■ CHAPTER - IV

Title: Extraction and characterization of Cerin and Friedelin from Quarcus suber, preparation of its derivatives and antimicrobial activity of each of them

Chapter IV deals with isolation, characterization and preparation of two antimicrobial derivatives from plant *Quarcus suber*. *Q. suber* is a plant commonly called Cork Oak, belongs to the family Fagaceae (Beech family). This particular plant mainly found in siliceous hills on the littoral [1]. This medium sized, evergreen tree is a primary source of cork for wine bottle stopper and other uses such as cork flooring [2]. It is a native of southwest Europe and northwest Africa. It grows up to 20 meters. The leaves are 4 to 7 cm long, lobed or toothed, dark green upper surface and pale lower surface. Sometimes the leaf margins curve downwards.

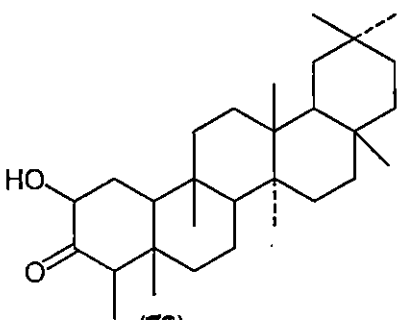
The tree forms a thick, rugged bark, containing high levels of suberin. The cork cambium layer of bark develops considerable thickness and at every 9 to 12 years cork can be harvested [3]. The harvesting of cork does not harm the tree. However, no trees are cut down during harvesting process. Only the bark is extracted, and a new layer of cork is produced, making it a renewable resource.

The plant has medicinal properties. Any gall produced on the tree is strong astringent and can be used in the treatment of Hemorrhages, chronic diarrhea, dysentery etc. The seeds are used as food [4]. It can be dried, ground into a powder and are used as a thickening in stews or mixed with cereals for making bread. It contains bitter tannins too [5]. Cerin and friedelin have been isolated from the plant which has both antibacterial as well as antifungal activity.

Section 1: Extraction and characterization of pentacyclic triterpenoids (Cerin and Friedelin) from *Quarcus suber*

1.1: Isolation of Cerin from bark cork:

3 kg of dried finely powdered cork was extracted with petroleum ether in a soxhlet apparatus for 72 hours. After removal of solvent, a brown gummy residue was separated out. The dried residue was dissolved in minimum volume of hot chloroform and kept overnight in dark. The desired compound Cerin was crystallized out from the solvent then filtered and once again recrystalized from hot chloroform. After that slightly yellow needle shaped crystals of Cerin were separated out from the solvent.

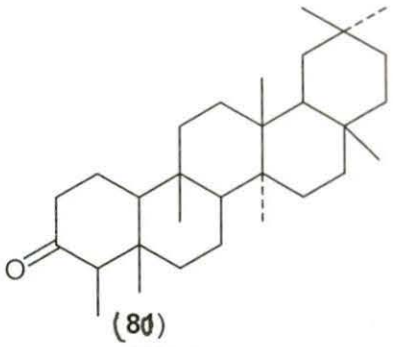


(79)
Cerin

1.2: Isolation of friedelin from bark cork:

3 kg of finely powdered cork was extracted with petroleum ether in a soxhlet apparatus for 18 hours. After removal of the solvent a white solid separated out. The solid was dissolved in minimum volume of benzene and chromatographed over silica gel column, developed with petroleum ether. Elution of the column with petroleum ether gave shining crystals of friedelin, m.p. 248^0-252^0C.

IR: 1720 cm^{-1} (-C=O)



(80)

Friedelin

1.3: General experimental detail:

The melting points were determined by open capillary method. The NMR spectra were recorded in $CDCl_3$ solutions at ambient temperature on a Bruker Avance 300 MHz-FT NMR spectrometer using 5 mm BBO probe. The chemical shift δ was given in ppm related to tetra methylsilane (TMS) as internal standard. The coupling constants (*J*) were reported in Hz.

The IR spectram was recorded in Shimadzu FT-IR spectrophotometer in KBr discs.

1.4: Plant material:

Fresh corks were collected from the local market and were cut into small pieces. These were then used for further study.

