CONTROL OF *BOTRYTIS CINEREA* (GREY MOULD DISEASE) BY METHANOLIC EXTRACT OF *PONGAMIA PINNATA L.*.

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The antifungal activity of *Pongamia pinnata* (L.) Pierre was evaluated against the phytopathogenic fungus *Botrytis cinerea* Pers., the causal agent of Botrytis grey mould disease in a number of agronomically important crops. Different methanolic concentrations of leaf, stem bark, seed and pod of *P. pinnata* were tested against *B. cinerea* in vitro to test its fungicidal properties. The 4 % concentration of methanolic leaf extract was found to be highly effective against the test fungus by inhibiting the fungal growth up to 75 %. Methanolic stem bark extract of *P. pinnata* inhibited fungal growth up to 69 % with 4 % of its concentration. Methanolic seed and pod extracts of the test plant also significantly inhibited the growth of *B. cinerea*. The highly fungicidal methanolic leaf extracts of *P. pinnata* were subjected to fractionally guided bioassays and partitioned using four organic solvents, viz., n-hexane, chloroform, ethyl acetate and n-butanol. Minimum Inhibitory Concentration (MIC) assay was performed with different concentrations (0.78 to 100 mg/ml) of the isolated fractions and the commercial synthetic fungicide Puslan (active ingredient metalaxyl + mancozeb 72 % WP). The assay proved that the n-hexane fraction and the synthetic fungicide were highly effective in retarding the mycelial germination with a MIC of 0.78 mg/ml. The other isolated fractions were comparatively less fungicidal than the n-hexane fraction.
Keywords: antifungal; grey mould; methanolic extract; MIC; Sukh chayn

Zur Bekämpfung von *Botrytis cinerea* (Grauschimmelfäule) mittels methanolischer Extrakte von *Pongamia pinnata L.*. Die fungizide Wirkung von *Pongamia pinnata* (L.) Pierre gegen den phytopathogenen Pilz *Botrytis cinerea Pers.*, den Erreger der Grauschimmelfäule bei einer Reihe von landwirtschaftlich wichtigen Kulturen, wurde untersucht. Unterschiedliche methanolische Konzentrationen aus Blatt, Stammrinde, Samen und Schote von *P. pinnata* wurden gegen *B. cinerea* in vitro getestet, um ihre fungiziden Eigenschaften zu testen. Die 4 %ige Konzentration des methanolischen Blattextrakts erwies sich als hochwirksam (Hemmung des Pilzwachstums bis zu 75 %). Der methanolische Stammrindenextrakt von *P. pinnata* hemmte bei einer 4 %igen Konzentration das Pilzwachstum um bis zu 69 %. Samen- und Schotenextrakte hemmten das Wachstum von *B. cinerea* auch ausreichend. Die stark fungiziden methanolischen Extrakte aus *S. pinnata* wurden fraktioniert geführten Bioassays unterzogen und mittels vier organischer Lösungsmittel (N-Hexan, Chloroform, Ethylacetat und n-Butanol) partitioniert. Ein MIC-Test (Minimale Hemm-Konzentration) wurde mit verschiedenen Konzentrationen (0,78 bis 100 mg/ml) der isolierten Fraktionen und dem kommerziellen synthetischen Fungizid Puslan (Wirkstoff Metalaxyl + Mancozeb 72 % WP) durchgeführt. Der Test bewies, dass die n-Hexan-Fraktion und das synthetische Fungizid hochwirksam waren bei der Verzögerung der Myzelkeimung bei einer MIC von 0,78 mg/ml. Die anderen isolierten Fraktionen zeigten eine vergleichsweise geringere fungizide Wirkung als die n-Hexan-Fraktion.
Schlagwörter: Fungizid, Grauschimmelfäule, methanolischer Extrakt, MIC, Sukh Chayn

