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EVALUATION OF ANTIFUNGAL POTENTIAL OF INDIGENOUS PLANT EXTRACTS AGAINST GREY MOULD AND HPLC AND LC-MS BASED IDENTIFICATION OF PHYTOCHEMICAL COMPOUNDS IN POLYGONUM AMPLEXICAULE D. DON EXTRACTS

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ABSTRACT

Grey mould disease is one of the highly destructive post-harvest strawberry disease caused by the fungus *Botrytis cinerea*. Several synthetic compounds are being used against *B. cinerea* but due to resistance development by synthetic fungicide many alternative management strategies have explored nowadays. In this study, antifungal potential of indigenous plant extracts against grey mould was evaluated and amongst these plant extracts HPLC and LC-MS based identification of phytochemical compounds in *Polygonum amplexicaule* was also done. In this regards, firstly *in vitro* evaluation of the antifungal properties of twelve plant extracts was undertaken against *B. cinerea* using fungal growth medium, of which five plants extracts (*P. amplexicaule, T. vulgaris, D. viscosa S. nigrum* and *E. globules*) indicated the percent mycelia inhibition in fungal growth is greater than 75% which were then used for *in vivo* experiment. *P. amplexicaule* showed (81%) the highest growth inhibition *in vitro* as well as in *in vivo* (80%) activity during storage conditions (on strawberry fruits) against the fungus. HPLC analysis of methanolic extracts of *P. amplexicaule* showed, the total phenolics 6.176 (µg GAE/mg SW) including 0.157 **(**µg GAE/mg SW) gallic acid and protocatechic acid. While total fluorescence were 1.85 (µg cate/mg SW) including catechin, procyanidin and epicatechin. Total hyrdoxycinnamates were found to be 7.696 (µg CAE/mg SW) comprising on chlorogenic acids, neochlorogenic acids and 4-caffeoylquinic acids. LC-MS based identification also showed the presence of acids like caffeic, and **gallic** acid. Other showed the presence of rutin, quercetin, catechin, kaemferol and myricetin. It was concluded that *Polygonum amplexicaule* extract has effective against grey mould amongst all indigenous plant extracts and detected known and unknown compounds from the plant are well known for antimicrobial activity. Therefore in future further investigation can carried out for synthesis of bio based fungicide from these compounds of Polygonum plant extract on commercial scale against post-harvest pathogens of strawberry.

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INTRODUCTION

Strawberry (*Fragaria ananassa* Duch.) fruits are tender and juicy which makes it susceptible to mold after harvest. The main strawberry pathogen is *Botrytis cinerea*, followed by *Rhizopus stolonifer*, *Mucor spp*., *Colletotrichum spp*., *Penicillium spp*., which are the main

pathogens responsible for strawberry postharvest rot (Feliziani and Romanazzi, 2016). Significantly, post-harvest diseases may lower the quality, taste and market value of fruit during storage and transportation. Mehmood *et al.* (2018a); Mehmood *et al.* (2018b) also reported that *Botrytis* fruit rot have 100% prevalence and *Alternaria* 17-55 % during pre-harvest stage which remain latent and become severe post-harvest diseases in major strawberry producing areas of Pakistan. Study conducted by Salami *et al.* (2010), average losses of strawberry fruits during post-harvest stages in Iran were found to be 28%. *Botrytis cinerea* affect both in pre and post-harvest processes. Their highly destructive nature found mostly in strawberry fruits in post-harvest (Elad *et al.*, 2007). *Botrytis cinerea* caused frequent loss in quality due to onset rots (Williamson *et al.*, 2007; Zhang *et al.*, 2007; Scaife, 2004; Ahmed *et al.*, 2018). In 2005, at central city of Taiwan, fruit rot was found in commercial strawberry fields of Fongyuan. Over the past 2 consecutive years, the disease incidence was increased upto 4 to 5% caused severe post-harvest losses (Ko *et al.*, 2008). The most common fungal pathogens may affect the strawberries viz. *Aspergillus niger*, *Alternaria alternata*, *Penicillium expansum, Colletotrichum, Phytopthora, Botrytis* and *Fusarium,* (Dignand, 2004). The traditional strategy for controlling strawberry postharvest rot relies on the application of fungicides during the crop's growing cycle. Common fungicides are applied around the flower and treatments can be repeated until harvest. Nowadays, there are many alternatives to conventional fungicides, which are characterized by a low impact on the environment and human health (Feliziani and Romanazzi, 2016). In many countries, the use of some fungicides (eg, benzimidazole) has been banned or is restricted under anti-resistance strategies. Mutants of *B. cinerea* that are resistant to fungicides have been isolated in laboratory studies and in the field for many active fungicide agents (Myresiotis *et al.*, 2007). Whereas, the growers mostly rely on fungicides to protect them from fungal diseases but farmer trainers advise them to avoid these fungicides and go forward towards the integrated disease management techniques. These techniques have been used successfully for several plants against plant diseases and proved as a non-phytotoxic. Plant extracts derived from plants are one of several non-chemical control alternatives that are very inspiring interesting because of their availability, non-toxicity and environmentally friendly nature. Many plant extracts have antifungal potential due to the presence of chemical compounds such as terpenoids, saponins, alkaloids, flavonoids. Abbey *et al.* (2019) briefly published some information about plant-based compounds and bio-control agents for *B. cinerea* control. As we study the mechanism of active constituents of plant extracts in disease suppression, they may either attack on the pathogen directly like anti-biotic or may boost systematic resistance in host plants ensuing in disease reduction. So it proves as an alternative plant disease management (Wongkaew and Sinsiri, 2014). The shelf life and consumption time of fruits and vegetables are minimal, higher concentrations of chemical sprays to overcome the field problems causes' high toxicity and contamination in the Product (Elad *et al.*, 2007). Polygonum is widely distributed in north of Pakistan in which anti-oxidant is high commonly known as masloon. The related study came with the thought and inspiration from the usage and advantages of *P. ampexicaule* related to vast medicinal attributes in treatment of joint fever, flue and gastrointestinal disorders (Qureshi *et al.*, 2007). A perusal of the literature revealed that antifungal activity of *P. amplexicaule, D. viscosa, S. aromaticum, A. indica* and *Eucalyptus spp*. against a number of pre- and post-harvest fungal pathogens (Thippeswamy *et al.*, 2013; Sattar *et al.*, 2014; Begum and Nath, 2015; Bashir *et al.*, 2020).