1.5: Preparation of plant extract:

The cork material was extracted with pet ether in a soxhlet apparatus for 72 hours. The solvent was recovered that yielded a deep brown gummy residue. This crude extract was dissolved in minimum volume of hot chloroform, crocked up and stored for 24 hours. Being less soluble in cold chloroform, cerin crystallized out at the bottom of the container as slightly yellowish crystals. It was then filtered over a sintered Buckner funnel and washed thrice with cold chloroform. All the washings and the supernatant liquid were then mixed and purified over a column of silica gel.

Section 2: Antimicrobial activity:

2.1 Material and Methods:

Details of materials and methods, maintenance of stock culture, spore germination bioassay technique and disc diffusion method have been mentioned in sections 2, of Chapter II.

2.2: Results

In the present study compound 'cerin', isolated from cork of *Quarcus suber,* was used as tenth compound to control some fungal and bacterial pathogens of plants. Five different concentrations of cerin were used for spore germination bioassay against three different plant pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*). From the results presented in table 4.1 it was evident that cerin was highly antifungal. No spores germinated even at the lowest concentration (100ppm) tested.

Table 4.1: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by Cerin (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germ tube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100
Fusarium equiseti	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100
Curvularia eragrostidis	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100

*mean of three replications

In case of disc diffusion test (table 4.2 & plate-10) also cerin showed high antifungal activity. All the five concentrations of the compound could check the growth of the fungi significantly.

Cerin showed poor antibacterial activity as evidenced from the results presented in table 4.3 and plate-10. No antibacterial activity was recorded by the compound cerin in case of *R. solanacearum, Xanthomonas sp.* and *P. syringae.* Only *E. carotovora* was controlled by cerin at concentration of 300ppm and above.

Table 4.2: Antifungal activity of Cerin

Fungal organism	Concentrations of compound (ppm)	Diameter of Inhibition zone(cm)*
Colletotrichum gloeosporioides	100	1.4
	200	1.6
	300	1.9
	400	2.0
	500	2.2
Fusarium equiseti	100	1.6
	200	1.7
	300	1.9
	400	2.1
	500	2.3
Curvularia eragrostidis	100	1.3
	200	1.5
	300	1.8
	400	2.0
	500	2.2

*mean of three replications;

Table 4.3: Antibacterial activity of Cerin

Bacterial organism	Concentrations of compound (ppm)	Diameter of Inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.4
	200	0.4
	300	0.4
	400	0.4
	500	0.4
Xanthomonas sp	control	-
	100	0.4
	200	0.4
	300	0.4
	400	0.4
	500	0.4
Pseudomonas syringae	Control	-
	100	0.4
	200	0.4
	300	0.4
	400	0.4
	500	0.4
Erwinia carotovora	Control	-
	100	0.4
	200	0.4
	300	0.8
	400	1.0
	500	1.2