Botrytis cinerea Pers. is a necrotrophic fungus which causes pre- and post-harvest diseases in at least 200 plant species including a range of agronomical important crops, such as grapevine, tomato, strawberry, cucumber, onion, potato, bulb flowers, chickpea and ornamental plants (Jarvis, 1977; Guinebretiere et al., 2000; Elad et al., 2007). It is the causal agent of grey mould decay which is a major threat to crop production worldwide (Hammer et al., 1999; Hahn et al., 2008). The disease is a serious concern in Pakistan, Bangladesh, India, Nepal, Australia and Argentina where 100 % chickpea yield losses have been reported under favourable conditions (Bakr et al., 1993; Dhar et al., 1993; Pande et al., 2001). There are some methods like good agronomical practices, chemical and biological control for grey mould disease. In good agronomical practices, crop rotation can be employed with non-host crops, non-planting of new crops near previous epidemic crops, and the use of disease free seeds (Gan et al., 2006). Tillage practices like burying infected residues and a voluntary control of suspected plants will also be beneficial (Navas-Cortes et al., 1998). Adopting some cultural practices, such as optimizing the planting date, proved to be very effective in reducing the fungal infection of plants, but these practices are insufficient under high disease pressure, especially when weather conditions are particularly conducive to disease development (Abdel-Monaim, 2011). The use of resistant cultivars is another practical and economical solution (Mabrouk and Belhadj, 2012). Most commonly applied chemicals as fungicides against *Botrytis cinerea* are Botran 75 WP, Decree 50 WDG and Ferbam 76 WDG (Sabaratnam, 2012). The inappropriate use of agrochemicals, especially fungicides, which were found to be of a higher carcinogenic risk than insecticides and herbicides together, such as Mancozeb, is found to be carcinogenic (Axelstad et al., 2011). Additionally, pathogen resistance to fungicides has rendered certain fungicides ineffective (Abdulrahman and Alkhail, 2005). Resistance of *B. cinerea* isolates from vegetable crops towards the major classes of commercial anti-botrytis fungicides: anilinopyrimidines, phenylpyrroles, hydroxyanilides, benzimimidazoles and dicarboximides have been recently confirmed (Myresiotis et al., 2007). To avoid use of synthetic fungicides, it is imperative to find alternative sources from naturally occurring compounds that are easily biodegradable and of low mammalian toxicity for a safe control of fungal pathogens (Adongo et al., 2012). Plants are the source of natural pesticides that make excellent leads for new pesticide developments (Arokiyaraj et al., 2008; Shanmugavalli et al., 2009). *Pongamia pinnata* (L.) Pierre belongs to the family Fabaceae. In Pakistan, this plant is locally known as "Sukh Chayn" and is cultivated throughout the country. The seeds of *P. pinnata* contain about 40 % of oil which can be converted to biodiesel by transesterification (Meher et al., 2006). Traditionally, different parts of *P. pinnata* such as bark, leaves, seeds, roots, flowers and stem have been utilized in the native medicine systems of different civilizations including Ayurveda, Unani and Sidhha systems of medicine (Chopade et al., 2008; Kumar et al., 2012). Flavones, isoflavones, chalcones, furanoflavonoids and pyranoflavonoids have been reported as the main phenolic constituents from various parts of *P. pinnata* (Tanaka et al., 1991; Yadav et al., 2004). Due to its strong chemical composition the present study is designed to check the antifungal potential of *P. pinnata* against *B. cinerea*.

MATERIALS AND METHODS

COLLECTING THE PLANT MATERIAL

Bark, leaf, seed and pod of *P. pinnata* were collected from Lahore College for Women University, Jail Road, Lahore, Pakistan in the months of February and March. The plant material was cleaned and thoroughly washed with tap water and was dried under sunlight and shade for 20 to 30 days varying for different parts of the plant. After drying, the plant material was finely ground into powder form and stored in air tight jars.

PROCUREMENT AND CULTURING OF THE TEST FUNGUS

B. cinerea Pers. was isolated from diseased root hair of onion in Fungal Biotechnology Laboratory, Department of Botany, Lahore College for Women University. The diseased onion root hair was isolated from the onion bulb and was placed on solidified 2 % MEA (Malt Extract Agar) medium. MEA (2 %) was prepared by adding 2 g of ME and 2 g of Agar to 100 ml of distilled water and was autoclaved at 121 °C at 1 bar for 30 minutes. This fungus was identified as *Botrytis cinerea* from previous literature by the presence of septate hyphae, hyaline conidia and at maturity greyish green colonies appeared.

SCREENING BIOASSAYS

Twenty grams of each powdered bark, leaf, seed and pod of *P. pinnata* were soaked in 100 ml of methanol, sealed with aluminum foils and left standing for 10 days at room temperature in April, 2013. Extracts of the testing plant materials were then obtained by filtering them with

Muslin cloth. The filtrates were allowed to evaporate for 3 days at room temperature. Two grams of gummy mass of each plant part were obtained after drying; these extracts were then diluted by adding 98 ml of distilled water. The stock extracts were then sealed with aluminum foil and were kept in the refrigerator at 4 °C for further use.

