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INSECTICIDAL PROPERTIES OF SECONDARY METABOLITES PRODUCED BY AN ENDOLICHENIC FUNGUS, *Penicillium pinophilum* AGAINST STORED GRAIN INSECT PESTS, *Callosobruchus maculatus*

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Abstract: Fungi produce secondary metabolites with insecticidal properties. To investigation the insecticidal activity from the isolation of secondary metabolites in fungus, *Penicillium pinophilum* and their toxicity against cowpea weevil, *Callosobruchus maculatus*. Pure fungi cultures were grown in small scale on PDA for two weeks. The fungi grown on PDA were used for further investigations and grown in large scale. After extracted to EtOAc and isolated bioactive compounds. The bioactive EtOAc extracts were partitioned with hexane, $CHCl_3$ and 50 % MeOH and bioactivity of the three fractions (hexane fraction, chloroform fraction and 50 % MeOH fraction) were evaluated for bioactivity separately using the insecticidal bioassay (Residual Film Bioassay) and anti-inflammatory bioassay (Heat Induced Human Red Blood Cell Bioassay). After bioactive $CHCl_3$ fraction and it sub-fractions were further purified using column chromatography and preparative TLC respectively. Pure compound 3 isolated from the chloroform extract of *P. pinophilum* was shown highly % mortality values (lowly LD_{50} values) and pure compounds 1, 2 and 3 were shown highly % inhibition values for above bio assays respectively. So results suggest pure compound 3 can be used as bio-insecticide against *C. maculatus* and pure compounds 1, 2 and 3 better than that aspirin as anti-inflammatory drugs.

Keywords: Insecticidal Bioassay, Anti-Inflammatory Bioassay, *P. pinophilum*, *C. maculates*

Introduction

Cowpea, (*Vigna unguiculata*) is one of the most nutritious grain legumes for human consumption worldwide. It is a valuable source of dietary protein, vitamins and minerals. Loss of seed yield in legume crops during storage due to different types of stored-grain insects, notably bruchid beetles, is a very serious problem for farmers and traders.[1] Cowpea weevil, *Callosobruchus maculatus* (Coleoptera: Bruchidae) is one of the most destructive pest species of cowpea.

***Callosobruchus maculatus*:** Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Coleoptera, Family: Chrysomelidae, Genus: Callosobruchus, Cowpea weevils, Growth temperature: 32 ºC, Life time: 10 days, Humidity: 60% - 90%. Eggs: Adult females oviposit their egg on the surface of the cowpea and eggs are cemented to the surface of cowpea. Larva and Pupa: The larvae and pupae are normally only found in cells bored within the cowpea. Adult: *C. maculatus* adults are 2.1-3.5 mm long. The females are maculated with four elytral spots and males are plain with no distinct spots.[11, 12]

Recent estimations have revealed that about 2.5 million tons of pesticides are used on crops and the worldwide damage caused by pesticides reaches $100 billion annually.The indiscriminate use and the excessive reliance on chemical pesticides (phosphine, organophosphates) in crop protection have resulted in serious problems linked to water and environmental contamination, phytotoxicity, and toxic hazards to human and non-target organisms. Selection of resistant insect strains, including *C. maculatus* against chemical pesticides is also a thorny problem in crop protection.[12] These concerns have resulted in a renewed interest being given to the search for new approaches to control insect pests without these negative draw-backs. In recent years, many research studies focused on the use of natural materials as low-risk pest control agents.[15, 16]

Bio-insecticides are certain types of insecticides derived from such natural materials as animals, plants, bacteria, fungi, viruses and certain minerals (Examples: *Bacillus thuringiensis, Azadirachtin, Bacillus popilliae*, Essential oils (citrus peel oil), Plants and plant extracts (*Aframomum melegueta, Aglaia elliptical, Annona spp*.)). Advantages of bio-insecticides: Do not persist long in the environment and have shorter shelf lives, they are effective in small quantities, safer to humans and animals compared to synthetic insecticides, they are very specific, often affecting only a single species of insect and have a very specific mode of action, slow in action and the timing of their application is relatively critical.[20]

