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Antifungal Activity of Camptothecin Extracted from *Mappia foetida* against Disease causing Pathogens in Pomegranate (*Punica granatum* L.)

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Camptothecin (CPT) is alkaloid known to have medicinal as well as pesticidal properties. *Mappia foetida* is one of the important source of camptothecin. Therefore an investigation was undertaken with an objective to extract CPT from *M. foetida* and to evaluate CPT against plant pathogenic fungi and bacteria in pomegranate. High-performance liquid chromatography (HPLC) based analysis revealed the CPT content of roots *of M. foetida* was 0.18%. Extracted CPT was tested against the leaf spot, wilt and bacterial blight diseases causing pathogens in pomegranate. *In vitro* bioassays of CPT isolated from *M. foetida* showed effective control of fungal pathogens like *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Half maximal effective concentration (EC_{50}) of CPT against mycelial growth of *A. alternata* was 250 µg/mL, while against *C. gloeosporioides* it was 500µg/mL CPT. Mycelial growth of *F. oxysporum* was effectively controlled upto 50 % with 250 µg/mL CPT. Bacterial blight caused by *Xanthomonas axonopodis* pv *punicae* could not be inhibited by CPT at the concentrations tested during *in vitro* assay.

Key words: Camptothecin, Bioassay. *Alternaria*, *Colletotrichum*, *Fusarium*, *Xanthomonas*.

Camptothecin (CPT) and its analogs are cytotoxic quinoline alkaloids having remarkable anti-tumor and antileukaemia activity. Semi-synthetic or synthetic drug based on camptothecin are used for chemotherapy, in cancerous treatment. It was found to inhibit growth and metastasis of gastric carcinoma (Oberlies and Kroll, 2004). It is also used in the treatment of various proliferative diseases. Two CPT drugs, Hycamtin and Camptosar, have received Food and Drug Administration (FDA or USFDA) approval for the treatment of ovarian and lung cancers and for colorectal cancer respectively (Oberlies and Kroll, 2004). CPTs also has promising potential as antiviral (HIV and herpes) (Li and Adair, 1994) antifungal (Candida) (Del Poeta *et al*., 1999), and antipsoriasis (Li and Adair, 1994) drugs as well as pesticides (Li, 2002; Yang *et al*., 2014).

The CPT is a quinoline alkaloid with planar pentacyclic ring structure (molecular weight 384.4 dalton), chemically soluble in Dimethyl sulfoxide (DMSO), methanol and water. The structure of CPT isolated from *Camptotheca acuminata* Decaisne was reported in 1966 (Wall *et al*., 1966; Oberlies and Kroll, 2004). To date commercial CPT synthesis is not feasible and supplies of CPT required to manufacture the drugs are now extracted from the *C. acuminata*, which has been listed as an endangered species in China since 1997 (Li *et al*., 2002; Zhang *et al*., 2004).

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Mappia foetida (syn. *Nothapodytes nimmoniana*) (family Icacinaceae) tree is commonly known as the "stinking tree" because of the foul smell it emanates during flowering. Thus it is neither used as timber nor eaten up by herbivores. Their plants have proved to contain high levels of camptothecin (CPT). Because of the enormous demand of CPT, it has been indiscriminate extracted from the source trees. The *M. foetida* plants are distributed in Western Ghats, North eastern India and in the foothills of Himalaya in India, Sri Lanka, Myanmar and Thailand (Wall *et al*., 1966; Li and Adair, 1994). Camptothecin content is influenced by the geographical conditions and their surroundings. Besides *M. foetida* which was found to give maximum yield of CPT, other sources of CPT included *Merriliodendron megacarpum* (family Icacinaceae), *Ophiorrhiza mungos* (family Rubiaceae), *Ervatamia heyneana* (family Apocynaceae) and *Mostuea brunonis* (family Loganiaceae) (Wall *et al*., 1966; Li and Adair, 1994).

The present study was undertaken with the objective to extract CTP from *M. foetida* in a simple way and to conduct *in vitro* antifungal/ antibacterial assay to determine whether CPT inhibits the fungi/bacterial pathogen that often infects the pomegranate and cotton. The results were expected to provide the procedure for isolation of CPT and its application in controlling diseases in economical important plants.

MATERIALS AND METHODS

Plant Materials

The plant materials comprised of roots of *M. foetida* collected from Mahabaleshwar, Maharashtra, India in the month of August 2011. The drug material was dried, powdered and stored at 25ºC in plastic bags till further use.

