

***In Vitro* Antioxidant and Anti-rhizopus Activity of Methanolic Seed Extract of *Camelina Sativa* L.**

Sanjay Mohan Gupta* and Kamal Kumar

Molecular Biology and Genetic Engineering Laboratory, Defence Institute of Bio-Energy Research, Haldwani - 263 139, India

*E-mail: smg555@rediffmail.com

ABSTRACT

The antifungal efficacy of seed extracts (propanol, ethanol, methanol and sterile deionised water) of *Camelina sativa* cv. Calena (EC643910) against *Rhizopus stolonifer* was investigated by agar disc diffusion method. Among all extracts, methanolic seed extract (Disc 3) showed significant activity against *R. stolonifer*. However, no activity was observed against rest of the extracts (Disc 1, 2 and 4). The inhibition zones were ~35 mm for methanolic extract and ~41 mm for standard drug, respectively. The total phenol content was observed 13.5 μmol , 23.3 μmol , 42.9 μmol and 3.4 $\mu\text{mol gDW}^{-1}$ in propanolic, ethanolic, methanolic and sterile deionised water extracts of *Camelina*, respectively. Likewise, the ascorbate peroxidase (APX) activity was observed highest in methanolic extract that was ~10.2, ~2.4 and ~5.7-folds higher as compared to propanolic, ethanolic and sterile deionised water extracts, respectively. Similarly, guaiacol peroxidase (GPX) activity was also observed highest in methanolic extract as compared to other extracts, which is ~7.3, ~1.9 and ~6.6-folds higher as compared to propanolic, ethanolic and sterile deionised water extracts, respectively. The findings of the study clearly speculate that the possibility of using methanolic seed extract of *Camelina* as a potential control measure against *R. stolonifer*, which may be used for the development of future herbal drug formulations.

Keywords: Agar disc diffusion assay; Antifungal and antioxidant activity; *Camelina* seed extracts; *Rhizopus stolonifer*; Soft rot disease

NOMENCLATURE

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
DMSO	Di-methyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
F-C	Folin-Ciocalteu
GPX	Guaiacol peroxidase
ITC	Isothiocyanates
LSD	Least significant difference
PDA	Potato dextrose agar
PMF	Proton motive force
PVP	Polyvinyl pyrrolidone
ROS	Reactive oxygen species

1. INTRODUCTION

Rhizopus stolonifer is a ubiquitous fungal pathogen causing soft rot disease in more than 300 plants including fruits, vegetable and ornamental crops¹. It was first described in 1818 and first recognised as a pathogen on sweet potato in 1890. The huge economic losses occur due to formation of watery soft rot of internal storage tissue of the vegetables and fruits during transportation². The characteristic features of this disease include the production of tufts of white hyphae which break through the surface of the root and produce large numbers of brown-black sporangiophores, which produces a characteristic

fermentation odor³. Infection can start from any part of the storage root but it usually visible first at the ends due harvest wounding. Thus, *R. stolonifer* is a problematic pathogen as it severely infects fresh wounds occurring during packing and shipping of most of the fruit and vegetable crops².

Effective methods to control this disease include the pre-treatment of fruit and vegetables with heat (29 °C) and humidity (90 per cent RH), use of decay control products and use of fungicides⁴. However, the development and use of resistant varieties of vegetables and fruits against *R. stolonifer* is also good options but it required more time. Among resistant varieties, beauregard is supposed to be mild resistant against rhizopus soft-rot disease, although severe losses occur during its transportation⁵. Till date, no cultivar has been found, which is completely resistant to this disease. However, curing and application of decay control products strategies are not effective and can give short-term relief. Therefore, the use of broad spectrum fungicide like Dicloran (also known as DCNA or Botran) is the only effective solution to this problem. But, now these days, it has been recommended to use natural bioactive compounds instead of using synthetic compounds due to safety and toxicity concerns that encouraged scientists to explore more medicinal plant resources⁶. Also, fungal pathogens have developed resistance to commonly broad spectrum fungicides due to their arbitrary use⁷. Therefore, there is urgent need to search for novel natural antifungal compounds from several unexplored plants including *Camelina sativa*⁸⁻¹⁰.

