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ANTIMICROBIAL AGENTS FROM *RUBIA CORDIFOLIA* AND *GLYCYRRHIZA GLABRA* AGAINST PHYTOPATHOGENS OF *GOSSYPIUM*

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ABSTRACT: The antimicrobial activity of medicinal plant extracts has been recognized for many years. In the present study, 55 plant methanolic extracts were investigated for antimicrobial activity against, thirteen phytopathogens of *Gossypium* using agar ditch diffusion method. Results from the *in vitro* antimicrobial assays indicated that six plant extracts exhibited antimicrobial activity, in which highest activity was observed from the root extracts of *R. cordifolia* and *G. glabra*. Qualitative phytochemical tests, Column chromatography and of these two active root extracts demonstrated the presence of phyto compounds VIZ, anthraquinones and flavonoids as major active constituents respectively.
KEYWORDS: Antimicrobial activity; *Gossypium*, Agar well diffusion method, Thin layer chromatography, Column chromatography.

INTRODUCTION

The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Ethno pharmacologists, botanists, microbiologists, and natural-product chemists are combing the Earth for phytochemicals and "leads" which could be developed for treatment of infectious diseases. While 25 to 50% of current pharmaceuticals are derived from plants, none are used as antimicrobials. Traditional healers have long used plants to prevent or cure infectious conditions; Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. This study attempts to summarize the current status of botanical screening efforts, as well as *in vitro* studies of their effectiveness and toxicity. The structure and antimicrobial properties of phytochemicals are also addressed. Since many of these compounds are currently available as unregulated botanical preparations and their use by the public is increasing rapidly.

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Gossypium hirsutum (cotton) which belongs to the family Malvaceae has reined as king of apparel fibers due to its easy wear quality. Today cotton is grown in 70 countries spread overall five continents with India as first in acreage (8.53m ha) and third in production (16m bales) after China and USA. Such a pride of place for cotton is a thing of the past because our cotton production has been gradually decreasing for the past couple of years. Such a frustrating situation has primarily been created by unexpectedly high incident of phytopathogens of cotton especially bacteria and fungi resulting in seedling disease, verticillium wilt, boll rot, leaf spot disease, bacterial blight etc.,

At present the use of pesticides and chemical fungicides are the only way out to keep these phytopathogens under control as a result of which about 50 percent of all the agrochemicals are being used on the cotton crop alone. It is but natural that under regular exposure to chemical fungicides the pathogens are likely to develop resistance, fungicides rendering even these poisons ineffective. Dismayed over this failure of chemical fungicides the cotton growers started making inevitable but excessive use of fungicides in terms of higher doses. But even with 3-4 times higher than recommended use of these fungicides the pathogens continue to devastate the cotton crop as a result of which cotton growers have lost faith in chemical control of pathogens. In such a situation every farmer will be surprised if somebody talks about a variety of fungicides

of plant origin to check these diseases and for monitoring cotton productivity without any damage to soil biosphere, cost effective, safe to human and animal health and also to protect national economy and he would jump to purchase such a variety of fungicide.

EXPERIMENTAL

Preparation of plant extracts: Fifty five plant species were collected and predicting to possess bioactive chemicals, plant species were selected based on the information available from literature (1-3) folklore (4) and through field observations. The plant materials were collected in and around Visakhapatnam District. Andhra Pradesh, India. The collected plant materials were washed thoroughly with running tap water and finally with sterile distilled water the material was chopped into small pieces and then air dried on a sterile blotter under shade for 20-30 days.

The completely shade dried plant materials were coarsely powdered and allowed to soxhlet extraction with methanol for 5-6 hrs at temperature not exceeding the boiling point of the solvent and then filtered through Whatman No-1 filter paper. The extracted liquid obtained was subjected to Rotary evaporator and subsequently concentrated under reduced pressure (in vacuum at 40c).The residue obtained were designated as crude extracts, were labeled and stored in refrigerator for further study (5). The dried plant extract residues obtained were re dissolved in 0.1% Dimethyl Sulfoxide (DMSO) to get different concentrations (100mg/ml, 300mg/ml, 500mg/ml) of crude extracts and filtration through a 0.45µm membrane filter and stored in sterile brown bottles in a freezer at 20^0C until bio-assayed.