However, there are no reports of their inhibitory activity plants against *B. cinerea* obtained from strawberry growing areas of Punjab in Pakistan and current research reports antifungal potential of extracts from native plants against target plants pathogen to sort out an environmentally friendly management strategy in future. Regarding our study, *P. amplexicaule* reveal significant antifungal potential and properties. The active fractions contains flavonoids and phenolics in leaves, shoots and rhizomes of plants which include quercetin, gallic acid, caffeic acid, rutin, catechin, myricetin and Kaempferol with established antioxidant activities. Kellactone and amplexicine are two novel antioxidants in that plant contains high amount of anti-oxidants (Tantry *et al.*, 2012). This study is useful in future prospect for safe postharvest management and strategies for strawberry fruits during storage and transport goods and enhance the growers productivity also consumer's desire for more natural, healthy and safe foods with respect to fungicides usage.

MATERIALS AND METHODS

Plant Material (Collection / Extraction)

Polygonum plants were searched in area at the high sea level above 6000 feet above the sea level nearby Neelem Valley in the months of March and April, uprooted, cleaned up, packed and brought to Fungal Plant Pathology Laboratory, PMAS-AAUR for further processing. Plant parts were washed twice with tap water and allowed them to dry at room temperature on paper towel. After drying leaves were crushed into crude powder form to extracts organic compounds through extraction method suggested by Ul-Haq *et al.* (2012). From them ground samples were put into flat bottom flasks (20 grams each) to which 80% of methanol was added in each flask. Flasks were placed at magnetic stirrer for 4 hours at 25 ^{0}C. The material was filtered after 48 hrs and left for fan dry in glass pans, allowed to evaporate the solvent to get concentrated dry material which was considered as 100 percent pure extract. Dried extracts were scratched with the help of sterilized surgical blades and was put in refrigerator for i*n vitro* trials. Doses and concentrations of prepared extracts were adjusted from the stock solution 1:1(w:v) accordingly. For poisoned food technique, each plant extract was added @ 3%, 6% and 9% concentration in autoclaved media.

Revival of Preserved Cultures of *Botrytis cinerea* for the Pathogenicity Test

Preserved cultures of *B. cinerea (BRID5* Islamabad) were obtained from Mycology laboratory of the university. Reactivation of cultures was accomplished by inoculating several granules of silica gel in the Czapek-dox medium. Fungal inoculum was prepared by harvesting the conidial spore mass of previously grown seven days old culture of pathogen on PDA media which was provided equal alternative dark and light hours of 12 hours at 28±2 ^{0}C . Suspension was made and adjusted @ 10^6 conidia/ml of inoculum suspension with the help of haemocytometer. Healthy Strawberry fruits were disinfected in 1% sodium hypochlorite for 5 mins and washed under tap water and left dried at room temperature, then inoculated by spore suspension (10 µ1) from the prepared spore suspension. Fruits were sprayed with sterile distilled water served as negative controls. Fruits were incubated at 25±2 ^{0}C in humid chamber at 70-100% relative humidity The pathogens were re-isolated after 5 days from the artificially diseased fruits using potato dextrose agar Petri dishes. Pathogenically tested cultures of *B. Cinerea* were grown at 25 ^{0}C for 7-14 days on PDA and allowed to fully mature and sporulate to produce enough conidial mass for the preparation of mycelial discs.

Plant Extracts Antifungal Activity (Poisoned Food Technique)

Poisoned food technique suggested by Balouiri *et al.* (2016) was done. The antifungal activity of plant extracts for growth inhibition percentage was measured at pre-defined concentrations and doses. Each plant extract was incorporated into the autoclaved PDA @ of 3% (3mL of extract in /97mL media), 6% (6ml of extract in /94ml media) and 9 % (9ml of extract in /91mL media) and were mixed well by using magnetic bar and magnetic stirrer. Then, the medium was dispensed into petri dishes and three replications of each treatment were maintained. After pre-incubation (overnight), the inoculation of the pathogen was done using mycelial disc (5mm) of *Botrytis cinerea* with the help sterilized cork borer and placing it in the center of the each plate. Diameters of fungal growth and their effect both in sample and controlled plates were measured and estimated by the following formula:

$$\mathrm{I}\ (\%) = \frac{\mathrm{Dc} - \mathrm{Dt}}{\mathrm{Dc}} \times 100$$

Where, I=Fungal growth inhibition (%)

Dc= Fungal growth of pathogen in control (Diameter in mm)

Dt= Fungal growth of pathogen in treatment (Diameter in mm)

Antifungal Activity Evaluation on Strawberry Fruits

Five best plants extract of *in vitro* test which exhibited percent mycelial growth inhibition >75% were particular for *in vivo* test; *P. amplexicaule, T. vulgaris, D. viscosa*, *S. nigrum* and *E. globules* in regarding study. Healthy and even sized strawberry fruits (without any symptoms of disease) were selected and remain untreated. Control and treatment sets of fruits were bathed in running water and surface sterilization with 0.1% sodium hypochlorite solution for 2 mins. Later, washed them with distilled water and left for dried-up on a filter paper. At 25 °C, culture was maintained and grown on PDA plates. From 7 to 8th day old culture, spore suspensions were prepared from the sporulation edges by removing spores with loops and suspend them in sterile distilled water. haemocytometer was used to determine spore concentration. Fruits were punctured with needle and inoculated by spraying of 10ul spore

suspension (10^6 conidia/ml) of *B. cinerea* and *A. alternta* (Tripathi *et al.*, 2008) and stored them at room temperature for 2hrs in order to promote the fungal inoculation (Asghari *et al.*, 2009). To get three final concentrations (3, 6 & 9%) for plant extracts as negative control; 20 µl (each concentration of each extract) was injected in every punctured spots using sterilized distilled water. However, positive control was obtained through submersion of 0.2% Dithane fungicide stored in isolated packages to prevent the loss of essential oils. At 4 °C for 7 days, treated and untreated (control) fruits in 3 replicates per treatment were incubated.

Measurements of Disease Infection and Disease Severity

Disease severity was measured through formula of DS = Σ (nxV)/ZxN x 100%, (DS = Disease severity, n = number of fruits in same category of each attack, V = each category score, N = nos. of fruits observed & Z = highest score attack. The disease score was measured as follows: 0 = no attack found, 1 = 0 < x < 20% fruit was attacked, 2 = 20 < x < 40% fruit was attacked, 3 = 40 < x < 60% fruit was attacked, 4 = 60 < x < 80% fruit was attacked, 5 = 80 < x < 100% fruit was attacked.