Some fungi produce many secondary metabolites with insecticidal properties. Therefore, the present study was focused to deduce bio-insecticide from extract of *P. pinophilum.*

***Penicillium pinophilum*:** reproduces asexually and non-motile spores (conidia) produced exogenously by constriction at the tips of special hyphen branches (conidiophores).[3] Growth temperature: 32 ºC, Growth time: 7 days, Colony nature: diameter 35-36 mm, deep, sub center somewhat raised, sulcate, dense and floccose, margin entre; low, narrow, Mycelium: white to orange white, Conidiogenesis moderate and grayish green, exudates soluble pigment absents and reverse pale orange to light orange, Enzyme production: dextranase.[4]

Objective

To investigation of the insecticidal activity from the isolation of secondary metabolites in Endolichenic fungus, *Penicillium pinophilum* and their toxicity against cowpea weevil, *Callosobruchus maculatus*.

Material and Methods

Test insect and rearing conditions

Adult cowpea weevils were collected from stores at piliyandala, Sri Lanka in July 2015 infested black eye peas cowpea variety. The stock culture of *C. maculatus* raised by placing 100 unsexed adults in two-liter jars half full of disinfected black eye peas cowpea seeds. Muslin cloth was used to cover the top of the jars so that cowpea weevils could not escape. These parent cowpea weevils were allowed to mate for seven days under laboratory conditions (30 – 33 °C and 60 % – 90 % relative humidity) and lay eggs, after which they were removed. A day after emerging, the insects were sexed by the examination of the elytral pattern; females are maculated with four elytral spots whereas males are plain with no distinct spots. The experiment was carried out in the laboratory at institute of chemistry Ceylon using reared population of *C. maculatus*.[12]

Isolation of the fungal strain

The lichen host, *Pseudocyphellaria sp*., was collected from the Hakgala Botanical Garden situated at an elevation of about 1745 m above mean sea level within the Hakgala Strict Natural Reserve in the Nuwara Eliya District, Sri Lanka. The collected lichen samples were kept in sterilized polythene packs and transported to the laboratory at the Department of Chemistry, University of Kelaniya and processed within 24 h.

Fungal isolations were carried out according to the surface sterilization method.9 Healthy lichen thalli were cleaned in tap water and surface sterilized by consecutive immersion for 10 s in 95 % ethanol, 3 min in 0.5 % sodium hypochloride and 30 s in 75 % ethanol. The thalli were surface dried with sterile filter papers and aseptically cut into small segments. Lichen segments of size 1 × 1 cm were then placed (20 pieces from each of the 5 replicate samples of the lichen species) on 2 % malt extract agar (MEA) supplemented with 0.01 % streptomycin. The plates were sealed with parafilm and incubated up to 14 days at room temperature under ambient light. Fungi grown from each lichen particle were isolated into pure cultures. Slides containing pure cultures were prepared using the sticky tape method and identified using identification keys.[9] The emergent fungi were sub-cultured on 2 % MEA, photographed and deposited as a living voucher in the Department of Chemistry, University of Kelaniya under the accession number US/PA/06.

Molecular identification of the isolated endolichenic fungus

The fungal identification was achieved by analysis of the ITS region of its rDNA.[9] The sequence data obtained from the fungus has been submitted to GenBank with accession number KC 427134.

Extraction of the secondary metabolites

Penicillium pinophilum was cultured on PDA for 2 weeks. After 2 weeks the solid PDA were cut into small squares using a steriale spatula and transferred to 1 L Erlenmeyer flask. The PDA pieces were submerged in EtOAc and shaken for overnight at 170 rpm using a vertical shaker. The extract was filtered under suction using a Whatman no 1 filter paper and dried with anhydrous Na_2SO_4 to remove water. The solvent was evaporated under reduced pressure using a rotator evaporator at 40 ˚C. The EtOAc extract was transferred to a vial and N_2 was passed through the samples to remove remaining solvent in the extract.