Microorganisms: Based on the disease index of *Gossypium* thirteen phytopathogenic microorganisms were selected to screen antimicrobial activity against the selected plant extracts, of these thirteen microorganisms, three are bacteria and ten are fungi listed in Table-1.1. All the thirteen microorganisms tested were purchased from Microbial Type Culture Collection and Gene Bank (MTTC), Chandigarh, India. All the pure cultures were obtained in lyophilized or freeze-dried form are reconstituted in sterile water and produced a suspension of the microbial cells. Inoculation was done with sterile inoculating loop to liquid broth medium. Liquid cultures are then incubated to allow cell replication and adequate growth of the culture, for use in bioassays. Following incubation, liquid cultures are refrigerated to store for further use. Typically, 24hrs will provide sufficient growth to allow visibly thick spread of the microbes for bioassay. The bacterial strains are maintained and tested on Nutrient agar (NA) and Potato Dextrose agar (PDA) for fungi.

***In vitro* antimicrobial assays:** The development of simple *in vitro* prescreens could offer initial idea of the biological activity of plant extracts and its compounds. Two types of media, solid (agar) and liquid (broth) media are generally required for culturing of microbes and bioassay studies. The antimicrobial activity was performed by Agar ditch or well/cup diffusion method (6-8) at desired concentrations diluted with DMSO (Dimethyl Sulfoxide) solvent which did not affect the growth of microorganisms.

Isolation and characterization of compounds: In the preliminary *in vitro* screening for antimicrobial activity, *Rubia cordifolia* and *Glycyrrhiza glabra* showed high antibacterial and antifungal activities even at very low concentrations. Although some phytochemical aspects have been recorded on these two plants, the author has considered it necessary to isolate the bioactive molecules in view of their medicinal and antimicrobial importance and also taxonomic markers view point. So, the extracts were subjected to column chromatography for separation of pure compounds by gradient elution method. The crude extract was chromatographed over a column of silica gel (G 100-200 MESH Acme) using eluents of increasing polarity of solvent mixtures hexane, hexane ethyl acetate, pure ethyl acetate and methanol. Fractions of 250ml were collected and monitored through silica gel TLC, the visualization of spots under UV light or iodine vapor or by spraying 5% methanolic sulphuric acid and heating at 110^0c. Fractions with similar spots were combined, the purification of each fraction was affected by extensive re-chromatography over small silica gel columns and re-crystallization from suitable solvents and the purity of the compounds was a curtained by homogeneity over silica gel (G60-120) TLC.

Identification and characterization of the compounds: For identification characterization and structural elucidation of the compounds spectroscopic methods like HPCL, GC/MS, UV, IR and NMR spectra were used, the IR spectral data has been recorded on KBr disc at Indian Institute of Chemical Technology (IICT) Hyderabad; GC/MS (GP 5050A) at Andhra University; ^{1}H and ^{13}C NMR recorded on MHz 400 at Central During Research Institute (CDRI), (SAIF), Lucknow and Indian Institute of Science (IISc), Bangalore. The compounds isolated were named with the prefix Rc and Gg signifying that these compounds were isolated from *R. cordifolia* and *G. glabra* plant species respectively.

RESULTS AND DISCUSSION

Out of several plants methanolic extracts screened eleven plant extracts showed significant antibacterial as well as antifungal activity as evidenced by a zone of inhibition. These eleven plant species were *Abutilon indicum, Cassia fistula, Cleome viscose, Datura metel, Glycyrrhiza glabra, Peltophorum pterocarpum, Psidium guajava, Quisqualis indica, Ricinus communis, Rubia cordifolia* and *Terminalia catappa.* Of all *R. cordifolia* and *G. glabra* produced the largest zone of inhibition against all the phytopathogens tested. Root extracts of *R. cordifolia* and *G. glabra* up to 5mg-l (W/V) concentrations showed significant activity against all tested pathogens. This is in agreement with the earlier studies of antimicrobial activity (9-11) on *R. cordifolia* (12-15) on *G. glabra*. They also isolated antimicrobial agents from the roots of both the plants.