HPLC of Plant Extracts with Highly Significant Antifungal Activity

Phytochemical analysis of five plant extracts with highest significant antifungal activity in *in vitro* and *in vivo* experiments amongst twelve plants was done through HPLC i.e., High performance liquid chromatography.

Extraction with Liquid Nitrogen

0.1g of dry plant tissues (dry powder) was taken with 2 replicates of five selected plant extracts. Liquid nitrogen was added in the sample slowly in previously frozen mortar & pestle at -20 ℃ one night before used. Crushing of material was done when bubbles started settling down. The whole process was repeated two or more times until fine powder obtained. The weight of the crushed sample was taken immediately using electric balance with high accuracy, delay in weighing could made paste of the powder because of liquid nitrogen and mortar, pestle was frozen and could be difficult to weigh. After weighing each sample was stored at -80 ℃ immediately until further use.

Extraction for Solid Phase

0.1 g air dried plant material, ground in liquid nitrogen, was extracted 3x with extraction solvent, 700ul/0.7ml, 80% MeOH (80% methanol/19.9% water), 0.1% HFO. Vortex for 10 sec and sonication was done for 20 min in small sonic bath with care that the tubes were tightly capped. Sonication was done for better extraction of compound through sonication waves. After sonication samples were vortexed for 10 sec again, then spinned in micro centrifuge at 10400rpm for 10 min rapid acceleration and deceleration by making sure tubes were balanced in rotor. Supernatants were transferred to a second labeled tube and was set aside. Supernatants were pooled and dried on vacuum centrifuge. Dried extracts were re-solvated in 1ml 0.1% HFOaq, centrifuged, and supernatants subjected to solid phase extraction (SPE) to clean them up. Oasis HLB 0.2g/6ml cartridges, was used to separate the compounds that were unwanted and could be stocked with HPLC columns, conditioned with 5ml MeOH then 4ml 0.1% HFOaq. After loading, the samples were washed with 4ml 0.1% HFOaq, then eluted with 2ml 0.1% HFO/MeOH. (all beds retained some colour). Eluant was collected and dried down in vacuum centrifuge, then resolvated in 0.5ml 10%MeOH/0.1%HFO, centrifuged.

Liquid Phase Extraction

In the final step 250 µL of supernatant was diluted with 500 µl 10% MeOH/0.1% HFO, vortexed, then sonication was done to dissolve the pellet and centrifuge the samples. The supernatant was then transferred to HPLC vials as after the first few injections of undiluted showed it was too concentrated.

The HPLC Conditions

HPLC system contains 1525 water pump with auto-sampler >PDA (photo diode array) detector and fluorescence detector (Waters, Milford, MA), by means of an Agilent Poroshell (2.7µ 3.0 x 75 mm) at room temperature with 0.5ml/min flow rate. Mobile phase 0.8% trifluoroacetic acid in water solvent A and 0.68% in acetonitrile solvent B with solvent elution gradient as follows: 0 min: 2% B, 2 min: 2% B, 22 min: 6% B, 30 min: 12% B, 60 min: 35% B, 62 min: 100% B, 64 min: 100% B, 65 min: 2% B; re-equilibrated 10 min before next injection. Injection volume was 30µL and detection was over 200 to 600nm on PDA. Extracting chromatograms at 280nm total phenolics, 360nm total flavanols, 520nm total anthocyanins, 320nm total hydroxycinnamates (HCA).

Table: 1. Colors of HPLC Pellets/Supernatants in Pre & Post solid Phase extraction and final concentrations of Five plant extracts.

ID	Wt/g	Extract	Color supernatant	Color of pellet	Post SPE color	Final conc. mg/ml
DV	0.1	cloudy, light green-brown	clear yellow green	yellow	Pale yellowish	0.0667
EU	0.1	cloudy, yellow green	clear yellow green	dark yellow-green	pale yellowish	0.0667
PE	0.1	faintly cloudy, orange	clear orange	dark orange	pale yellowish	0.0667
SN	0.1	cloudy, dark green	clear green	dark green	pale yellowish	0.0667
TV	0.1	faintly cloudy, yellow-green	clear yellow green	almost no pellet	pale yellowish	0.0667

(DV= *Dodonaea viscosa*, EU= *Eucalyptus globulus*, PE= - *Polygonum amplexicaule*, SN= *Solanum nigrum,* TV= *Thymus vulgaris)*

Fluorescence was examined by using 324nm as emission wavelengths and 228nm as excitation, which independently detects catechin, epicatechin, procyanidin B1, B2 and C1. To identify peaks and quantify specific compounds, retention times and UV-Vis profiles were paralleled to pure standards. Chlorogenic acid (5-O-caffeoylquinic acid), catechin, epicatechin, phloridzin, quercetin, 4-O-caffeoylquinic acid, quercitrin, cyanidin-3-glucoside, rutin, isoquercetin were purchased and used as standards from Sigma-Aldrich Canada Co. (Oakville, Ontario). Standards for procyanidin B1, B2 & C1 were purchased from Indofine Chemical Co. (Hillsborough, NJ). Under same conditions of 10% methanol, 30 µL injection, quantified the respective phenolic compounds in the extracts and standards were used to calibrate the HPLC.

Phytochemical Analysis by High Resolution Mass Spectrometry

Five samples of ground material including polygonum were analyzed by high resolution Mass Spectrometry after *in vitro* and *in vivo* antifungal evaluation. All samples were extracted with an acidified, organic liquid extraction method. Samples were evaluated using a Q-Executive Orbit rap. Components of the material were identified by accurate mass, making comparison with published literature and possible, matching MS/MS fragmentation profiles with published spectra. Confirmation of compound identities was done by comparison with standards or isolation/purification of the compounds from the material.

Sample Preparation

Two hundred mg sub samples were mined with 1ml (78:18:2), acetonitrile, water, acetic acid. Samples were vortexes for 30sec and sonicited in a water bath for 20 minutes at 30 ℃. Samples were removed and placed into a Thermo mixer for 20 min operating at 1400rpm, 30 ℃. A 100 µL aliquot was removed and diluted in water 80:20: acetonitrile and placed at 4 ℃ for 30mins. Samples were then centrifuged at 10k rpm at 4 ℃ for 10 mins. The supernatant transferred into polypropylene HPLC for analysis by LC-MS//MS.

