations of extracts 50µg, 100µg and 250µg in triplicates and one tube kept as control. Normal saline was used to prepare a turbid suspension of test bacteria. The dilution of the test bacteria was done continuously in the normal saline until the turbidity matched that of 0.5 Mc-Farlands standard by visual comparison. At that point, microorganism has a concentration of about 1.5 x 10^8 cfu/ml. 0.1 ml of this suspension was transferred into the test tubes containing broth with different concentrations of extracts (Jain *et al.*, 2012). The tubes were incubated at 37^o C for 24 h. The minimum inhibitory concentration (MIC) was regarded as the lowest concentration that inhibited the visible growth. To determine minimum bactericidal concentration (MBC) 100µl of culture was spread on nutrient agar plates from each of the above tubes. The concentration at which no growth (no visible colonies) was observed was taken as MBC for particular extract.

III. RESULT AND DISCUSSION:

1) PRELIMINARY PHYTOCHEMICAL ANALYSIS

Preliminary phytochemical screening of various extracts of *G. asitica* Leaf (Table 1), and fruit (Table 2), *C. reflexa stem* (Table 3), and *flower* (Table 4) was done for Carbohydrate, protein, saphonin, tannins, flavonoid, alkaloid, glycoside and phytosterol. Roy *et al.* (2013) in their study for *C.reflexa* found tannins, phlobatannins, saponins, terpenoids, alkaloids and poly phenols in leaf extracts prepared with methanol, ethanol and aqueous solvents.

Grewia asiatica Leaves Extracts						
S.No.		Methanol	Ethyl acetate	Acetone	Aqueous(Cold)	Aqueous(Hot)
1.	Carbohydrate test					
a.	Molish's test	-	-	-	+	+
b.	Fehling's test	-	-	-	-	-
2.	Protein test					
a.	Biuret test	-	-	-	-	-
b.	Xanthoprotein	-	-	-	-	-
3.	Saphonin	-	-	-	-	+
4.	Tannins	-	-	-	-	-
5.	Flavanoid	-	-	-	+	+
6.	Alkaloid test					
a.	Mayer's test	+	+	+	-	-
b.	Wegner's test	+	+	+	-	-
c.	Hager's test	-	-	-	-	-
7.	Glycoside	+	+	+	+	+
8.	Phytosterol	-	+	-	+	+

Table 1: Preliminary phytoconstituents analysis of *Grewia asiatica* Leaves

Grewia asiatica Fruit Extracts			
S.No.		Aqueous(Cold)	Aqueous(Hot)
1.	Carbohydrate test		
a.	Molish's test	++	++
b.	Fehling's test	++	++
2.	Protein test		
a.	Biuret test	+	+
b.	Xanthoprotein	+	+
3.	Saphonin	-	+
4.	Tannins	-	-
5.	Flavanoid	+	+
6.	Alkaloid test		
a.	Mayer's test	-	-
b.	Wegner's test	-	-
c.	Hager's test	-	+
7.	Glycoside	+	+
8.	Phytosterol	+	+

Table 2: Preliminary phytoconstituents analysis of *Grewia asiatica* Fruit

*Cuscuta r*eflexa Stem Extracts						
S.No.		Methanol	Ethyl acetate	Acetone	Aqueous(Cold)	Aqueous(Hot)
1.	Carbohydrate test					
a.	Molish's test	+	+	+	+	+
b.	Fehling's test	+	-	-	+	+
2.	Protein test					
a.	Biuret test	-	-	-	-	-
b.	Xanthoprotein	-	-	-	-	-
3.	Saphonin	++	+	+	-	+
4.	Tannins	+	-	-	-	+
5.	Flavanoid	+	-	-	+	+
6.	Alkaloid test					
a.	Mayer's test	-	-	-	-	-
b.	Wegner's test	+	+	-	-	+
c.	Hager's test	-	-	-	-	-
7.	Glycoside	+	+	+	+	+
8.	Phytosterol	+	-	-	+	+

Table 3: Preliminary phytoconstituents analysis of *Cuscuta r*eflexa Stem

Cuscuta reflexa Flower			
S.No.		Aqueous(Cold)	Aqueous(Hot)
1.	Carbohydrate test		
a.	Molish's test	+	+
b.	Fehling's test	+	+
2.	Protein test		
a.	Biuret test	+	+
b.	Xanthoprotein	+	+
3.	Saphonin	+	+
4.	Tannins	+	+
5.	Flavanoid	+	+
6.	Alkaloid test		
a.	Mayer's test	-	+
b.	Wegner's test	-	-
c.	Hager's test	-	-
7.	Glycoside	+	+
8.	Phytosterol	+	-