Camelina sativa L. Crantz, (*Brassicaceae*) is an oilseed bio-fuel crop that native to northern Europe and central Asia. It has been traditionally cultivated for vegetable oil and animal feed^{10,11}. Camelina seed contains potential secondary metabolites namely, phytoalexin as camalexin¹²; phenols as sinapine (sinapic acid) and phytic acid¹³; antioxidants as tocopherols¹¹, flavonols as quercetin¹⁴; omega-3 fatty acids¹⁵ and glucosinolates¹⁶. The seeds, leaves and roots extracts of camelina contained several anti-insect compounds as natural pesticides that showed strong antimicrobial activity towards a variety of pathogens¹⁷.

This paper reports the antifungal and antioxidant activities of different seed extracts of *Camelina sativa* cv. Calina (EC643910) against *Rhizopus stolonifer* pathogen. Thus, the present study will suggest the possibility of using camelina extracts as a potential control measure against *R. stolonifer*, which may further used for the development of future herbal drug formulations.

2. MATERIALS AND METHODS

2.1 Extracts Preparation

Seeds were collected from the mature *Camelina sativa* cv. Calina (EC643910) plant grown under natural conditions at Defence Institute of Bio-Energy Research (DIBER), Haldwani. Seeds were surface sterilised (70 per cent ethanol for 5 min) and washed thoroughly with sterile deionised water. After drying, seeds were ground to fine powder using mixer grinder (REMI, India). The fine seed powders (5 g) was mixed with 20 ml of 90 per cent aqueous solvents (propanol, ethanol, methanol and sterile deionised water) and allowed to incubate for 48 h at room temperature (RT) with gentle shaking in dark, separately. After incubation, the extracts were centrifuged at 10000 g for 15 min and obtained supernatants were completely evaporated in a centrivap concentrator (Labconco, Biogentek, India). The dried powders were separately dissolved in 500 µl of Di-methyl sulphoxide (DMSO) by keeping the tubes in water bath at 37 °C for 1 h. An aliquot of 10 µl of each solvent extract was applied on disc 1 to 4 (6 mm, Himedia), separately. Standard antifungal drug (Clotrimazole; 100 µg/ml) was applied on disc 5 as positive control, while DMSO solvent applied on disc 6 as negative control.

2.2 Estimation of Anti-rhizopus Activity

Pathogenic culture of *Rhizopus stolonifer* was obtained from G. B. Pant University of Agriculture and Technology, Pantnagar, India. *In vitro* anti-rhizopus activity was carried out against standard drugs (Clotrimazole) and different Camelina seed extracts (propanol, ethanol, methanol and sterile deionised water) by agar disc diffusion assay method¹⁸. A 100 µl aliquots of *R. stolonifer* cultures (about 10⁸ cfu/ml) were evenly spreaded on to PDA (Potato dextrose agar; Himedia) plates (90 mm diameter) using a sterile spreader, respectively. The plates were left at RT for 15 min to allow the agar surface to dry. The sterile discs containing the standard drug and test compounds were placed on the inoculated plates. The PDA plates were incubated at 28 °C for 24 h to 48 h, respectively. Cleared zones were measured as anti-rhizopus activity of

standard drug and test compounds against pathogenic *R. stolonifer* culture. The antifungal activity was evaluated by measuring the diameter of the inhibition zone¹⁸. The antifungal activity was scored following Baur's method that suggests the microbicidal activity is classified into resistant if the zone of inhibition in mm is less than 7; if it is 7 mm - 9 mm intermediate; if the inhibition is 10 or more it is sensitive. The experiments were performed in triplicate under strict aseptic conditions and the data were calculated as mean ± SD.

2.3 Estimation of Antioxidant Activity

2.3.1 Total Phenolic Content

Phenol content was estimated according to the Folin-Ciocalteu reagent (F-C) method¹⁹. The absorbance was measured at 765 nm and total phenol content (mM µM⁻¹ gallic acid equivalent) was calculated using gallic acid (50 µM to 250 µM) standard in 95 per cent (v/v) methanol.

2.3.2 Ascorbate Peroxidase Assay

APX activity was determined following standard method of Nakano and Asada²⁰. The reaction mixture (2 ml) consisted of 50 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 1 mM H₂O₂ (hydrogen peroxide) and 100 µl enzyme extract. The H₂O₂-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). APX activity was measured in terms of µmol mg protein⁻¹ min⁻¹.