LC-MS Analysis

All MS data were obtained from Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific), amalgamated to an Agilent 1290 HPLC system with the Zorbax Eclipse Plus RRHD C18 column (2.1×50mm, 1.8 µm; Agilent) preserved at 35°C. Mobile phase watered with acetonitrile 0.1% formic acid A & B (Optima grade, Fisher Scientific NJ, USA). Mobile phase B held at 0% for 0.5min, before increasing it to 100% over 3mins and 100% for 1.5 min, before returning it to 0% over 0.5 min. 5 µL injections were used with a flow rate of 0.3ml/min. The following settings were used for positive; HESI (heated electrospray ionization); 3.9 kV capillary voltage, 400 °C capillary temperature, 17 units sheath gas, 8 units auxiliary gas, 450 °C probe heater temperature, RF level 45 (S-Lens). For negative ionization HESI, all condition was identical with the acceptation of capillary voltage -3.5 kV. The extracts were profiled by data-dependent acquisition LC-MS//MS method in both ionization modes i.e. positive and negative. MS scan of 35000 resolution method at AGC automatic gain control (1×10^{6}). Maximum injection time is 128ms while mass range *m/z* is 100-800. 5 most intense ions in each full MS scan (dynamic exclusion 7s) were particular for MS//MS performed at 17500, AGC (1×10^{6}). Maximum injection time is 64ms while normalized collision energy is *30/45*. Isolation window for MS//MS was 1.2amu. MS data was reconnoitered by Xcalibur software which was used to calculate chemical formula based on accurate mass. When possible,

determined chemical formula was putatively identified by searching of Metlin and Knapsack databases.

Statistical Analysis

The statistical analysis of *vitro* and *vivo* trials was carried out by Statistix10 software. The analysis of variance was determined using 2-factorial CRD design and all pair wise comparisons were made using LSD test.

RESULTS

***In Vitro*; Plant Extracts Antifungal Activity (Poisoned Food Technique)**

Results of the experiment revealed that all applied plant extracts were effective to manage the pathogen in *in-vitro* conditions, but their effectiveness varied with the change in concentrations in media as compared to control group of the experiment. On the radial growth of *B. cinerea*, effects of different concentrations of these plant extracts are shown in Figure 1. In a dose-dependent manner, all of 12 plant extracts were establish to prevent the growth of pathogens.

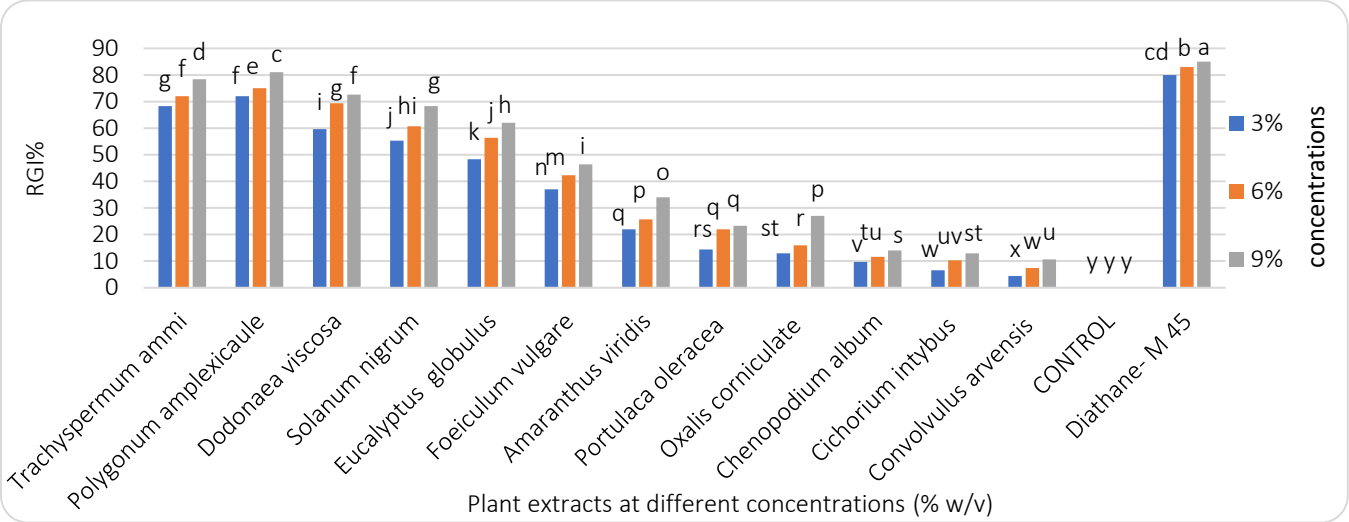

Figure.1. Effect of plant extracts applied at three (3%, 6% and 9%) concentrations on growth inhibition of *Botrytis cinerea* through poisoned food technique. Letters represent significant differences in growth inhibition at applied concentrations (P<0.05).

The highest radial growth was observed in the control as the results indicated that without plant extracts application. Polygonum extract had the highest value *i.e.,*81% @ 9% concentration while, the minimum antifungal activity was observed @ of 3% by Convulus where growth inhibition was only 4.4% after 6 days of incubation as compared to control.

As depicted in Figure 2, Polygonum was applied @ 3%, 6% and 9% concentrations and radial growth rate was noted after interval of 48 hours for 6 days. It was found that gradual increase in growth inhibition was observed with increase in concentrations from 3% to 6% while at 9% there was no fungal growth observed until day 3, after that very slow growth rate was observed at day 6. The extract was found to be effective at all concentrations to inhibit the growth of the pathogen.

Grey Mould Inhibition Activity of Five Plant Extracts on Strawberry Fruits

As observed, the control plants demonstrated disease right after 3 days of inoculation whereas in case of Dithane and plant extracts, no symptoms of the disease were seen. Seven days after inoculation it was observed that Polygonum and Thymus extract could lessen infections by 80% whereas Dithane abridged infection by 86.86% (Figure 3). Disease severity was significantly higher in case of three other plant extracts and control (no-treatment) than in the treatment of Polygonum (9%) or Dithane, suggests that the custom Polygonum 9% could reduce the disease.