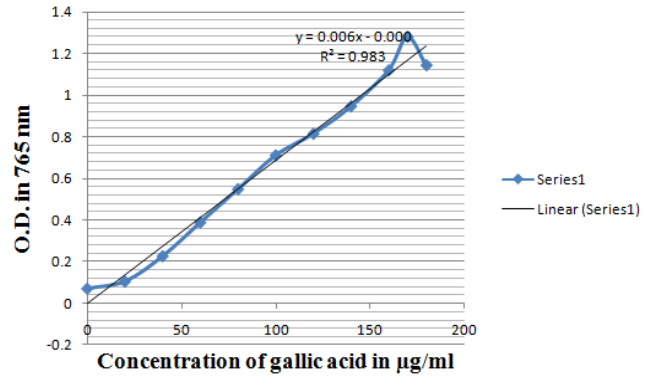
Table 4: Preliminary phytoconstituents analysis of *Cuscuta r*eflexa Flower

2) PHENOLIC AND FLAVANOID ESTIMATION

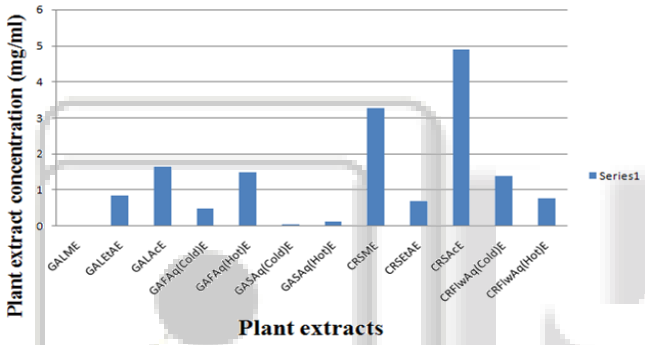
Graphical presentation of data obtained for the total phenolics and flavanoids estimated for G. asitica leaf, fruit and seeds and C. reflexa stem and flower per gram dry weight, are shown in Graph 1, 2, 3 and 4.

Among different extracts prepared for different parts of G. asitica, GALAcE contained maximum phenolics of 3.26 mg/gm galic acid and in C. reflexa, CRSAcE contained maximum phenolics of 4.9mg/gm galic acid .Present study revealed flavanoid contents highest in CRSAcE is 17.85 mg/ml quercetin equivalent.as compaired to GALEtAE which is 8.1 mg/ml quercetin equivalent.