The root methanolic extracts of *R. cordifolia* and *G. glabra* roots had potent antimicrobial activity than that of its corresponding water extracts. The solvent control of methanol, DMSO and water did not show any effect on microbial growth. Standard synthetic fungicide bavistin and antibacterial drugs penicillin had varied activity against all tested pathogens (Table 1.2). From the MIC assays of fifty five plant species six plant species (*Cassia fistula, Glycyrrhiza glabra, Peltophorum pterocarpum, Psidium guajava, Rubia cordifolia* and *Terminalia catappa*) showed both antifungal and antibacterial activities even at very lower concentrations ranging 5mg/ml to 10mg/ml (Table 1.3). Shelf life stability of all the active extracts was same and stable for both dry and solvent state during the period of testing ie. up to 20 months revealing the retention of original antimicrobial activity even with prolonged shelf life, when proper care was taken.

In this study methanolic extracts of *T. catappa* leaves showed good inhibition against all the tested pathogens. The reports (16-18) also conferred the leaf extracts of *T. catappa* against both bacteria and fungi. *P. guajava* leaf methanolic extracts showed considerable inhibitory activity and no inhibition by *C. papaya* leaf extracts against the tested phytopathogens. This is in agreement with the previous reports by the several workers (19-20).

In the previous findings *P. pterocarpum* and *S. cumini* showed promising activity against both Gram positive and Gram negative human pathogenic bacteria (21). In this study leaf methanolic extracts of *P. peltophorum* showed a range of antimicrobial activity and *S. cumini* showed moderate activity against the phytopathogens tested.

The root extracts of *R. cordifolia* and *G. glabra* spotted on the TLC plates precoated with silica gel- G with best resolving solvent system showed four and five prominent spots respectively. Therefore the extracts subjected to column chromatography over silica gel, using different solvent systems fractions of mixtures were eluted. Four biologically active compounds (Table 1.4) Rc I, Rc II, Rc III, Rc IV from *R. cordifolia* and Gg I, Gg II, Gg III from *G. glabra* were obtained by further chromatographic and re-crystallization techniques.

Physical and spectral datas are as follows

Rc I- Nordamnacanthal (figure.1) (22). Yellow color compound obtained from the fraction of Hexane: Ethyl acetate of (98:2) mp 220^0-223^0C. Rf value 0.76 (Hexane: Ethyl acetate in 90:10 ratio mobile phase). IR $^{Neat}{}_{Max}$ spectrum showed absorption at 1666.25, 1628.67, 1591.46, 1568.55, 1431.88, 1189.18, 1032.68, 905.02, 882.80, 806.03, 753. 45. ^{1}H NMR ($CDCl_3$, 400MHz) spectrum showed multiplet between 7.15 (H,s C_4-H), 7.73 and 8.2 (2H,m, C_5-C_6, C_7- and C_8-H), 10.4 (H,s,CHO and C_2), 12.6 and 13.98 (H,s,OH Phenolic and C_1 and C_3).

Rc II- Rubiadin (figure.1) (23). Orange color compound obtained from fraction of Hexane: Ethyl acetate (95:5) mp 295^0-305^0C. Rf value 0.39 (Hexane: Ethyl acetate 15% mobile phase). IR $^{Neat}{}_{Max}$ spectrum showed absorption at 3389.15, 1657.55, 1619.50, 1580.76, 1336.25, 1304.48, 1116.50, and 706.96. ^{1}H NMR ($CDCl_3$, 400MHz) spectrum showed multiplet between 2.05 (3H, s, CH3), 7.2 (H,s, CH_4-7), 7.82 (2H,m,C_6-H,C_7-H). MS m/z: 254 (M^+, 100), 240, 226 (M^+,-CO), 225 (M^+,-CHO), 197,152, 115, 105.