Evaluation of bioactivity of crude extract using selected bioassay

Determination of Insecticidal Bioassay using Residual Film method

Mortality Test

The amounts of 50 µl, 100 µl, 200 µl, 350 µl and 500 µl of each extract dissolved in CH_2Cl_2 (1 mg/ml) were applied separately to clean glass vials. The vials were slowly rotated on a horizontal plane to apply the extracts on the inner surface. Vials were air dried to remove the remaining solvent and then three pairs of one-day old *C. maculatus* were introduced into them and screw caps of the vials were tightened (place at 32 ºC). After that percentage mortality of *C. maculatus* was recorded daily for 3 days.[12]The calculation of mortality rate was corrected for control mortality according to Abbott's formula: $Mc = \left(\frac{Mo-Me}{100-Me}\right) \times 100\%$

Where, Mo = Observed mortality rate of treated insects (%), Me = mortality rate of control (%), and Mc = corrected mortality rate (%).

Determination of Anti-inflammatory activity using Heat Induced Human Red Blood membrane stability bioassay

Preparation of Red Blood cells (RBCs) suspension

The blood was collected from healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10 % v/v suspension with normal saline.[18, 19]

Heat induced haemolysis

The reaction mixture (2 ml) consisted of 1 ml test sample of different doses (0.4 - 0.025 mg/ml) and 1 ml of 10 % RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 540 nm. The experiment was performed in triplicates for all the test samples.[18, 19] The Percentage inhibition of Haemolysis was calculated as follows:

$$\% \text{ Inhibition of hemolysis} = \left(\frac{\text{Absorbance for control} - \text{Absorbance for test sample}}{\text{Absorbance for control}}\right) \times 100\%$$

Solvent solvent partitioning of crude extract

A portion (3.60 g) of crude was dissolved in 100 mL of 80% methanol in water and partitioned with 150 mL of hexane three times. The hexane fractions were combined and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure using rotary evaporator at 40 ˚C. The aqueous methanol fraction was diluted to 60 % aqueous methanol by addition of appropriate volume of water and partitioned with 150 mL of $CHCl_3$ three times. The $CHCl_3$ fractions were combined and dried with anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure using rotary evaporator at 40 ˚C. Each fractions were transferred to glass vials and dried the fractions by passing N_2 gas. They were labeled as PC/M (methanol fraction), PC/C (chloroform fraction) and PC/H (hexane fraction). The anti-insecticidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Bioassay guided fraction of Chloroform fraction of *P. pinophilum*

A portion (2.7 g) of the bioactive $CHCl_3$fraction was subjected to column chromatography on silica gel (81.0 g) by elution with CH_2Cl_2 followed by increasing amounts of MeOH in CH_2Cl_2, which afforded 38 fractions. These fractions were combined on the basis of their TLC profiles to give seven fractions; F/1 (116.3 mg), F/2 (160.0 mg), F/3 (76.7 mg), F/4 (476.8 mg), F/5 (71.7 mg), F/6 (33.7 mg) and F/7 (175.8 mg). The anti-insectisidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Purification of F/1 using Sephadex LH-20 Column

A portion of (110.0 mg) fraction F/1 was further fractionated by gel permeation chromatography over a column of Sepadex LH-20 (4.0 g) made up in hexane, and eluted with hexane contacting increasing amounts of CH_2Cl_2, CH_2Cl_2 only, followed by CH_2Cl_2 containing increasing amounts of MeOH, and finally 100 % MeOH. Twenty-two fractions (10 ml each) were collected and combined on the basis of their TLC profiles. The anti-insecticidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Fractionation of F/1/A using Silica gel column Chromatography and Purification using Preparative TLC

A protion (70 mg) of the resulting active fraction F/1/A was next chromatographed on silica gel to give three active fraction F/1/A/1 (12.3 mg), F/1/A/2 (20 mg) and F/1/A/3 (13.1 mg). Final purification of each of these three fractions by normal phase preparative TLC (2 % MeOH in CH_2Cl_2) led to the isolation of three pure compounds, 1 (8 mg), 2 (12 mg) and 3 (10 mg).