ANTIFUNGAL BIOASSAY WITH EXTRACTS

Different v/v concentrations from 1 % to 5 % from two grams of methanolic gummy mass of each plant extract were prepared. To 95 ml, 90 ml, 85 ml, 80 ml and 75 ml of MEA media, 5 ml, 10 ml, 15 ml, 20 ml and 25 ml of each plant extract were added to make various concentrations, viz. 1 %, 2 %, 3 %, 4 % and 5 %, respectively, whereas, in the control treatment no plant extract was added. Antibacterial capsules (Terramycin 250 mg, Oxytetracycline hydrochloride) (Pfizer; Karachi, Pakistan) were added in each experimental concentration and control medium by the ratio of 5 mg/100 ml and gently shaken. Each concentration and control treatment was replicated thrice by pouring them in three 9 x 9 cm Petri plates. The media was allowed to solidify for some hours after pouring. Five millimeter of mycelial discs of pure culture of B. cinerea Pers. were separated by using sterilized cork borer and were carefully put in the center of each Petri-plate of the set using sterilized forceps. After inoculation, each Petri-plate was carefully packed and sealed and the whole set was incubated at 20 °C for 7 days. After one week of incubation, the fungal growth diameter around the inoculum was measured by calculating average of three diameters taken at right angles for each colony with the help of a transparent ruler. Percentage growth inhibition of each fungal colony was calculated by using the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in Treatment} - \text{Growth in Control}}{\text{Growth in Control}} \times 100$$

FRACTIONATION OF GUIDED BIOASSAYS

The leaf extracts of *P. pinnata* were found to be the most effective in inhibiting the growth of *B. cinerea*. Therefore, the extract was selected for fractionation. Different organic fractions of methanolic leaf extract of *P. pinnata* were isolated by using various organic solvents in increasing order of polarity. The isolation of fractions was done by following the protocol given by Jabeen et al. (2013). One hundred grams of leaf powder of *P. pinnata* were soaked in 250 ml of methanol (MeOH) for three days and then extracted thoroughly with a muslin cloth. The extract was then evaporated under vacuum on rotary evaporator (R-210; Büchi Labortechnik AG, Flawil, Switzerland) at 40 °C to give 6.8 g of gummy mass. This methanolic extract (6.8 g) was further partitioned in a separating funnel by using 100 ml each of four organic solvents in increasing order of polarity, i.e., n-hexane, chloroform, ethyl acetate and n-butanol at room temperature in the laboratory. The fractions were then allowed to evaporate at room temperature for 5 days till a gummy mass of fractions was obtained, i.e., n-hexane (1.6 g), chloroform (0.1 g), ethyl acetate (1.15 g) and n-butanol (3.15 g).

MINIMUM INHIBITORY CONCENTRATION (MIC) BIOASSAYS

For the determination of MIC broth serial dilution assay was used (Jabeen et al., 2011) with little modifications. Hundred milligrams of each isolated fraction (n-hexane, chloroform, ethyl acetate and n-butanol) of the methanolic leaf extract of *P. pinnata* and the synthetic fungicide Puslan (active ingredient metalaxyl + mancozeb 72 % WP; Tarzan Markaz by Four Brother Group, Lahore City, Pakistan) were used in this assay. This 100 mg of each fraction and fungicide were separately dissolved in 1 ml of pure dimethysulfoxide (DMSO) and 1 ml of distilled water and were serially diluted in distilled water to make 8 concentrations from 0.78 mg/ml to 100 mg/ml of each isolated fraction along with fungicide. The two control treatments viz. distilled water and DMSO were maintained without any addition of test plant material. MEA (2 %) was added to 7 days old pure culture of B. cinerea and 1 x 105/ml conidial concentration were added in each test tube. Data was recorded after 24, 48 and 72 hours of incubation period with the aid of inverted microscope.