2.3.3 Guaiacol Peroxidase Assay

GPX activity was estimated according to the method of Urbanek²¹, *et al.* with slight modifications. The activity was measured in a reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0), 5 mM guaiacol and 15 mM H₂O₂. The reaction was initiated by adding 100 µl enzyme extract and the activity was determined by monitoring increase in absorbance at 470 nm ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) for 2 min at intervals of 15 s. The slope of the rate assay (ΔA) was used to determine the enzyme activity, that was expressed as µmol mg protein⁻¹ min⁻¹.

2.4 Statistical Analysis

The all treatments and controls of the experiments were carried out in triplicates. The CropStat for Windows (7.2.2007.2 module) was used for analysis of variance (ANOVA) for experimental data. The treatment means were compared by least significant difference (LSD) test at a significance level of P≤0.05. Duncans multiple range test (DMRT) was also performed to study the significance of differences between mean values.

3. RESULTS AND DISCUSSION

In present study, four extracts (propanol, ethanol, methanol and sterile deionised water) of Camelina seed were tested for their antifungal activity against *R. stolonifer* pathogen by agar disc diffusion method. Among these, dried supernatant of methanolic seed extract (Disc 3) showed significant, activity against *R. stolonifer* (Table 1; Fig. 1). However, no activity was observed against rest of the extracts (Disc 1, 2

and 4) and in negative experimental control (Disc 6) against *R. stolonifer*. Methanolic Camelina seed extracts (Disc 3) showed inhibition zone ~35 mm, respectively (Table 1), which is quite comparable to the inhibition zone (~41 mm) of the standard antifungal drug (Clotrimazole; 100 µg/ml; Disc 5) (Table 1; Fig. 1).

Table 1. Antifungal activity of *Camelina sativa* cv. Calena (EC643910) seed extracts (propanolic, ethanolic, methanolic and sterile deionised water) against *Rhizopus stolonifer*. Standard antifungal drug (Clotrimazole; 100 µg/ml) and DMSO were applied on disc 5 and 6, as positive and negative control, respectively. The experiments were conducted in triplicates and the data were calculated as mean ± SD

Disc no.	Test compounds	Antifungal activity (Diameter of inhibition zone in mm)
Dried supernatant of Camelina seed extracts dissolved in DMSO		
1	Propanol	-
2	Ethanol	-
3	Methanol	35±0.2
4	Sterile Water	-
Experimental Controls		
5	Standard drug	41±0.4
6	DMSO	-

Major highlights of the results: Among all *Camelina* seed extracts tested, methanolic seed extract (Disc 3) showed significant antifungal activity against *Rhizopus stolonifer*.



Figure 1. *In vitro* antifungal activity of *Camelina sativa* cv. Calena (EC643910) seed extracts [propanolic (disc 1), ethanolic (disc 2), methanolic (disc 3) and sterile deionised water (disc 4)] against *Rhizopus stolonifer*. Standard antifungal drug (Clotrimazole; 100 µg/ml) was applied on disc 5 as positive control. The DMSO solvent was also applied on disc 6 as negative control.

Plants are unlike humans lacks defence immune systems therefore; they produce a wide range of secondary metabolites that provide defence against various pathogens⁷. It has been proven that extracts of many species of the *Brassicaceae* family including *Camelina* showed antimicrobial activities due to the presence of various bioactive compounds like camalexin, sinapic acid, phytic acid, flavonols, glucosinolates and many more¹⁷. The first studied phytoalexin is a camalexin that was firstly isolated from the leaves of the crucifer *C. sativa*, which provides an exceptional model for the investigation of phytoalexins in defence responses¹². Several *in vitro* studies showed the antimicrobial activity of camalexin against various pathogens²²⁻²³. Actually, camalexin disrupts cell membrane of bacterial and fungal pathogens²⁴. However, phenolic compounds like sinapic, phytic acids and derivatives of flavonols (quercetin) have been considered as potent antioxidant with antimicrobial, anti-inflammatory and anticancer properties by many workers^{14,25,26}. It is speculated that the mechanism of flavonoid antimicrobial activity is due to their ability to inhibition of microbial adhesion and inactivation of cell envelope transport proteins¹⁴. Similarly, flavonoids also disrupt the fluidity of the microbial membranes²⁷. In addition to these, *Camelina* seeds accumulate significant levels of glucosinolates, which are derived from amino acids biosynthesis and are responsible for their bitter taste and act as natural pesticides against various pathogens¹⁶. They are further converted by the endogenous thioglucosidase myrosinase into isothiocyanates (ITCs) and nitriles that inhibit and kills pathogens¹⁶. Also, *Camelina* seed contains exceptionally high level of omega-3 fatty acids and antioxidants such as tocopherols, syringaldehyde and many more^{15,28} as second line of plant defence mechanisms. It has been proven that the solubility of most of the secondary metabolites is highest in methanol as compared to other solvents. Therefore, the observed significant activity of methanolic seed extracts of *Camelina* (Table 1) against *R. stolonifer* clearly suggests the presence of novel bioactive compounds.