Chemical analysis

M. foetida root tissues (500 mg) were vortexed in 5 mL methanol and sonicated in bath sonicator by subjecting them to ultrasonic waves for duration of 5 min (18 kHz -1 MHz). Paper filtration was done twice to remove the solid plant extract. Methanol was evaporated overnight in a Petri dish; dissolved in 1.5 mL methanol and refrigerated (at +4°C) for further activity checking.

Methanol extract from root was subjected to HPLC analysis to estimate its CPT concentration. Custom HPLC purification was performed in Doctors' Analytical Laboratories Pvt. Ltd. Pune. Analysis was done in RP18 column with mobile phase used being combination of acetonitrile (45%) and water (55%). Flow rate through column was adjusted to 1 mL/min with injection volume being 20 µl. Various concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 100 ppm) of standard CPT were also simultaneously run on column. The results were detected at 360 nm. Thereafter it was run and the peak was obtained. The graph of 'area under the peak' and 'concentration' was plotted. The area of the peak of unknown sample was then extrapolated and the unknown concentration was calculated. The NMR data of these compounds was in agreement with that reported by Lin and Cordell (1990). General extraction and isolation methods described by Li *et al*., (2005) and Lin and Cordell (1990) were used.

Isolation of Pathogen

Nutrient agar (NA) medium was used for isolation of bacteria associated with bacterial blight leaf samples of pomegranate (*Punica granatum* L.) and cotton (*Gossypium hirsutum* L.). Similarly, potato dextrose agar medium was used for isolation of fungal pathogen from leaf spots and wilt disease samples of pomegranate. Bacteria were isolated by serial dilution method and fungal pathogens were isolated by bit isolation method.

Alternaria alternata, *Colletotrichum gloeosporioides*, *Xanthomonas axonopodis* pv. *punicae* were isolated from infected leaves and fruits; while *F. oxysporum* was isolated from the infected roots of *P. granatum*. Additional *Xanthomonas* spp. from cotton i.e. *X. axonopodis* pv. *malvacearum* was taken for within species comparison therefore it was isolated from the infected leaves of cotton grown in experimental research farm in Mahatma Phule Krishi Vidyapeeth campus, Rahuri in 2011-12. The fungal isolates were purified and maintained on potato dextrose agar (PDA) medium at 28ºC; while the bacterial isolates were cultured on nutrient agar medium and maintained on YCGA (Yeast Charcoal Glucose agar) medium.

Pathogens Analysis

CPT isolated from the *M. foetida* was evaluated against the three plant pathogenic fungi *i.e. A. alternata*, *C. gloeosporioides* and *F. oxysporum* and one bacterial pathogen *X. axonopodis* pv. *punicae*. The CPT extracted from

the root of *M. foetida* was evaluated for their ability to inhibit fungi and bacteria at three concentrations *i.e.* 250, 500, and 1000 ppm by food poison method against the every test pathogen.

In addition to a negative control, these three recommended fungicides *i.e.* Mancozeb (2500µg/mL) (manganese ethylene bis(dithocarbamate) (polymeric) complex with zinc salt), copper fungicide copper oxychloride (coc-2500µg/mL) and captan {2-[(trichloromethyl) sulfanyl]-3a,4,7,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione}; as well as two recommended antibiotics i.e. streptocycline (250 µg/mL) and 2-bromo, 2-nitropropane, 1,3, diol (bronopol)_(250µg/mL) were taken as positive control. These chemicals were recommended in our previous work (Raghuwanshi *et al*., 2013). For fungal culture, final concentrations were made in molten (50ºC) potato dextrose agar and for bacterial cultures final concentrations were made in molten (50ºC) nutrient agar and 20 mL aliquots were poured into each petridish (90 mm in diameter). Within 6 h after pouring each of the PDA plates was inoculated at centre with one of the three fungi and the NA plates was inoculated with either of the bacterium by streaking methods. Fungal cultures taken for inoculation were two weeks old while bacterial cultures were 48h old. These inoculated plates were incubated at 28ºC. Four replicate plates were inoculated for each treatment. Growth of all pathogens on each plate was observed daily for seven days. Colony radii were measured along four vertical radial directions in ease of fungal pathogen while growth of bacterial pathogen was observed along the sticking. The mean of the four measurements was calculated as the growth rate on each plate. The mean and standard error were calculated from the four replicates of each treatment.