STATISTICAL ANALYSIS

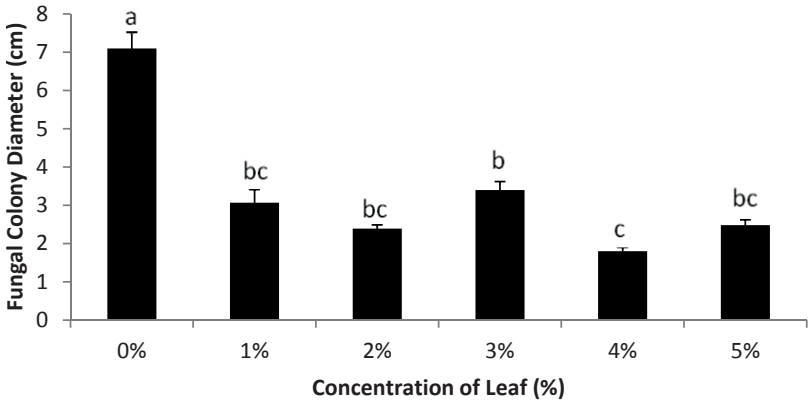
All the collected data were analyzed statistically by applying Analysis of Variance ANOVA at 5 % significance level followed by Duncan's Multiple Range Test (Steel et al., 1997).

RESULTS AND DISCUSSION

Botanical derivatives like essential oils and plant extracts have been used to control *Botrytis cinerea* Pers. (Vio-Michaelis et al., 2012; Adebayo et al., 2013). Plants are the sources of natural pesticides

that make excellent leads for new pesticide development (Arokiyaraj et al., 2008; Shanmugavalli et al., 2009). Natural antifungal compounds have been found to be comparatively much safer than synthetic ones in terms of toxicity and foodstuffs (Hanekamp and Kwakman, 2004). *P. pinnata* has been reported for its antimicrobial properties in literature (Brijesh et al., 2006; Wagh et al., 2007; Joseph and Singh, 2008; Chandrashekar and Prasanna, 2010). In the current investigation, plant extracts of *P. pinnata* have been found to be antifungal against *B. cinerea*. Different applied concentrations of methanolic leaf extract (1 to 5 %) significantly reduced the target fungal growth, 4 % concentration were found to be highly effective retarding fungal growth of up to 74.65 %, while 3 % showed minimum growth retardation, i.e., up to 52.15 % as compared to control treatment (Fig. 1). The findings are supported by literature (Chandrashekar and Prasanna, 2010), in which antifungal and antibacterial activity of leaf extracts (petroleum ether, chloroform and acetone) of *P. pinnata* was observed by employing agar cup diffusion technique. Significant antifungal activity was found against *Aspergillus niger* and *Candida albicans* by the petroleum ether extract and chloroform extract of the leaf of *Derris indica* (Biswal et al., 2011). The present study was further supported by literature (Yasmeen et al., 2002) which presented the antifungal activity of aqueous leaf extract of *P. pinnata* against *Botrytis ricini* (*Amphobotrys ricini*) using poisoned food technique. The aqueous leaf extract inhibited the fungal growth by more than 50 %.

Fig. 1: Effects of methanolic leaf extracts of *P. pinnata* against in vitro growth of *B. cinerea*. Vertical bars show standard error of means of three replicates. Values with different alphabetical letters show significant difference as determined by Duncan's multiple range test.



Among different applied concentrations of methanolic bark extract (1 to 5 %), 4 % was found to be highly effective against *B. cinerea* which inhibited the growth up to 68.87 % while the minimum retardation shown by the same extract was 2 %, inhibiting growth up to 59.3 % compared to control treatment (Fig. 2). This was supported by literature (Sajid et al., 2012) which evaluated antimicrobial activity of different solvent extracts (absolute methanol, aqueous methanol, absolute ethanol, aqueous ethanol, absolute acetone, aqueous acetone, and deionized water) from bark, leaf and seed of *P. pinnata*. In his study, aqueous methanolic bark extract exhibited strongest antimicrobial activity against *Aspergillus oryzae*, *A. niger*, *Fusarium solani*, *Pseudomonas stutzeri*, *P. aeroginosa* and *Escherichia coli*. Following leaf and bark, methanolic seed extracts of the tested plant were found to be effective. Methanolic seed extract (5 %) inhibited the tested fungal growth up to 66.2 % while the minimum inhibition shown by 1 % that prevented fungal growth up to 44.27 % compared to the control treatment (Fig. 3). Our findings were further supported by findings of Wagh et al. (2007), in which fungicidal and bactericidal activities of seed oil of *P. pinnata* against two strains of each bacteria and fungi were studied. The seed oil proved to be highly antimicrobial against *Staphylococcus aureus* and *Aspergillus fumigatus*. The study was further supported by literature (Adebayo et al., 2013) which states that the essential oils of *Origanum vulgare L.* and *Monarda didyma L.* inhibited the growth of *B. cinerea* at high concentration of essential oil, i.e, 200 µg/ml. The overall minimum antifungal activity shown by the pod extracts among the four tested parts of the tree was the weakest. But it cannot be assumed that the pod extract was proved to be least effective or near to be non-effective against *B. cinerea* as 4 % of its concentration inhibited the *B. cinerea* growth up to 62.39 % while the concentration showing minimum antifungal activity was 2 % retarding growth up to 26.76 % compared to control treatment (Fig. 4). The antifungal activity of *P. pinnata* in the present study is due to the antimicrobial phytochemical compounds present in various parts of the tree. The presence of such antimicrobial compounds is confirmed by many researches. Karanjin, chemically a furanoflavonoid is the