The antioxidant potential of *Camelina* seed extracts (propanol, ethanol, methanol and sterile deionised water) was also measured in terms of the total phenol content and APX and GPX enzyme activities. The total phenol content was observed 13.5, 23.3, 42.9 and 3.4 µmol gDW⁻¹ in propanolic, ethanolic, methanolic and sterile deionised water extracts of *Camelina*, respectively, (Fig. 2(a)). The methanolic seed extract of *Camelina* contained highest (~2 to 13-folds) total phenolic content as compared to other test extracts (Fig. 2(a)) that suggest its potential antifungal activity.

In addition, the enzymatic antioxidants like APX, GPX *etc* play essential role in protecting cells from oxidative damage caused by ROS in plants. Therefore, we estimated the APX and GPX activities in different camelina seed extracts. APX specific activity was observed 0.8 µmol, 3.5 µmol, 8.6 µmol, and 1.5 µmol mg protein⁻¹ min⁻¹ in propanolic, ethanolic, methanolic and sterile deionised water seed extracts of *Camelina*, respectively (Fig. 2(b)). Like phenolic content, it was observed highest in methanolic seed extract, which was ~10.2, ~2.4 and ~5.7-folds higher as compared to propanolic, ethanolic and sterile deionised water extracts of

Camelina, respectively (Fig. 2(b)). Similarly, GPX activity was observed 10.5 $\mu\text{mol mg protein}^{-1} \text{min}^{-1}$ in propanolic, ethanolic, methanolic and sterile deionised water seed extracts of Camelina, respectively (Fig. 2(c)). It was also observed highest in methanolic seed extract as compared to other extracts, which is ~ 7.3 , ~ 1.9 and ~ 6.6 -folds higher as compared to propanolic, ethanolic