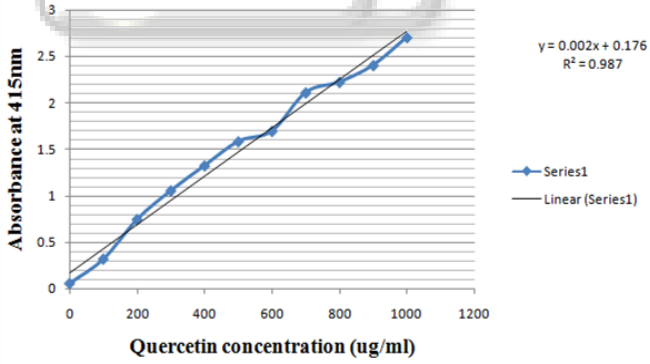
GRAPH-1 Gallic acid standard curve



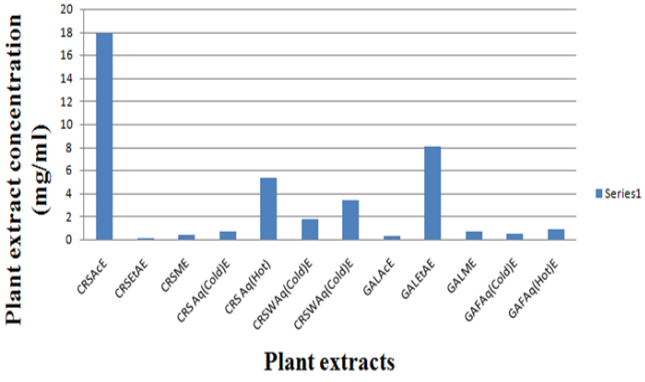
GRAPH-2 TOTAL PHENOLIC ESTIMATION



GRAPH -3 Quercetin standard curve



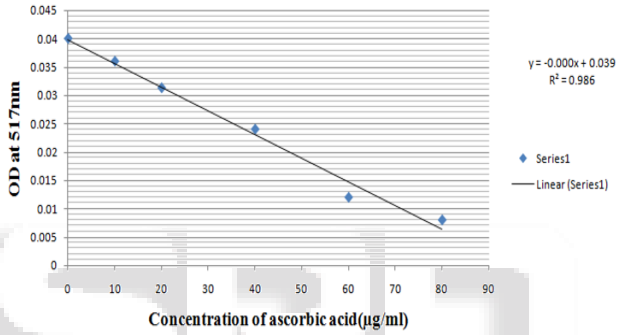
GRAPH -4 Total Flavanoid estimation



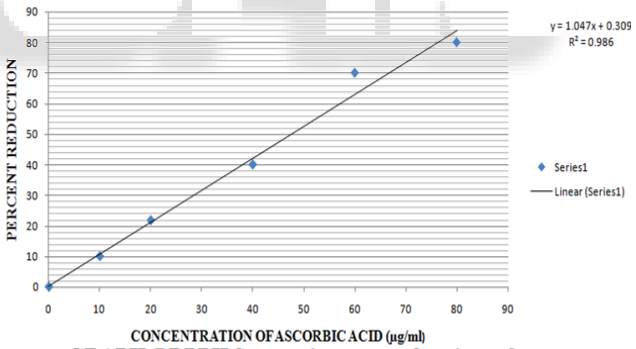
3) DPPH SCAVENGING ASSAY:

In complex systems, various different mechanisms may contribute to oxidative processes such as generation of different reactive oxygen species from various target structures such as carbohydrates, proteins and lipids. Thus, it is necessary to characterize the plant extracts by different antioxidant assays. The DPPH test provides information on the activity of test compound/ extract with a suitable free radical. The hydrogen donating ability of *Grewia asitica* leaf, fruit and seed and *Cuscuta reflexa* stem and flower were estimated in presence of DPPH stable radical. The free radical scavenging and antioxidant activity found in *G. asitica and C. reflexa may* be associated with their main phytochemical compounds like flavonoids, phenols and tannins. Both *G. asitica* plant parts as well as *C.reflexa* stem and flower extracts were having good percentage reduction values (Graph 5, 6, 7). Thus it can be concluded that these plants can be used as potent antioxidants for treating damages caused due to free radical generation in cells and resulting diseases.

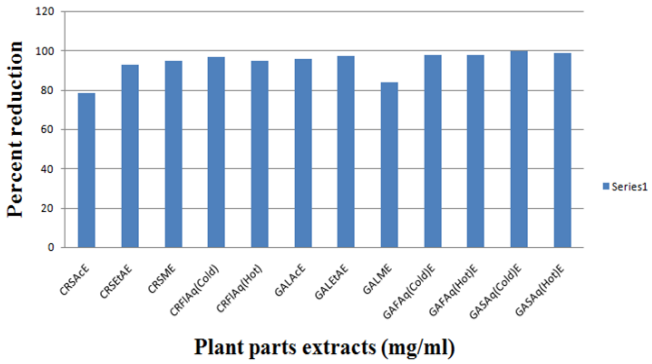
GRAPH- 5 Standard curve of Ascorbic acid against DPPH



GRAPH- 6 PERCENT REDUCTION BY ASCORBIC ACID



GRAPH-7 DPPH Scavenging assay of various plant extracts



4) ANTIBACTERIAL ACTIVITY

All the plant extracts showed varying degree of antibacterial activity against the test organisms. Agar well diffusion assay

was used for evaluating the zone of inhibition. GALAcE produced maximum zone of inhibition against Gram positive and Gram negative bacteria under taken for the study such as *Bacillus cereus* (30±2mm), *Citrobacter* environmental isolates (19.5±2mm) and *Staphylococcus aureus* (16±2mm) followed by GALME against *Escherichia coli* (11.5±2mm), *Bacillus cereus* (11.5±2mm) *and Pseudomonas mendocena* (10±2 mm). CRSME developed zone of inhibition against *Bacillus cereus* (6±2 mm) only whereas CRSAcE developed zone of inhibition against *E.coli* environmental isolates and *Bacillus cereus* of 5±2mm (Table 6). Such study has also been conducted which showed significant antibacterial activity against *Staphylococcus aureus, Shigella boydii, Pseudomonas aeruginosa, Shigella dysenteries* and *E.coli* for CRS methanol extract (125µg/ml) (Pal *et al*., 2006). MIC and MBC against *E. coli* were performed for various solvent extracts of GAL and CRS (Table 8). MIC and MBC of GALME was 100 mg/ml and for GALAcE was 250mg/ml. MIC and MBC concentrations against *E.coli* were 100mg/ml and 250 mg/ml with CRSAcE. Such study was performed using *G. asitica* fruit ethanol extract which has given antibacterial activity against *B.cereus*, *Staphylococcus aureus* and *Enterococcus faecalis* (Gupta *et al*., 2012; Praveen *et al*., 2012). Gram positive bacteria are usually more sensitive to, crude extracts and bioactive constituents because of the specific structure of their cell walls.