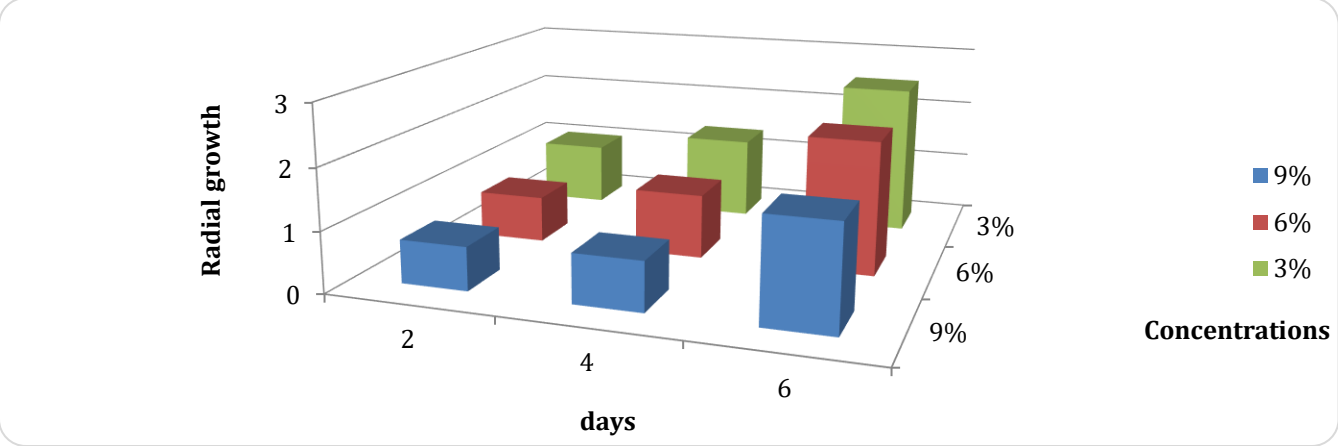

Figure 2. Effect of *Polygonum amplexicaule* extract, applied at 3%, 6% and 9% concentrations on growth inhibition (%) of grey mould (*Botrytis cinerea*) on strawberry fruits.

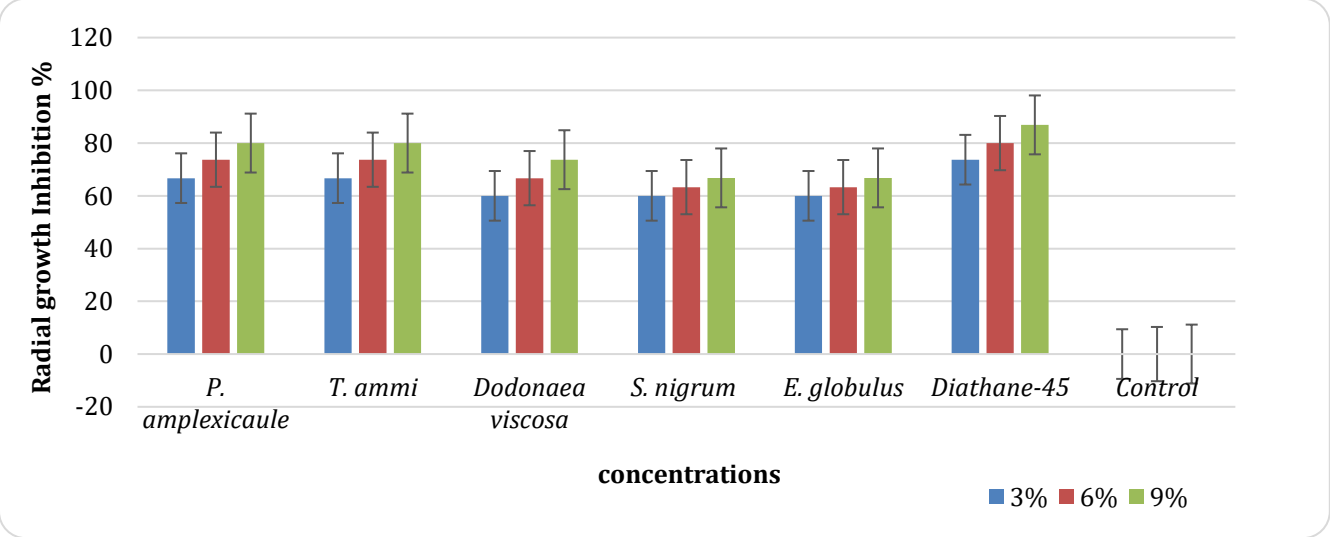

Figure.3. Effect of plant extracts on growth inhibition of *Botrytis cinerea* applied at three (3%, 6% and 9%) concentrations during *in vivo* experiment on strawberry fruits.

Identification of Antifungal Compounds in Methanolic extracts of *P. amplexicaule* by HPLC

Analysis of methanolic extract of *Polygonum amplexicaule* revealed total phenolics 6.176 ug GAE/mg SW) including 0.157(ug GAE/mg SW) gallic acid and protocatechic acid. While total flouresence were 1.85 (ug catE/mg SW) including catechin, procyanidin and Epicatechin. Total hyrdoxycinnamates were found 7.696 (ug CAE/mg SW) comprising 4-caffeoylquinic acid, chlorogenic and neo-chlorogenic acid.

Identified Minor Compounds of *Polygonum amplexicaule*

Extracts of *P. amplexicaule* were analyzed in both (positive & negative) ionization modes and base peak chromatograms of *P. amplexicaule leaves* extracts were include 6-dimethoxyflavone, 5,7,4'-trihydroxy-3, penduletin, aliarin 4'-methyl ether, kaempferol 7,4'-dimethyl ether and viscosol during Mobile phase B was alleged at 0% (0.5 min), before increasing to 100% (3 min). Mobile phase B was seized at 100% (1.5 min), before returning to 0% B (0.5 min). Injections of 5 µL were used with a flowrate of 0.3ml/min. Major phenolic compounds were identified as Cinchonain-catechin, Epicatechin, gallic acid, gallocatechin 3-Dehydroquinic acid and have been confirmed from literature, Studies of the phytochemicals of this specific material are sparse, however, studies of related species were critical to allow a high number of identification in this material and many other minor compounds were listed below in table 3.

Table 2. Quantification of total phenolics of plant extracts of *P. amplexicaule* at 280nm (a), of fluorescence compounds at 228ex/324em (b) and of hyrdoxycinnamates at 320nm wavelength(c).