*mean of three replications; - indicates no inhibition zone formed

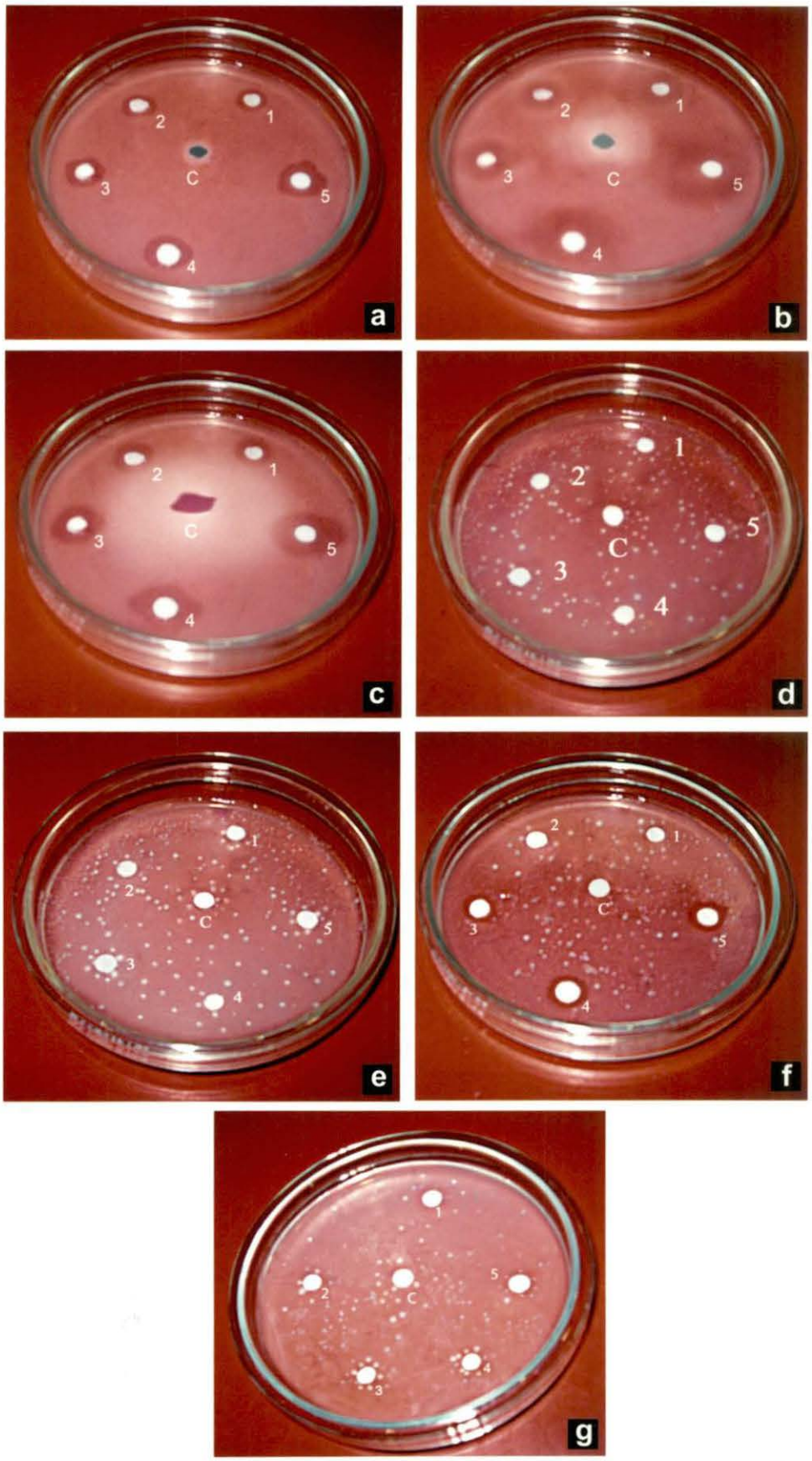


Plate 10: Disc diffusion test for anti microbial activity of compound Cerin, at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