Results and Discussion

Evaluation of bioactivity of crude, fractions and pure compounds using selected bioassay

Determination of Insecticidal Assays using residual film method

The toxic effects of the crude extract, chloroform fraction and pure compound 3 were evaluated against, *C. maculatus* by using the residual film method. Crude extract, chloroform fraction and pure compound 3 at different doses revealed toxicity. But any of hexane and methanol fractions, pure compounds 1 and 2 did not showed any toxic effect to *C. maculatus*.

The mortality percentages of *C. maculatus* were counted after 1st, 2nd and 3rd days at all doses 50, 100, 200, 350 and 500 µg/µl respectively. Then the percentages of corrected mortality were calculated by using Abbott's formula. Mortality percentage of *C.maculatus* treated with the crude extract, chloroform fraction and pure compound 3 isolated from the chloroform extract of *P. pinophilum* respectively by Film Residue method are shown in figure 1, 2 and 3.

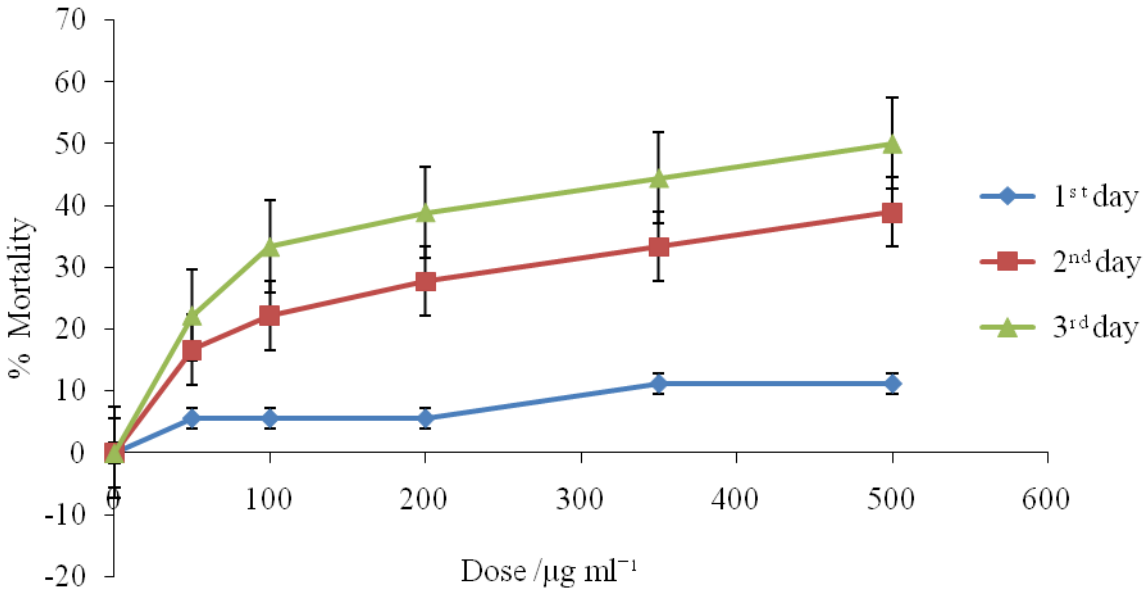

Figure 1: Mortality percentage of *C. maculatus* treated with the crude extract of *P.pinophilum*. Note: Each data point represents the mean of 3 replicates.