(a)Total phenolics of plant extracts at 280nm				
Total (µg GAE/mg SW)	Arbutin(µg GAE/mg SW)	Gallic acid (µg GAE/mg SW)	protocatechuic acid (µg GAE/mg SW)	t-cinnamic acid (µg GAE/mg SW)
6.176	-	0.157	0.038	-
(b) Fluorescence Compounds at 228ex/324em				
total fluor (µg catE/mg SW)	Catechin µg catE/mg SW	procyanidin b2 (µg catE/mg SW)	Epicatechin (µg epi/mg SW)	
1.85	1.017	-	0.021	
©Quantification of hyrdoxycinnamates at 320nm				
total hca (µg CAE/mg SW)	Chlorogenic acid (µg CA/mg SW)	neochlorogenic acid (uµ CAE/mg SW)	4-caffeoylquinic acid (µg CAE/mg SW)	caffeic acid (µg CAE/mg SW)
7.969	7.042	0.260	0.196	-

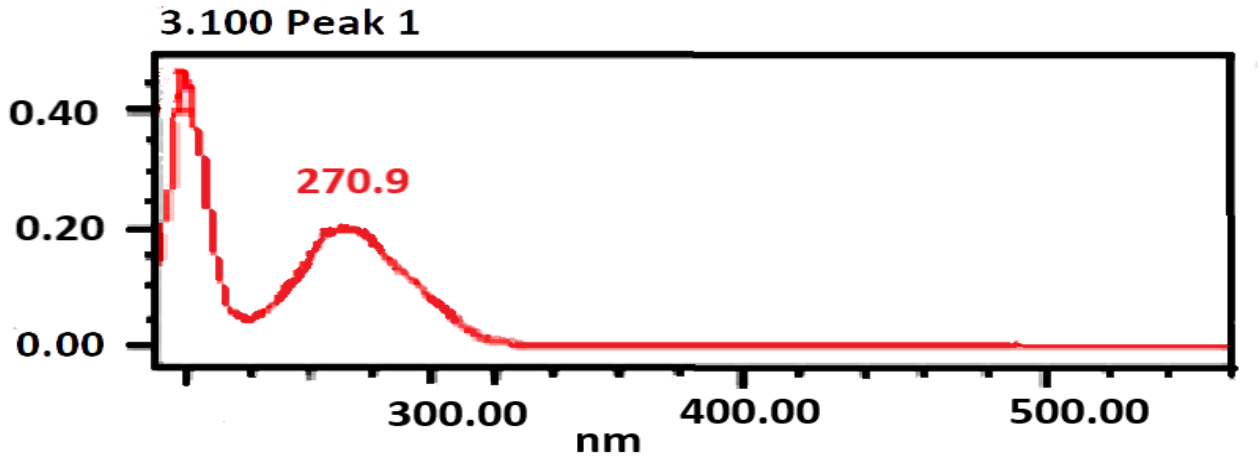

Figure 4. Chromotograph of *P.amplexcule* extracts showing elution at 280 nm of gallic acid (Phenolics) with RT: 3.100.

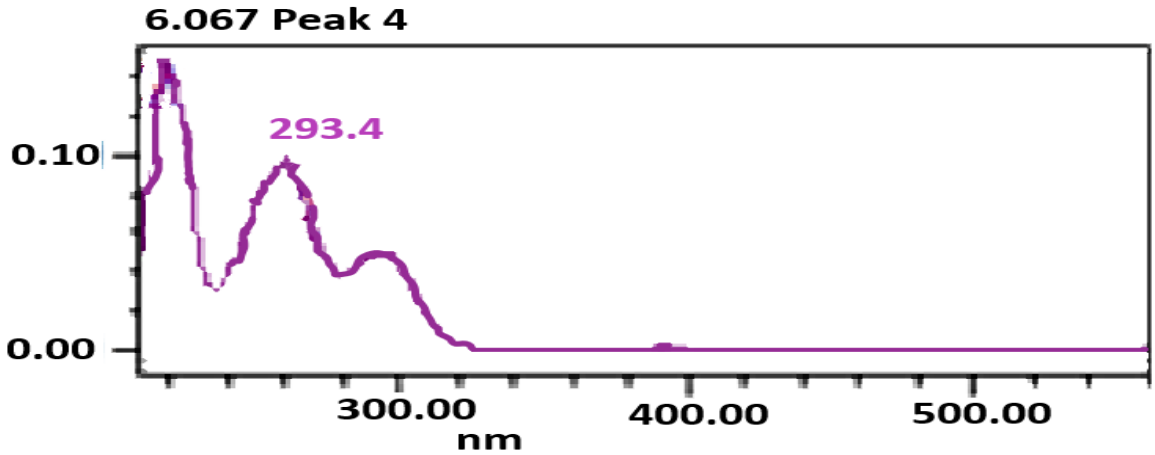

Figure 5. Chromotograph of *P.amplexcule* extracts showing elution at 280nm of protocatechic acid (Phenolics) with RT:6.067.

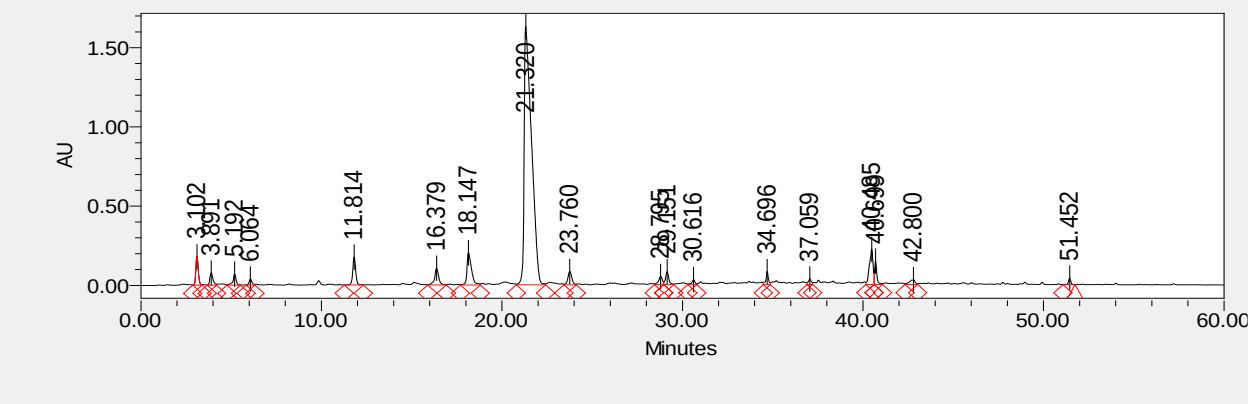

Figure 6. Overall Profile of Chromatograph of *P. amplexcule* extracts compounds showing elution at 280nm.