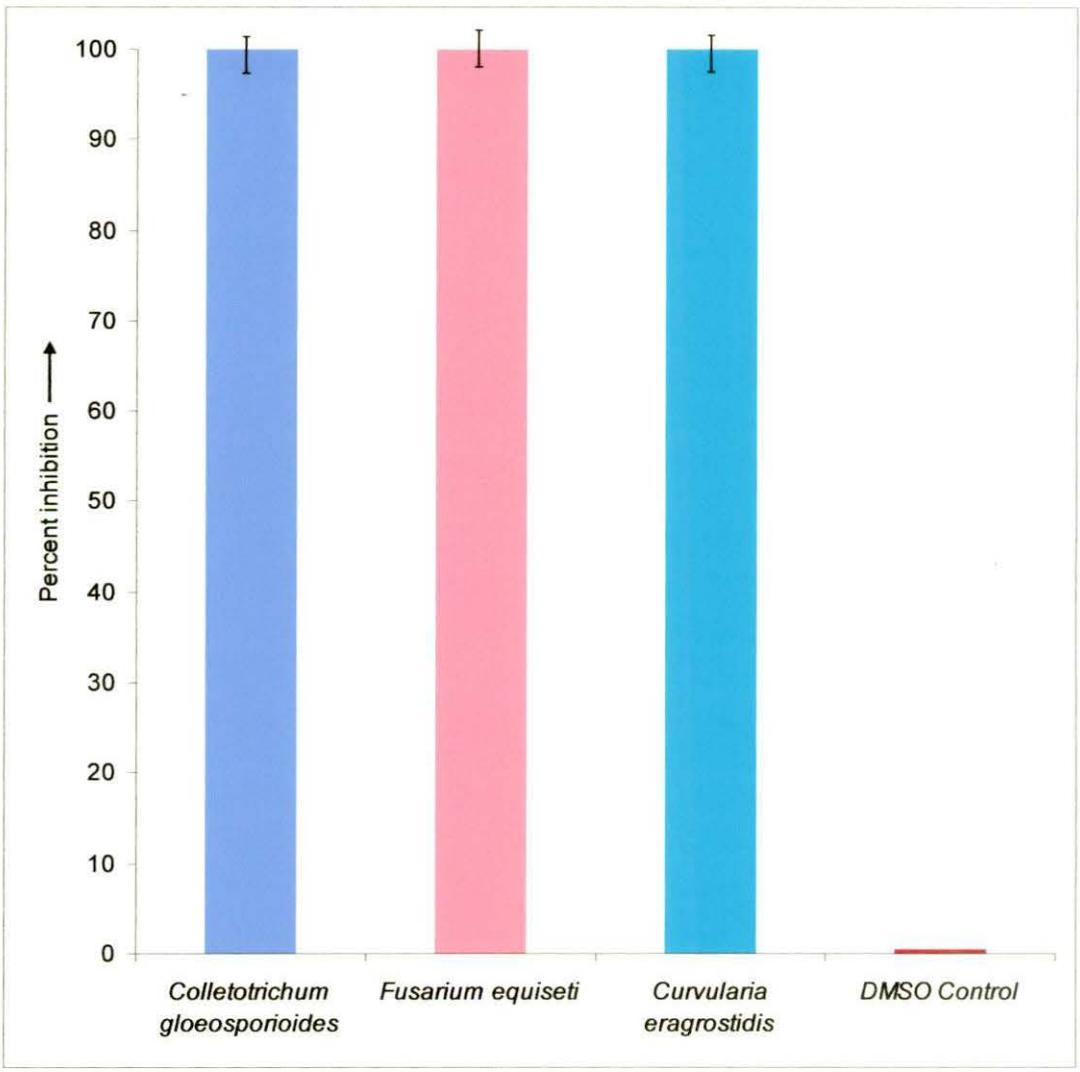


Fig. 37: Percent inhibition of spore germination at 300 ppm concentration of Cerin.

The second compound 'friedelin' was also isolated from cork of *Quarcus suber,* to control some fungal and bacterial pathogens of plants. Five different concentrations of friedelin were used for spore germination bioassay against the three different fungal plant pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*). From the results presented in table 4.4 it was evident that friedelin was also highly antifungal and no spore germination was found to occur even at the lowest concentration (100ppm) tested.

Table 4.4: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by Friedelin (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germ tube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100
Fusarium equiseti	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100
Curvularia eragrostidis	100	-	-	100
	200	-	-	100
	300	-	-	100
	400	-	-	100
	500	-	-	100

*mean of three replications

From the results presented in the table 4.5 & plate 11, it was found that the antifungal activity of friedelin was very high. In disc diffusion tests all the five concentrations of friedelin could effectively control the growth of the fungi as evidenced by larger antifungal zones.

Table 4.5: Antifungal activity of Friedelin

Fungal organism	Concentrations of compounds(ppm)	Diameter of inhibition zone(cm)*
Colletotrichum gloeosporioides	100	0.9
	200	0.9
	300	1.0
	400	1.3
	500	1.4
Fusarium equiseti	100	1.5
	200	1.7
	300	1.8
	400	2.0
	500	2.1
Curvularia eragrostidis	100	1.3
	200	1.5
	300	1.6
	400	1.8
	500	2.0

*mean of three replications; - indicates no inhibition zone formed

Like cerin, friedelin also showed poor antibacterial activity as evidenced from the results presented in table 4.6 and plate-11. In case of *R. solanacearum* and *E. carotovora* two concentrations (400 and 500ppm) of Friedelin showed antibacterial activity. In case of *Xanthomonas sp.* and *P. syringae* bacterial growth was inhibited significantly at concentrations of 300ppm and above.