Rc III- Hydroxy-1 methyl-2 anthraquinone (figure 1) (22). Orange colour compound obtained from fraction of Hexane: Ethyl acetate (95:5) mp 180^0-190^0C. Rf value 0.19 (In 20% hexane ethyl acetate mobile phase). IR $^{Neat}{}_{Max}$ spectrum showed absorption at 1631.52, 1587.33, 1454.87, 1331.46, 1289.61, 1010.46, 851.63, 826.14, 751.93, and 712.14. ^{1}H NMR ($CDCl_3$, 400MHz) spectrum showed multiplet between 2.26 (3H, s, CH3), 7.42, 7.62 (Hd, J 3-4=7.5Hz, C_3 and C_4-H), 7.68, 8.17 (2H,m, C_5-, C_6-C_7 and C_8-H) and 12.84 (H,s,OH and C_1). MS m/z: 240 (M^+, 100), 238, 227,210 (M^+,-CO), 212, 209 (M^+,-CHO), 182 (M^+ 2CO), 182,184, 153 (M^+-C3O3H), 155 and 76.

Rc IV- Xanthopurpurin (figure.1) (24). Yellowish orange colored compound obtained from the fraction of Hexane: Ethyl acetate (95:5) mp 260^0-270^0C. IR $^{Neat}{}_{Max}$ spectrum showed absorption at 3420.89 (OH), 1662.37, 1631.60 (C=O), 1587.87 (aromatic C=C). ^{1}H NMR ($CDCl_3$, 400MHz) spectrum showed multiplet between 7.27 (1H, d, J=2.4 Hz), 7.76-7.84 (2H, m), 8.23-8.30 (2H, m). MS m/z is 240 (M^+, 100), 212, 184, 127, 84, 71 and 57.

Gg I- Glycyrrhizin or glycyrrhizinic acid (figure.2). White crystalline powder obtained from the fraction of Hexane: Ethyl acetate (98: 2) mp 295-302^0C. ^{1}HNMR (CDCl3, 400 MHz) spectrum showed multiplet between 1.6, 2.15 and 7.2.

Gg II- Glabridin (figure. 2) (25-26). Red brown powder obtained from the fraction of Hexane: Ethyl acetate (95: 5) mp 154- 156^0C. ^{1}HNMR (DMSO, 400 MHz) spectrum showed multiplet between 6.82 (d, 1H, J=8.3 Hz), 6.38 (dd, 1H, J= 8.4 Hz), 6.3 (d, 1H, J=9.9 Hz), 6.31 (d, 1H, J= 2.6 Hz), 2.85 (m, 1H), 1.43 (s, 3H). MS at m/z is 325.3 (M^+ +H), 189.21, 149.15, 130.60, 123.03, 110.01 and 82.97.

Gg III- 18-β Glycyrrhetinic acid (figure.2) white crystalline powder obtained from the fraction of Hexane: Ethyl acetate (95: 5) mp 292- 295^0C. ^{1}HNMR (CDCl3, 400 MHz) spectrum showed multiplet between 7.2, 5.7, 3.2, 2.8, 2.7, 2.3, 2.1, 1.9, 1.8, 1.6, 1.5, 1.4, 1.1. MS m/z is 471.53 (M^+, 100), 469.34, 457.46, 409.37, 337.33, 317.24, 277.26, 212.22, 191.06, 189.21, 122.22.

In conclusion, it is observed that to standardize the botanical identity of the drug in crude form and the bioactive compounds in pure form revealed that the antimicrobial activity of *R. cordifolia* aqueous and methanolic e xtracts are largely due to the presence of anthraquinones and their synergistic effect. Now a day's new approach by plant pathologists in reducing the threshold level of disease by using cost effective and eco-friendly management option in agronomy is the target. In this concern discovery of aqueous root extracts of *R. cordifolia, G. glabra* and their

bioactive compounds as biological fungicides against the tested phytopathogens are highly recommendable. The results of the present studies may be helpful in formulating the plant based natural fungicides in controlling common destructive diseases of *Gossypium*.