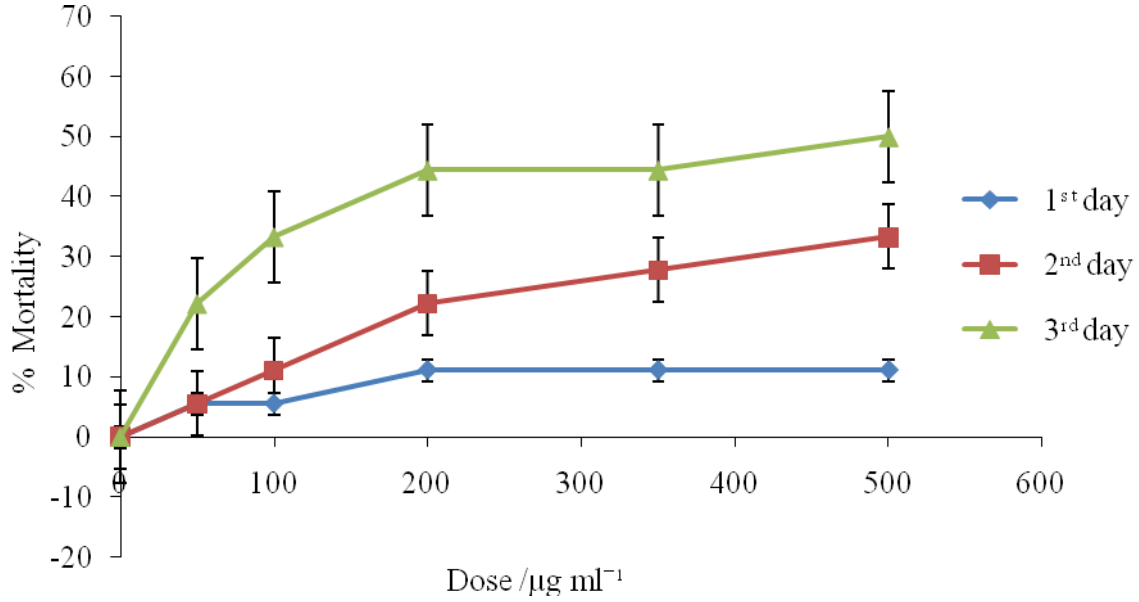

Figure 2: Mortality percentage of *C. maculatus* treated with the chloroform fraction of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates.

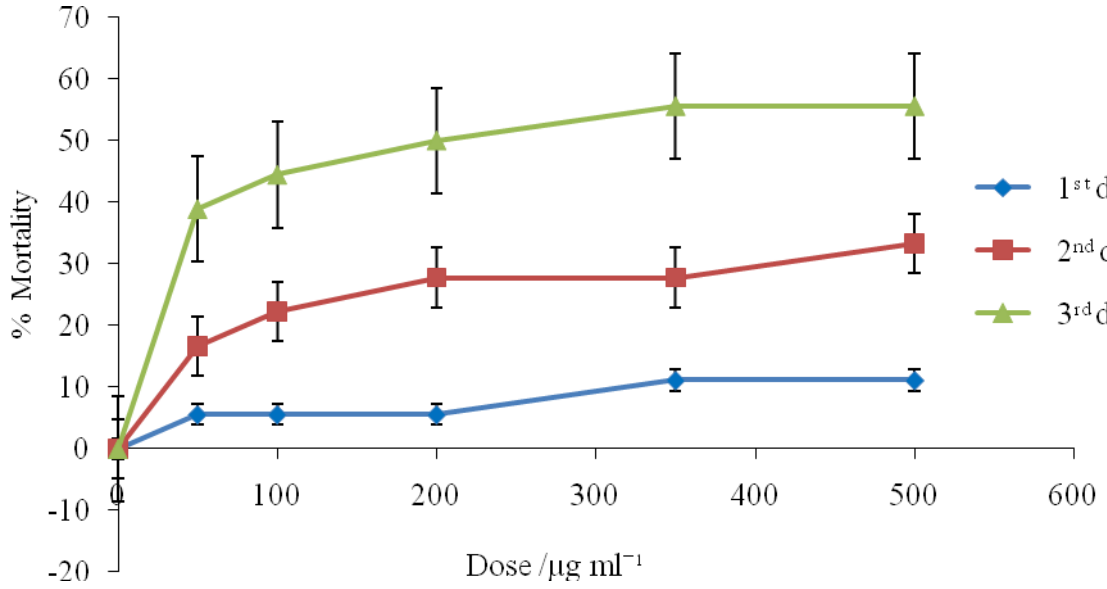

Figure 3: Mortality percentage of *C. maculatus* treated with the pure compound 3 of *P.pinophilum*. Note: Each data point represents the mean of 3 replicates.