Table 4.6: Antibacterial activity of Friedelin

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.4
	200	0.4
	300	0.4
	400	1.2
	500	1.5
Xanthomonas sp	control	-
	100	0.4
	200	0.4
	300	1.2
	400	1.5
	500	2.0
Pseudomonas syringae	Control	-
	100	0.4
	200	0.4
	300	1.3
	400	1.8
	500	2.0
Erwinia carotovora	Control	-
	100	0.4
	200	0.4
	300	0.4
	400	1.2
	500	1.9

*mean of three replications ; - indicates no inhibition zone formed

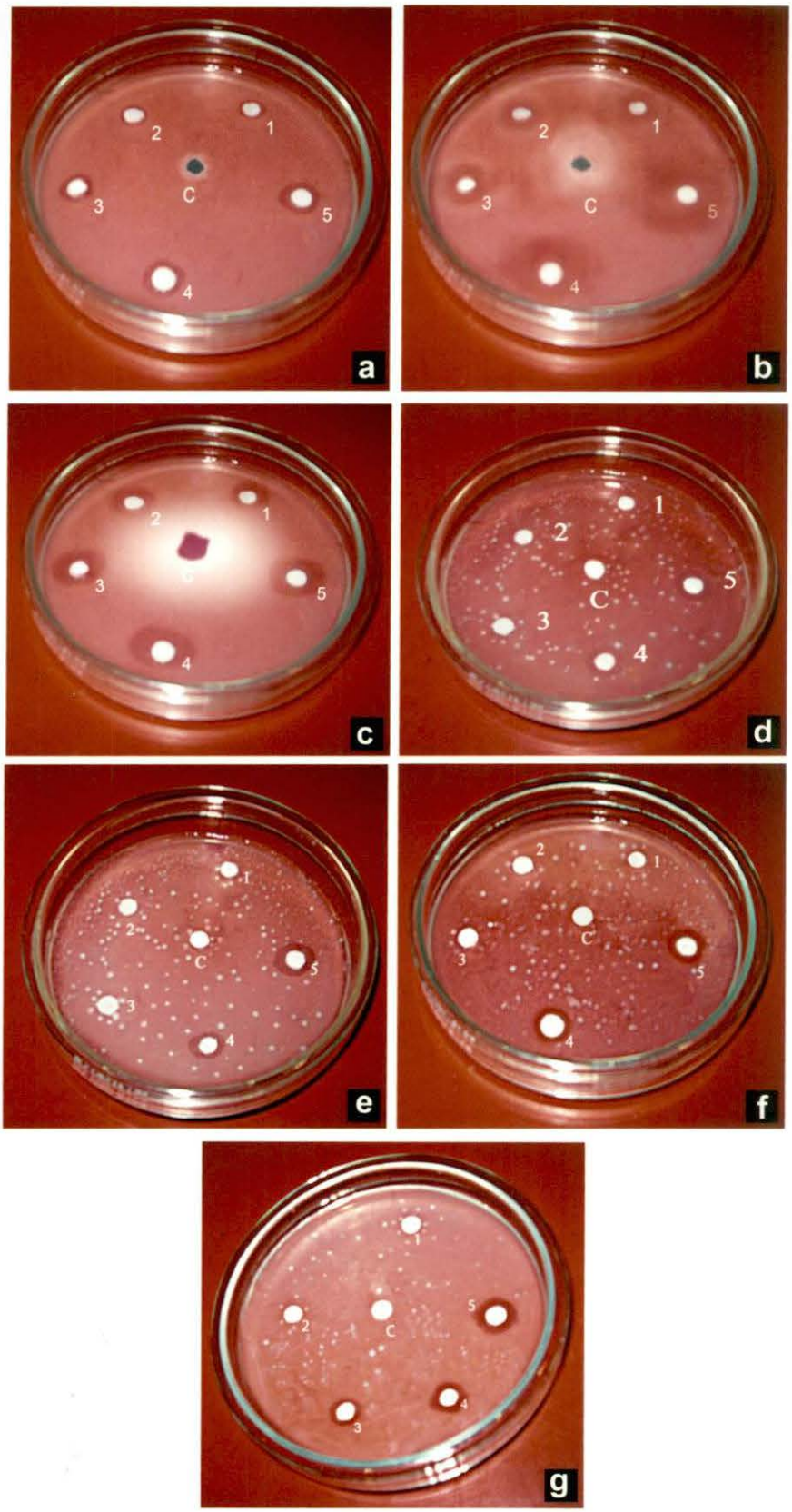


Plate 11: Disc diffusion test for anti microbial activity of compound Friedelin at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora* (g) *Xanthomonas* sp.

* 1=100ppm, 2=200 ppm, 3=300 ppm, 4=400 ppm, 5=500ppm, c=DMSO control.