RESULTS AND DISCUSSION

Pomegranate cultivation in India is threatened by various diseases like leaf and fruit spots, wilt and bacterial blight (Raghuwanshi *et al*., 2004; Jadhav and Sharma, 2009). Leaf and fruit spots caused by *C. gloeosporioides*, *A. alternata*, *X. axonopodis* pv. *punicae* and wilt caused by *F. oxysporum* are major fungal and bacterial diseases in *P. granatum* cultivation. Leaf and fruit spot diseases severity increase constantly under humid weather conditions which prevail particularly in rainy season for maximum period as a result plant suffers more due to these diseases during rainy seasons and causes yield losses upto cent percent under favourable conditions. Similarly incidence of wilt disease also increases considerably during rainy season. Prevailing of soil moisture above field capacity in and around the rhizosphere of the plant resulted in considerable increase in the severity of the disease. *F. oxysporum* is cosmopolitan, soil borne inhibiting in all types of soil in every regions and every location. It is a weak pathogen infecting through wounds under moist conditions. *X. axonopodis* pv *malvacearum* causing leaf blight disease in cotton was selected for comparative study. *X. malvacearum* is a major bacterial pathogen affecting all parts of the cotton plant which affects the yield under favourable conditions (Monga *et al*., 2011).In the present study efficiency of CPT as an antifungal/antibacterial agent against these pathogens was investigated.

HPLC analysis

Methanol extract was subjected to custom HPLC purification was performed in Doctors' Analytical Laboratories Pvt. Ltd. Pune. Results of the extracts from root sample of unknown concentration were compared with that of standard CPT of known concentrations. The CPT concentration in the root extract was found to be 60ppm. Roots of *M. foetida* plants were found to contain high levels of CPT. Methanol extracts from dried roots could be successfully used for getting as high as 0.18% CPT content.

Antifungal Activity of CPT

CPT significantly inhibited *Alternaria* growth at all concentrations (Table 1). Maximum inhibition of *Alternaria* growth was noticed at CPT–1000 µg/mL (PL 0.005). Colonies exposed to CPT (1000 µg/mL) were inhibited 86.34% over positive control. The mycelium growth was 3.62 mm under the CPT-1000µg/mL treatment; while it was 4.50 mm in positive control and 26.5 mm in negative control. CPT-1000 µg/mL was more effective than recommended chemical mancozeb-2500µg/mL. CPT-250 µg/mL and CPT-500µg/mL inhibited mycelium growth up to 72.83% and 76.60% respectively over negative control; while it was less effective than positive control. On day 7, colonies in all CPT treatments were 72.83 to

83.01% smaller than those of the negative control. Thus, it was estimated that the EC_{50} of CPT for *A. alternata* was 250 µg/mL.

C. gloeosporioides grew slower than *A. alternata* and *F. oxysporum* under control conditions in the experiment with colony covering agar surface upto 25.25 mm in 7 days (Table 2). However, this fungus was strongly inhibited by CPT than *A. alternata*. CPT – 1000µg/mL significantly inhibited the growth of *C. gloeosporioides* by 90.10% over control and 50.0%over positive control. CPT at 500 µg/mL inhibited growth of *C. gloeosporioides* by 82.18% over control.. The EC_{50} of CPT for *C. gloeosporioides* was estimated to be 250 µg/mL because mycelia growth was 48.51% smaller than negative control on day seven in CPT- 250 µg/mL. Mycelial growth of *C. gloeosporioides* in CPT – 500µg/mL treatment was 82.18% smaller than negative control on day seven. .CPT – 500µg/mL and CPT-1000 µg/mL restricted the growth of *C. gloeosporioides* better than positive control.

Table 1. Effect of CPT isolated from roots of *M. foetida* on growth of fungi at different concentrations

S. No	Particular µg /mL of water	Mean diameter of fungi (mm)	Percent inhibition of fungal growth over control	Percent inhibition of fungal growth over positive control
1.	CPT - 250	7.2	72.83	- 60.0
2.	CPT -500	6.25	76.60	- 38.89
3.	CPT -1000	3.62	86.34	19.55
4.	Mancozeb - 2500 (positive control)	4.50	83.01	-
5.	Negative control -	26.5	-	-
	SE±	1.585	-	-
	CD at 5%	3.378	-	-

F. oxysporum exhibited the fastest growth rate in all experimental fungi under control conditions and shown significant sensitivity to CPT and positive control (Table 3). CPT-250µg/mL inhibited the growth of *F. oxysporum* by 69.65% over control and maximum inhibition of *F. oxysporum* growth was observed at CPT 1000µg/mL ($P<.005$). Colonies exposed to CPT-1000 µg/mL were inhibited by 89.65% over negative control and 25% over positive control *F. oxysporum* cover 36.25 mm agar surface on days 7 under the control conditions in the experiment while other two fungal pathogens grew little slower than this pathogen. The colony of *F. oxysporum* in CPT (250 µg/mL) was 69.65% small than control on day seven. Thus EC_{50} of CPT for *F. oxysporum* was estimated to be 250µg/mL. CPT-1000µg/mL inhibited the *F. oxysporum* growth 25% better than the positive control.