The results revealed that, the crude extract, chloroform fraction and pure compound 3 possessed the highest toxicity at doses of 500 and 350 µg/µl, but showed the moderate toxicity at doses of 200 and 100 µg/µl, where as the lowest toxic effect at dose of 50 µg/µl against *C. maculat* LD_{50} values of the crude extract, chloroform fraction and pure compound 3 isolated from the chloroform extract of *P. pinophilum* respectively against *C. maculatus* stored grain insect pests after 1st, 2nd and 3rd days of treatment are shown in figure No: 4.

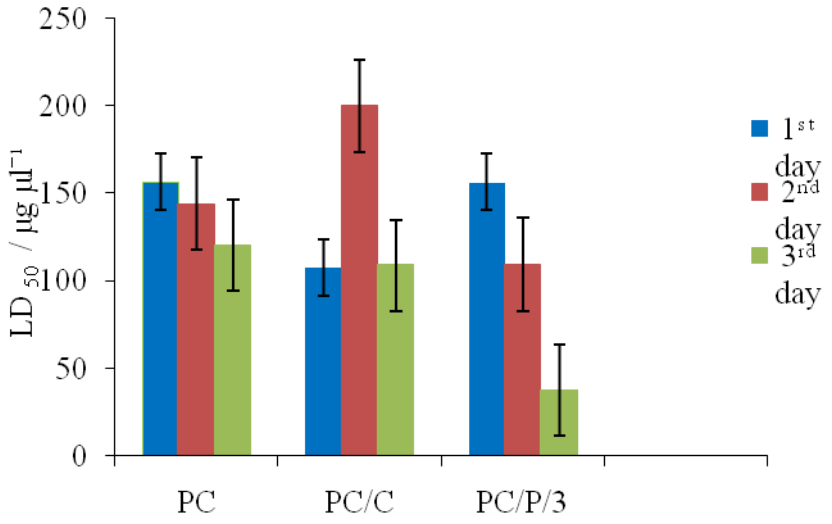

Figure 4: LD_{50} values of the crude extract, chloroform fraction and pure compound 3 of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates. PC- crude extract, PC/C- chloroform fraction and PC/P/3- pure compound 3.

The results of the probity analysis for the estimation of LC_{50} values at 1st, 2nd and 3rd days for the mortality of *C. maculatus* are presented in figure No: 4. The LC_{50} values of crude extract of *P. pinophilum* at 1st day after treatment is 156.52 µg/µl, at 2nd day after treatment is 143.98 µg/µl and at 3rd day after treatment is 120.24 µg/µl respectively. The results indicated that the crude of *P. pinophilum* at 3rd day at treatment was the most toxic against *C. maculatus*.

The LC_{50} values of chloroform fraction of *P. pinophilum* at 1st day after treatment is 107.84 µg/µl, at 2nd day after treatment is 200.16 µg/µl and at 3rd day after treatment is 108.88 µg/µl respectively. The results indicated that the chloroform fraction of *P.pinophilum* at 1st day at treatment was the most toxic against *C. maculatus*. The LC_{50} values of the pure compound 3 isolated from the chloroform extract of *P. pinophilum* at 1st day after treatment is 156.52 µg/µl, at 2nd day after treatment is 109.50 µg/µl and at 3rd day after treatment is 37.46 µg/µl respectively. The results indicated that the pure compound 3 isolated from the chloroform extract of *P. pinophilum* at 3rd day at treatment was the most toxic against *C. maculatus*.