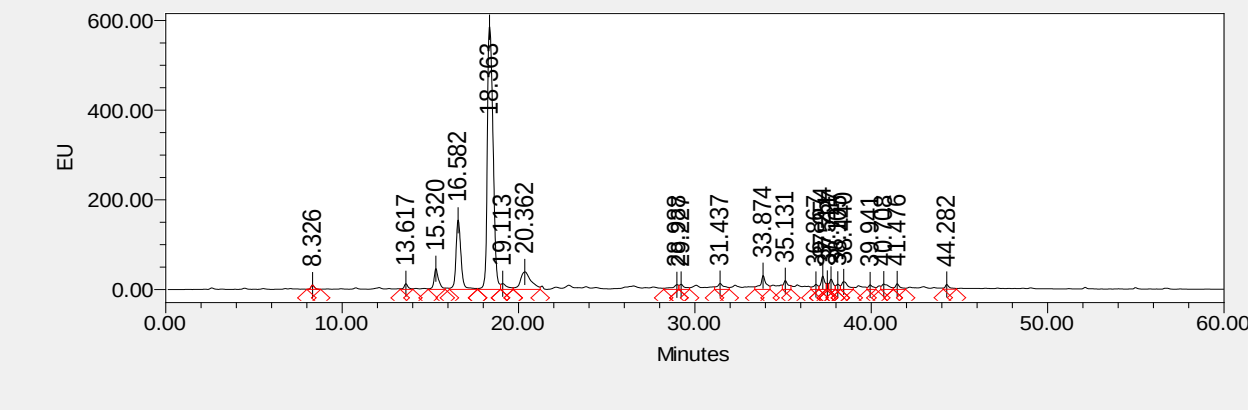

Figure 7. Overall Profile of Chromatograph of *P. amplexcule* extracts compounds showing 320nm.

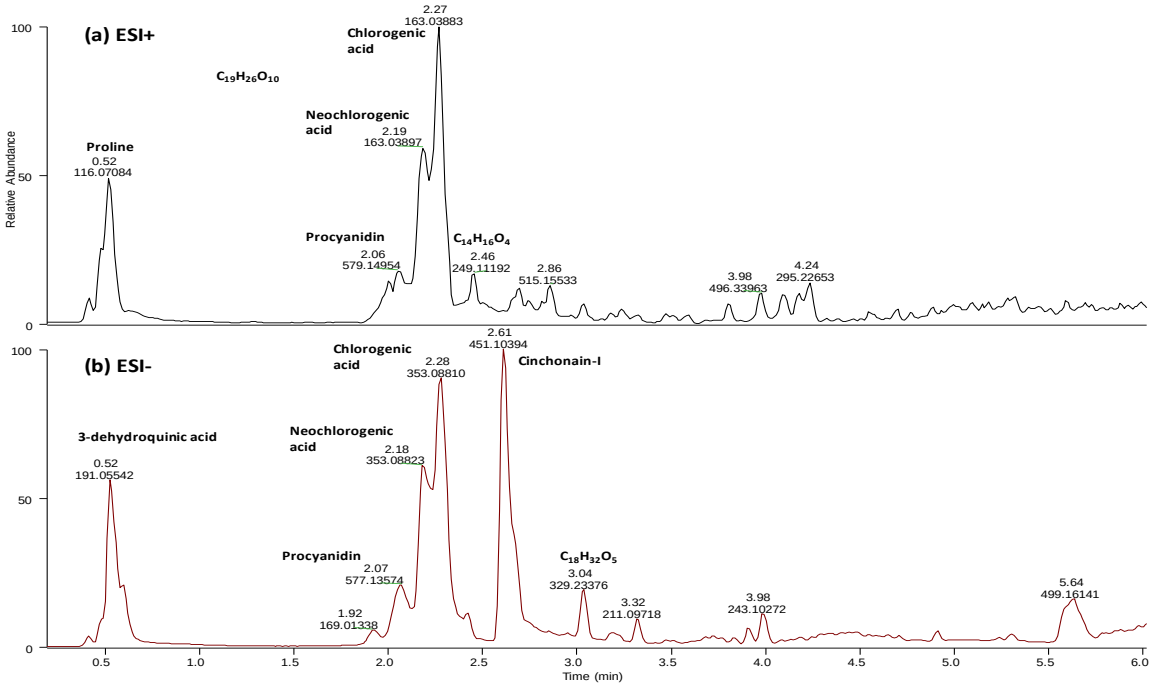

Figure 8: Base peak chromatograms of *P. amplexicaule* extracts analyzed in (a) positive ESI and (b) negative ESI ionization mode.

Table 3. Identified compounds with chemical characteristics found in extracts of *P. amplexicaule.*

Name	Formula	Ion type	theo mass	exp mass	Ppm	RT
3-Dehydroquinic acid	$C_7H_{12}O_6$	[M-H] -	191.05611	191.05542	-3.62	0.52
Cinchonain-catechin	$C_{39}H_{32}O_{15}$	[M-H] -	739.16684	739.16779	1.28	2.42
Cinchonain-I	$C_{24}H_{20}O_9$	[M-H] -	451.10346	451.10394	1.07	2.61
Gallic acid	$C_7H_6O_5$	[M-H] -	169.01425	169.01370	-3.23	1.92
Procyanidin	$C_{30}H_{26}O_{12}$	[M-H] -	577.13515	577.13510	-0.09	2.23
Gallocatechin	$C_{15}H_{14}O_7$	[M-H]-	305.06668	305.06590	-2.54	2.31
Catechin	$C_{15}H_{14}O_6$	[M-H] -	289.07176	289.07140	-1.25	2.31
Mono-O Galloyl procyanidin I	$C_{37}H_{30}O_{16}$	[M-H]-	729.14611	729.14530	-1.11	2.36
Rumejaposide	$C_{21}H_{22}O_{11}$	[M-H]-	449.10893	449.10840	-1.19	2.39
Procyanidin	$C_{30}H_{26}O_{12}$	/o0	577.13515	577.13490	-0.43	2.4
Epicatechin	$C_{15}H_{14}O_6$	[M-H]-	289.07176	289.07150	-0.90	2.49
Mono-O-galloyl procyanidin II	$C_{37}H_{30}O_{16}$	[M-H]-	729.14611	729.14646	0.48	2.52
Unknown	$C_{21}H_{24}O_{10}$	[M-H]-	435.12967	435.12920	-1.08	2.63
N-trans-feruloyl tyramine	$C_{18}H_{19}NO_4$	[M-H]-	312.12413	312.12360	-1.70	2.82
Quinic Acid	$C_7H_{12}O_6$	[M-H]-	191.05611	191.05542	-3.62	0.52
Neohlorogenic acid	$C_{16}H_{18}O_9$	[M-H]-	353.08781	353.08823	1.20	2.18
Chlorogenic acid	$C_{16}H_{18}O_9$	[M-H]-	353.08781	353.08807	0.75	2.27
Unknown	$C_{18}H_{34}O_5$	[M-H]-	329.23335	329.23376	1.25	3.03
Choline	C_5H1_3NO	[M+H]+	104.10699	104.10745	4.41	0.48