	Zone of inhibition(mm)			
Cultures	GAFME	GAFEtAE	GAFAcE	DMSO (Control)
E.coli	11.5±2	-	14±2	-
E.coli envt.	-	-	15±2	-
Bacillus cereus	11.5±2	12±2	30±2	-
Citrobacter envt.	-	-	19.5±2	-
Klebsella	-	7±2	12±2	-
Pseudomonas mendocina	10±2	-	13±2	-
Salmonella typhimurium	-	-	10±2	-
Staphylococcus aureus	-	-	16±2	-

Table 6: Antibacterial activity of *Grewia asitica* leaf extracts
(Conc. 50 mg/ml DMSO)

	Zone of inhibition(mm)			
Cultures	CRSME	CRSEtAE	CRSAcE	DMSO (Control)
E.coli	-	-	-	-
E.coli envt.	-	-	5±2	-
Bacillus cereus	6±2	-	5±2	-
Citrobacter envt.	-	-	-	-
Klebsella	-	-	-	-
Pseudomonas mendocina	-	-	-	-
Salmonella typhimurium	-	-	-	-
Staphylococcus aureus	-	-	-	-

Table 7: Antibacterial activity of *Cuscuta reflexa stem* extracts
(Conc. 50 mg/ml DMSO)

E.COLI	GALAcE	GALME	GALEtAE	CRSME
MIC	-	100mg/ml	100mg/ml	100mg/ml
MBC	-	250mg/ml	250mg/ml	250mg/ml

Table 8: MIC and MBC of Grewia asitica and Cuscuta reflexa extracts

IV. SUMMARY

G. asitica and *C.reflexa* plant parts have been used as traditional medicines for treatment of various diseases. The objective of this work was to evaluate the antimicrobial activity, phytochemical screening and antioxidant properties of methanol, ethanol, ethyl-acetate and aqueous soluble extracts of leaves, fruits and seeds of *G. asitica* and stem and flower of *C. reflexa*. Agar well diffusion assay was used for evaluating the zone of inhibition. GALAcE produced maximum zone of inhibition against Gram positive and Gram negative bacteria under taken such as *Bacillus cereus* (30±2mm), *Citrobacter* environmental isolates (19.5±2mm) and *Staphylococcus aureus* (16±2mm) followed by GALME against *Escherichia coli* (11.5±2mm), *Bacilus cereus* (11.5±2mm) and *Pseudomonas mendocena* (10±2 mm). CRSME developed weak zone of inhibition against *Bacillus cereus* (6±2 mm), similarly CRSAcE also developed small zone of inhibition against *E. coli* environmental isolates and *Bacillus cereus* of 5±2mm. MIC and MBC against *E. coli* were performed for various solvent of GAL *and* CRS. MIC and MBC of GALME was 100 mg/ml and for GALAcE was 250mg/ml. While, MIC and MBC concentrations against *E.coli* were 100mg/ml and 250 mg/ml with CRSAcE. Among different extracts prepared for different parts of *G.asitica*, GALAcE contained maximum phenolics of 3.26 mg/gm galic acid and in C. reflexa, CRSAcE contained maximum phenolics of 4.9mg/gm galic acid. Present study revealed flavanoid contents highest in CRSAcE is 17.85 mg/ml quercetin equivalent. DPPH scavenging assay was performed to evaluate antioxidant activity of various plant part extracts. It was estimated that all types of extracts from *C. reflexa* stem and flower and G. asitica leaf fruit and seed had high antioxidant activity. DPPH scavenging assay percent

reduction of GASAq(cold)E was evaluated as 99.44 as compared to CRFlAq(cold)E (96.66). Thus this study indicates *G. asitica* and *C.reflexa* to be very useful in treating various stress diseases and infections.

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