Table 2. Effect of CPT isolated from roots of *M. foetida* on growth of *C. gloeosporioides* fungi at different concentrations

S. No	Particular µg /mL of water	Mean diameter of fungi (mm)	Percent inhibition of fungal growth over control	Percent inhibition of fungal growth over positive control
1.	CPT - 250	13.00	48.51	- 160.0
2.	CPT -500	4.5	82.18	10.00
3.	CPT -1000	2.5	90.10	50.00
4.	Copper oxychloride - 2500 (positive control)	5.0	80.20	-
5.	Negative control -	25.25	-	-
	SE±	0.7472	-	-
	CD at 5%	1.5926	-	-

Bioassays showed that the CPT can effectively inhibit the fungal pathogen *in vitro*. CPT can effectively inhibit mycelia grown by 50%

(EC_{50}) at relative low concentrations approximately < 250 µg/mL for *A. alternata*, and *F. oxysporum*, while *C. gloeosporioides* needs 500 µg/mL concentration for inhibition of 50 % (EC_{50}) fungal growth. Higher concentration of CPT more

Table 3. Effect of CPT isolated from roots of *M. foetida* on growth of *F. oxysporum* fungi at different concentrations

S. No	Particular µg /mL of water	Mean diameter of fungi (mm)	Percent inhibition of fungal growth over control	Percent inhibition of fungal growth over positive control
1.	CPT - 250	11.0	69.65	- 120.0
2.	CPT -500	5.50	84.83	- 10.0
3.	CPT -1000	3.75	89.65	25.0
4.	Copper oxychloride - 2500 (positive control)	5.0	86.20	-
5.	Negative control -	36.25	-	-
	SE±	1.7368	-	-
	CD at 5%	3.7020	-	-

effectively inhibited mycelia growth, but the minimal inhibitory concentration varied among the fungal pathogen. Fungi varied in their sensitivity to the CPT and fungicides. Mancozeb successfully suppressed the growth of *A. alternata* while copper oxychloride controlled *C. gloeosporioides* and *F. oxysporum* successfully. The pathogens grew fast in negative control plate but the all three fungal pathogens were inhibited by CPT at relatively low concentrations except *C. gloeosporioides.* These results are in conformity with the result obtained by Li *et al* (2005). They reported that CPT isolated from *Camptotheca* effectively controlled fungal pathogen *in vitro* includes *A. alternata*, *Epicoccum nigrum, Pestalotia guepinii, Drechslera* sp. and *Fusarium avenaceum*. CPT showed more potent antifungal activity than some recently discovered natural antifungal products. Carpinella *et al*. (2003) reported that most potent antifungal compounds were vanillin, 4–hydroxy-3-metoxycinnamalehyde, and (α)–pinoresinol isolated from *Melia azedarach* L. (Meliaceae) controlled *Fusarium verticillioides* at higher concentration [with minimal inhibition concentrations (MICs) of 600, 400 and 1000µg/mL respectively]. The MIC of CPT in present investigation against *F. oxysporum* was L 250µg/mL.

Antibacterial activity of CPT

In vitro bioassay of CPT was also carried out against *X. axonopodis* pv. *punicae* and *X. axonopodis* pv. *malvacearum* at various three different concentrations. The results showed (Table 4 and 5) infectiveness of CPT against *Xanthomonas* spp. The growth of both *Xanthomonas* spp. was observed in all three concentration of CPT whereas, no growth was observed in positive control.

Table 4. Effect of CPT isolated from roots of *M. foetida* on growth of plant pathogenic bacteria *X. axonopodis* pv. *punicae* at different concentrations

S. No.	Particular µg /mL of water	Growth of bacteria
1.	CPT - 250	√
2.	CPT -500	√
3.	CPT -1000	√
4.	Streptocycline (positive control)	√
5.	Bronopol	-
6.	Captan	-
7.	Negative control -	√

Table 4. Effect of CPT isolated from roots of *M. foetida* on growth of plant pathogenic bacteria *X. axonopodis* pv. *punicae* at different concentrations

S. No.	Particular µg /mL of water	Growth of bacteria
1.	CPT - 250	√
2.	CPT -500	√
3.	CPT -1000	√
4.	Streptocycline (positive control)	-
5.	Negative control -	√

√ = Growth of plant pathogenic bacteria observed

CONCLUSION

It could be concluded that methanol extract from root tissues of *M. foetida* had high level of CPT and could be used as an alternate source of CPT instead of *C. acuminata*. CPT showed better antifungal activity over positive control under *in vitro* conditions and therefore could be used for field trials to control leaf and fruit spots and wilt diseases of pomegranate. After critical evaluation of CPT against fungal pathogens an excellent effective and easy applicable formulation may come out in near future. Judicious use of CPT under field conditions may reduce environmental pollution and pesticide residues in the pomegranate fruit. Further economical viability of *M. foetida* extract as a source of CPT for controlling plant pathogens also needs to be investigated.