From the insecticidal activity results, pure compound 3 isolated from the chloroform extract of *P. pinophilum* was shown insecticidal activity against cowpea weevil, *C.maculatus* by residual film bioassay (at day of LD_{50} of 3rd and 37.46 µg/µl) and so it can be used as bio-insecticide.

Determination of Anti-inflammatory Assay using Human Red blood cell membrane stability method

The HRBC membrane stabilization has been used as a method to study the *in-vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane[18, 19] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorder. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.[19] In recent years, the search for phytochemicals possessing anti-inflammatory property has been on the therapy of various chronic and infectious diseases. Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular, diseases, cancer, and aging etc.[20]Denaturation of proteins is a well-

documented cause of inflammation. The inflammatory drugs (aspirin, ibuprofen, naproxen etc) have shown dose dependent ability to thermally induced protein denaturation. Similar results were observed from many reports from plant extract. The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage.[19]

Heat Induced Human Red Blood Cell Bioassay activity of the crude extract of *P.pinophilum* and the resulting hexane, chloroform and methanol fractions respectively compared with the aspirin are shown in figure 5.

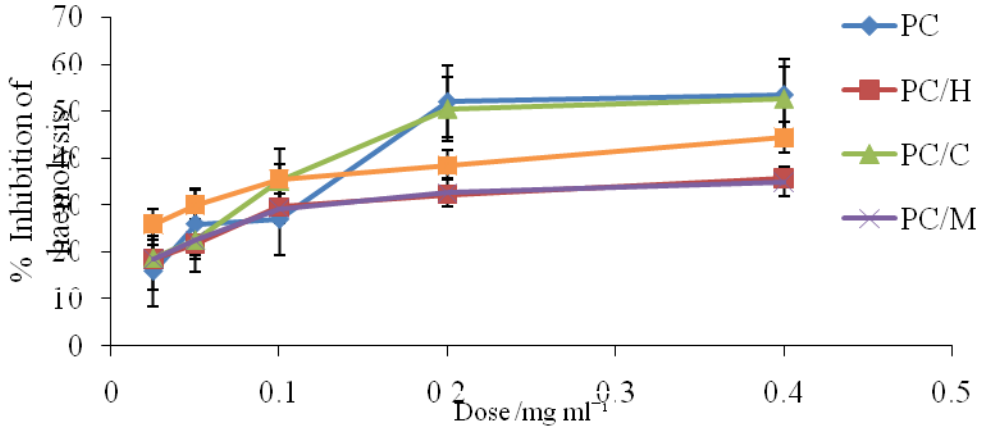

Figure 5: Anti-inflammatory assay activity of the crude extract, hexane, chloroform and 50 % methanol fractions of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates. PC- crude extract, PC/H- hexane fraction, PC/C- chloroform fraction and PC/M- 50 % methanol fraction.

The percentage of membrane stabilization form isolated of secondary metabolites in fungus and aspirin were done at 0.4, 0.2, 0.1, 0.050, 0.025 mg/ml. In the anti-inflammatory assay, aspirin showed the best results (44.44 % at 0.4 mg/ml).

The activity increased in a dose dependant manner compared to aspirin. Crude extract of P. pinophilum and hexane, $CHCl_3$ and 50 % MeOH fractions were showed the maximum inhibition of haemolysis at 53%, 35%, 52% and 35 % respectively at dose of 0.4 mg/ml. The results revealed that the crude extract of *P. pinophilum* and $CHCl_3$ fraction have the highest activity.

Heat Induced Human Red Blood Cell Bioassay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* respectively compared with aspirin are shown in figure 6.