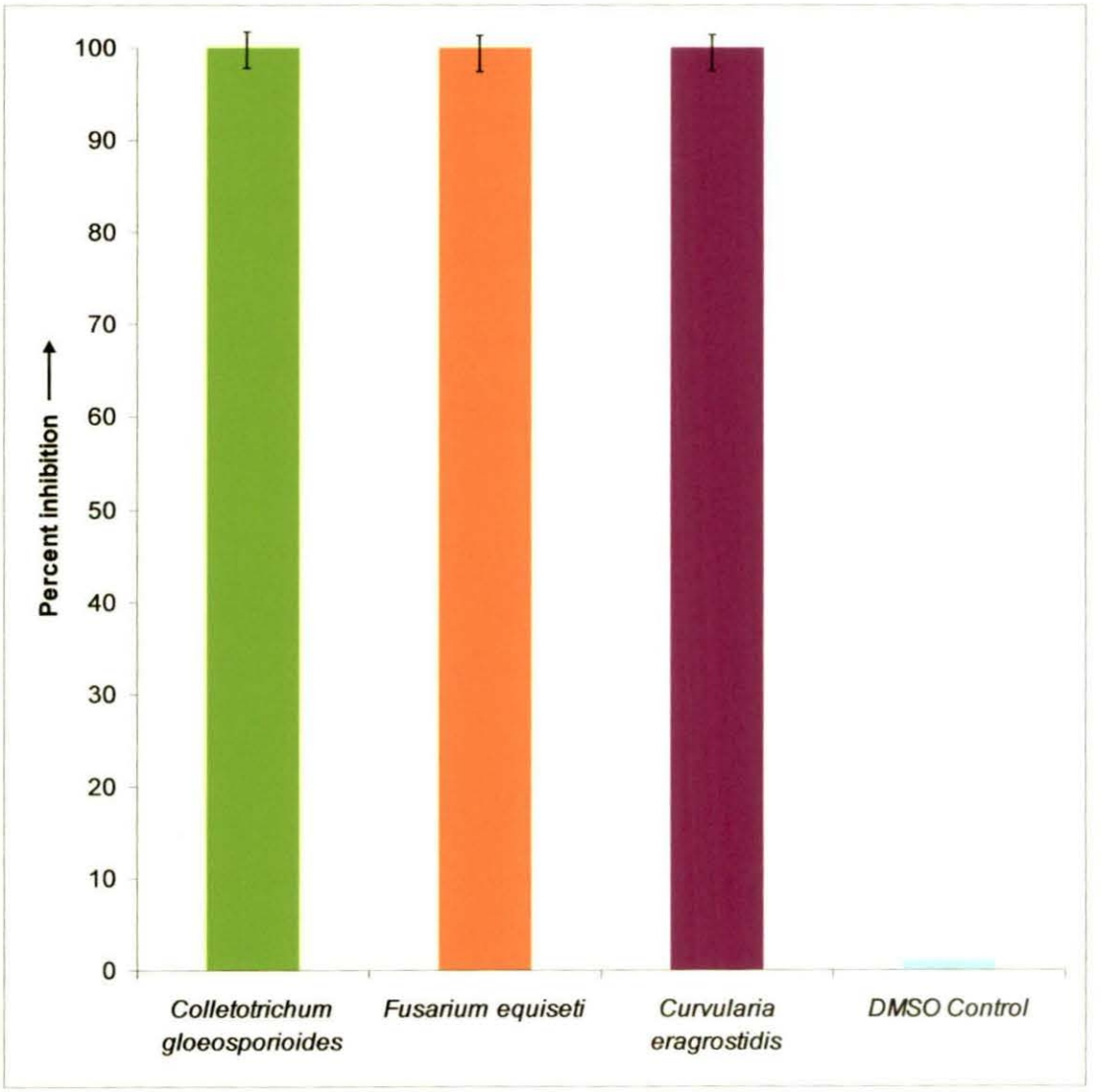


Fig. 38: Percent inhibition of spore germination at 300 ppm concentration of Friedelin.

Phytotoxicity of Cerin and Friedelin:

Phytotoxicity of the two compounds was tested in tomato plants (of Priya variety popularly cultivated in the present study area). To test phytotoxicity, tomato plants were grown in earthen pots [size 15cm (diameter) and 15 cm (height)]. The pots were maintained in the experimental garden of the Department of Botany with normal watering. Thirty such pots containing plants (each 10 cm in height) were kept in a net house. The plants were grouped into three sets and each set contained 10 plants each. Two compounds (at 100 ppm concentration) were sprayed in two sets separately and the third set was sprayed with sterile distilled water. The plants were observed up to seven days. No phytotoxicity was observed at 100ppm concentration of the compounds when compared with the control set which was sprayed with the sterile distilled water. (table-4.7)

Table 4.7: Phytotoxicity of cerin and friedelin at 100ppm concentration

Compounds	Concentrations (ppm)	Morphological & Physiological abnormalities abnormalities.			
		1day	3day	5day	7day
cerin	100	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated
friedelin	100	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated
Control	Sterile distilled water	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated	Plants alive, no significant changes Root-germinated Shoot-germinated

Plate 12: Tomato plants used in different *In vivo* studies (Priya variety)

2.3: Discussion