major active constituent of *P. pinnata* and also has been analysed from its leaves by employing HPLC method on methanolic leaf extract (Katekhaye et al., 2012). Pongarotene, a new rotenoid was also isolated from its seed (Simin et al., 2002). HPLC of aqueous methanolic extract of bark, leaf and seed was done (Sajid et al., 2012) and presence of protocatechuic, ellagic, ferulic, gallic, gentisic, 4-hydroxybenzoic and 4-hydroxycinnamic acids in bark (1.50 to 6.70 mg/100 g dry weight); sorbic, ferulic, gallic, salicylic and p-coumaric acids in leaves (1.18 to 4.71 mg/100 g dry weight); vanillic, gallic and tannic acids in seeds (0.52 to 0.65 mg/100 g dry weight) as the main phenolic acids was reported. Presence of flavonoids was also reported with highest concentrations found in stem bark followed by leaves and seeds. The petroleum ether and chloro-

Fig. 2: Effects of methanolic bark extracts of *P. pinnata* against in vitro growth of *B. cinerea*. Vertical bars show standard error of means of three replicates. Values with different alphabetical letters show significant difference as determined by Duncan's multiple range test.

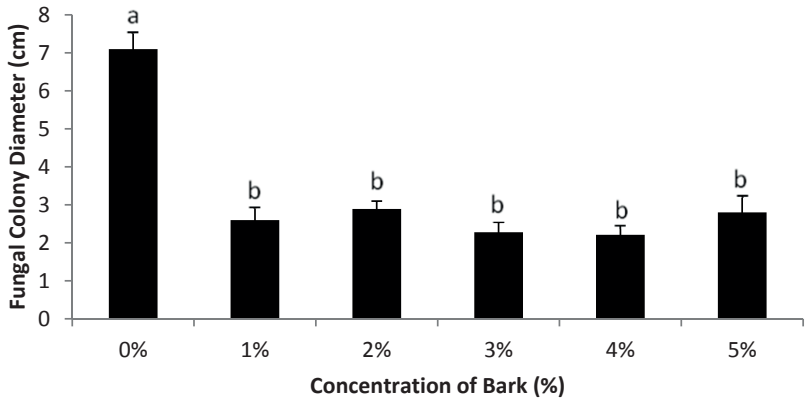


Fig. 3: Effects of methanolic seed extracts of *P. pinnata* against in vitro growth of *B. cinerea*. Vertical bars show standard error of means of three replicates. Values with different alphabetical letters show significant difference as determined by Duncan's multiple range test.

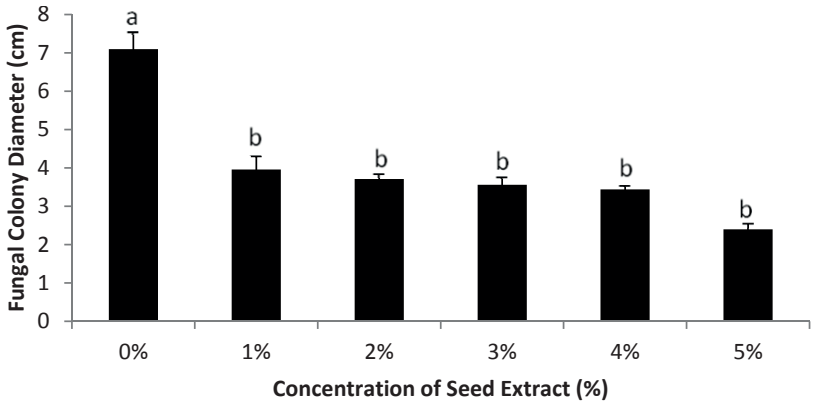
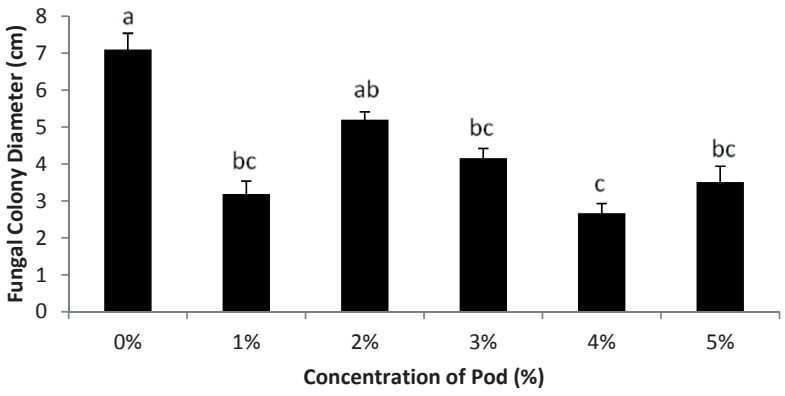