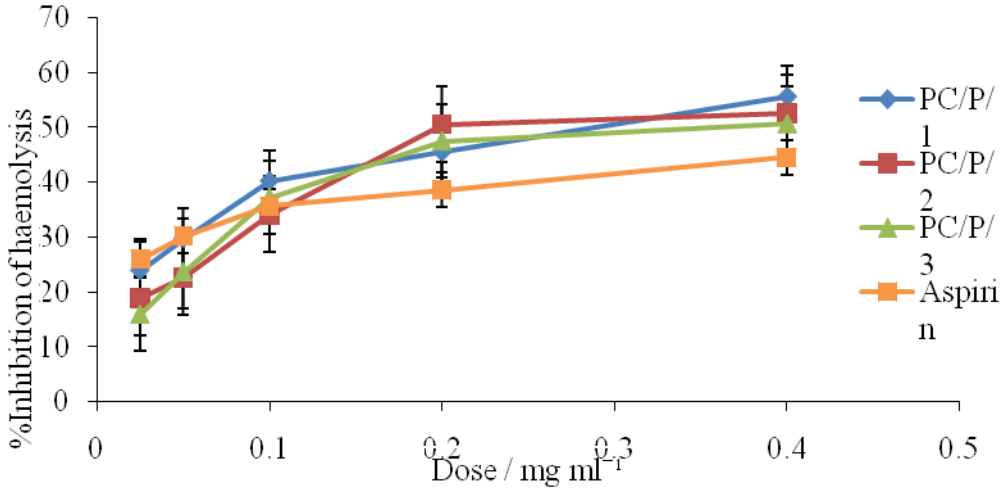

Figure 6: Anti-inflammatory assay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum.* Note: Each data point represents the mean of 3 replicates. PC/P/1- pure compound 1, PC/P/2- pure compound 2 and PC/P/3- pure compound 3.

The 1, 2 and 3 pure compounds are effective in inhibiting the heat induced hemolytic of HRBC at different doses. They showed the maximum inhibition of hemolysis 55, 52 and 50 % respectively at dose of 0.4 mg/ml. The results revealed that the 1, 2 and 3 pure compounds have the highest activity than that aspirin (at doses of 0.4 and 0.2 mg/ml) for Heat Induced Human Red Blood Cell Bioassay.

Heat Induced Human Red Blood Cell Bioassay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* respectively compared with aspirin. From the anti-inflammatory activity results, pure compounds 1, 2 and 3 were showed higher % inhibition values than that aspirin value (at doses of 0.4 and 0.2 mg/ml). So pure compounds 1, 2 and 3 better than that aspirin as anti-inflammatory drugs.

Conclusion

The EtOAc fractions of the endolichen fungus, isolated from the lichen *Pseudocyphellaria* showed highest anti-insectisidal and anti-inflammatory activity. Therefore, bioassay guidline fraction of the EtOAc extract was carried out. The chloroform fraction indicates that it contains more bioactive compounds than other two fractions. Therefore, chloroform fraction was further separated and three pure compounds were isolated. Based on results, it can be concluded that pure compound 3 isolated from the chloroform extract of *P. pinophilum* was showed higher % mortality value (at day of % mortality of 3rd and 55.56) and lower LD $_{50}$ value (at day of LD $_{50}$ of 3rd and 37.46 µg/µl). Chemical insecticides are costly and not sustainable in the long run due to environmental contamination, the use of this extract of fungus would be cost effective and sustainable, especially considering that this fungus is easy to grow. The pure compound 3 isolated from the chloroform extract of fungus, many of which is selective and has little or no harmful effect on non-target organisms and the environment. So this compound would play a role in the future of management of stored-grain insects, including *C. maculatus*.

Pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* were effectively inhibiting the heat induced hemolysis (at doses of 0.4 and 0.2 mg/ml). These results provide evidence for membrane stabilization as an additional mechanism of their ant-inflammatory effect. The anti-inflammatory activity was comparable with standard aspirin.

These finding provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an anti-inflammatory agent from pure compounds of *P. pinophilum* fungus. These pure compounds (1, 2 and 3) of fungus by anti-inflammatory results appear as interesting and promising and may be effective as potential sources of novel anti-inflammatory drugs.

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