DISCUSSION

As the production of biological-active phytochemicals, the plants are considered potent. These phytochemicals shows only activities depending upon the plant product nature. This specific study booms the antifungal potential of several indigenous plants of Pakistan and *P. amplexicaule* (locally known as masloon) which showed maximum inhibition of growth of *B. cinerea* during *in vitro* and *in vivo* experiments had high contents of phenolics and flavones *i.e.,* catechin acid, gallic acid, Procyanidin, which have major antifungal activity against *B. cinerea* related with strawberry fruit**.** Similar results reported by Sattar *et al.* (2018) that *Polygonum* gave 98.5% growth inhibition of mycelial growth of tested pathogen at 0.2% concentration and found highly effective from all tested plant extracts as compared to control. Plant extracts are one of several non-chemical control alternatives that are inspiring great interest due to their availability, non-toxicity and environmental friendliness. The antifungal activity of *Polygonium* was found to be highly effective than the other three. *Polygonium amplexicaule* which were applied in three doses and showed more effective results at 50 µL compared to the other two doses of 10 and 25 µL. It can be concluded that *P. amplexicaule* and *D. viscosa* can be used as biofungicides to control apple blue mold on apple fruits and their use will be safe (Sattar *et al.*, 2014). Antifungal activity of *P. amplexicaule*, *D. viscosa*, *S. aromaticum*, *A. indica* and *Eucalyptus spp*. against a number of pre and post-harvest fungal pathogens (Thippeswamy *et al.*, 2013; Sattar *et al.*, 2014; Begum and Nath, 2015; Bashir *et al.*, 2020). However, there are no reports of their inhibitory activity of *Polygonium amplexicaule* against *B. cinerea* obtained from strawberry growing areas of Punjab in Pakistan and current research reports antifungal potential of extracts from native plants against target plants pathogen to sort out an environmentally friendly management strategy in future. This indigenous plant is located in the adjacent area of Rawalpindi and Islamabad and are easily accessible. Impact of plant extracts on other fungi will

be studied to see if they can be more useful for control other fungal diseases. Many plant extracts have antifungal potential due to the presence of chemical compounds such as terpenoids, saponins, alkaloids, flavonoids. These bio-active anti-oxidants are eligible against diseases relating cross-linking of microbial enzymes, inhibits the pathogen's cellulases, pectinases and xylanases, chelation of metal ions relevant for enzymatic activities also for cell walls tightening, leading to the foundation of a physical barrier against attack of pathogens (Mierziak *et al.*, 2014). Some active constituents may impact on pathogens directly or indirectly or as the part of systematic resistance in host plant significantly reduction in disease**.** Flavonoid compounds found in *P. amplexicaule* like Catechin and caffeic acid, rutin, Gallic acid, myricetin. Kaempferol & Quercetin transported at the infection site and tempt the hypersensitivity reaction which is the first defense mechanism engaged by the infected plants resulting in death of programmed cells. As noticed, the flavonoids are incorporated into the cell walls of adjacent & necrotic cells also contribute in tissue tightening of the plant structures by moderating auxin (IAA) activity, which differentiates the tissues, callus promotion, tylose formation and vascular system closure to prevent pathogenic infection. They are directly involved in the reticence of pathogen's enzymes, especially to those digesting plant cell wall, by chelating metals vital for their activity (Mierziak *et al.*, 2014). From phytochemical analysis based Identification were reported manifesting different antifungal compounds. Due to the presence of catechin, caffeic acid, gallic acid, quercetin, rutin, kaempferol and myricetin, the antioxidants revealed in leaf, shoot, rhizome and in their fractions (Begum and Nath, 2015). It has been reported that the genus polygonum contains metabolites like avicularin, flavonoids, plantaginin, quercetin and taxifolin (Isobe *et al.*, 1980; Isobe *et al.*, 1981). Promising results were found as concerns flavonoids and phenolics. Caffeic acid, Catechin, rutin, Gallic acid, myricetin. Quercetin and Kaempferol was found. It was found that *P. amplexicaule* had a broad spetrum of antifungal activity and having phenolic compounds like phenolic-acid flavonoids plus their derivatives; anthraquinones, tannins, stilbenes (Yang *et al.*, 2010). Polygonum analysis contains the presence of emodin and quercetin as antioxidants (Lin *et al.*, 2010). Discovery of secondary metabolites holding diverse pharmacological properties. Moreover, when used pharmacologically these secondary metabolites considered comparably less toxic than synthetic equivalents so these can be used as safe alternatives (Muhammad and Muhammad, 2005). *P. amplexicaule*, an herbaceous perennial plant grows upto 4 ft high and produces rose-red or white flowers in summer season so the leaves become pointed and broader. Also beneficial for human related problems like inflammation, fractures, dysentery, hemorrhage, relieving pain, promoting blood circulation and as a diuretic. The plants are very high in antioxidants and contain two novel antioxidant amplexicine and kellactone (Tantry *et al.*, 2012). Polygonum (genus) high polyphenolic content has associated with certain biological activities (Gong *et al.*, 2002). Recently, 12 phenolic compounds have been secluded from this plant including P-hydroxy phenethyl alcohol, 5, 7-dihydroxychromone, dihydro-kaempferol, vanillin and isovanillic acid (Xiang *et al.*, 2011).

CONCLUSION

In this work *in-vitro* and *in vivo* experiments for determination of antifungal potential and letting the identification of effective plant extracts, forming the concentrations required for reticence of a specific pathogen are major steps towards the development of synthetic bio fungicides. This research provides the detailed insight in this regard. *P. amplexicaule* extracts showed significant antifungal potential both *in vitro* (81%) and *In vivo* (80%) against grey mould disease of strawberry. Active fractions contain phenolics and flavonoids with proven antioxidant and antifungal activities**.** Therefore, extracts of this plant could be investigated and used in treatment of fruits and vegetables as such in various concentrations against postharvest pathogens on commercial level and its synthetic equivalents might be effective against such pathogens.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.

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