Table 1.1 Common pathogen index of *Gossypium hirsutum* L. crop

S.No	Pathogen	MTCC Code	Disease
1	*Erwinia herbicola*	B 110	Lint degradation
2	*Agrobacterium tumefaciens*	B 431	Crown gal
3	*Xanthomonas campestris*	B 2286	Bacterial blight
4	*Slerotium rolfsii*	F 288	Stem and root rot
5	*Thielaviopsis basicola*	F 1467	Seedling disease complex
6	*Rhizoctonia solani*	F 4633	Leaf spot, boll rot and seedling disease
7	*Verticillium dahliae*	F 1351	Verticillium wilt
8	*Alternaria alternata*	F 2723	Leaf spot
9	*Phoma exigua Desmaz*	F 2315	Stem canker
10	*Cochliobolus spicifer*	F 2112	Leaf spot
11	*Tiarosporella phaseolina*	F 2165	Charcoal rot
12	*Fusarium oxysporum*	F 1755	Fusarium wilt, boll rot and seedling disease
13	*Aspergillus flavus*	F 1884	Lint contamination

Table 1.2 Synthetic Antibiotics Reference Standards Antibiotic concentrations in 50µl of well loaded solution

S.No	Organism	DIZ[a]	MIC[C]	DIZ[a]	MIC[P]	DIZ[a]	MIC[B]
1	*E. herbicola*	20	1	20	0.5	-	-
2	*A. tumefaciens*	20	1.5	20	1.5	-	-
3	*X. campestris*	20	2	20	2	-	-
4	*S. rolfsii*	-	-	-	-	40	15
5	*T. basicola*	-	-	-	-	40	12
6	*R. solani*	-	-	-	-	40	25
7	*V. dahliae*	-	-	-	-	40	50
8	*A. alternata*	-	-	-	-	40	10
9	*P. e. Desmaz*	-	-	-	-	40	1.5
10	*C. spicifer*	-	-	-	-	40	2
11	*T. phaseolina*	-	-	-	-	40	1
12	*F. oxysporum*	-	-	-	-	40	100
13	*A. flavus*	-	-	-	-	40	10

DIZ[a:] Zone of inhibition including 6mm well diameter, is the mean of three replicates.

MIC[C] and MIC[P]: Minimum inhibition concentration of antibiotics Chloramphenicol and Penicillin drugs respectively at mg/ml.

MIC[B]: Minimum inhibition concentration of Bavistin at µg/ml.

-: Indicates no inhibition.

Table 1.3 Minimum Inhibition Concentrations of Some Plant Extracts

S.no	Organism	*R.cordifolia*			*G.glabra*			*T.catappa*			*P.guajava*			*C.fistula*			*P.pterocarpum*		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	*E.herbicola*	10	11	13	9	11	12	0	8	10	10	13	14	9	10	13	9	11	12
2	*A.tumefaciens*	0	0	9	9	10	11	9	10	11	9	10	11	8	9	10	0	9	9
3	*X.campestris*	9	11	15	0	9	10	0	0	10	0	10	11	8	9	11	0	10	11
4	*S.rolfsii*	14	18	20	15	17	19	0	8	10	0	0	8	0	0	0	9	10	12
5	*T.basicola*	10	13	19	10	12	15	0	9	10	8	9	10	0	8	12	0	10	11
6	*R.solani*	15	17	19	14	18	20	12	14	16	7	8	9	0	9	10	0	9	10
7	*V.dahliae*	17	18	20	15	17	19	9	10	14	10	13	14	10	11	13	0	10	11
8	*A.alternata*	11	14	16	10	15	17	10	12	14	10	12	15	11	11	12	0	9	11
9	*P.e.Desmaz*	14	18	20	15	19	21	0	10	13	0	8	9	10	10	11	0	9	10
10	*C.spicifer*	12	14	16	10	14	17	9	10	12	10	13	15	11	12	15	10	13	15
11	*T.phaseolina*	15	18	20	15	17	19	0	0	9	13	14	15	0	10	12	12	13	15
12	*F.oxysporum*	10	12	15	11	13	15	11	12	15	13	15	16	9	10	11	10	11	14
13	*A.flavus*	15	16	17	13	15	17	11	13	15	7	9	10	0	0	8	0	9	10

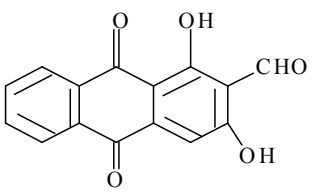
A, B, C- Concentrations of plant methanolic extracts at 5mg/ml, 10mg/ml, 15g/ml respectively.
Diameter of zone of inhibition in mm includes well diameter 6mm.