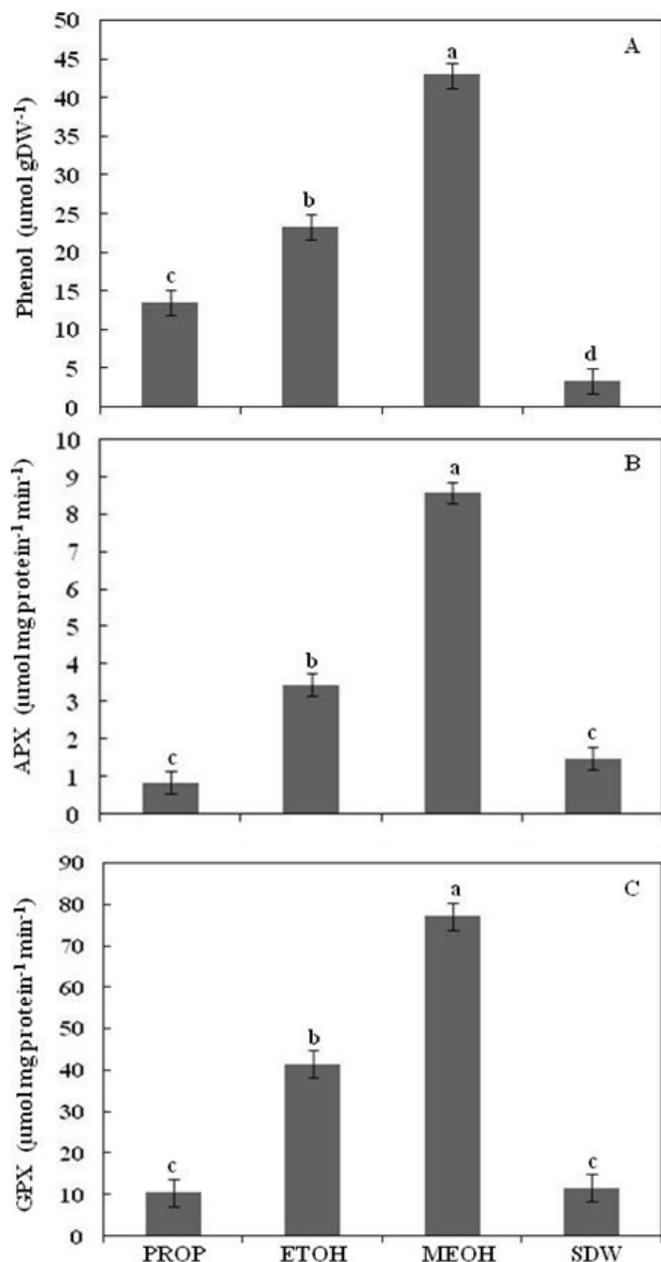


Figure 2. Antioxidant properties of *Camelina sativa* cv. Calena (EC643910) seed extracts [propanolic (PROP); ethanolic (ETOH); methanolic (MEOH) and sterile deionised water (SDW)]; (A) total phenol content; (B) ascorbate peroxidase (APX) enzyme activity; and (C) guaiacol peroxidase enzyme activity. Bar shown means ($n = 3$) \pm SE are statistically significant ($P \leq 0.05$) according to least significant difference (LSD) test. Different letters used in each column indicate significant differences at $P \leq 0.05$, according to Duncans multiple range test (DMRT).

and sterile deionised water extracts of Camelina, respectively (Fig. 2(c)). However, no significant ($P \leq 0.05$) difference in APX and GPX activity was observed among propanolic and deionised water seed extracts of Camelina.

It is well proven that the antioxidant enzymes like APX, GPX *etc* exerts its biological activity by inhibiting microbial growth that helped various nutraceutical industries²⁹. The antimicrobial properties of these enzymatic antioxidants were already studied by many researchers^{30,31}. The enzymatic antioxidants disrupt the membrane proton motive force (PMF) that leads to leakage and lysis of intracellular constituents of the pathogens. It also modulated the perturbation of cell homeostasis; inhibition of enzymes of electron transport, and oxidative phosphorylation; coagulation of cytoplasmic constituents and effects on various macromolecular biosynthetic processes in pathogens³³. Also, it has been suggested that enzymatic antioxidant activity played important role in polymerisation of lignin and suberin (as structural barriers), cross-linking of wall protein²⁹ and dimerisation of antimicrobial phenols that are toxic to pathogens^{33,34}. Likewise in our case, the total phenolic content and APX and GPX antioxidant enzyme activity was observed highest (~ 2 to 13-folds) in methanolic seed extract of Camelina as compared to other extracts that suggest its potential antioxidant property against *R. stolonifer*.

4. CONCLUSION

The findings of the study suggest, the methanolic seed extract of Camelina is a potential source of natural bioactive antimicrobial compounds against *R. stolonifer*. However, detail research works on purification, characterisation, pre-clinical and human trials of identified bioactive compounds from Camelina seed extracts are required for the development of future herbal drug formulations against *R. stolonifer*.

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CONTRIBUTORS

Dr Sanjay Mohan Gupta did his PhD (Biochemistry), in 2007 from University of Lucknow, Lucknow. He has isolated and characterised ethylene regulated and ripening related 27 novel genes from banana pulp tissue by differential-display (DDRT-PCR) method. Presently, he is working as Scientist 'D' in DIBER, Haldwani and involved in research project investigating the antifungal and antioxidant activity of different leaf, seed extract and seed oil of *Camelina sativa* and stinging plants against various pathogenic microorganisms. He has been awarded with Laboratory Scientist of the Year -2010 by DRDO. Recently, he has also been awarded the 'National Technology Day-2015 by DRDO.

Dr Kamal Kumar did his PhD in Botany in 2015 from Magadh University, Bodh Gaya and worked on "ecological studied of *V. faba* under abiotic stress in Nalanda, Bihar. He joined DRDO in 2012 as JRF and presently working as SRF and involved in the abiotic stress tolerance, Phytochemical and toxicity studies in many plants.