Fig. 4: Effects of methanolic pod extracts of *P. pinnata* against in vitro growth of *B. cinerea*. Vertical bars show standard error of means of three replicates. Values with different alphabetical letters show significant difference as determined by Duncan's multiple range test.



form extracts showed the presence of phytosterols and saponins. The chloroform and ethanolic extracts showed the flavonoids and fixed oils (Biswal et al., 2011). The leaves of *P. pinnata* were selected for further bioassays and various organic fractions (n-hexane, chloroform, ethyl acetate and n-butanol) were isolated from crude methanolic leaf extract. The four isolated organic fractions showed significant variations in antifungal activity against *B. cinerea* due to different chemical nature of the four organic solvents. It is likely that different phytochemical compounds were getting dissolved in the organic solvents on the basis of their polarities that resulted in variable activity of the extracts of different organic solvents. MIC of the isolated fractions of the methanolic leaf extracts of *P. pinnata* was checked against the test fungus and was compared with a commercial fungicide, i.e., Puslan 72 WP (Table 1). The MIC assay was conducted through broth serial dilution method; various concentrations were made ranging from 0.78 to 100 mg/ml (Rangasamy et al., 2007). N-hexane highly inhibited the in vitro germination of B. cinerea. Following n-hexane, n-butanol was observed to be highly effective in inhibiting the in vitro growth of the target fungus, Ethyl acetate and chloroform fraction were found to be least effective, whereas, the synthetic fungicide was observed to show best results in growth retardation of the fungus after 72 hours of incubation. In the light of MIC assay, it can be concluded that n-hexane fractions of methanolic leaf extract of *P. pinnata* can be used to isolate antifungal compounds against *B. cinerea*.

Table 1: Results of MIC (Minimum Inhibitory Concentration) assay of different organic fractions isolated from methanolic leaf extract of *Pongamia pinnata* and Puslan against *Botrytis cinerea* after 24, 48 and 72 hours of incubation period; mycelium present (+), mycelium absent (-)

Fractions	Concentration (mg/ml)							
	100	50	25	12.5	6.25	3.12	1.56	0.78
	24 hours after incubation							
Control (H_2O)	+	+	+	+	+	+	+	+
Control (DMSO)	–	–	–	–	–	–	–	–
n-hexane fraction	–	–	–	–	–	–	–	–
Chloroform fraction	–	–	–	–	–	–	–	–
Ethyl acetate fraction	–	–	+	+	+	+	+	+
n-butanol	–	–	+	+	+	+	+	+
Puslan	–	–	–	–	–	–	–	–
	48 hours after incubation							
Control (H_2O)	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+
n-hexane fraction	–	–	_	–	–	–	–	–
Chloroform fraction	–	–	+	+	+	+	+	+
Ethyl acetate fraction	–	+	+	+	+	+	+	+
n-butanol fraction	–	+	+	+	+	+	+	+
Puslan	–	–	–	–	–	–	–	–
	72 hours after incubation							
Control (H_2O)	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+
n-hexane fraction	–	–	–	–	–	–	–	–
Chloroform fraction	–	+	+	+	+	+	+	+
Ethyl acetate fraction	–	+	+	+	+	+	+	+
n-butanol fraction	–	+	+	+	+	+	+	+
Puslan	–	–	–	–	–	–	–	–

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