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REFERENCES

1. Carpinella, M.C., Giorda, L.M., Ferrayoli, C.G., Palacios, S.M. Antifungal effects of different organic extracts from *Melia azedarach* L. on phytopathogenic fungi and their isolated active compounds. *J. Agric. Food Chem*. 2003; **51**: 2506-2511.
2. Del Poeta, M, Chen, F., Von Hoff, D., Dykstra, C.C., Wani, M.C., Manikumar, G., Heitman, J., Wall, M.E., Perfect, J.R. Comparison of *in vitro* activities of camptothecin and nitidine derivatives against fungal and cancer cells. *Antimicrob. Agents Chemother.* 1999; **43**: 2862-2868.
3. Jadhav, V.T., Sharma, K.K.: Integrated management of diseases in pomegranate. pp. 48-52. In: *2nd International symposium on Pomegranate and minor including Mediterranean fruits*. Univ Agril. Sci, Dharwad 2009.
4. Li, S., Zhang, Z., Cain, A., Wang, B., Long, M., Taylor, J. Antifungal activity of camptothecin, trifolin and hyperoside isolated from *Camptotheca acuminata. J. Agric. Food Chem.* 2005; **53**: 32-37.
5. Li, S.Y. Utilizing camptotheca products for termite control. U.S. Patent Appl. US20020018762 and PCT Int. Appl. WO 0200020, 2002.
6. Li, S.Y., Adair, K.T. (eds.): *Camptotheca acuminata Decaisne*, Xi Shu, Chinese Happy tree: A promising antitumor and antiviral tree for the 21st Century. The Tucker Center College of Forestry Stephen F. Austin State University, Nacogdoches, Texas, USA. 1994; pp. xviii + 249 pp.
7. Li, S.Y., Yi, Y.J., Zhang, Z.Z., Beasley, R.S. Camptothecin accumulation and variation in *Camptotheca decaisne*. *Planta Med.* 2002; **68**: 1010-1016.
8. Lin, L.Z., Cordell, G.A. 13C-NMR assignments of camptothecine and 10-hydroxy camptothecine. *J. Nat. Prod.* 1990; **53**: 186-189.
9. Monga, D., Kranthi, K.R., Gopalakrishnan, N., Mayee, C.D., Venugopalan, M.V., Balasubramanya, R.H., Kranthi, S., Singh, S.: Changing scenario of cotton diseases in India-the challenge ahead. pp. 272-280. In: *World Cotton Research Conference-5*, Mumbai, India, Excel India Publishers. 2011.
10. Oberlies, N.H., Kroll, D.J. Camptothecin and taxol: historic achievements in natural products research. *J. Nat. Prod.* 2004; **67**: 129-135.
11. Raghuwanshi, K.S., Dake, G.N., Sawant, D.M., Pharande, A.L. Chemical control of leaf and fruit spot of pomegranate in *Hastabahar*. *J. Maharashtra Agric. Univ.* 2004; **30**(1): 56-58.
12. Raghuwanshi, K.S., Hujare, B.A., Chimote, V.P., Borkar, S.G. Characterization of *Xanthomonas axonopodis* pv. *punicae* isolates from western Maharashtra and their sensitivity to chemical treatments. *Bioscan* 2013; **8**(3): 845-850.
13. Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T., Sim, G.A. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *J. Amer. Chem. Soc.* 1966; **88**: 3888-3890.
14. Yang, L., Zhang, Z.J., Liu, Y.Q., Zhao, C.Y., Wang, M.J., Nan, X., Feng, G. Evaluation of insecticidal activity of camptothecin analogs against *Brontispa longissima*. *Med. Chem. Res.* 2014; **23**(2): 980-986.
15. Zhang, Z.Z., Li, S.Y., Zhang, S.M., Liang, C., Gorenstein, D., Beasley, R.S. New Camptothecin and ellagic acid analogues from the root bark of *Camptotheca acuminata. Planta Med*. 2004; **70**(12): 1216-1221.