Table 1.4 Antimicrobial activity of pure compounds eluted from column.

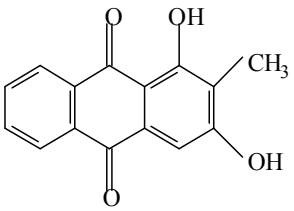
S.No	Organism	Rc I	Rc II	Rc III	Rc IV	Gg I	Gg II	Gg III
1	*E.herbicola*	35	20	31	12	0	28	20
2	*A.tumefaciens*	37	22	35	17	21	34	25
3	*X.campestris*	34	25	32	18	23	34	27
4	*S.rolfsii*	26	13	30	14	0	23	15
5	*T.basicola*	30	20	33	21	21	38	30
6	*R.solani*	27	15	25	16	20	25	29
7	*V.dahliae*	40	30	37	25	21	38	30
8	*A.alternata*	28	25	34	22	9	29	18
9	*P.e.Desmaz*	30	13	25	12	12	30	32
10	*C.spicifer*	31	15	38	17	18	35	30
11	*T.phaseolina*	10	15	16	8	6	15	9
12	*F.oxysporum*	26	14	28	9	0	31	10
13	*A.flavus*	27	14	30	15	19	29	27

Concentrations of pure compounds eluted from column at 1mg/ml. Diameter of zone of inhibition in mm includes well diameter 6mm.

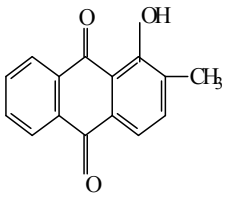
Figure.1 Antimicrobial agents from *Rubia cordifolia*



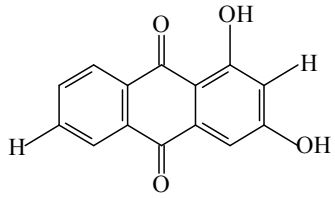
Rc I-Nordamnacanthal



RcII- Rubiadin

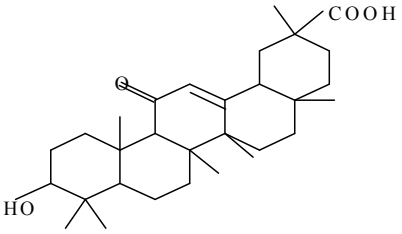


Rc III-Hydroxy-1-methyl-2 anthraquinone

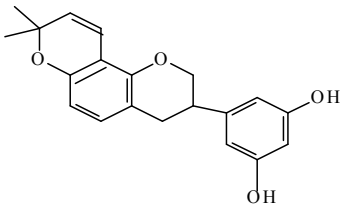


RcIV- Xanthopurpurin

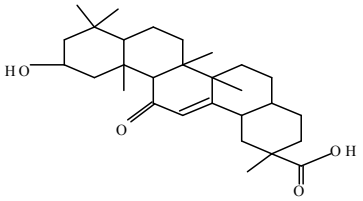
Figure.2 Antimicrobial agents from *Glycyrrhiza glabra*



Gg I-Glycyrrhizin



Gg II - Glabridin



Gg III -18-B - Glycyrrhetinicacid

ABBREVATIONS AND NOMENCLATURE
Rc- *Rubia cordifolia*
Gg- *Glycyrrhiza glabra*
DMSO- Dimethyl Sulfoxide
Mp- Melting point
IR- Infra red
^{1}H NMR- Proton Nuclear Magnetic Resonance
$CDCl_3$- Duteriated Chloroform
MHz- Mega Hertz

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