In Chapter IV isolation, characterization and preparation of two antimicrobial derivatives from the plant *Quarcus suber* have been described. It is a plant commonly called Cork oak belongs to the family Fagaceae [1]. Through phytochemical extraction of *Quarcus suber*, two derivatives Cerin and Friedelin have been prepared from dried and finely powdered cork, extracted with petroleum ether in a Soxhlet apparatus for 72 hours and was characterized. Cerin and Friedelin, both the derivatives were very significant to inhibit the spore germination of fungi (*Colletotrichum gloeosporioides, Fusarium equiseti and Curvularia eragrostidis).*

From the results of 'spore germination bioassay' cerin showed its highly antifungal activity. No spore germination was observed at any of the five concentrations (100, 200, 300, 400 & 500ppm) tested. Lowest concentration (100ppm) of the compound showed cent percent inhibition of spore germination. In disc diffusion method mycelial growth of the three fungi (*Colletotrichum gloeosporioides, Fusarium equiseti and Curvularia eragrostidis*) was also controlled by Cerin significantly even at 100ppm concentration. In contrast to the antifungal activity, Cerin could not show its efficacy towards the three bacteria (*R. solanacearum, Xanthomonas sp.* and *P. syringae*). Growth of *E. carotovora* was controlled by Cerin only at higher concentrations (300,400,500ppm) as evident from disc diffusion test (table-4.3). Friedelin, like the cerin did not allow the spores of the three fungi (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*) to germinate at lowest concentration tested. Results of the disc diffusion test also showed significant antifungal activity of the compound. Friedelin, in comparison to Cerin, showed smaller diameter of inhibition zones in disc diffusion method. Friedelin also showed insignificant antibacterial activity at lower concentrations (100 & 200ppm) but all the four bacteria (*R. solanacearum, E. carotovora Xanthomonas* sp. and *P. syringae*) tested was controlled by Friedelin at higher concentrations (400 & 500ppm). Thus friedelin was more antifungal than antibacterial.

In several respects our results were supported by different workers. Ramesh *et al.* (2008) isolated Friedelin, epi-Friedelin, n-Octacosanol, α-Amyrin, Sitosterol, Sitosterol-3-α-D-glucopyranoside and luteoforol from *Bridelia crenulaa* Roxb. The aqueous and

methanolic extracts and their fractions were tested against ten human pathogenic bacteria and four fungal strains. They observed that inhibitory activities were maximum in the chloroform-methanol (1:1) fraction of the methanolic extract against *E.coli, K.pneumoniae* and *P.aeruginasa*, which were responsible for the pathogenesis of urinary tract infection. Their study provided scientific evidences for the efficacy of the use of triterpedoids. Khan *et al.* (2008) extracted crude from the leaves, stem bark, stem heart wood, root bark and root heart wood of *Euroschinus papuanus* and isolated fractions on partitioning with petrol, dichloromethane, ethyl acetate and butanol and studied antibacterial and antifungal activity. They observed that ethylacetate fractions of the stem heart wood, dichloromethane fraction of root bark and butanol fraction of root heart wood exhibited excellent antibacterial activity and butanol fraction of leaves, stem heartwood and root bark exhibited antifungal activity. Prusky *et al*, (1982) and Rahmani *et al* (2006) reported that leaf extract of *Datura metel, X. strumarium* exhibited 100% of inhibition of spore germination of *A.alata*. Spore germination of *P.theae* was completely inhibited by leaf extract of *X.strumarium* and *D.stamoniun*. The antifungal potentiality of several plant extracts through spore germination bioassay were reported by the authors. Ramesh *et al* (2002) performed a phytochemical investigation of the various extracts of the leaves of *Begonia malabarica* L. (Begoniaceae) resulted in the isolation and identification of six known compounds, viz. friedelin, epi-friedelinol, beta-sitosterol, luteolin, quercetin and beta-sitosterol-3-beta-D-glucopyranoside. The aqueous and organic solvent extracts were also tested against ten human pathogenic bacteria and four fungal strains by the agar-well diffusion method. All the extracts were devoid of antifungal activity against the tested fungi. The hexane extract did not show any activity. The aqueous extracts showed activity against the Gram-negative bacteria except *Vibrio parahaemolyticus*. The chloroform and methanol extracts showed activity against all the tested bacteria.

Duraipandiyan *et al* (2006) designed an experiment to evaluate the antifungal activity of *Azima tetracantha* extracts and isolated compound friedelin and used against fungi. Antifungal activity was carried out using broth micro dilution method and fractions were collected using (silica gel) column chromatography. The antifungal activity of *Azima tetracantha* crude extracts and isolated compound (friedelin) were evaluated using

the micro dilution method. Hexane extract showed some antifungal activity. The compound also exhibited antifungal activity against same fungi. They showed lowest MIC against *Trichophyton rubrum* and *Curvularia lunata.* In both the cases it was 62.5 µg/ml. They suggested that Friedelin was a promising antifungal agent. Shing *et al.* (2001) reported that the hexane extract of *Heliotropium marifolium* yielded a mixture of triterpenoids: β-sitosterol, stigmasterol, β-amyrin, friedelan-3β-ol (epifriedelenol), cycloartenone, β-amyrin acetate, friedelin and epifriedenyl acetate. Isolated triterpenoid and reference antibiotics (gentamycin/mycostatin) were tested against selected pathogenic bacteria and fungi, e.g. *Escherichia coli, Staphylococcus aureus, Aspergillus niger* and *Penicillium chrysogenum*. Joseph *et al* (2010) reported that the 50% methylene chloride in hexane fresh column fraction of the extract of leaves of *Ficcus racemosa* was found to be antifungal. The extract inhibited the growth of several plant pathogens (*Curvularia sp, Colletotrichum gloeosporioides, Alternaria sp, Fusarium sp*).

Phytotoxicity test of cerin and friedelin at 100ppm concentration showed no toxicity on the plants tested up to seven days. Hence the compounds which were effective in controlling pathogens at 100ppm concentration may be recommended for controlling the pathogens *in vivo* or in field conditions.

SECTION 3: References

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